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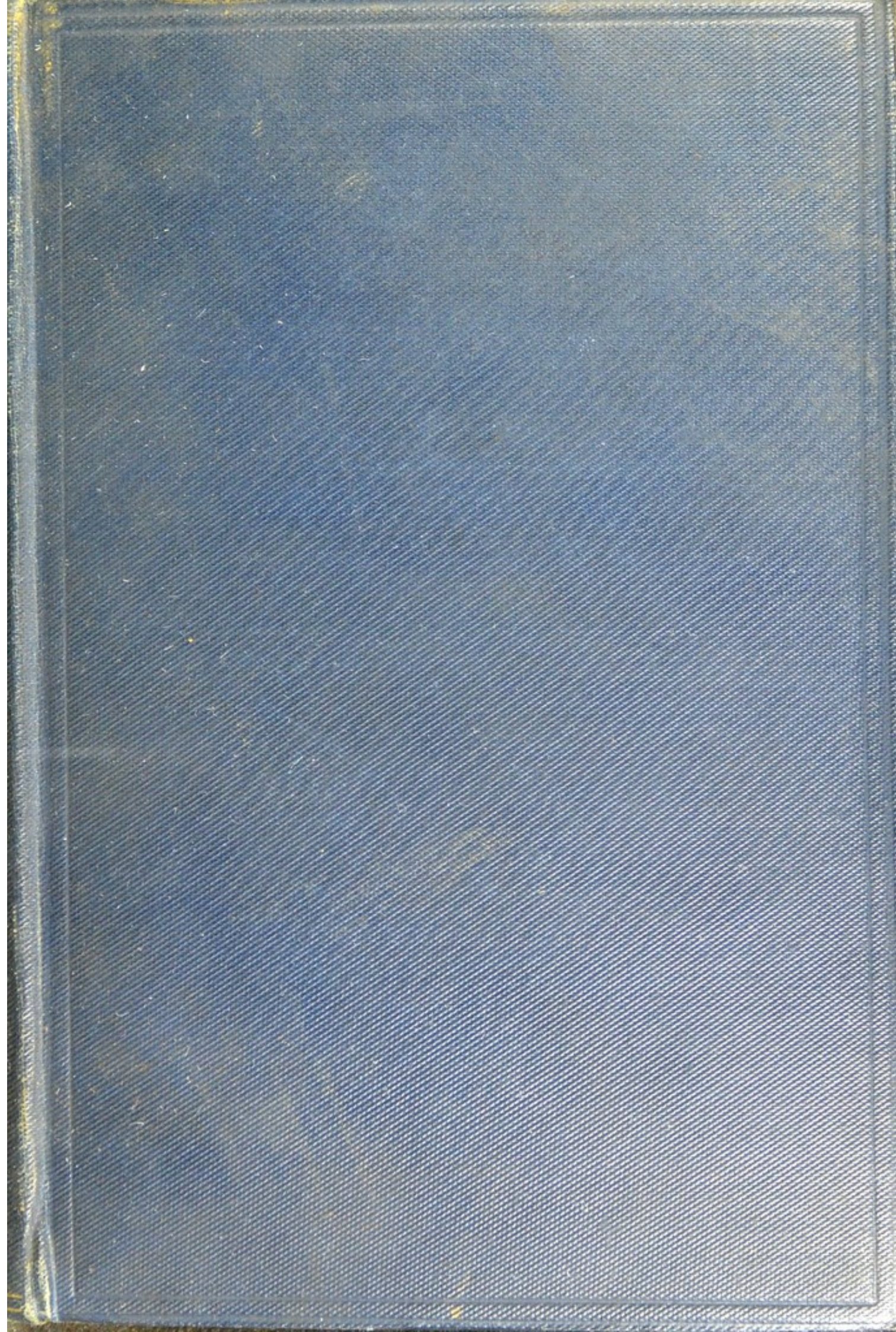
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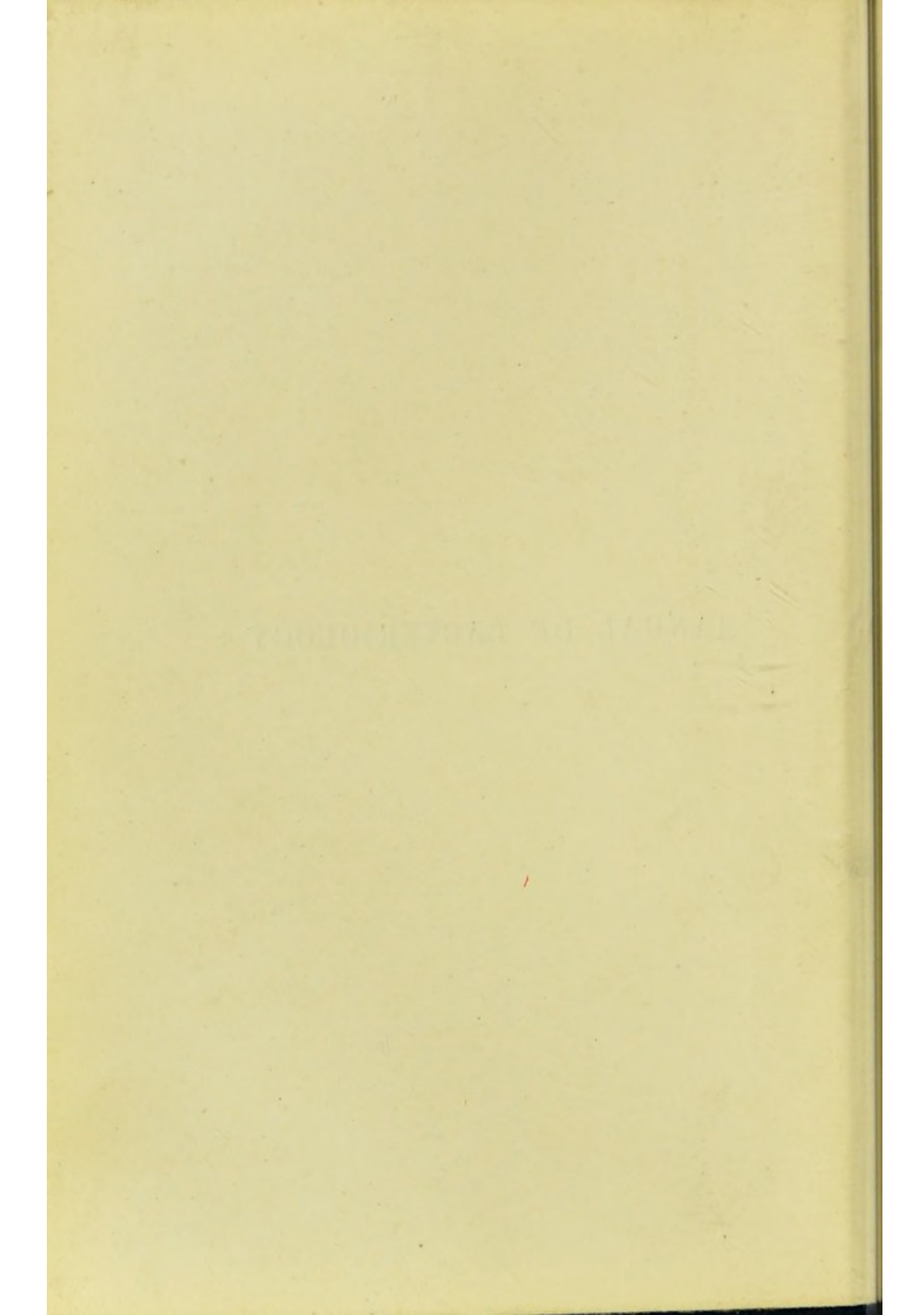
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MANUAL OF BACTERIOLOGY



# MANUAL OF BACTERIOLOGY

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*FIFTH EDITION*

*WITH ONE HUNDRED & SEVENTY-FOUR ILLUSTRATIONS*

*IN THE TEXT*

*AND SIX COLOURED PLATES*

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THE HISTORY OF THE  
CITY OF GLASGOW

FROM THE EARLIEST PERIODS  
TO THE PRESENT

BY  
JAMES WATSON

THE HISTORY OF THE  
CITY OF GLASGOW

THE HISTORY OF THE  
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## PREFACE TO THE FIFTH EDITION.

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DURING the three years which have elapsed since the publication of the previous edition, important additions to knowledge have been made in nearly every department of bacteriological research. We have again endeavoured to incorporate these as fully as possible, having in view our primary object, namely, to supply a Manual for students and practitioners of medicine.

It is impossible to refer in detail to the new matter introduced, as this occurs in practically every chapter. It may, however, be mentioned that in dealing with technique, we have introduced a new chapter in which the more recent methods of investigating the properties of serum and allied subjects are described. While it is necessary for students of medicine to have a general knowledge of such subjects, those commencing independent bacteriological research may benefit from the details given in this department.

We have also in the present edition grouped together in a new chapter the pathological conditions with which spirochaetes are associated, but as the biological relationships of these organisms to kindred forms are still not completely determined, we have retained this chapter in its former position.

We have transferred the consideration of Yellow Fever to the Appendices, which include diseases of protozoal origin and conditions in which the nature of the infective agent is still unknown. In further following this principle, we have added

new Appendices dealing with Acute Poliomyelitis, Phlebotomus Fever, and Typhus Fever.

Several new illustrations will be found in the text, and we have also added a series of coloured plates which have been reproduced from drawings by Mr. Richard Muir of the Department of Pathology, University of Edinburgh.

*November 1910.*



## PREFACE TO THE FIRST EDITION.

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THE science of Bacteriology has, within recent years, become so extensive, that in treating the subject in a book of this size we are necessarily restricted to some special departments, unless the description is to be of a superficial character. Accordingly, as this work is intended primarily for students and practitioners of medicine, only those bacteria which are associated with disease in the human subject have been considered. We have made it a chief endeavour to render the work of practical utility for beginners, and, in the account of the more important methods, have given elementary details which our experience in the practical teaching of the subject has shown to be necessary.

In the systematic description of the various bacteria, an attempt has been made to bring into prominence the evidence of their having an etiological relationship to the corresponding diseases, to point out the general laws governing their action as producers of disease, and to consider the effects in particular instances of various modifying circumstances. Much research on certain subjects is so recent that conclusions on many points must necessarily be of a tentative character. We have, therefore, in our statement of results aimed at drawing a distinction between what is proved and what is only probable.

In an Appendix we have treated of four diseases; in two of these the causal organism is not a bacterium, whilst in the other two its nature is not yet determined. These diseases have been

included on account of their own importance and that of the pathological processes which they illustrate.

Our best thanks are due to Professor Greenfield for his kind advice in connection with certain parts of the work. We have also great pleasure in acknowledging our indebtedness to Dr. Patrick Manson, who kindly lent us the negatives or preparations from which Figs. 163-168 have been executed.

As we are convinced that to any one engaged in practical study, photographs and photomicrographs supply the most useful and exact information, we have used these almost exclusively in illustration of the systematic description. These have been executed in the Pathological Laboratory of the University of Edinburgh by Mr. Richard Muir. The line drawings were prepared for us by Mr. Alfred Robinson, of the University Museum, Oxford.

To the volume is appended a short Bibliography, which, while having no pretension to completeness, will, we hope, be of use in putting those who desire further information on the track of the principal papers which have been published on each of the subjects considered.

*June 1897.*



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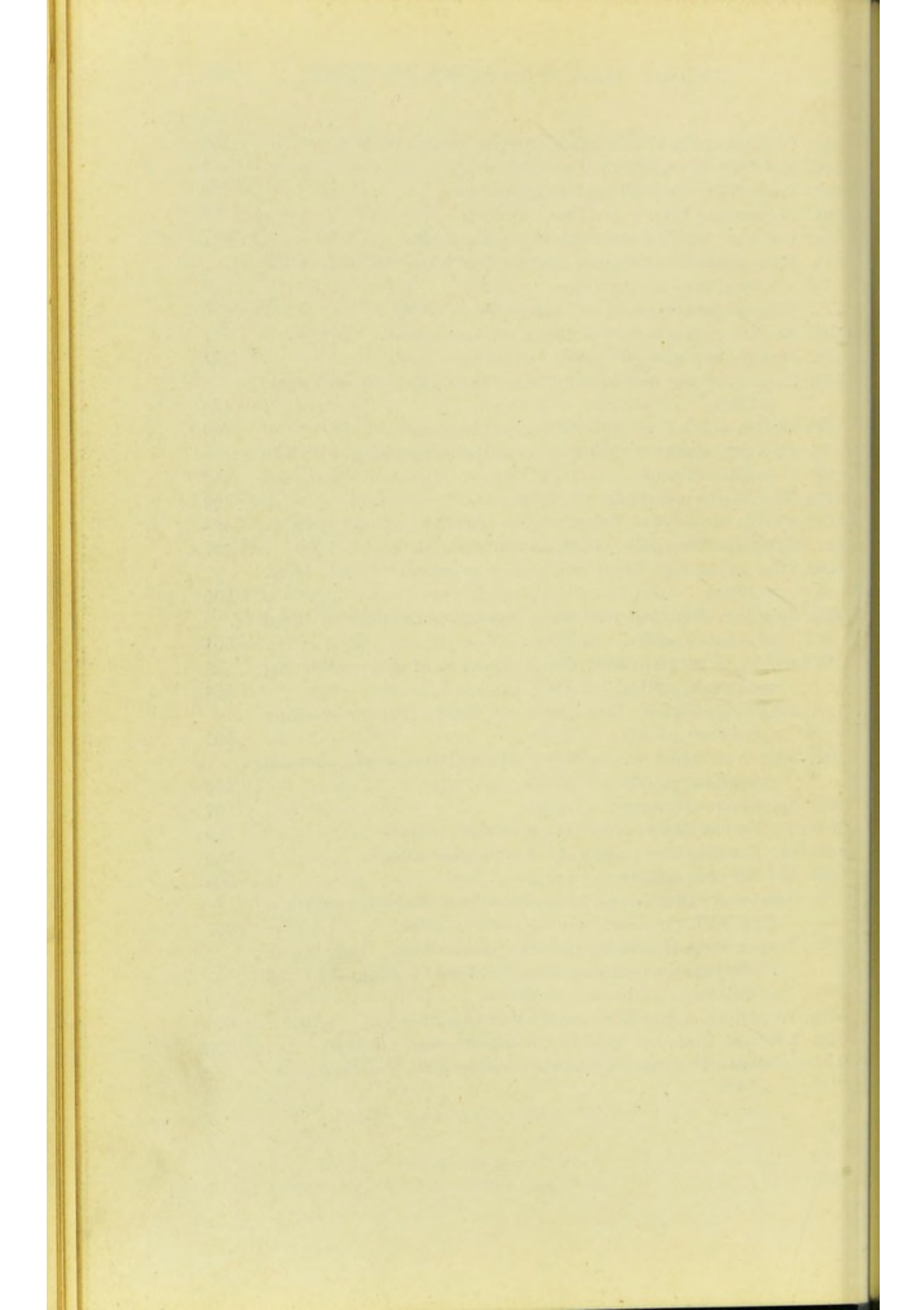
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## PLATE I.

FIG. 1. Film of pus, containing staphylococci and streptococci. Stained by Gram's method.  $\times 1000$  diameters.

FIG. 2. Fraenkel's pneumococcus in sputum, from a case of acute pneumonia. Rd. Muir's method of capsule staining.  $\times 1000$  diameters.

FIG. 3. Meningococcus in epidemic cerebro-spinal fever, from lumbar puncture fluid, showing some involution forms. Leishman's stain.  $\times 1000$  diameters.

FIG. 4. Film from a scraping of throat in Vincent's angina, showing fusiform bacilli and spirochaetes.  $\times 1000$  diameters.

FIG. 5. Gonorrhœal pus, showing gonococci (stained red) and staphylococci. Gram's method.  $\times 1000$  diameters.

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Fig. 3. Meningococcus in epidemic cerebro-spinal fever, from lumbar puncture fluid, showing some involution forms. Teichmann's stain.  $\times 1000$  diameters.

Fig. 4. Film from a scraping of throat in Vincent's angina, showing fusiform bacilli and spirilla.  $\times 1000$  diameters.

Fig. 5. Gonorrheal pus, showing gonococci (stained red) and staphylococci. Gram's method.  $\times 1000$  diameters.



PLATE I.



FIG. 1.

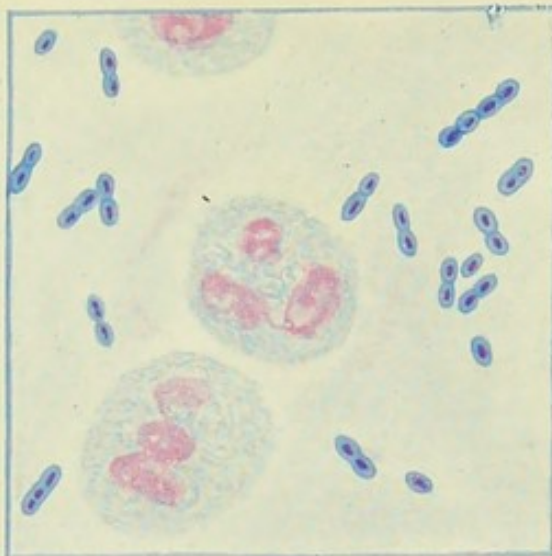


FIG. 2.

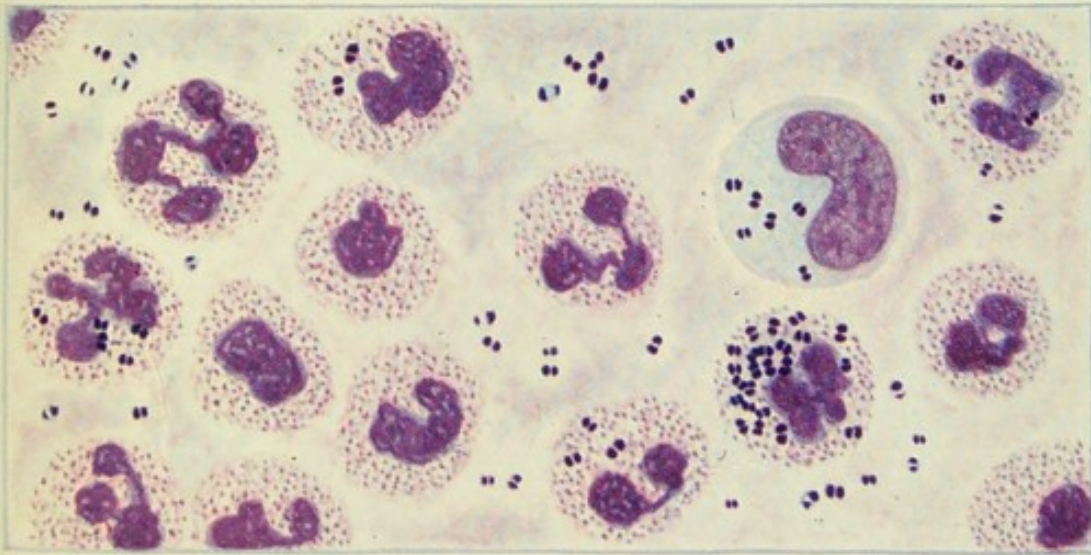


FIG. 3.

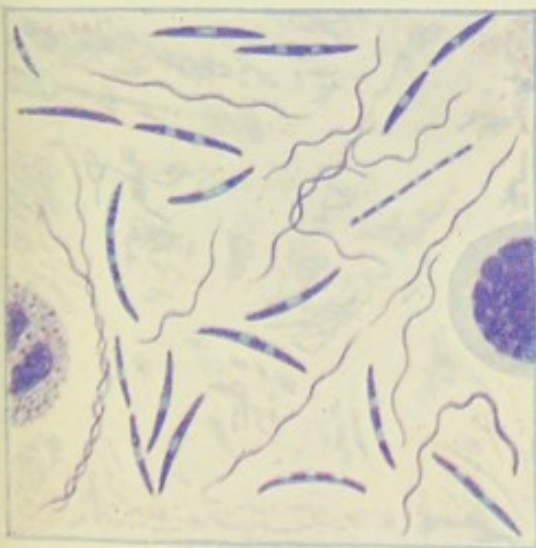


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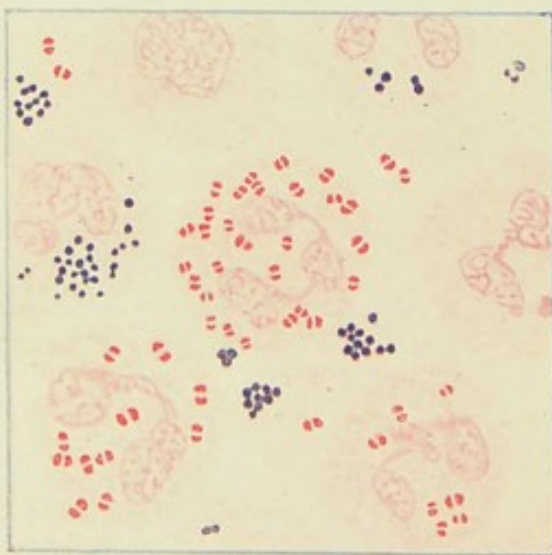


FIG. 5.





PLATE II.

FIG. 6. *Spirochæte pallida* in section of spleen of child ; case of congenital syphilis. Levaditi's stain.  $\times 1000$  diameters.

FIG. 7. Tubercle bacillus and other bacteria in sputum ; case of chronic phthisis. Ziehl-Neelsen stain.  $\times 1000$  diameters.

FIG. 8. Section of leprous skin, showing numerous clumps of bacilli (stained red) in the cutis. Carbol-fuchsin and methylene-blue.  $\times 80$  diameters.

FIG. 9. Section of leprous granulation tissue, showing large numbers of bacilli, chiefly contained within cells. Carbol-fuchsin and methylene-blue.  $\times 1000$  diameters.

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FIG. 6

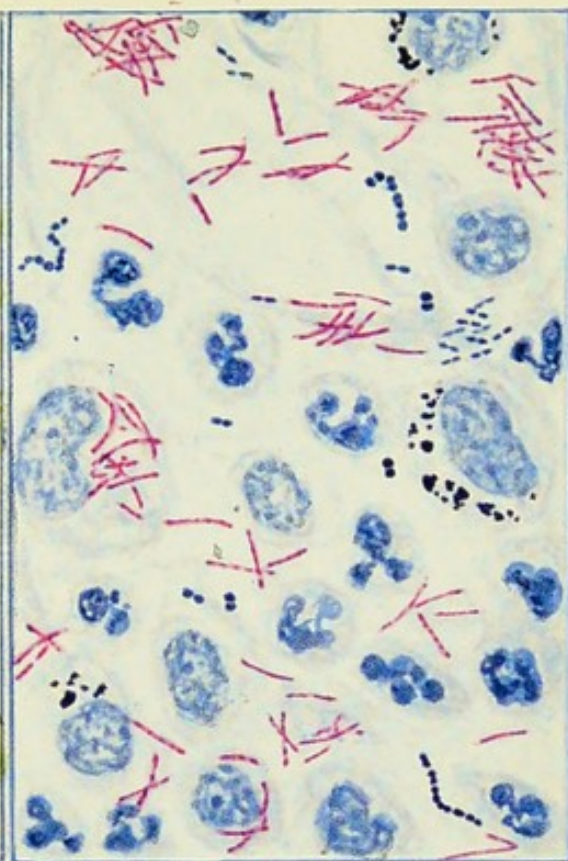


FIG. 7.

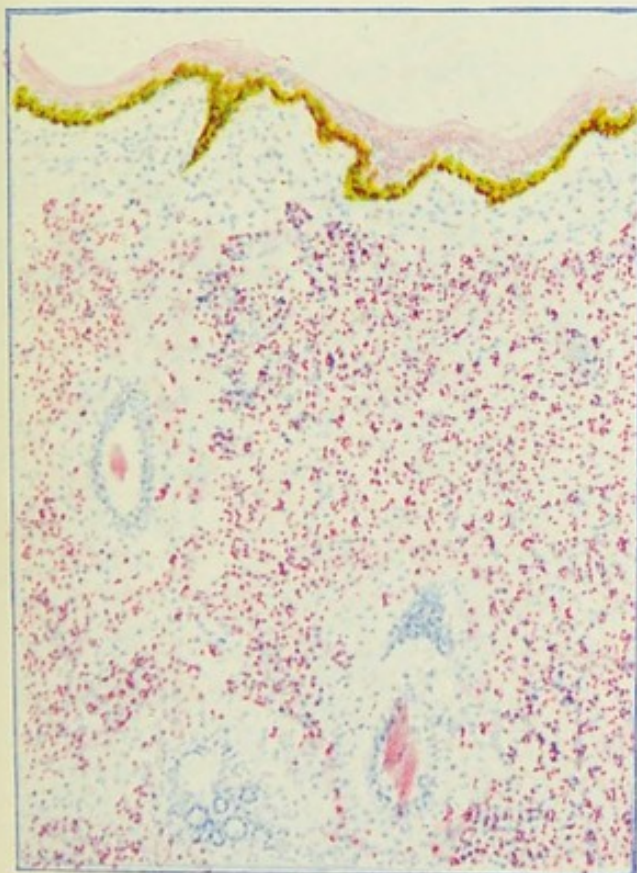


FIG. 8.

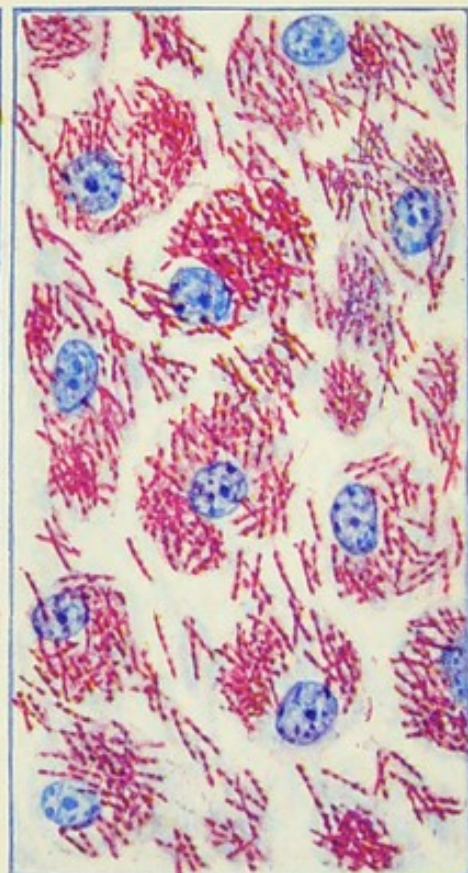


FIG. 9.





PLATE III.

FIG. 10. *Streptothrix actinomyces*, from agar culture. Gram's method.  
× 1000 diameters.

FIG. 11. Anthrax bacilli, from 4-days' agar culture, showing spores.  
Carbol-fuchsin and methylene-blue. × 1000 diameters.

FIG. 12. *Bacillus diphtheriæ*, from a 12-hours' blood serum culture.  
Neisser's stain modified. × 1000 diameters.

FIG. 13. *Bacillus diphtheriæ*, from a 5-days' blood serum culture, showing  
involution forms. Neisser's stain modified.  
× 1000 diameters.

FIG. 14. Pseudo-diphtheria bacillus (Hofmann's), from young agar  
culture. Neisser's stain modified. × 1000 diameters.

FIG. 15. Typhoid bacillus, from a 24-hours' agar culture, showing  
flagella. Rd. Muir's method. × 1000 diameters.

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Neisser's stain modified. × 1000 diameters.

Fig. 13. *Bacillus diphteriae*, from a 5-days' blood serum culture, showing involution forms. Neisser's stain modified.  
× 1000 diameters.

Fig. 14. *Pseudo-diphteria bacillus* (Hofmann's), from young agar culture. Neisser's stain modified. × 1000 diameters.

Fig. 15. *Typoid bacillus*, from a 24-hours' agar culture, showing flagella. Rd. Muir's method. × 1000 diameters.



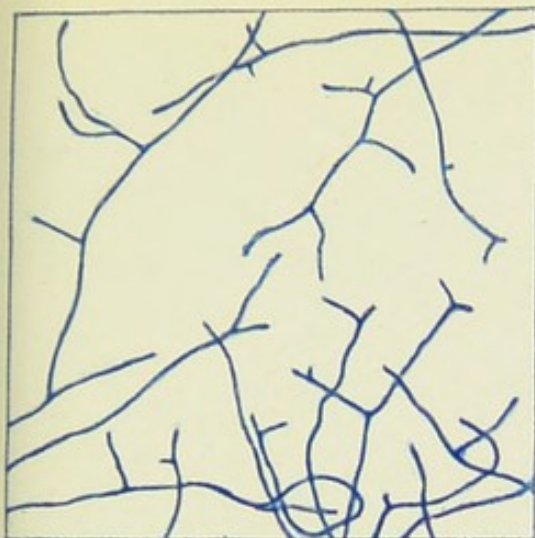


FIG. 10.

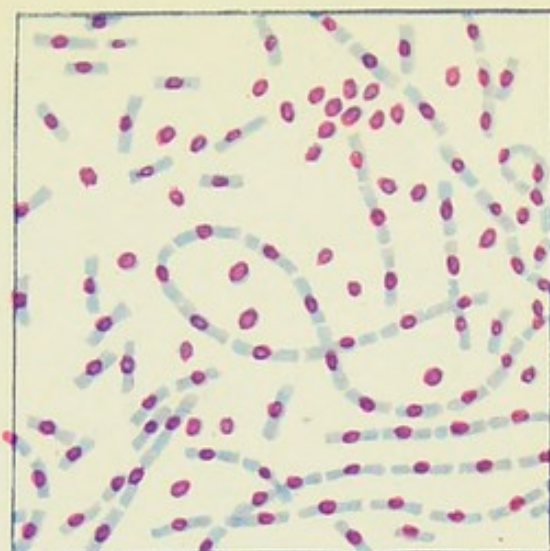


FIG. 11.

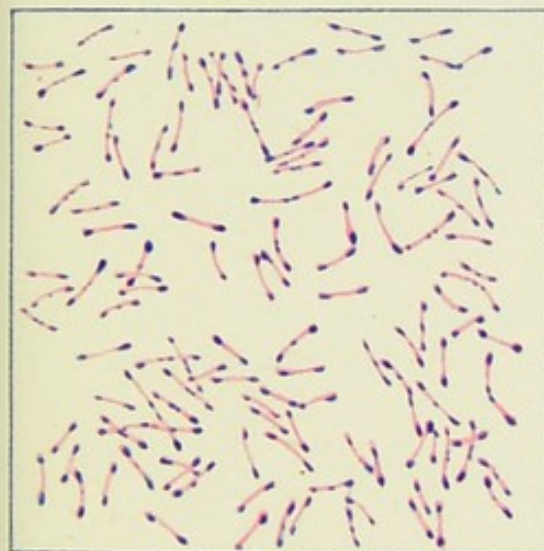


FIG. 12.

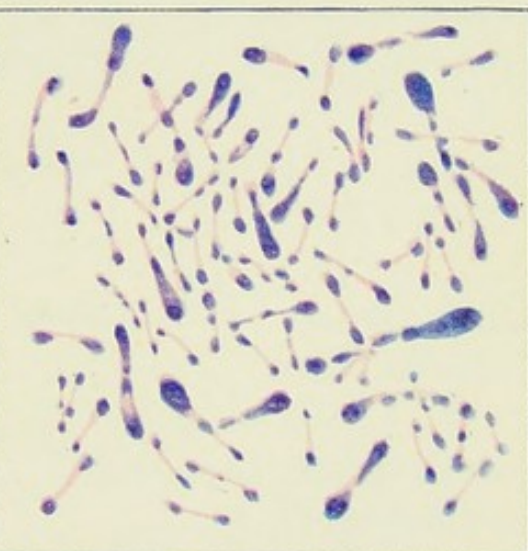


FIG. 13.



FIG. 14.

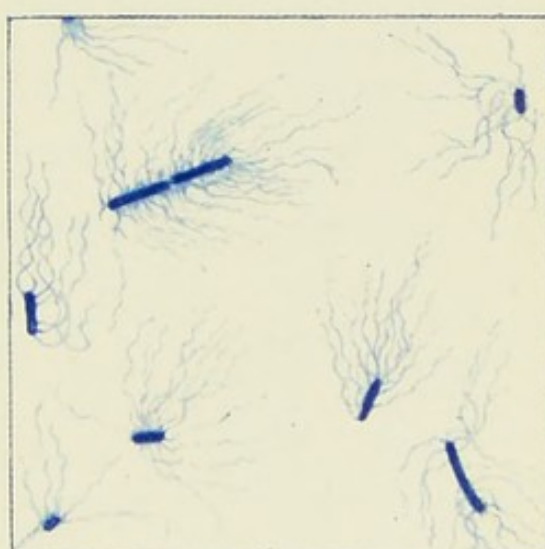


FIG. 15.





PLATE IV.

FIG. 16. Negri bodies in nerve cells in rabies (hippocampus of dog).  
Alcoholic eosin and methylene-blue.  $\times 1000$  diameters.

FIG. 17. *Bacillus pestis*, showing involution forms, from a salt-agar  
culture.  $\times 1000$  diameters.

FIG. 18. Blood film, showing the spirochæte of relapsing fever.  
Leishman's stain.  $\times 1000$  diameters.

FIG. 19. The cholera spirillum, from a 12-hours' agar culture, showing  
flagella.  $\times 1000$  diameters.

FIG. 20. *Bacillus tetani*, showing spores.  $\times 1000$  diameters.

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Fig. 20. *Bacillus tetani*, showing spores.  $\times 1000$  diameters.



PLATE IV.

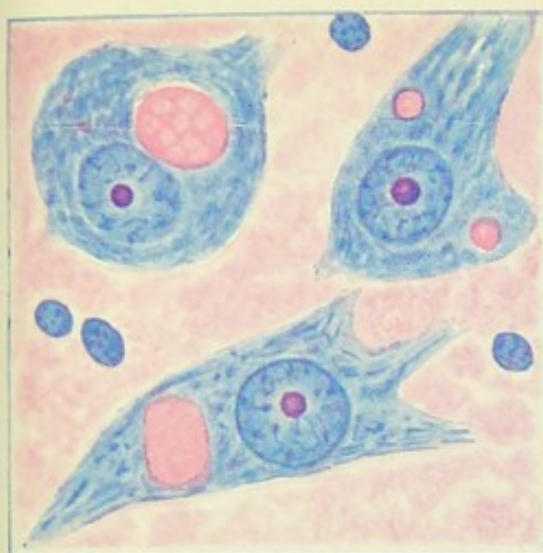


FIG. 16.

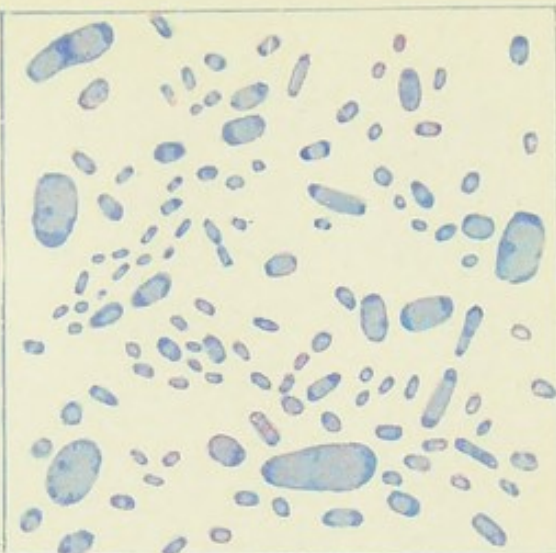


FIG. 17.

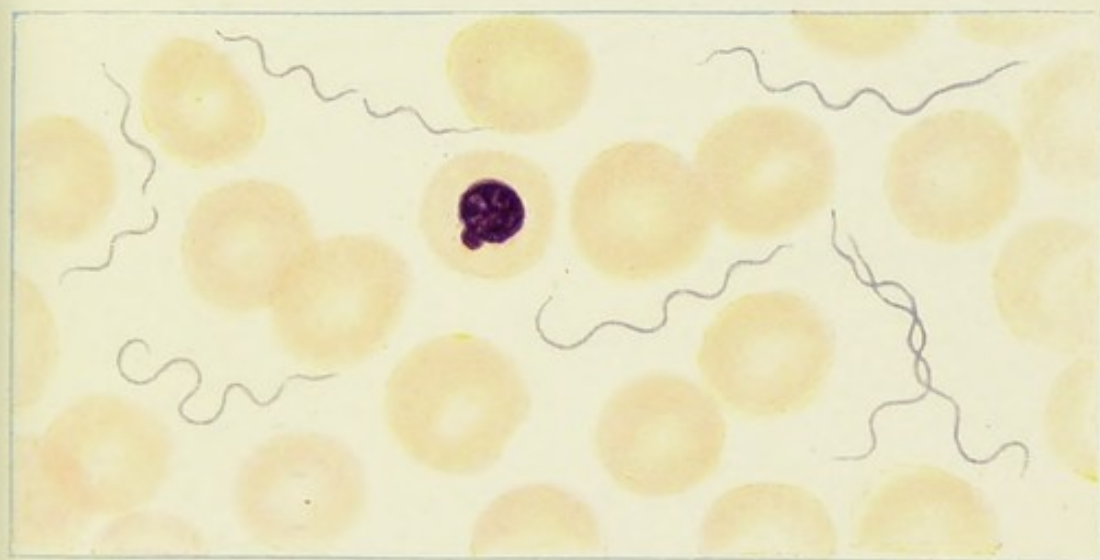


FIG. 18.



FIG. 19.

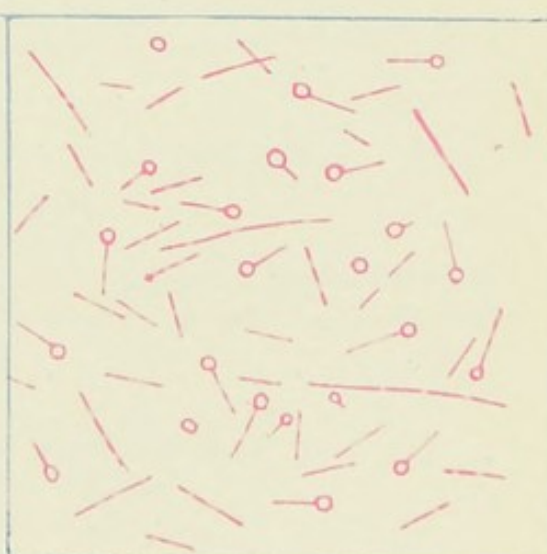


FIG. 20.





## PLATE V.

FIG. 21. THE PARASITE OF MILD TERTIAN MALARIA.

*Cycle I.* (Schizogony). Asexual cycle in the human blood.

- a. Sporozoite entering red blood corpuscle and forming young trophozoite.
- b. Young trophozoite in red blood corpuscle.
- c. Young trophozoite in red blood corpuscle, with accumulation of pigment.
- d. Large pigmented trophozoite.
- e. Mature schizont.
- f. Commencing segmentation of schizont.
- g. Further stage of segmentation.
- h. Segmented schizont ; formation of merozoites.
- i. Disintegration of red blood corpuscle, setting free the merozoites.
- j. Young merozoite entering red blood corpuscle.
- k. Macrogametocyte, or female sporont.
- l. Microgametocyte, or male sporont.

*Cycle II.* (Sporogony). Sexual cycle in the mosquito.

In the cavity of  
the stomach  
of the mosquito.

- |   |    |  |
|---|----|--|
| { | m. | Microgametocyte.   |
| { | n. | Macrogametocyte.   |
| { | o. | Formation of microgametes from the microgametocyte.        |
| { | p. | Free microgamete.  |
| { | q. | Microgamete entering the macrogametocyte.                  |
| { | r. | Zygote or ookinete.  |
|   | s. | Sporocyst.   |
|   | t. | Formation of sporoblasts in the sporocyst.                 |
|   | u. | Formation of sporozoites from sporoblasts.                 |
|   | v. | Rupture of sporocyst, setting free the sporozoites.        |
|   | w. | Free sporozoites in the body fluid.                        |
|   | x. | Accumulation of sporozoites in the salivary gland.         |
|   | y. | Sporozoites passing from gland duct into the blood of man. |

FIG. 22. THE PARASITE OF MALIGNANT MALARIA.

- a. Young trophozoite entering red blood corpuscle.
- b. Do. in red corpuscle.
- c. Multiple infection of red corpuscle.
- c'. Multiple infection with chromatic stippling in cellular protoplasm ; a similar cell is seen lying beneath a,—it contains a pigmented trophozoite.
- d. Pigmented trophozoite.
- e. Segmented schizont, cluster of merozoites.
- f. Macrogametocyte, "female crescent."
- g. Microgametocyte, "male crescent."
- h. Red blood corpuscle with chromatic stippling.
- i. Large mono-nucleated phagocyte containing malarial pigment.

- FIG. 21. THE PARASITE OF MILD TERTIAN MALARIA.
- Cycle I. (Schizogony). Asexual cycle in the human blood.
- Sporozoite entering red blood corpuscle and forming young trophozoite.
  - Young trophozoite in red blood corpuscle.
  - Young trophozoite in red blood corpuscle, with accumulation of pigment.
  - Large pigmented trophozoite.
  - Mature schizont.
  - Commencing segmentation of schizont.
  - Further stage of segmentation.
  - Segmented schizont; formation of merozoites.
  - Disintegration of red blood corpuscle, setting free the merozoites.
  - Young merozoite entering red blood corpuscle.
  - Macrogametocyte, or female sporont.
  - Microgametocyte, or male sporont.

- Cycle II. (Sporogony). Sexual cycle in the mosquito.
- In the mosquito to give rise to the asexual cycle.
- Microgametocyte.
  - Macrogametocyte.
  - Formation of microgametes from the microgametocyte.
  - Free microgamete.
  - Microgamete entering the macrogametocyte.
  - Zygote or ookinete.
  - Sporozoyst.
  - Formation of sporoblasts in the sporozoyst.
  - Formation of sporozoites from sporoblasts.
  - Rupture of sporozoyst, setting free the sporozoites.
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  - Pigmented trophozoite.
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  - Microgametocyte, "male crescent."
  - Red blood corpuscle with chromatic stippling.
  - Large mono-nucleated phagocyte containing malarial pigment.



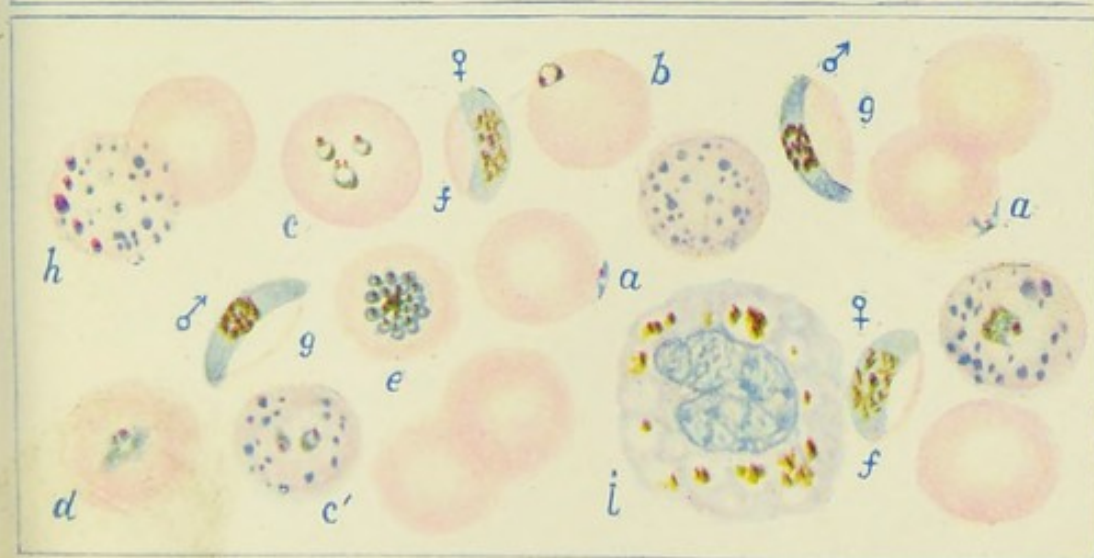
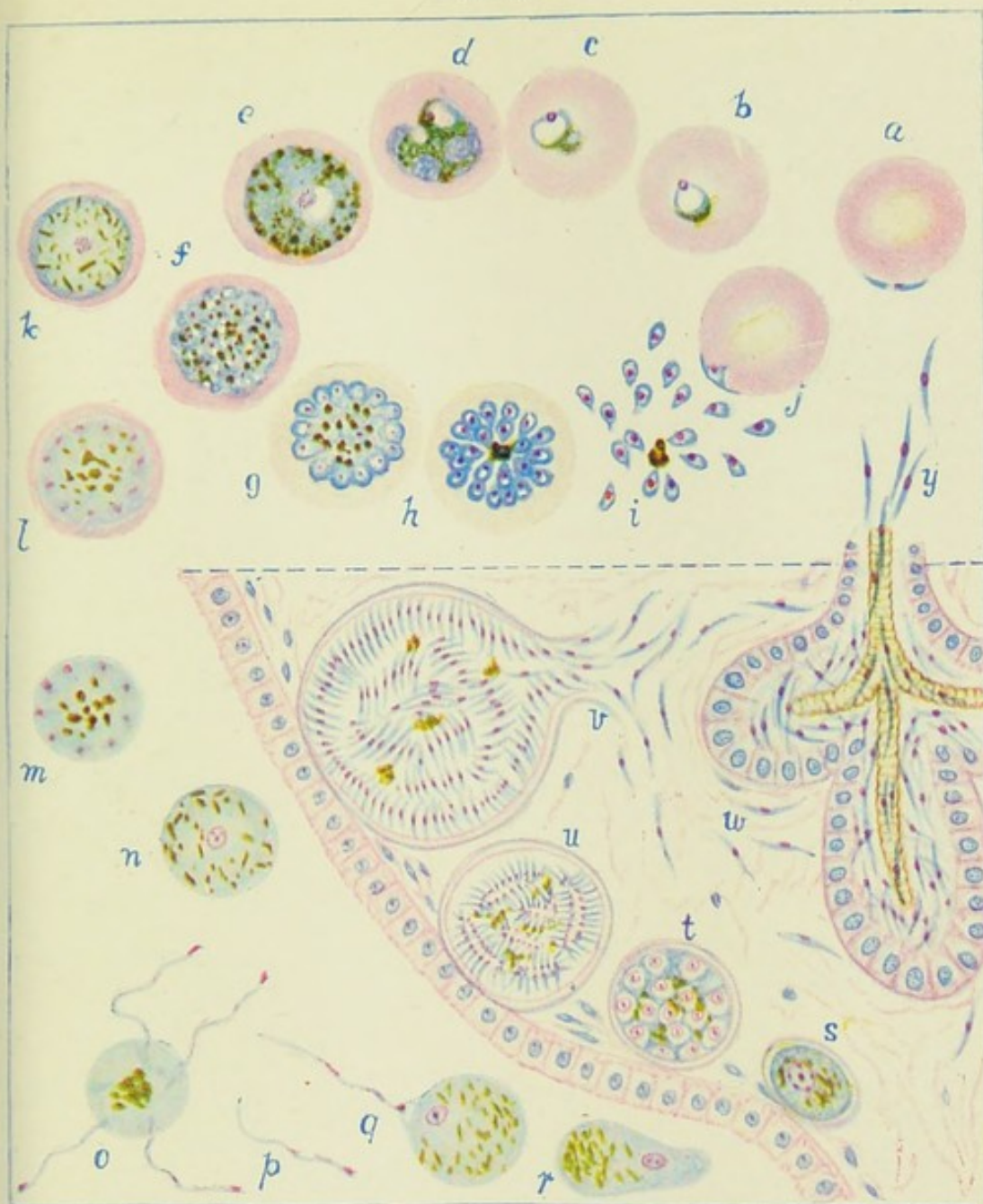






PLATE VI.

FIG. 23. *Entamoeba histolytica* in pus, from tropical abscess of liver.

Wet fixed film. Stained by Benda's method.

× 1000 diameters.

FIG. 24. Leishman-Donovan bodies, from the spleen of a case of

Kála-ázar.

× 1000 diameters.

FIG. 25. Blood film, showing *Trypanosoma Gambiense*. Leishman's stain.

× 1000 diameters.

PLATE VI

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Wet fixed film. Stained by Benda's method.  
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Fig. 25. Blood film, showing *Trypanosoma Gambiense*. Leishman's stain.  
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PLATE VI.



FIG. 23.

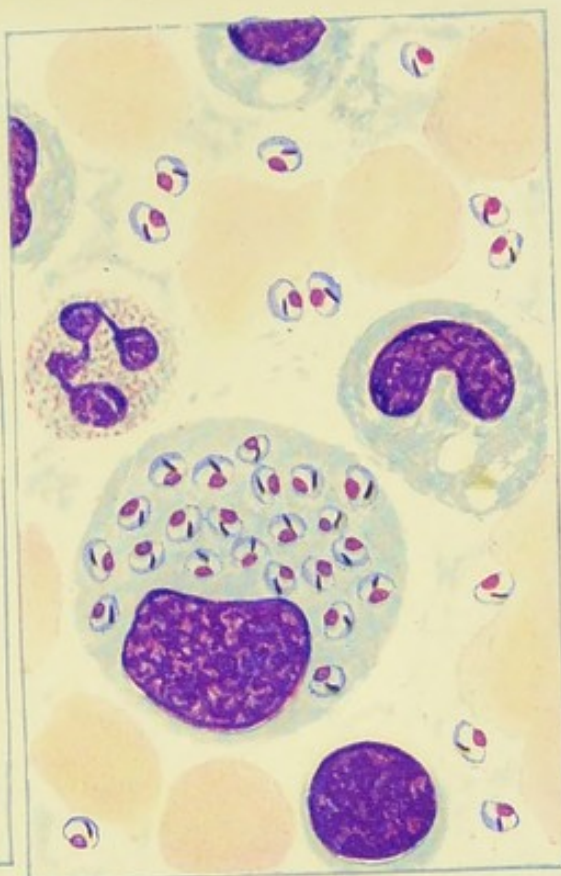


FIG. 24.

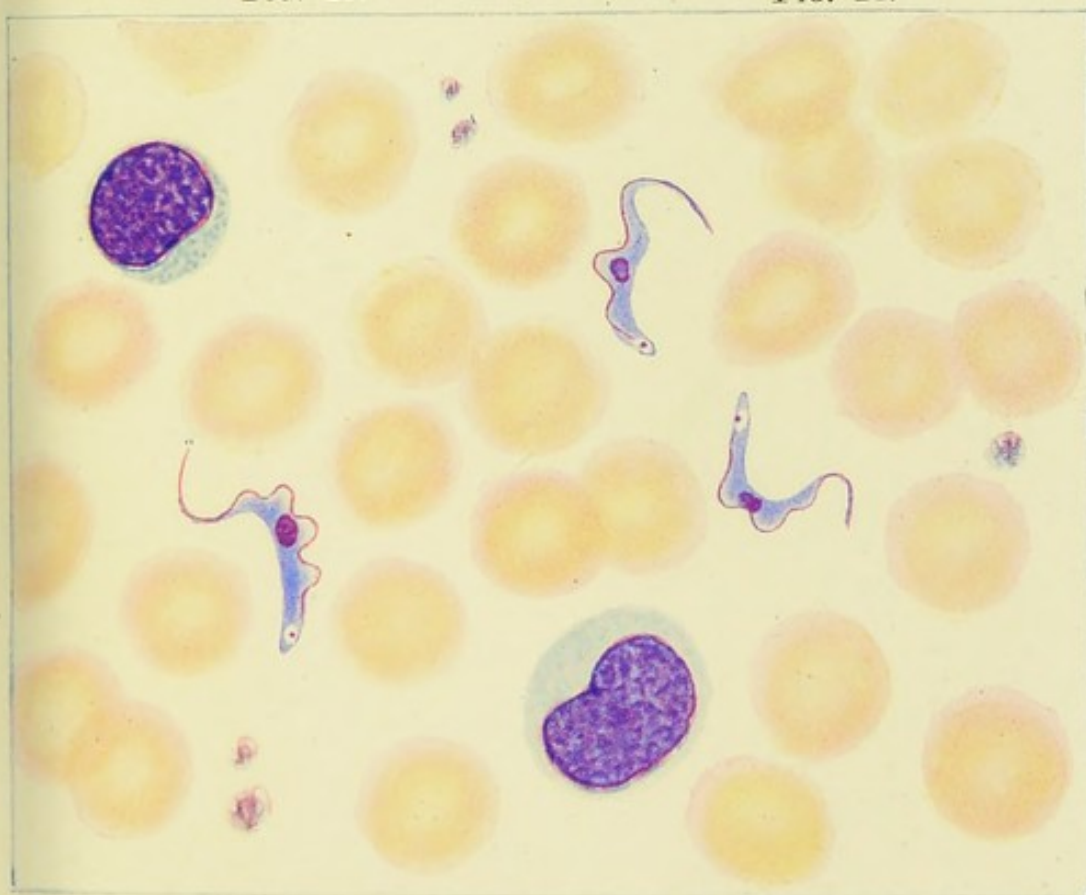
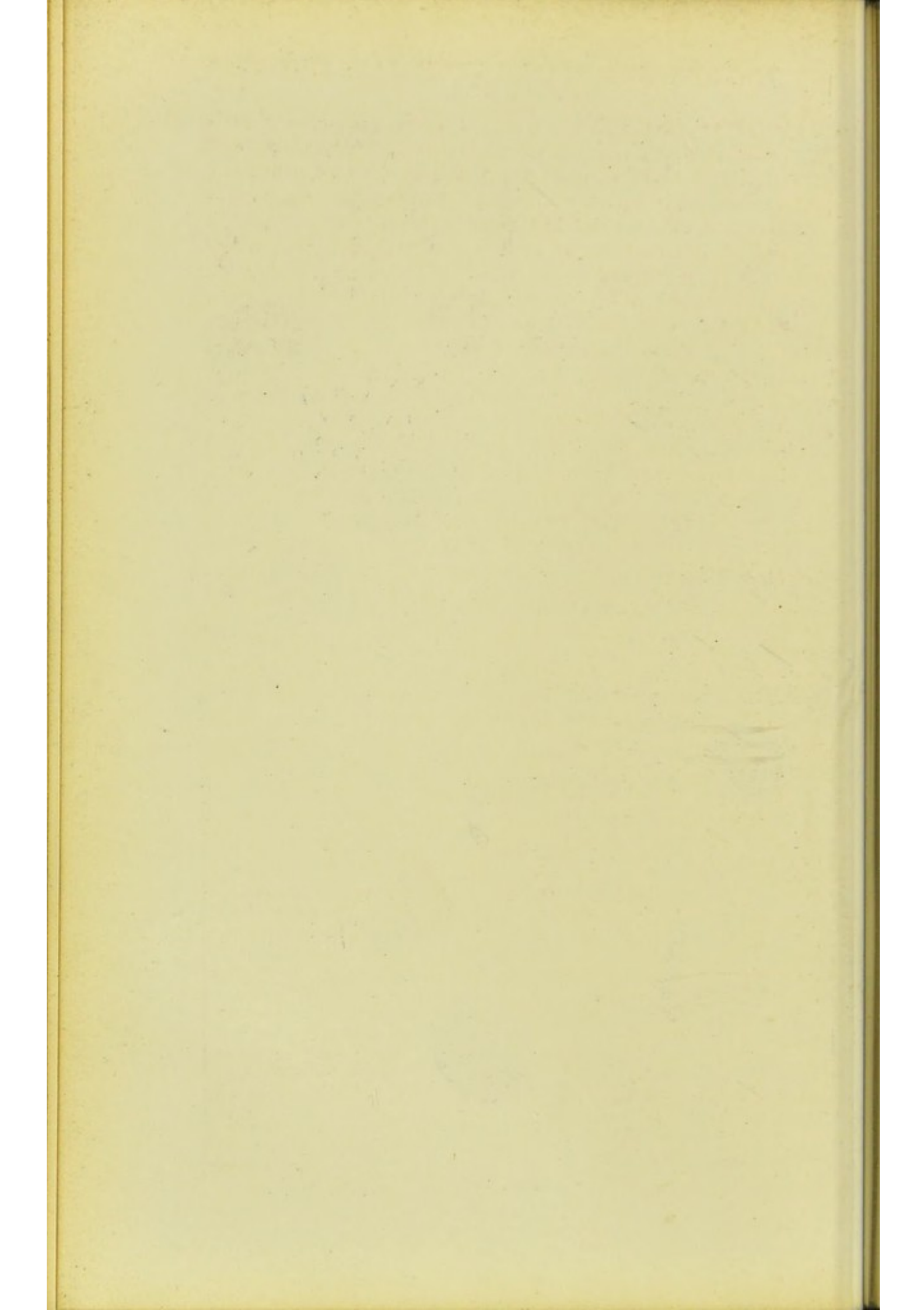


FIG. 25.





# MANUAL OF BACTERIOLOGY

## CHAPTER I.

### GENERAL MORPHOLOGY AND BIOLOGY.

**Introductory.**—At the bottom of the scale of living things there exists a group of organisms to which the name of bacteria is usually applied. These are apparently of very simple structure, and may be subdivided into two sub-groups, a lower and simpler and a higher and better-developed.

The *lower forms* are the more numerous, and consist of minute unicellular masses of protoplasm devoid of chlorophyll, which multiply by simple fission. Some are motile, others non-motile. Their minuteness may be judged of by the fact that in one direction at least they usually do not measure more than  $1\ \mu$  ( $\frac{1}{25000}$  inch). These forms can be classified according to their shapes into three main groups—(1) A group in which the shape is globular. The members of this are called *cocci*. (2) A group in which the shape is that of a straight rod—the proportion of the length to the breadth of the rod varying greatly among the different members. These are called *bacilli*. (3) A group in which the shape is that of a curved or spiral rod. These are called *spirilla*. The full description of the characters of these groups will be more conveniently taken later (p. 12). In some cases, especially among the bacilli, there may occur under certain circumstances changes in the protoplasm whereby a resting stage or spore is formed.

The *higher forms* show advance on the lower along two lines. (1) On the one hand, they consist of filaments made up of simple elements such as occur in the lower forms. These filaments may be more or less septate, may be provided with a



sheath, and may show branching either true or false. The minute structure of the elements comprising these filaments is analogous to that of the lower forms. Their size, however, is often somewhat greater. The lower forms sometimes occur in filaments, but here every member of the filament is independent, while in the higher forms there seems to be a certain interdependence among the individual elements. For instance, growth may occur only at one end of a filament, the other forming an attachment to some fixed object. (2) The higher forms, moreover, present this further development, that in certain cases some of the elements may be set apart for the reproduction of new individuals.

**Terminology.**—The term bacterium of course in strictness only refers to the rod-shaped varieties of the group, but as it has given the name bacteriology to the science which deals with the whole group, it is convenient to apply it to all the members of the latter, and to reserve the term bacillus for the rod-shaped varieties. Other general words, such as germ, microbe, micro-organism, are used as synonymous with bacterium, though these are often made to include the smallest organisms of the animal kingdom.

While no living organisms lower than the bacteria are known (though certain facts regarding ultra-microscopic forms of life make the occurrence of such possible), the upper limits of the group are difficult to define, and it is further impossible in the present state of our knowledge to give other than a provisional classification of the forms which all recognise to be bacteria. The division into lower and higher forms, however, is fairly well marked, and we shall therefore refer to the former as the lower bacteria, and to the latter as the higher bacteria.

**Morphological Relations.**—The relations of the bacteria to the animal kingdom on the one hand and to the vegetable on the other constitute a somewhat difficult question. It is best to think of there being a group of small, unicellular organisms, which may represent the most primitive forms of life before differentiation into animal and vegetable types had occurred. This would include the flagellata and infusoria, the myxomycetes, the lower algæ, and the bacteria. To the lower algæ the bacteria show many similarities. These algæ are unicellular masses of protoplasm, having generally the same shapes as the bacteria, and largely multiply by fission. Endogenous sporulation, however, does not occur, nor is motility necessarily associated with the possession of flagella. Also their protoplasm differs from that of the bacteria in containing chlorophyll and another blue-green pigment called phycocyan. From the morphological resemblances, however, between these algæ and the bacteria, and from the fact that fission plays a predominant part in the multiplication of both, they have been grouped together in one class as the Schizophyta



or splitting plants (German, Spaltpflanzen). And of the two divisions forming these Schizophyta the splitting algæ are denominated the schizophyceæ (German, Spaltalgen), while the bacteria or splitting fungi are called the schizomycetes (German, Spaltpilzen). The bacteria are, therefore, often spoken of as the schizomycetes. Certain bacteria which have been described as containing chlorophyll ought probably to be grouped among the schizophyceæ.

#### GENERAL MORPHOLOGY OF THE BACTERIA.

**The Structure of the Bacterial Cell.**—On account of the minuteness of bacteria the investigation of their structure is attended with great difficulty. When examined under the microscope, in their natural condition, *e.g.* in water, they appear merely as colourless refractile bodies of the different shapes named. Spore formation and motility, when these exist, can also be observed, but little else can be made out. For their proper investigation advantage is always taken of the fact of their affinities for various dyes, especially those which are usually chosen as good stains for the nuclei of animal cells. Certain points have thus been determined. The bacterial cell consists of a sharply contoured mass of protoplasm which reacts to, especially basic, aniline dyes like the nucleus of an animal cell—though from this fact we cannot deduce that the two are identical in composition. A healthy bacterium when thus stained presents the appearance of a finely granular or almost homogeneous structure. The protoplasm is surrounded by an envelope which can in some cases be demonstrated by overstaining a specimen with a strong aniline dye, when it will appear as a halo round the bacterium. This envelope may sometimes be seen to be of considerable thickness. Its innermost layer is probably of a denser consistence, and sharply contours the contained protoplasm, giving the latter the appearance of being surrounded by a membrane. It is only, however, in some of the higher forms that a definite membrane occurs. Sometimes the outer margin of the envelope is sharply defined, in which case the bacterium appears to have a distinct capsule, and is known as a capsulated bacterium (*vide* Fig. 1, No. 4; and Fig. 62). The cohesion of bacteria into masses depends largely on the character of the envelope. If the latter is glutinous, then a large mass of the same species may occur, formed of individual bacteria embedded in what appears to be a mass of jelly. When this occurs, it is known as a *zooglæa* mass. On the other hand, if the envelope has not this cohesive property the separation of individuals may easily take place, especially in a fluid medium



in which they may float entirely free from one another. Many of the higher bacteria possess a sheath which has a much more definite structure than is found among the lower forms. It resists external influences, possesses elasticity, and serves to bind the elements of the organism together.

**Reproduction among the Lower Bacteria.**—When a bacterial cell is placed in favourable surroundings, it multiplies; as has been said, this, in the great majority of cases, takes place by simple fission. In the process a constriction appears in the middle and a transverse unstained line develops across the protoplasm at that point. The process goes on till two individuals can be recognised, which may remain for a time attached to one another, or become separate, according to the character of the envelope, as already explained. In most bacteria growth and multiplication go on with great rapidity. A bacterium may reach maturity and divide in from twenty minutes to half an hour. If division takes place only every hour, from one individual after twenty-four hours 17,000,000 similar individuals will be produced. As shown by the results of artificial cultivation, others, such as the tubercle bacillus, multiply much more slowly. Sometimes division proceeds so rapidly that the young individuals do not reach the adult size before multiplication again occurs. This may give rise to anomalous appearances.

From investigations by Graham-Smith and others, it appears that the consistence of the envelope may have an importance in modifying the naked-eye and low-power appearances presented by bacterial colonies which constitute a feature in the identification of species (see p. 137). Graham-Smith, working with bacilli, differentiates four groups—a “loop-forming,” in which the envelope is so tough that, after division, rupture but rarely occurs (*b. anthracis*); a “folding” group, in which the envelope is so flexible and extensile that the members of a chain can be folded on one another as successive divisions take place (*b. pestis*); a “snapping” group, in which partial rupture of the envelope occurs on division (*b. diphtheriæ*); and a “slipping” group, where the envelope readily breaks, and successively developed bacilli slip past each other (*v. cholerae*).

When bacteria are placed in unfavourable conditions as regards food, etc., growth and multiplication take place with difficulty. In the great majority of cases this is evidenced by changes in the appearance of the protoplasm. Instead of its maintaining the regularity of shape seen in healthy bacteria, various aberrant appearances are presented. This occurs especially in the rod-shaped varieties, where flask-shaped or dumb-bell-shaped individuals may be seen. The regularity in structure



and size is quite lost. The appearance of the protoplasm also is often altered. Instead of, as formerly, staining well, it does not stain readily, and may have a uniformly pale homogeneous appearance, while in an old culture only a small proportion of the bacteria may stain at all. Sometimes, on the other hand, a degenerated bacterium contains intensely stained granules or globules which may be of large size. Such aberrant and degenerate appearances are referred to as *involution forms*. That these forms really betoken degenerative changes is shown by the fact that, on their being again transferred to favourable conditions, only slight growth at first takes place. Many individuals have undoubtedly died, and the remainder which live and develop into typical forms may sometimes have lost some of their properties.

**Reproduction among the Higher Bacteria.**—Most of the higher bacteria consist of thread-like structures more or less septate and often surrounded by a sheath. The organism is frequently attached at one end to some object or to another individual. It grows to a certain length and then at the free end certain cells, called gonidia, are cast off from which new individuals are formed. These gonidia may be formed by a division taking place in the terminal element of the filament such as has occurred in the growth of the latter. In some cases, however, division takes place in three dimensions of space. The gonidia have a free existence for a certain time before becoming attached, and in this stage are sometimes motile. They are usually rod-like in shape, sometimes pyriform. They do not possess any special powers of resistance.

**Spore Formation.**—In certain species of the lower bacteria, under certain circumstances, changes take place in the protoplasm which result in the formation of bodies called spores, to which the vital activities of the original bacteria are transferred. Spore formation occurs chiefly among the bacilli and in some spirilla. Its commencement in a bacterium is indicated by the appearance in the protoplasm of a minute highly refractile granule unstained by the ordinary methods. This increases in size, and assumes a round, oval, or short rod-shaped form, always shorter but often broader than the original bacterium. In the process of spore formation the rest of the bacterial protoplasm may remain unchanged in appearance and staining power for a considerable time (*e.g.* *b. tetani*), or, on the other hand, it may soon lose its power of staining and ultimately disappear, leaving the spore in the remains of the envelope (*e.g.* *b. anthracis*). This method of spore formation is called *endogenous*. Bacterial spores are always non-motile. The spore may appear in the centre of the bacterium, or it may be at one extremity, or a short distance from one extremity (Fig. 1, No. 11). In structure



the spore consists of a mass of protoplasm surrounded by a dense membrane. This can be demonstrated by methods which will be described, the underlying principle of which is the prolonged application of a powerful stain. The membrane is supposed to confer on the spore its characteristic feature, namely, great capacity of resistance to external influences such as heat or noxious chemicals. Koch, for instance, in one series of experiments, found that while the bacillus anthracis in the unspored form was killed by a two minutes' exposure to 1 per cent carbolic acid, spores of the same organism resisted an exposure of from one to fifteen days.

When a spore is placed in suitable surroundings for growth, it again assumes the original bacillary or spiral form. The capsule dehisces either longitudinally, or terminally, or transversely. In the last case the dehiscence may be partial, and the new individual may remain for a time attached by its ends to the hinged spore-case, or the dehiscence may be complete and the bacillus grow with a cap at each end consisting of half the spore-case. Sometimes the spore-case does not dehisce, but is simply absorbed by the developing bacterium.

It is important to note that in the bacteria spore formation is rarely, if ever, to be considered as a method of multiplication. In at least the great majority of cases only one spore is formed from one bacterium, and only one bacterium in the first instance from one spore. Sporulation is to be looked upon as a *resting stage* of a bacterium, and is to be contrasted with the stage when active multiplication takes place. The latter is usually referred to as the *vegetative stage* of the bacterium. Regarding the signification of spore formation in bacteria, there has been some difference of opinion. According to one view, it may be regarded as representing the highest stage in the vital activity of a bacterium. There is thus an alternation between the vegetative and spore stage, the occurrence of the latter being necessary to the maintenance of the species in its greatest vitality. Such a rejuvenescence, as it were, through sporulation, is known in many algæ. In support of this view there are certain facts. In many cases, for instance, spore formation only occurs at temperatures specially favourable for growth and multiplication. There is often a temperature below which, while vegetative growth still takes place, sporulation will not occur; and in the case of *b. anthracis*, if the organism be kept at a temperature above the limit at which it grows best, not only are no spores formed, but the strain may lose the power of sporulation. Furthermore, in the case of bacteria preferring



the presence of oxygen for their growth, an abundant supply of this gas may favour sporulation. It is probable that even among bacteria preferring the absence of oxygen for vegetative growth, the presence of this gas favours sporulation. Some facts relating to the cases in which two spores are formed in one bacterium have been adduced to support the view that sporulation may represent a degenerate sexual process. Here a partial fission of a cell has been observed followed by a re-fusion of the protoplasmic moieties and the formation of a spore at each end of the rod. The second view with regard to sporulation is that a bacterium only forms a spore when its surroundings, especially its food supply, become unfavourable for vegetative growth; it then remains in this condition until it is placed in more suitable surroundings. Such an occurrence would be analogous to what takes place under similar conditions in many of the protozoa. Often sporulation can be prevented from taking place for an indefinite time if a bacterium is constantly supplied with fresh food (the other conditions of life being equal). The presence of substances excreted by the bacteria themselves plays, however, a more important part in making the surroundings unfavourable than the mere exhaustion of the food supply. A living spore will always develop into a vegetative form if placed in a fresh food supply. With regard to the rapid formation of spores when the conditions are favourable for vegetative growth, it must be borne in mind that in such circumstances the conditions may really very quickly become unfavourable for a continuance of growth, since not only will the food supply around the growing bacteria be rapidly exhausted, but the excretion of effete and inimical matters will be all the more rapid.

We must note that the usually applied tests of a body developed within a bacterium being a spore are (1) its staining reaction, namely, resistance to ordinary staining fluids, but capacity of being stained by the special methods devised for the purpose (*vide* p. 109); (2) the fact that the bacterium containing the spore has higher powers of resistance against inimical conditions than a vegetative form. It is important to bear these tests in mind, as, in some of the smaller bacteria especially, it is very difficult to say whether they spore or not. There may appear in such organisms small unstained spots, the significance of which it is very difficult to determine.

**The Question of Arthrosporous Bacteria.**—It is stated by Hueppe that among certain organisms, *e.g.* some streptococci, certain individuals may, without endogenous sporulation, take on a resting stage. These become



swollen, stain well with ordinary stains, and they are stated to have higher power of resistance than the other forms; further, when vegetative life again occurs, it is from them that multiplication is said to take place. From the fact that there is no new formation within the protoplasm, but that it is the whole of the latter which participates in the change, these individuals have been called *arthrospores*. The existence of such special individuals amongst the lower bacteria is extremely problematical. They have no distinct capsule, and they present no special staining reactions, nor any microscopic features by which they can be certainly recognised, while their alleged increased powers of resistance are very doubtful. All the phenomena noted can be explained by the undoubted fact that in an ordinary growth there is very great variation among the individual organisms in their powers of resistance to external conditions.

**Motility.**—As has been stated, many bacteria are motile. Motility can be studied by means of hanging-drop preparations (*vide* p. 69). The movements are of a darting, rolling, or vibratile character. The degree of motility depends on the species, the temperature, the age of the growth, and on the medium in which the bacteria are growing. Sometimes the movements are most active just after the cell has multiplied, sometimes it goes on all through the life of the bacterium, sometimes it ceases when sporulation is about to occur. Motility is associated with the possession of fine wavy thread-like appendages called flagella, which for their demonstration require the application of special staining methods (*vide* Fig. 1, No. 12; and Fig. 108). They have been shown to occur in many bacilli and spirilla, but only in a few species of cocci. They vary in length, but may be several times the length of the bacterium, and may be at one or both extremities or all round. When terminal they may occur singly or there may be several. The nature of these flagella has been much disputed. Some have held that, unlike what occurs in many algæ, they are not actual prolongations of the bacterial protoplasm, but merely appendages of the envelope, and have doubted whether they are really organs of locomotion. There is now, however, little doubt that they belong to the protoplasm. By appropriate means the central parts of the latter can be made to shrink away from the peripheral (*vide infra*, "plasmolysis"). In such a case movement goes on as before, and in stained preparations the flagella can be seen to be attached to the peripheral zone. It is to be noted that flagella have never been demonstrated in non-motile bacteria, while, on the other hand, they have been observed in nearly all motile forms. There is little doubt, however, that all cases of motility among the bacteria are not dependent on the possession of flagella, for in some of the special spiral forms, and in most



of the higher bacteria, motility is probably due to contractility of the protoplasm itself.

**The Minuter Structure of the Bacterial Protoplasm.**—Many attempts have been made to obtain deeper information as to the structure of the bacterial cell, and especially as to its behaviour in division. These have largely turned on the interpretation to be put on certain appearances which have been observed. These appearances are of two kinds. First, under certain circumstances irregular deeply-stained granules are observed in the protoplasm, often, when they occur in a bacillus, giving the latter the appearance of a short chain of cocci. They are often called metachromatic granules (*vide* Fig. 1, No. 16) from the fact that by appropriate procedure they can be stained with one dye, and the protoplasm in which they lie with another; sometimes, when a single stain is used, such as methylene blue, they assume a slightly different tint from the protoplasm.

For the demonstration of the metachromatic granules two methods have been advanced. Ernst recommends that a few drops of Löffler's methylene blue (*vide* p. 104) be placed on a cover-glass preparation and the latter passed backwards and forwards over a Bunsen flame for half a minute after steam begins to rise. The preparation is then washed in water and counter-stained for one to two minutes in watery Bismarck-brown. The granules are here stained blue, the protoplasm brown. Neisser stains a similar preparation in warm carbol-fuchsin, washes with 1 per cent. sulphuric acid, and counter-stains with Löffler's blue. Here the granules are magenta, the protoplasm blue. The general character of the granules thus is that they retain the first stain more intensely than the rest of the protoplasm does.

A second appearance which can sometimes be seen in specimens stained in ordinary ways is the occurrence of a concentration of the protoplasm at each end of a bacterium, indicated by these parts being deeply stained. These deeply stained parts are sometimes called polar granules (*vide* Fig. 1, No. 16, the bacillus most to the right) (German, Polkörnerchen or Polkörner).

With regard to the significance that is to be attached to such appearances, much depends on whether they are constantly present under all circumstances, or only occasionally, when the organism is grown in special media or under special growth conditions. Some bacteria, however stained, show evidence of having the protoplasm somewhat granular, *e.g.* the diphtheria bacillus. In other cases this granular condition is only seen when the organism has been grown under bad conditions, or where the food supply is becoming exhausted. Some have thought that the appearances might be due to a process allied to mitosis and might signify approaching division, but of this there is no evidence.

In perfect healthy and young bacteria, appearances of granule formation and of vacuolation may be accidentally produced by physical means in the occurrence of what is known as *plasmolysis*. To speak generally, when a mass of protoplasm surrounded by a fairly firm envelope of a colloidal nature is placed in a solution containing salts in greater concentration than that in which it has previously been living, then by a process of osmosis the water held in the protoplasm passes out through the membrane, and, the protoplasm retracting from the latter, the appearance of vacuolation is presented. Now, in making a



dried film for the microscopic examination of bacteria, the conditions necessary for the occurrence of this process may be produced, and the appearances of vacuolation and, in certain cases, of Polkörner may thus be brought about. Plasmolysis in bacteria has been extensively investigated,<sup>1</sup> and has been found to occur in some species more readily than in others. Furthermore, it is often most readily observed in old or otherwise enfeebled cultures.

Bütschli, from a study of some large sulphur-containing forms, concludes that the greater part of the bacterial cell may correspond to a nucleus, and that this is surrounded by a thin layer of protoplasm which in the smaller bacteria escapes notice, unless when, as in the bacilli, it can be made out at the ends of the cells. Fischer, it may be said, looks on the appearances seen in Bütschli's preparations as due to plasmolysis.

**The Chemical Composition of Bacteria.**—In the bodies of bacteria many definite substances occur. Some bacteria have been described as containing chlorophyll, but these are properly to be classed with the schizophyceæ. Sulphur is found in some of the higher forms, and starch granules are also described as occurring. Many species of bacteria, when growing in masses, are brilliantly coloured, though few bacteria associated with the production of disease give rise to pigments. In some of the organisms classed as bacteria a pigment named bacterio-purpurin has been observed in the protoplasm, and similar intracellular pigments probably occur in some of the larger forms of the lower bacteria and may occur in the smaller; but it is usually impossible to determine whether the pigment occurs inside or outside the protoplasm. In many cases, for the free production of pigment abundant oxygen supply is necessary; but sometimes, as in the case of *spirillum rubrum*, the pigment is best formed in the absence of oxygen. Sometimes the faculty of forming it may be lost by an organism for a time, if not permanently, by the conditions of its growth being altered. Thus, for example, if the *b. pyocyaneus* be exposed to the temperature of 42° C. for a certain time, it loses its power of producing its bluish pigment. Pigments formed by bacteria often diffuse out into, and colour, the medium for a considerable distance around.

Comparatively little is known of the nature of bacterial pigments. Zopf, however, has found that many of them belong to a group of colouring matters which occur widely in the vegetable and animal kingdoms, namely, the lipochromes. These lipochromes, which get their name from the colouring matter of animal fat, include the colouring matter in the petals of Ranunculaceæ, the yellow pigments of serum and of the yolks of eggs, and many bacterial pigments. The lipochromes are characterised by their solubility in chloroform, alcohol, ether, and

<sup>1</sup> Consult Fischer, "Untersuchungen über Bakterien," Berlin, 1894; "Ueber den Bau der Cyanophyceen und Bakterien," Jena, 1897.



petroleum, and by their giving indigo-blue crystals with strong sulphuric acid, and a green colour with iodine dissolved in potassium iodide. Though crystalline compounds of these have been obtained, their chemical constitution is entirely unknown, and even their percentage composition is disputed.

Some observations have been made on the chemical structure of bacterial protoplasm. Nencki isolated from the bodies of certain putrefactive bacteria proteid bodies which, according to Ruppel, appear to have been allied to peptone, and which differed from nucleo-proteids in not containing phosphorus, but many of the proteids isolated by other chemists have been allied in their nature to the protoplasm of the nuclei of cells. Buchner in certain researches obtained bodies of this nature allied to the vegetable caseins, and he adduces evidence to show that it is to these that the characteristic staining properties are due. Various observers have isolated similar phosphorus-containing proteids from different bacteria. Besides proteids, however, substances of a different nature have been isolated. Thus cellulose, fatty material, chitin, wax-like bodies, and other substances have been observed. There are also found various mineral salts, especially those of sodium, potassium, and magnesium. The amount of different constituents varies according to the age of the culture and the medium used for growth, and certainly great variation takes place in the composition of different species.

**The Classification of Bacteria.**—There have been numerous schemes set forth for the classification of bacteria, the fundamental principle running through all of which has been the recognition of the two sub-groups and the type forms mentioned in the opening paragraph above. In the attempts to still further subdivide the group, scarcely two systematists are agreed as to the characters on which sub-classes are to be based. Our present knowledge of the essential morphology and relations of bacteria is as yet too limited for a really natural classification to be attempted. To prepare for the elaboration of the latter, Marshall Ward suggested that in every species there should be studied the habitat, best food supply, condition as to gaseous environment, range of growth temperature, morphology, life history, special properties, and pathogenicity. Some recent attempts to carry out such a plan will be referred to in connection with the principles of general bacteriological diagnosis (p. 135).

We must thus be content with a provisional and incomplete classification. We have said that the division into lower and



higher bacteria is recognised by all, though, as in every other classification, transitional forms have to be accounted for. In subdividing the bacteria further, the forms they assume constitute at present the only practicable basis of classification. The lower bacteria thus naturally fall into the three groups mentioned, the cocci, bacilli, and spirilla, though the higher are more difficult to deal with. Subsidiary, though important, points in still further subdivision are the planes in which fission takes place and the presence or absence of spores. The recognition of actual species is often a matter of great difficulty. The points to be observed in this will be discussed later (p. 137).

**I. The Lower Bacteria.**<sup>1</sup>—These, as we have seen, are minute unicellular masses of protoplasm surrounded by an envelope, the total vital capacities of a species being represented in every cell. They present three distinct type forms, the coccus, the bacillus, and the spirillum; endogenous sporulation may occur. They may also be motile.

1. *The Cocci.*—In this group the cells range in different species from  $\cdot 5 \mu$  to  $2 \mu$  in diameter, but most measure about  $1 \mu$ . Before division they may increase in size in all directions. The species are usually classified according to the method of division. If the cells divide only in one axis, and through the consistency of their envelopes remain attached, then a chain of cocci will be formed. A species in which this occurs is known as a *streptococcus*. If division takes place irregularly, the resultant mass may be compared to a bunch of grapes, and the species is often called a *staphylococcus*. Division may take place in two axes at right angles to one another, in which case cocci adherent to each other in packets of four (called *tetrads*) or sixteen may be found, the former number being the more frequent. To all these forms the word *micrococcus* is often generally applied. The individuals in a growth of micrococci often show a tendency to remain united in twos. These are spoken of as *diplococci*, but this is not a distinctive character, since every coccus as a result of division becomes a diplococcus, though in some species the tendency to remain in pairs is well marked. The adhesion of cocci to one another depends on the character of the capsule. Often this has a well-marked outer limit (*micrococcus tetragenus*), sometimes it is of great extent, its diameter being many times that of the coccus (*streptococcus mesenteriodes*). It is especially among the streptococci and staphylococci that the phenomenon of the formation of arthrospores is said to occur. In none of

<sup>1</sup> For the illustration of this and the succeeding systematic paragraphs, vide Fig. 1.



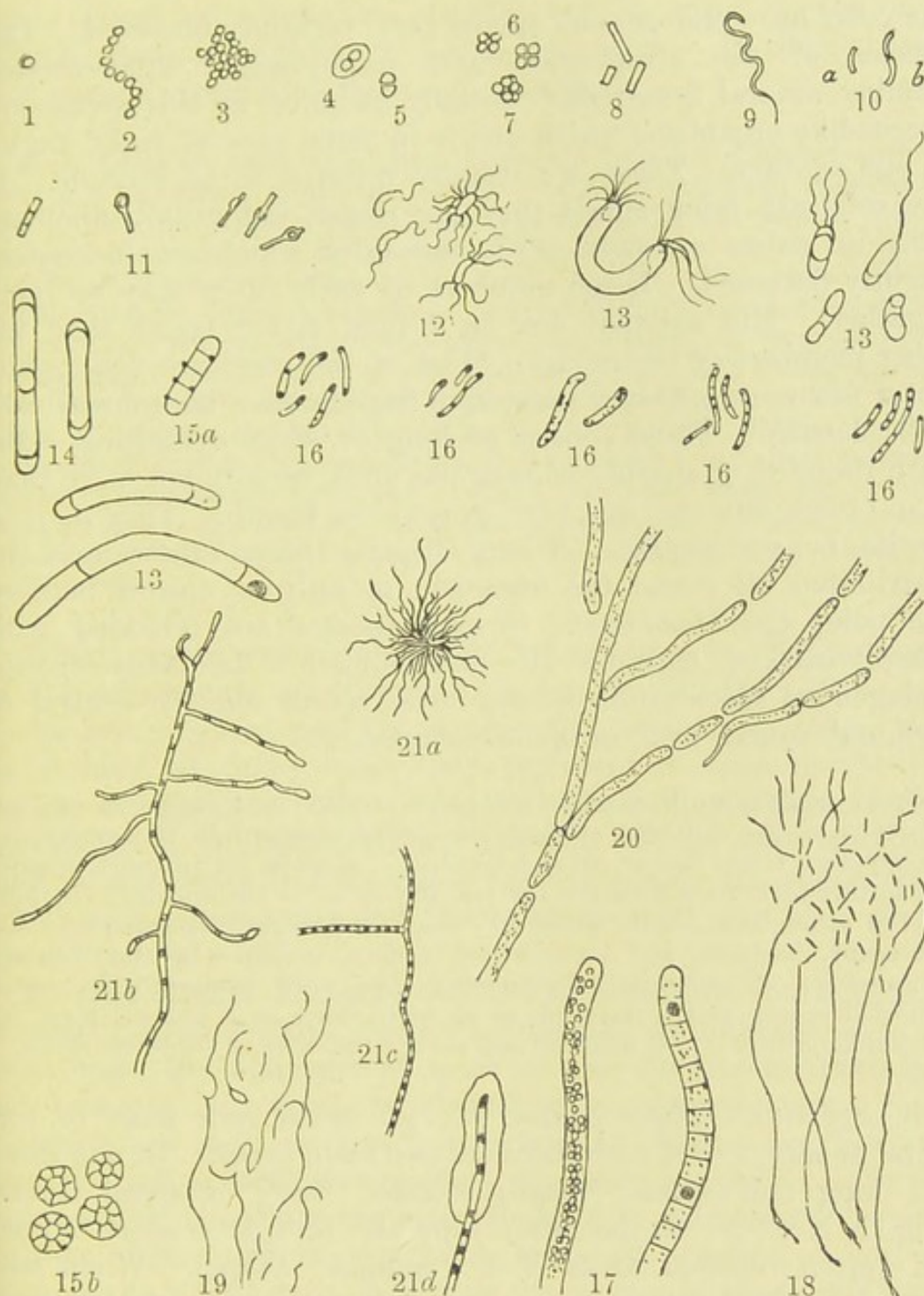


FIG. 1.—1. Coccus. 2. Streptococcus. 3. Staphylococcus. 4. Capsulated diplococcus. 5. "Biscuit"-shaped coccus. 6. Tetrads. 7. Sarcina form. 8. Types of bacilli (1-8 are diagrammatic). 9. Non-septate spirillum  $\times 1000$ . 10. Ordinary spirillum—(a) comma-shaped element; (b) formation of spiral by comma-shaped elements  $\times 1000$ . 11. Types of spore formation. 12. Flagellated bacteria. 13. Changes in bacteria produced by plasmolysis (after Fischer). 14. Bacilli with terminal protoplasm (Bütschli). 15. (a) Bacillus composed of five protoplasmic meshes; (b) protoplasmic network in micrococcus (Bütschli). 16. Bacteria containing metachromatic granules (Ernst, Neisser)—some contain polar granules. 17. *Beggiatoa alba*. Both filaments contain sulphur granules—one is septate. 18. *Thiothrix tenuis* (Wino-gradski). 19. *Leptothrix innominata* (Miller). 20. *Cladothrix dichotoma* (Zopf). 21. *Streptothrix actinomyces* (Boström), (a) colony under low power; (b) filament showing true branching; (c) filament containing coccus-like bodies; (d) filament with club at end.



the cocci have endogenous spores been certainly observed. The species of the streptococci and staphylococci differentiated number several hundreds. Usually included in this group are coccus-like organisms which divide in three axes at right angles to one another. These are usually referred to as *sarcinae*. If the cells are lying single they are round, but usually they are seen in cubes of eight with the sides which are in contact slightly flattened. Large numbers of such cubes may be lying together. The *sarcinae* are, as a rule, rather larger than the other members of the group. Most of the cocci are non-motile, but a few motile species possessing flagella have been described.

2. *Bacilli*.—These consist of long or short cylindrical cells, with rounded or sharply rectangular ends, usually not more than  $1\ \mu$  broad, but varying very greatly in length. They may be motile or non-motile. Where flagella occur, these may be distributed all round the organism, or only at one or both of the poles (*pseudomonas*). Several species are provided with sharply-marked capsules (b. *pneumoniae*). In many species endogenous sporulation occurs. The spores may be central or terminal, round, oval, or spindle-shaped.

Great confusion in nomenclature has arisen in this group in consequence of the different artificial meanings assigned to the essentially synonymous terms bacterium and bacillus. Migula, for instance, applies the former term to non-motile species, the latter to the motile. Hueppe, on the other hand, calls those in which endogenous sporulation does not occur, bacteria, and those where it does, bacilli. In the ordinary terminology of systematic bacteriology the word bacterium has been almost dropped, and is reserved, as we have done, as a general term for the whole group. It is usual to call all the rod-shaped varieties bacilli.

3. *Spirilla*.—These consist of cylindrical cells more or less spiral or wavy. Of such there are two main types. In one there is a long non-septate, usually slender, wavy or spiral thread (Fig. 1, No. 9). In the other type the unit is a short curved rod (often referred to as of a "comma" shape). When two or more of the latter occur, as they often do, end to end with their curves alternating, then a wavy or spiral thread results. An example of this is the cholera microbe (Fig. 1, No. 10). This latter type is of much more frequent occurrence. Among the first group motility is often not associated, as far as is known, with the possession of flagella. The cells here apparently move by an undulating or screw-like contraction of the protoplasm. Most of the motile spirilla, however, possess flagella. Of the latter there may be one or two, or a bunch containing as many as twenty, at one or both poles. Division



takes place as among the bacilli, but in some of the non-septate forms a longitudinal fission may occur. In some species endogenous sporulation has been observed.

Three terms are used in dividing this group, to which different authors have given different meanings. These terms are spirillum, spirochæte, vibrio. Migula makes "vibrio" synonymous with "microspira," which he applies to members of the group which possess only one or two polar flagella; "spirillum" he applies to similar species which have bunches of polar flagella, while "spirochæte" is reserved for the long unflagellated spiral cells. Hueppe applies the term "spirochæte" to forms without endospores, "vibrio" to those with endospores in which during sporulation the organism changes its form, and "spirillum" to the latter when no change of form takes place in sporulation. Flugge, another systematist, applies "spirochæte" and "spirillum" indiscriminately to any wavy or corkscrew form, and "vibrio" to forms where the undulations are not so well marked. It is thus necessary, in denominating such a bacterium by a specific name, to give the authority from whom the name is taken.

Within recent years great doubt has arisen as to whether many of the non-septate spirillary forms, *e.g.* *Spirochæte pallida*, are to be looked on as bacteria at all,—the view being taken that in, it may be, many cases they represent a stage in the life history of what are really protozoa. The ultimate classification of this group of bacteria must thus be left an open question, and at present it is convenient to denominate the non-septate spiral rods *Spirochæte*, and those whose vital unit is a single curved rod *Spirilla*.

**II. The Higher Bacteria.**—These show advance on the lower in consisting of definite filaments branched or unbranched. In most cases the filaments at more or less regular intervals are cut by septa into short rod-shaped or curved elements. Such elements are more or less interdependent on one another, and special staining methods are often necessary to demonstrate the septa which demarcate the individuals of a filament. There is further often a definite membrane or sheath common to all the elements in a filament. Not only, however, is there this close organic relationship between the elements of the higher bacteria, but there is also interdependence of function; for example, one end of a filament is frequently concerned merely in attaching the organism to some other object. The greatest advance, however, consists in the setting apart among most of the higher bacteria of the free terminations of the filaments for the production of new individuals, as has been described (p. 5). There are various classes under which the species of the higher bacteria are grouped; but our knowledge of them is still somewhat



limited, as many of the members have not yet been artificially cultivated. The *beggiatoa* group consists of free swimming forms, motile by undulating contractions of their protoplasm. For the demonstration of the rod-like elements of the filaments special staining is necessary. The filaments have no special sheath, and the protoplasm contains sulphur granules. The method of reproduction is doubtful. The *thiothrix* group resembles the last in structure, and the protoplasm also contains sulphur granules; but the filaments are attached at one end, and at the other form gonidia. A *leptothrix* group is usually described which closely resembles the thiothrix group, except that the protoplasm does not contain sulphur granules. It cannot, however, be with certainty said whether such organisms can be sufficiently differentiated from the bacilli to warrant their being placed among the higher bacteria. In the *cladothrix* group there is the appearance of branching, which, however, is of a false kind. What happens is that a terminal cell divides, and on dividing again, it pushes the product of its first division to one side. There are thus two terminal cells lying side by side, and as each goes on dividing, the appearance of branching is given. Here, again, there is gonidium formation; and while the parent organism is in some of its elements motile, the gonidia move by means of flagella. The highest development is in the *streptothrix* group, to which belongs the streptothrix actinomyces, or the actinomyces bovis, and several other important pathogenic agents. Here the organism consists of a felted mass of non-septate filaments, in which true dichotomous branching occurs. Under certain circumstances threads grow out, and produce chains of coccus-like bodies from which new individuals can be reproduced. Such bodies are often referred to as spores, but they have not the same staining reactions nor resisting powers of so high a degree as ordinary bacterial spores. Sometimes, too, the protoplasm of the filaments breaks up into bacillus-like elements, which may also have the capacity of originating new individuals. In the streptothrix actinomyces there may appear a club-shaped swelling of the membrane at the end of the filament, which has by some been looked on as an organ of fructification, but which is most probably a product of a degenerative change. The streptothrix group, though its morphology and relationships are much disputed, may be looked on as a link between the bacteria on the one hand, and the lower fungi on the other. Like the latter, the streptothrix forms show the felted mass of non-septate branching filaments, which is usually called a mycelium. On the other hand, the breaking



up of the protoplasm of the streptothrix into coccus- and bacillus-like forms, links it to the other bacteria.

#### GENERAL BIOLOGY OF THE BACTERIA.

There are five prime factors in the growth of bacteria which must be considered, namely, food supply, moisture, relation to gaseous environment, temperature, and light.

**Food Supply.**—The bacteria are chiefly found living on the complicated organic substances which form the bodies of dead plants and animals, or which are excreted by the latter while they are yet alive. Seeing that, as a general rule, many bacteria grow side by side, the food supply of any particular variety is, relatively to it, altered by the growth of the other varieties present. It is thus impossible to imitate the complexity of the natural food environment of any species. The artificial media used in bacteriological work may therefore be poor substitutes for the natural supply. In certain cases, however, the conditions under which we grow cultures may be better than the natural conditions. For while one of two species of bacteria growing side by side may favour the growth of the other, it may also in certain cases hinder it, and therefore, when the latter is grown alone it may grow better. Most bacteria seem to produce excretions which are unfavourable to their own vitality, for, when a species is sown on a mass of artificial food medium, it does not in the great majority of cases go on growing till the food supply is exhausted, but soon ceases to grow. Effete products diffuse out into the medium and prevent growth. Such diffusion may be seen when the organism produces pigment, *e.g.* *b. pyocyaneus* growing on gelatin. In supplying artificial food for bacterial growth, the general principle ought to be to imitate as nearly as possible the natural surroundings, though it is found that there exists a considerable adaptability among organisms. With the pathogenic varieties it is usually found expedient to use media derived from the fluids of the animal body, and in cases where bacteria growing on plants are being studied, infusions of the plants on which they grow are frequently used. Some bacteria can exist on inorganic food, but most require organic material to be supplied. Of the latter, some require proteid to be present for their proper nourishment, while others can derive their nitrogen from a non-proteid such as asparagin. All bacteria require nitrogen to be present in some form, and many require to derive their carbon from carbohydrates. Mineral salts, especially sulphates, chlorides, and



phosphates, and also salts of iron are necessary. Occasionally special substances are needed to support life. Thus some species, in the protoplasm of which sulphur granules occur, require sulphuretted hydrogen to be present. In nature the latter is usually provided by the growth of other bacteria. When the food supply of a bacterium fails, it degenerates and dies. The proof of death lies in the fact that when it is transferred to fresh and good food supply it does not multiply. If the bacterium forms spores, it may then survive the want of food for a very long time. It may here be stated that the reaction of the food medium is a matter of great importance. Most bacteria prefer a slightly alkaline medium, and some, *e.g.* the cholera spirillum, will not grow in the presence of the smallest amount of free acid.

**Moisture.**—The presence of water is necessary for the continued growth of all bacteria. The amount of drying which bacteria in the vegetative stage will resist varies very much in different species. Thus the cholera spirillum is killed by two or three hours' drying, while the staphylococcus pyogenes aureus will survive ten days' drying, and the bacillus diphtheriæ still more. In the case of spores the periods are much longer. Anthrax spores will survive drying for several years, but here again moisture enables them to resist longer than when they are quite dry. When organisms have been subjected to such hostile influences, even though they survive, it by no means follows that they retain all their vital properties.

**Relation to Gaseous Environment.**—The relation of bacteria to the oxygen of the air is such an important factor in the life of bacteria that it enables a biological division to be made among them. Some bacteria will only live and grow when oxygen is present. To these the title of *obligatory aerobes* is given. Other bacteria will only grow when no oxygen is present. These are called *obligatory anaerobes*. In still other bacteria the presence or absence of oxygen is a matter of indifference. This group might theoretically be divided into those which are preferably aerobes, but can be anaerobes, and those which are preferably anaerobes, but can be aerobes. As a matter of fact such differences are manifested to a slight degree, but all such organisms are usually grouped as *facultative anaerobes*, *i.e.* preferably aerobic but capable of existing without oxygen. Examples of obligatory aerobes are *b. proteus vulgaris*, *b. subtilis*; of obligatory anaerobes, *b. tetani*, *b. oedematis maligni*, while the great majority of pathogenic bacteria are facultative anaerobes. With regard to anaerobes, hydrogen and nitrogen are indifferent



gases. Many anaerobes, however, do not flourish well in an atmosphere of carbon dioxide. Very few experiments have been made to investigate the action on bacteria of gas under pressure. A great pressure of carbon dioxide is said to make the *b. anthracis* lose its power of sporing, but it seems to have no effect on its vitality or on that of the *b. typhosus*. In the case of the *bacillus pyocyaneus*, however, it is said to destroy life.

**Temperature.**—For every species of bacterium there is a temperature at which it grows best. This is called the “optimum temperature.” There is also in each case a maximum temperature above which growth does not take place, and a minimum temperature below which growth does not take place. As a general rule the optimum temperature is about the temperature of the natural habitat of the organism. For organisms taking part in the ordinary processes of putrefaction the temperature of warm summer weather ( $20^{\circ}$  to  $24^{\circ}$  C.) may be taken as the average optimum, while for organisms normally inhabiting animal tissues  $35^{\circ}$  to  $39^{\circ}$  C. is a fair average. The lowest limit of ordinary growth is from  $12^{\circ}$  to  $14^{\circ}$  C., and the upper is from  $42^{\circ}$  to  $44^{\circ}$  C. In exceptional cases growth may take place as low as  $5^{\circ}$  C., and as high as  $70^{\circ}$  C. Some organisms which grow best at a temperature of from  $60^{\circ}$  to  $70^{\circ}$  C. have been isolated from dung, the intestinal tract, etc. These have been called *thermophilic* bacteria. It is to be noted that while growth does not take place below or above a certain limit, it by no means follows that death takes place outside such limits. Organisms can resist cooling below their minimum or heating beyond their maximum without being killed. Their vital activity is merely paralysed. Especially is this true of the effect of cold on bacteria. The results of different observers vary; but if we take as an example the cholera vibrio, Koch found that while the minimum temperature of growth was  $16^{\circ}$  C., a culture might be cooled to  $-32^{\circ}$  C. without being killed. With regard to the upper limit, few ordinary organisms in a spore-free condition will survive a temperature of  $57^{\circ}$  C., if long enough applied. Many organisms lose some of their properties when grown at unnatural temperatures. Thus many pathogenic organisms lose their virulence if grown above their optimum temperature, and some chromogenic forms, most of which prefer rather low temperatures, lose their capacity of producing pigment, *e.g.* *spirillum rubrum*.

**Effect of Light.**—Of recent years much attention has been paid to this factor in the life of bacteria. Direct sunlight is



found to have a very inimical effect. It has been found that an exposure of dry anthrax spores for one and a half hours to sunlight kills them. When they are moist, a much longer exposure is necessary. Typhoid bacilli are killed in about one and a half hours, and similar results have been obtained with many other organisms. In such experiments the thickness of the medium surrounding the growth is an important point. Death takes place more readily if the medium is scanty or if the organisms are suspended in water. Any fallacy which might arise from the effect of the heat rays of the sun has been excluded, though light plus heat is more fatal than light alone. In direct sunlight it is chiefly the green, violet, and, it may be, the ultra-violet rays which are fatal. Diffuse daylight has also a bad effect upon bacteria, though it takes a much longer exposure to do serious harm. A powerful electric light is as fatal as sunlight. Here, as with other factors, the results vary very much with the species under observation, and a distinction must be drawn between a mere cessation of growth and the condition of actual death. Some bacteria, especially occurring on the dead bodies of fresh fish, are phosphorescent.

**Conditions affecting the Movements of Bacteria.**—In some cases differences are observed in the behaviour of motile bacteria, contemporaneous with changes in their life history. Thus, in the case of *Bacillus subtilis*, movement ceases when sporulation is about to take place. On the other hand, in the *Bacillus* of symptomatic anthrax, movement continues while sporulation is progressing. Under ordinary circumstances motile bacteria appear not to be constantly moving, but occasionally to rest. In every case the movements become more active if the temperature be raised. Most interest, however, attaches to the fact that bacilli may be attracted to certain substances and repelled by others. Schenk, for instance, observed that motile bacteria were attracted to a warm point in a way which did not occur when the bacteria were dead and therefore only subject to physical conditions. Most important observations have been made on the attraction and repulsion exercised on bacteria by chemical agents, which have been denominated respectively *positive* and *negative chemiotaxis*. Pfeffer investigated this subject in many lowly organisms, including *Bacterium termo* and *Spirillum undula*. The method was to fill with the agent a fine capillary tube, closed at one end, to introduce this into a drop of fluid containing the bacteria under a cover-glass, and to watch the effect through the microscope. The general result was to indicate that motile bacteria may be either attracted or



repelled by the fluid in the tube. The effect of a given fluid differs in different organisms, and a fluid chemiotactic for one organism may not act on another. Degree of concentration is important, but the nature of the fluid is more so. Of inorganic bodies salts of potassium are the most powerfully attracting bodies, and in comparing organic bodies the important factor is the molecular constitution. These observations have been confirmed by Ali-Cohen, who found that while the vibrio of cholera and the typhoid bacillus were scarcely attracted by chloride of potassium, they were powerfully influenced by potato juice. Further, the filtered products of the growth of many bacteria have been found to have powerful chemiotactic properties. It is evident that all these observations have a most important bearing on the action of bacteria, though we do not yet know their true significance. Corresponding chemiotactic phenomena are shown also by certain animal cells, *e.g.* leucocytes, to which reference is made below.

**The Parts played by Bacteria in Nature.**—As has been said, the chief effect of bacterial action in nature is to break up into more simple combinations the complex molecules of the organic substances which form the bodies of plants and animals, or which are excreted by them. In some cases we know some of the stages of disintegration, but in most cases we know only general principles and sometimes only results. In the case of milk, for instance, we know that lactic acid is produced from the lactose by the action of the bacillus acidi lactici and of other bacteria, and that from urea ammonium carbonate is produced by the micrococcus ureæ. That the very complicated process of putrefaction is due to bacteria is absolutely proved, for any organic substance can be preserved indefinitely from ordinary putrefaction by the adoption of some method of killing all bacteria present in it, as will be afterwards described. This statement, however, does not exclude the fact that molecular changes take place spontaneously in the passing of the organic body from life to death. Many processes not usually referred to as putrefactive are also bacterial in their origin. The souring of milk, already referred to, the becoming rancid of butter, the ripening of cream and of cheese, are all due to bacteria.

A certain comparatively small number of bacteria have been proved to be the causal agents in some disease processes occurring in man, animals, and plants. This means that the fluids and tissues of living bodies are, under certain circumstances, a suitable pabulum for the bacteria involved. The



effects of the action of these bacteria are analogous to those taking place in the action of the same or other bacteria on dead animal or vegetable matter. The complex organic molecules are broken up into simpler products. We shall study these processes more in detail later. Meantime we may note that the disease-producing effects of bacteria form the basis of another biological division of the group. Some bacteria are harmless to animals and plants, and apparently under no circumstances give rise to disease in either. These are known as saprophytes. They are normally engaged in breaking up dead animal and vegetable matter. Others normally live on or in the bodies of plants and animals and produce disease. These are known as parasitic bacteria. Sometimes an attempt is made to draw a hard-and-fast line between the *saprophytes* and the *parasites*, and obligatory saprophytes or parasites are spoken of. This is an erroneous distinction. Some bacteria which are normally saprophytes can produce pathogenic effects (*e.g.* bacillus oedematis maligni), and it is consistent with our knowledge that the best-known parasites may have been derived from saprophytes. On the other hand, the fact that most bacteria associated with disease processes, and proved to be the cause of the latter, can be grown in artificial media, shows that for a time at least such parasites can be saprophytic. As to how far such a saprophytic existence of disease-producing bacteria occurs in nature, we are in many instances still ignorant.

**The Methods of Bacterial Action.**—The processes which bodies undergo in being split up by bacteria depend, first, on the chemical nature of the bodies involved, and, secondly, on the varieties of the bacteria which are acting. The destruction of albuminous bodies which is mostly involved in the wide and varied process of putrefaction can be undertaken by whole groups of different varieties of bacteria. The action of the latter on such substances is analogous to what takes place when albumins are subjected to ordinary gastric and intestinal digestion. In these circumstances, therefore, the production of albumoses, peptones, etc., similar to those of ordinary digestion, can be recognised in putrefying solutions, though the process of destruction always goes further, and still simpler substances, *e.g.* indol, and, it may be, crystalline bodies of an alkaloidal nature, are the ultimate results. The process is an exceedingly complicated one when it takes place in nature, and different bacteria are probably concerned in the different stages. Many other bacteria, *e.g.* some pathogenic forms,



though not concerned in ordinary putrefactive processes, have a similar digestive capacity. When carbohydrates are being split up, then various alcohols, ethers, and acids are produced. During bacterial growth there is not infrequently the abundant production of such gases as sulphuretted hydrogen, carbon dioxide, methane, etc. For an exact knowledge of the destructive capacities of any particular bacterium there must be an accurate chemical examination of its effects when it has been grown in artificial media the nature of which is known. The precise substances it is capable of forming can thus be found out. Many substances, however, are produced by bacteria, of the exact nature of which we are still ignorant, for example, the toxic bodies which play such an important part in the action of many pathogenic species.

Many of the actions of bacteria depend on the production by them of *ferments* of a very varied nature and complicated action. Thus the digestive action on albumins probably depends on the production of a peptic ferment analogous to that produced in the animal stomach. Ferments which invert sugar, which split up sugars into alcohols or acids, which coagulate casein, which split up urea into ammonium carbonate, also occur.

Such ferments may be diffused into the surrounding fluid, or be retained in the cells where they are formed. Sometimes the breaking down of the organic matter appears to take place within, or in the immediate proximity of, the bacteria, sometimes wherever the soluble ferments reach the organic substances. And in certain cases the ferments diffusing out into the surrounding medium probably break down the constituents of the latter to some extent, and prepare them for a further, probably intracellular, disintegration. Thus, in certain putrefactions of fibrin, if the process be allowed to go on naturally, the fibrin dissolves and ultimately great gaseous evolution of carbon dioxide and ammonia takes place, but if the bacteria, shortly after the process has begun, are killed or paralysed by chloroform, then only a peptonisation of the fibrin occurs, without the further splitting up and gaseous production. That a purely intracellular digestion may take place is illustrated by what has been shown to occur in the case of the micrococcus ureæ, which from urea forms ammonium carbonate by adding water to the urea molecule. Here, if after the action has commenced the bacteria are filtered off, no further production of ammonium carbonate takes place, which shows that no ferment has been dissolved out into the urine. If now the bodies of the bacteria be extracted with absolute alcohol or ether,



which of course destroy their vitality, a substance is obtained of the nature of a ferment, which, when added to sterile urine, rapidly causes the production of ammonium carbonate. This ferment has evidently been contained within the bacterial cells. In the investigation of the phenomena of the ferment action of bacteria, it has been noted in certain cases that the ferments formed depend on the food supply offered to the bacterium. Thus in one case a bacterium growing in starch forms diastase, which it does not do when grown on sugar.

In considering the effects of bacteria in nature, it must be recognised that some species are capable of building up complex substances out of simple chemical compounds. Examples of these are found in the bacteria which in the soil make nitrogen more available for plant nutrition by converting ammonia into nitrites and nitrates. Winogradski, by using media containing non-nitrogenous salts of magnesium, potassium, and ammonium, and free of organic matter, has demonstrated the existence of forms which convert, by oxidation, ammonia into nitrites, and of other forms which convert these nitrites into nitrates. Both can derive their necessary carbon from alkaline carbonates. Other bacteria, or organisms allied to the bacteria, exist which can actually take up and combine into new compounds the free nitrogen of the air. These are found in the tubercles which develop on the rootlets of the leguminosæ. Without such organisms the tubercles do not develop, and without the development of the tubercles the plants are poor and stunted. Bacteria thus play an important part in the enrichment and fertilisation of the soil.

**The Occurrence of Variability among Bacteria.**—The question of the division of the group of bacteria into definite species has given rise to much discussion among vegetable and animal morphologists, and at one time very divergent views were held. Some even thought that the same species might at one time give rise to one disease,—at another time to another. There is, however, now practical unanimity that bacteria show as distinct species as the other lower plants and animals, though, of course, the difficulty of defining the concept of a species is as great in them as it is in the latter. Still, we can say that among the bacteria we see exhibited (to use the words of De Bary) “the same periodically repeated course of development within certain empirically determined limits of variation” which justifies, among higher forms of life, a species to be recognised. What at first raised doubts as to the occurrence of species among the bacteria was the observation in certain cases of what is known as *pleomorphism*. By this is meant that one species may assume at different times different forms, *e.g.* appear as a coccus, a bacillus, or a leptothrix. Undoubtedly, many of the cases where this was alleged to have been observed occurred before the elaboration of the modern technique for the obtaining of pure cultures, but at the present day there are cases where evidence appears to exist of the occurrence of pleomorphism. This is especially the case with certain bacilli, and it may lead to such forms being classed among the higher bacteria. Pleomorphism is, however, a rare condition, and with regard to the bacteria as a whole we may say that each variety tends to conform to a definite type of structure and function which is peculiar to it and to it alone. On the other hand, slight variations from such type can occur in each. The size may vary



a little with the medium in which the organism is growing, and under certain similar conditions the adhesion of bacteria to each other may also vary. Thus cocci, which are ordinarily seen in short chains, may grow in long chains. The capacity to form spores may be altered, and such properties as the elaboration of certain ferments or of certain pigments may be impaired. Also the characters of the growths on various media may undergo variations. As has been remarked, variation as observed consists largely in a tendency in a bacterium to lose properties ordinarily possessed, and all attempts to transform one bacterium into an apparently closely allied variety (such as the *b. coli* into the *b. typhosus*) have failed. This of course does not preclude the possibility of one species having been originally derived from another, or of both having descended from a common ancestor, but we can say that only variations of an unimportant order have been observed to take place, and here it must be remembered that in many cases we can have forty-eight or more generations under observation within twenty-four hours.

## CHAPTER II.

### METHODS OF CULTIVATION OF BACTERIA.

**Introductory.**—In order to study the characters of any species of bacterium, it is necessary to have it growing apart from every other species. In the great majority of cases where bacteria occur in nature, this condition is not fulfilled. Only in the blood and tissues in some diseases do particular species occur singly and alone. We usually have, therefore, to remove a bacterium from its natural surroundings and grow it on an artificial food medium. When we have succeeded in separating it, and have got it to grow on a medium which suits it, we are said to have obtained a *pure culture*. The recognition of different species of bacteria depends, in fact, far more on the characters presented by pure cultures and their behaviour in different food media, than on microscopic examination. The latter in most cases only enables us to refer a given bacterium to its class. Again, in inquiring as to the possible possession of pathogenic properties by a bacterium, the obtaining of pure cultures is absolutely essential.

To obtain pure cultures, then, is the first requisite of bacteriological research. Now, as bacteria are practically omnipresent, we must first of all have means of destroying all extraneous organisms which may be present in the food media to be used in the vessels in which the food media are contained, and on all instruments which are to come in contact with our cultures. The technique of this destructive process is called sterilisation. We must therefore study the *methods of sterilisation*. The growth of bacteria in other than their natural surroundings involves further the *preparation of sterile artificial food media*, and when we have such media prepared we have still to look at the technique of the *separation of micro-organisms from mixtures of these, and the maintaining of pure cultures when the latter have been obtained*. We shall here find that different methods are necessary according as we are dealing with *aerobes*



or *anaerobes*. Each of these methods will be considered in turn.

### THE METHODS OF STERILISATION.

To exclude extraneous organisms, all food materials, glass vessels containing them, wires used in transferring bacteria from one culture medium to another, instruments used in making autopsies, etc., must be sterilised. These objects being so different, various methods are necessary, but underlying these methods is the general principle that all bacteria are destroyed by heat. The temperature necessary varies with different bacteria, and the vehicle of heat is also of great importance. The two vehicles employed are hot air and hot water or steam. The former is usually referred to as "dry heat," the latter as "moist heat." As showing the different effects of the two vehicles, Koch found, for instance, that the spores of *Bacillus anthracis*, which were killed by moist heat at  $100^{\circ}\text{C}$ ., in one hour, required three hours' dry heat at  $140^{\circ}\text{C}$ . to effect death. Both forms of heat may be applied at different temperatures—in the case of moist heat above  $100^{\circ}\text{C}$ ., a pressure higher than that of the atmosphere must of course be present.

#### *A. Sterilisation by Dry Heat.*

**A. (1) Red Heat or Dull Red Heat.**—Red heat is used for the sterilisation of the platinum needles which, it will be found, are so constantly in use. A dull heat is used for cauteries, the points of forceps, and may be used for the incidental sterilisation of small glass objects (cover-slips, slides, occasionally when necessary even test-tubes), care of course being taken not to melt the glass. The heat is obtained by an ordinary Bunsen burner.

**A. (2) Sterilisation by Dry Heat in a Hot-Air Chamber.**—The chamber (Fig. 2) consists of an outer and inner case of sheet iron. In the bottom of the outer there is a large hole. A Bunsen is lit beneath this, and thus plays on the bottom of the inner case, round all the sides of which the hot air rises and escapes through holes in the top of the outer case. A thermometer passes down into the interior of the chamber, half-way up which its bulb should be situated. It is found, as a matter of experience, that an exposure in such a chamber for one hour to a temperature of  $160^{\circ}\text{C}$ ., is sufficient to kill all the organisms which usually pollute articles in a bacteriological laboratory, though circumstances might arise where this would



be insufficient. This means of sterilisation is used for the glass flasks, test-tubes, plates, Petri's dishes, the use of which will be described. Such pieces of apparatus are thus obtained sterile and dry. It is advisable to put glass vessels into the chamber before heating it, and to allow them to stand in it after sterilisation till the temperature falls. Sudden heating or cooling is apt to cause glass to crack. The method is manifestly unsuitable for food media.

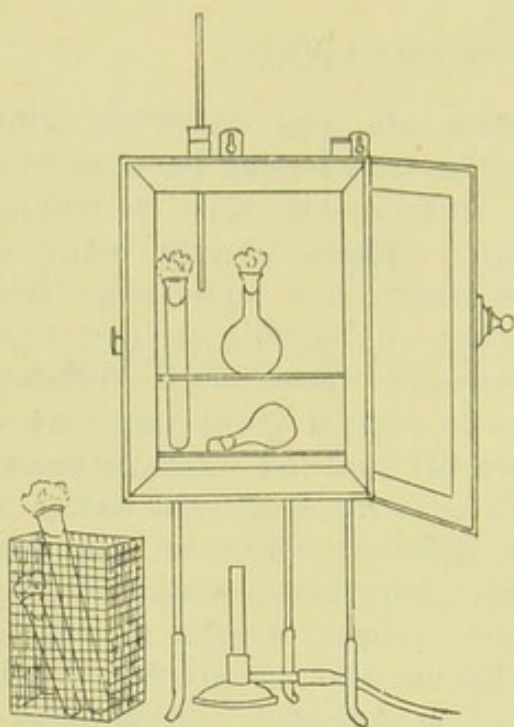


FIG. 2.—Hot-air steriliser.

minutes is sufficient to kill ordinary germs if no spores be present, and this method is useful for sterilising distilled or tap water which may be required in various manipulations. It is best to sterilise knives and instruments used in autopsies by boiling in water to which a little sodium carbonate has been added to prevent rusting. Twenty minutes' boiling will here be sufficient. The boiling of any fluid at  $100^{\circ}\text{C}$ . for one and a half hours will ensure sterilisation under almost any circumstances.

**B. (2) By Steam at  $100^{\circ}\text{C}$ .**—This is by far the most useful means of sterilisation. It may be accomplished in an ordinary potato steamer placed on a kitchen pot. The apparatus ordinarily used is "Koch's steam steriliser" (Fig. 3). This consists of a tall metal cylinder on legs, provided with a lid, and covered externally by some bad conductor of heat, such as felt or asbestos. A perforated tin diaphragm is fitted in the interior at a little distance above the bottom, and there is a tap at the bottom

*B. Sterilisation by Moist Heat.*

**B. (1) By Boiling.**—The boiling of a liquid for five

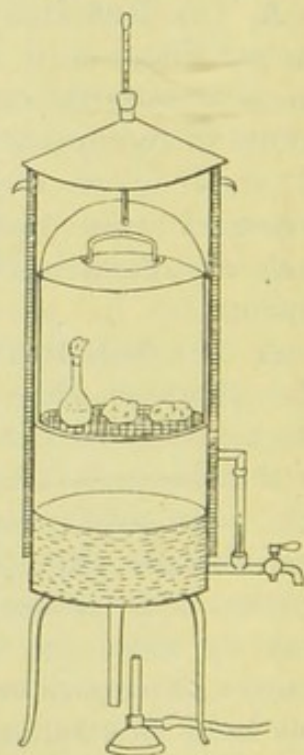


FIG. 3.—Koch's steam steriliser.



by which water may be supplied or withdrawn. If water to the depth of 3 inches be placed in the interior and heat applied, it will quickly boil, and the steam streaming up will surround any flask or other object standing on the diaphragm. Here no evaporation takes place from any medium, as it is surrounded during sterilisation by an atmosphere saturated with water vapour. It is convenient to have the cylinder tall enough to hold a litre flask with a funnel 7 inches in diameter standing in its neck. The funnel may be supported by passing its tube through a second perforated diaphragm placed in the upper part of the steam chamber. With such a "Koch" in the laboratory a hot-water filter is not needed. As has been said, one and a half hour's steaming will sterilise any medium, but in the case of media containing gelatin such an exposure is not practicable, as, with long boiling, gelatin tends to lose its physical property of solidification. The method adopted in this case is to *steam for twenty minutes on each of three succeeding days*.

This is a modification of what is known as "Tyndall's intermittent sterilisation." The fundamental principle of this method is that all bacteria in a non-spored form are killed by the temperature of boiling water, while if in a spored form they may not be thus killed. Thus by the sterilisation on the first day all the non-spored forms are destroyed—the spores remaining alive. During the twenty-four hours which intervene before the next heating, these spores, being in a favourable medium, are likely to assume the non-spored form. The next heating kills these. In case any may still not have changed their spored form, the process is repeated on a third day. Experience shows that usually the medium can now be kept indefinitely in a sterile condition.

Steam at  $100^{\circ}\text{C}$ . is therefore available for the sterilisation of all ordinary media. In using the Koch's steriliser, especially when a large bulk is to be sterilised, it is best to put the medium in while the apparatus is cold, in order to make certain that the whole of the food mass reaches the temperature of  $100^{\circ}\text{C}$ . The period of exposure is reckoned from the time boiling commences in the water in the steriliser. At any rate allowance must always be made for the time required to raise the temperature of the medium to that of the steam surrounding it.

**B. (3) Sterilisation by Steam at High Pressure.**—This is the most rapid and effective means of sterilisation. It is effected in an autoclave (Fig. 4). This is a gun-metal cylinder supported in a cylindrical sheet-iron case; its top is fastened down with screws and nuts, and is furnished with a safety valve, pressure-gauge, and a hole for thermometer. As in the Koch's steriliser, the contents are supported on a perforated diaphragm. The



source of heat is a large Bunsen beneath. The temperature employed is usually  $115^{\circ}\text{C}$ . or  $120^{\circ}\text{C}$ . To boil at  $115^{\circ}\text{C}$ ., water requires a pressure of about 23 lbs. to the square inch

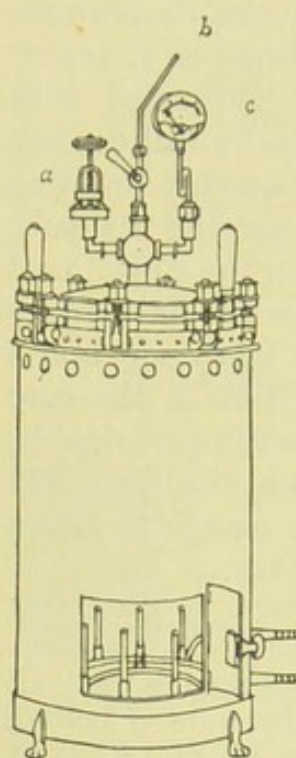


FIG. 4.—Autoclave.

- a. Safety-valve.
- b. Blow-off pipe.
- c. Gauge.

(*i.e.* 8 lbs. plus the 15 lbs. of ordinary atmospheric pressure). To boil at  $120^{\circ}\text{C}$ ., a pressure of about 30 lbs. (*i.e.* 15 lbs. plus the usual pressure) is necessary. In such an apparatus the desired temperature is maintained by adjusting the safety-valve so as to blow off at the corresponding pressure. One exposure of media to such temperatures for a quarter of an hour is amply sufficient to kill all organisms or spores. Here, again, care must be taken when gelatin is to be sterilised. It must not be exposed to a temperature above  $105^{\circ}\text{C}$ ., and is best sterilised by the intermittent method. Certain precautions are necessary in using the autoclave. In all cases it is necessary to allow the apparatus to cool well below  $100^{\circ}\text{C}$ . before opening it or allowing steam to blow off, otherwise there will be a sudden development of steam when the pressure is removed, and fluid media will be blown out of the flasks. Sometimes the instrument is

not fitted with a thermometer. In this case care must be taken to expel all the air initially present, otherwise, a mixture of air and steam being present, the pressure read off the gauge cannot be accepted as an indication of the temperature. Further, care must be taken to ensure the presence of a residuum of water when steam is fully up, otherwise the steam is superheated, and the pressure on the gauge again does not indicate the temperature correctly.

**B. (4) Sterilisation at Low Temperatures.**—Most organisms in a non-spored form are killed by a prolonged exposure to a temperature of  $57^{\circ}\text{C}$ . This fact has been taken advantage of for the sterilisation of blood serum, which will coagulate if exposed to a temperature above that point. Such a medium is sterilised on Tyndall's principle by exposing it for an hour at  $57^{\circ}\text{C}$ . for eight consecutive days, it being allowed to cool in the interval to the room temperature. The apparatus shown in Fig. 5 is a small hot-water jacket heated by a Bunsen placed beneath it, the temperature being controlled by a gas regulator. To ensure that the temperature all around shall be the same,



the lid also is hollow and filled with water, and there is a special gas burner at the side to heat it. This is the form originally used, but serum sterilisers are now constructed in which the test-tubes are placed in the sloped position, and in which inspissation (*vide* p. 40) can afterwards be performed at a higher temperature.

#### THE PREPARATION OF ORDINARY CULTURE MEDIA.

The general principle to be observed in the artificial culture of bacteria is that the medium used should approximate as closely as possible to that on which the bacterium grows naturally. In the case of pathogenic bacteria the medium therefore should resemble the juices of the body. The serum of the blood satisfies this condition, and is often used, but its application is limited by the difficulties in its preparation and preservation. Other media have been found which can support the life of all the pathogenic bacteria isolated. These consist of proteids or carbohydrates in a fluid, semi-solid, or solid form, in a transparent or opaque condition. The advantage of having a variety of media lies in the fact that growth characters on particular media, non-growth on some and growth on others, etc., constitute specific differences which are valuable in the identification of bacteria. The most commonly used media have as their basis a watery extract of meat. Most bacteria in growing in such an extract cause only a grey turbidity. A great advance resulted when Koch, by adding to it gelatin, provided a transparent solid medium in which growth characteristics of particular bacteria become evident. Many organisms, however, grow best at a temperature at which this nutrient gelatin is fluid, and therefore another gelatinous substance called agar, which does not melt below  $98^{\circ}\text{C.}$ , was substituted. Bouillon made from meat extract, gelatin, and agar media, and the modifications of these, constitute the chief materials in which bacteria are grown.

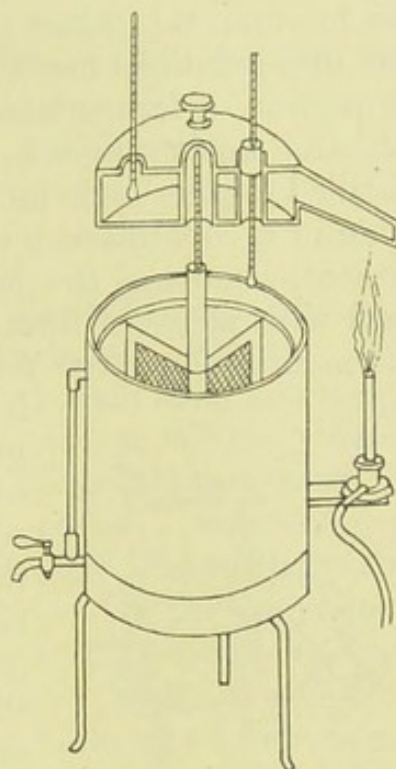


FIG. 5.—Steriliser for blood serum.



*Preparation of Meat Extract.*

The flesh of the ox, calf, or horse is usually employed. Horse-flesh has the advantage of being cheaper and containing less fat than the others; though generally quite suitable, it has the disadvantage for certain purposes of containing a larger proportion of fermentable sugar. The flesh must be freed from fat, and finely minced. To a pound of mince add 1000 c.c. distilled water, and mix thoroughly in a shallow dish. Set aside in a cool place for twenty-four hours. Skim off any fat present, removing the last traces by stroking the surface of the fluid with pieces of filter paper. Place a clean linen cloth over the mouth of a large filter funnel, and strain the fluid through

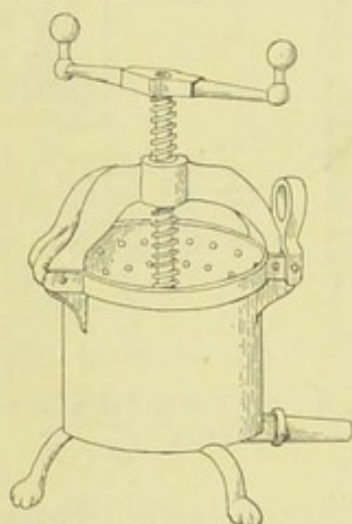


FIG. 6.—Meat press.

it into a flask. Pour the minced meat into the cloth, and, gathering up the edges of the latter in the left hand, squeeze out the juice still held back in the contained meat. Finish this expression by putting the cloth and its contents into a meat press (Fig. 6), similar to that used by pharmacists in preparing extracts; thus squeeze out the last drops. The resulting sanguineous fluid contains the soluble albumins of the meat, the soluble salts, extractives, and colouring matter, chiefly hæmoglobin. It is now boiled thoroughly for two hours, by which process the albumins coagulable

by heat are coagulated. Strain now through a clean cloth, boil for another half-hour, and filter through white Swedish filter paper (best, C. Schleicher u. Schull, No. 595). Make up to 1000 c.c. with distilled water. The resulting fluid ought to be quite transparent, of a yellowish colour without any red tint. If there is any redness, the fluid must be reboiled and filtered till this colour disappears, otherwise in the later stages it will become opalescent. A large quantity of the extract may be made at a time, and what is not immediately required is put into a large flask, the neck plugged with cotton wool, and the whole sterilised by methods B (2) or (3). This extract contains very little albuminous matter, and consists chiefly of the soluble salts of the muscle, certain extractives, and altered colouring matters, along with any slight traces of soluble proteid not coagulated by heat. It is of acid reaction. We have now to see how, by the addition of proteid



and other matter, it may be transformed into proper culture media.

1. **Bouillon Media.**—These consist of meat extract with the addition of certain substances to render them suitable for the growth of bacteria.

(1) (a). **Peptone Broth or Bouillon.**—This has the composition:—

Meat extract <sup>1</sup>	.	.	.	1000 c.c.
Sodium chloride	.	.	.	5 grms.
Peptone albumin	.	.	.	10 „

Boil till the ingredients are quite dissolved, and neutralise with a saturated solution of sodium hydrate. Add the latter drop by drop, shaking thoroughly between each drop and testing the reaction by means of litmus paper. Go on till the reaction is slightly but distinctly alkaline. Neutralisation must be practised with great care, as under certain circumstances, depending on the relative proportions of the different phosphates of sodium and potassium, what is known as the amphoteric reaction is obtained, *i.e.* red litmus is turned blue, and blue red, by the same solution. The sodium hydrate must be added till red litmus is turned slightly but distinctly blue, and blue litmus is not at all tinted red. After alkalisation, allow the fluid to become cold, filter through Swedish filter paper into flasks, make up to original volume with distilled water, plug the flasks with cotton wool, and sterilise by methods B (2) or (3) (pp. 28, 29). This method of neutralisation is to be recommended for all ordinary work.

In this medium the place of the original albumins of the meat is taken by peptone, a soluble proteid not coagulated by heat. Here it may be remarked that the commercial peptone albumin is not pure peptone, but a mixture of albumoses (see footnote, p. 193) with a variable amount of pure peptone. The addition of the sodium chloride is necessitated by the fact that alkalisation precipitates some of the phosphates and carbonates present. Experience has shown that sodium chloride can quite well be substituted. The reason for the alkalisation is that it is found that most bacteria grow best on a medium slightly alkaline to litmus. Some, *e.g.* the cholera vibrio, will not grow at all on even a slightly acid medium.

**Standardisation of Reaction of Media.**—While the above procedure of dealing with the reaction of a medium is sufficient for ordinary work, it has been thought advisable to have a more

<sup>1</sup> Some workers, instead of meat extract as made above, use Liebig's extract of beef, 2 grammes to the litre.



exact method for making media to be used in growing organisms, the growth characteristics of which are to be described for systematic purposes. Such a method should also be used in studying the changes in reaction produced in a medium by the growth of bacteria. It, however, involves considerable difficulty, and should not be undertaken by the beginner. It entails the preparation of solutions of acid and alkali which may be used for determining the original reaction of the medium, and for accurately making it of a definite degree of alkalinity. Normal<sup>1</sup> and decinormal solutions of sodium hydrate and hydrochloric acid are used.

**Preparation of Standard Solutions.**—The first requisites here are normal solutions of acid and alkali. The latter is prepared as follows: 85 grammes of pure sodium bicarbonate are heated to dull redness for ten minutes in a platinum vessel and allowed to cool in an exsiccator; just over 54 grammes of sodium carbonate should now be present. Any excess is quickly removed, and the rest being dissolved in one litre of distilled water, a normal solution is obtained. A measured quantity is placed in a porcelain dish, and a few drops of a .5 per cent. solution of phenol-phthaleine in neutral methylated spirit is added to act as indicator. The alkali produces in the latter a brilliant rose-pink, which, however, disappears on the least excess of acid being present. The mixture is boiled and a solution of hydrochloric acid of unknown strength is run into the dish from a burette till the colour goes and does not return after very thorough stirring. The strength of the acid can then be calculated, and a normal solution can be obtained. From these two solutions any strength of acid or alkali (such as the decinormal solution of NaOH mentioned below) may be derived.

As Eyre has suggested, the reaction of a medium may be conveniently expressed by the sign + or - to indicate acid or alkaline respectively, and a number to indicate the number of cubic centimetres of normal acid or alkaline solution necessary to make a litre of the medium neutral to phenol-phthaleine. Thus, for example, "reaction = -15," will mean that the medium is alkaline, and requires 15 c.c. of normal HCl to make a litre neutral. It has been found that when a medium such as bouillon reacts neutral to litmus, its reaction to phenol-

<sup>1</sup> A "normal" solution of any salt is prepared by dissolving an "equivalent" weight in grammes of that salt in a litre of distilled water. If the metal of the salt be monovalent, *i.e.* if it be replaceable in a compound by one atom of hydrogen (*e.g.* sodium), an equivalent is the molecular weight in grammes. In the case of NaCl, it would be 58.5 grammes (atomic weight of Na=23, of Cl=35.5). If the metal be bivalent, *i.e.* requiring two atoms of H for its replacement in a compound (*e.g.* calcium), an equivalent is the molecular weight in grammes divided by two. Thus in the case of CaCl<sub>2</sub> an equivalent would be 55.5 grammes (atomic weight of Ca=40, of Cl<sub>2</sub>=71).



phthaleine, according to the above standard, is on the average +25. Now, as litmus was originally introduced by Koch, and as nearly all bacterial research has been done with media tested by litmus, it is evidently difficult to say exactly what precise degree of alkalinity is the optimum for bacterial growth. It is probably safe to say, however, that when a medium has been rendered neutral to phenol-phthaleine by the addition of NaOH, the optimum degree is generally attained by the addition of from 10 to 15 c.c. of normal HCl per litre, *i.e.* the optimum reaction is from +10 to +15. In other words, the optimum reaction for bacterial growth lies, as Fuller has pointed out, about midway between the neutral point indicated by phenol-phthaleine and the neutral point indicated by litmus.

The only objection to the use of phenol-phthaleine is that its action is somewhat vitiated if free  $\text{CO}_2$  be present. This can be obviated by boiling any medium, before it is tested, in the porcelain dish into which titration takes place. The soda solutions are best stored in bottles such as that shown in Fig. 42, having on the air inlet a little bottle filled with soda lime and fitted with tubes as in the large one. The  $\text{CO}_2$  of the air which passes through is thus removed.

*Method.*—The following procedure includes most of the improvements introduced by Eyre. The medium with all its constituents dissolved is filtered and then heated for about forty-five minutes in the steamer, the maximum acidity being reached after this time. Of the warm medium take 25 c.c. and put in a porcelain dish, add 25 c.c. distilled water, and 1 c.c. phenol-phthaleine solution. Run in decinormal soda till neutral point is reached, indicated by the first trace of pink colour, the mixture being kept hot.<sup>1</sup> Repeat process thrice, and take the mean; this divided by 10 will give the amount ( $x$ ) of *normal* soda required to neutralise 25 c.c. of medium; then  $40x$  = amount necessary to neutralise a litre; and  $40x - 10$  = amount of normal soda necessary to give a litre its optimum reaction. Then measure the amount of medium to be dealt with, and add the requisite amount of soda solution.

Eyre uses a soda solution of ten times normal strength, which is delivered out of a 1 c.c. pipette divided into hundredths; this

<sup>1</sup> The beginner may find considerable difficulty in recognising the first tint of pink in the yellow bouillon. A good way of getting over this is to take two samples of the medium, adding the indicator to one only; then to run the soda into these from separate burettes; for each few drops run into the medium containing the indicator the same amount is run into the other. Thus the recognition of the first permanent change in tint will be at once recognised by comparing the two lots of solution.



obviates, to a large extent, the error introduced by increasing the bulk of the medium if a weaker neutralising solution be used.

1 (b). **Glucose Broth.**—To the other constituents of 1 (a) there is added 1 or 2 per cent. of grape sugar. The steps in the preparation are the same. Glucose being a reducing agent, no free oxygen can exist in a medium containing it, and therefore glucose broth is used as a culture fluid for anaerobic organisms.

1 (c). **Glycerin Broth.**—The initial steps are the same as in 1 (a), but *after filtration* 6 to 8 per cent. of glycerin (sp. grav. 1.25) is added. This medium is especially used for growing the tubercle bacillus when the products of the growth of the latter are required.

2. **Gelatin Media.**—These are simply the above broths, with gelatin added as a solidifying body.

2 (a). **Peptone Gelatin :—**

Meat extract	.	.	.	.	1000 c.c.
Sodium chloride	.	.	.	.	5 grms.
Peptone albumin	.	.	.	.	10 „
Gelatin	.	.	.	.	100–150 „

(The “gold label” gelatin of Coignet et Cie, Paris, is the best.) The gelatin is cut into small pieces, and added with the other constituents to the extract; they are then thoroughly melted on

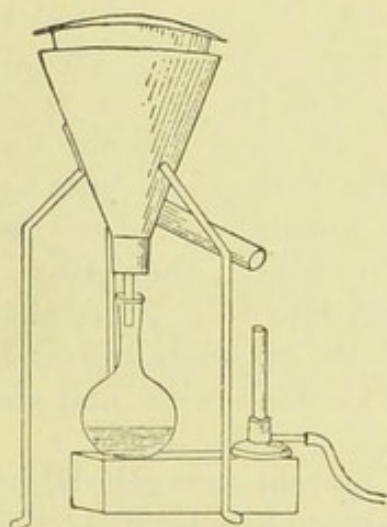


FIG. 7.—Hot-water funnel.

a sand bath, or in the “Koch.” The fluid medium is then rendered slightly alkaline, as in 1 (a), and filtered through filter paper. As the medium must not be allowed to solidify during the process, it must be kept warm. This is effected by putting the flask and funnel into a tall Koch’s steriliser, in which case the funnel must be supported on a tripod or diaphragm, as there is great danger of the neck of the flask breaking if it has to support the funnel and its contents. The filtration may also be carried out in a funnel with water-jacket which is heated, as

shown in Fig. 7. Whichever instrument be used, before filtering shake up the melted medium, as it is apt while melting to have settled into layers of different density. Sometimes what first comes through is turbid. If so, replace it in the unfiltered



part: often the subsequent filtrate in such circumstances is quite clear. A litre flask of the finished product ought to be quite transparent. If, however, it is partially opaque, add the white of an egg, shake up well, and boil thoroughly over the sand bath. The consequent coagulation of the albumin carries down the opalescent material, and, on making up with distilled water to the original quantity and refiltering, it will be found to be clear. The flask containing it is then plugged with cotton wool and sterilised, best by method B (2), p. 28. If the autoclave be used the temperature employed must not be above 105° C., and exposure not more than a quarter of an hour on three successive days. Too much boiling, or boiling at too high a temperature, as has been said, causes a gelatin medium to lose its property of solidification. The exact percentage of gelatin used in its preparation depends on the temperature at which growth is to take place. Its firmness is its most valuable characteristic, and to maintain this in hot summer weather, 15 parts per 100 are necessary. A limit is placed on higher percentages by the fact that, if the gelatin be too stiff, it will split on the perforation of its substance by the platinum needle used in inoculating it with a bacterial growth; 15 per cent. gelatin melts at about 24° C. For ordinary use in British laboratories 10 per cent. gelatin is a sufficient strength.

2 (b). **Glucose Gelatin.**—The constituents are the same as 2 (a), with the addition of 1 to 2 per cent. of grape sugar. The method of preparation is identical. This medium is used for growing anaerobic organisms at the ordinary temperatures.

3. **Agar Media (French, "gélose").**—The disadvantage of gelatin is that at the blood temperature (38° C.), at which most pathogenic organisms grow best, it is liquid. To get a medium which will be solid at this temperature, agar is used as the stiffening agent instead of gelatin. Unlike the latter, which is a proteid, agar is a carbohydrate. It is derived from the stems of various seaweeds growing in the Chinese seas, commercially classed together as "Ceylon Moss." For bacteriological purposes the dried stems of the seaweed may be used, but there is in the market a purified product in the form of a powder, which is preferable.

3 (a). **"Ordinary" Agar.**—This has the following composition:—

Meat extract	.	.	.	.	.	1000 c.c.
Sodium chloride	.	.	.	.	.	5 grms
Peptone albumin	.	.	.	.	.	10 „
Agar	.	.	.	.	.	15 „



Cut up the agar into very fine fragments (in fact till it is as nearly as possible dust), add to the meat extract with the other ingredients, and preferably allow to stand all night. Then boil gently in a water bath for two or three hours, till the agar is thoroughly melted. The process of melting may be hastened by boiling the medium in a sand bath and passing through it a stream of steam generated in another flask; the steam is led from the second flask by a bent glass tube passing from just beneath the cork to beneath the surface of the medium (Eyre). After melting, render slightly alkaline with sodium hydrate solution, make up to original volume with distilled water, and filter. Filtration here is a very slow process, and is best carried out in a tall Koch's steriliser. In doing this, it is well to put a glass plate over the filter funnel to prevent condensation water from dropping off the lid of the steriliser into the medium. If a slight degree of turbidity may be tolerated, it is sufficient to filter through a felt bag or jelly strainer. Plug the flask containing the filtrate, and sterilise either in autoclave for fifteen minutes or in Koch's steriliser for one and a half hours. Agar melts just below  $100^{\circ}\text{C}$ ., and on cooling solidifies about  $39^{\circ}\text{C}$ .

3 (b). **Glycerin Agar.**—To 3 (a) after filtration add 6 to 8 per cent. of glycerin and sterilise as above. This is used especially for growing the tubercle bacillus.

3 (c). **Glucose Agar.**—Prepare as in 3 (a), but add 1 to 2 per cent. of grape sugar along with agar. This medium is used for the culture of anaerobic organisms at temperatures above the melting-point of gelatin. It is also an excellent culture medium for some aerobes, *e.g.* the *b. diphtheriæ*.

These bouillon, gelatin, and agar preparations constitute the most frequently used media. Growths in bouillon do not usually show any characteristic appearances which facilitate classification, but such a medium is of great use in investigating the soluble toxic products of bacteria. The most characteristic developments of organisms take place on the gelatin media. These have, however, the disadvantage of not being available when growth is to take place at any temperature above  $24^{\circ}\text{C}$ . For higher temperatures agar must be employed. Agar is, however, never so transparent. Though quite clear when fluid, on solidifying it always becomes slightly opaque. Further, growths upon it are never so characteristic as those on gelatin. It is, for instance, never liquefied, whereas some organisms, by their growth, liquefy gelatin and others do not—a fact of prime importance.



## SPECIAL CULTURE MEDIA.

An enormous variety of different media has been brought forward for use in cases either where special difficulty is experienced in getting an organism to grow, or where some special growth characteristic is to be studied. It is impossible to do more than give the chief of these.

*Peptone Solution.*

A simple solution of peptone (Witte) constitutes a suitable culture medium for many bacteria. The peptone in the proportion of 1 to 2 per cent., along with .5 per cent. NaCl, is dissolved in distilled water by heating. The fluid is then filtered, placed in tubes, and sterilised. The reaction is usually distinctly alkaline, which condition is suitable for most purposes. For special purposes the reaction may be standardised. In such a solution the cholera vibrio grows with remarkable rapidity. It is also much used for testing the formation of indol by a particular bacterium; and by the addition of one of the sugars to it the fermentative powers of an organism may be tested (p. 80). Litmus may be added to show any change in reaction.

*Media containing an Indicator.*

*Litmus Media.*—To any of the ordinary media litmus (French, tournesol) may be added to show change in reaction during bacterial growth. The litmus is added, before sterilisation, as a strong watery solution (*e.g.* the Kubel-Tiemann solution, *vide* p. 48) in sufficient quantity to give the medium a distinctly bluish tint. During the development of an acid reaction the colour changes to a pink, and may subsequently be discharged.

*Neutral Red Media.*—This dye has been introduced as an aid in determining the presence or absence of members of the *b. coli* group, especially in the examination of water. The media found most suitable are agar or bouillon containing .5 per cent. of lactose, to which .5 per cent. of a 1 per cent. watery solution of neutral red is added. The alkaline medium is of a yellowish brown colour which on the presence of acid passes into a deep rose red. Sometimes there subsequently occurs a change to a fluorescent green, caused apparently by a change in the composition of the dye, as the fluorescence is not discharged by addition of alkali.



*Blood Serum Media.*

**Koch's Blood Serum.**—Koch introduced this medium, and it is prepared as follows: Plug the mouth of a tall cylindrical glass vessel (say of 1000 c.c. capacity) with cotton wool, and sterilise by steaming it in a Koch's steriliser for one and a half hours. Take it to the place where a horse, ox, or sheep is to be killed. When the artery or vein of the animal is opened, allow the first blood which flows, and which may be contaminated from the hair, etc., to escape; fill the vessel with the blood subsequently shed. Carry carefully back to the laboratory without shaking, and place for twenty-four hours in a cool place, preferably an ice chest. The clear serum will separate from the clotted blood. If a centrifuge is available, a large yield of serum may be obtained by centrifugalising the freshly drawn blood. If coagulation has occurred, the clot must first be thoroughly broken up. With a sterile 10 c.c. pipette, transfer this quantity of serum to each of a series of test-tubes which must previously have been sterilised by dry heat. The serum may, with all precautions, have been contaminated during the manipulations, and must be sterilised. As it will coagulate if heated above  $68^{\circ}\text{C}$ ., advantage must be taken of the intermittent process of sterilisation at  $57^{\circ}\text{C}$ . [method B (4)]. It is therefore kept for one hour at this temperature on each of eight successive days. It is always well to incubate it for a day at  $37^{\circ}\text{C}$ . before use, to see that the result is successful. After sterilisation it is "inspissated," by which process a clear solid medium is obtained. "Inspissation" is probably an initial stage of coagulation, and is effected by keeping the serum at  $65^{\circ}\text{C}$ . till it stiffens. This temperature is just below the coagulation point of the serum. The more slowly the operation is performed the clearer will be the serum. The apparatus used for the purpose is one of the various forms of serum steriliser (*e.g.* Fig. 8), generally a chamber with water-jacket heated with a Bunsen below. The temperature is controlled by a gas regulator, and such an apparatus can, by altering the temperature, be used either for sterilisation or inspissation. As is evident, the preparation of this medium is tedious, but its use is necessary for the observation of particular characteristics in several pathogenic bacteria, notably the tubercle bacillus. Pleuritic and other effusions may be prepared in the same way, and used as media, but care must be taken in their use, as we have no right to say that pathological effusions have the same chemical composition as normal serum.

If blood be collected with strict aseptic precautions, then



sterilisation of the serum is unnecessary. To this end the mouth of the cylinder used for collecting the blood, instead of being plugged with wool, has an indiarubber bung inserted in it through which two bent glass tubes pass. The outer end of one of these is of convenient length, and, before sterilisation, a large cap of cotton wool is tied over it; the other tube is plugged with a piece of cotton wool. In the slaughter-house the cap is removed and the tube is inserted into the blood-vessel as a cannula. The cylinder is thus easily filled. Another method is to conduct the blood to the cylinder by means of a sterilised cannula and indiarubber tube, the former being inserted in the blood-vessel. In every case the serum must be incubated before use, to make sure that it is sterile.

### Coagulated Blood Serum.

—If fresh serum be placed in sterile tubes and be steamed in the sloped position for an hour, it coagulates, and there is thus obtained a solid medium very useful for the growth of the diphtheria bacillus for diagnostic purposes.

**Löffler's Blood Serum.**—This is the best medium for the growth of the *b. diphtheriæ*, and may be used for other organisms. It has the following composition: Three parts of calf's or lamb's blood serum are mixed with one part ordinary neutral peptone bouillon made from veal with 1 per cent. of grape sugar added to it. Though this is the original formula, it can be made from ox or sheep serum and beef bouillon without its qualities being markedly impaired. Sterilise by method B (4) as above (p. 30).

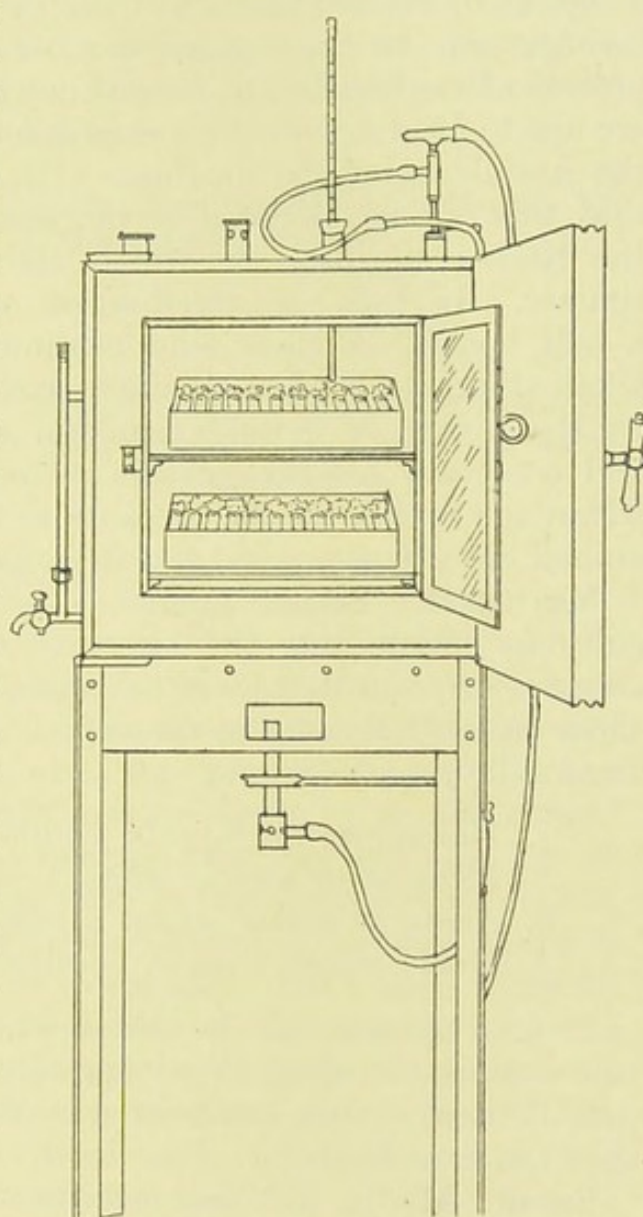


FIG. 8.—Blood serum inspissator.



**Alkaline Blood Serum** (Lorrain Smith's Method).—To each 100 c.c. of the serum obtained as before, add 1 to 1.5 c.c. of a 10 per cent. solution of sodium hydrate and shake gently. Put sufficient of the mixture into each of a series of test-tubes, and, laying them on their sides, sterilise by method B (2). If the process of sterilisation be carried out too quickly, bubbles of gas are apt to form before the serum is solid, and these interfere with the usefulness of the medium. Dr. Lorrain Smith informs us that this can be obviated if the serum be solidified high up in the Koch's steriliser, in which the water is allowed only to simmer. In this case sterilisation ought to go on for one and a half hours. A clear solid medium (consisting practically of alkali-albumin) is thus obtained, and he has found it of value for the growth of the organisms for which Koch's serum is used, and especially for the growth of the *b. diphtheriæ*. Its great advantage is that aseptic precautions in obtaining blood from the animal are not necessary, and it is easily sterilised.

**Marmorek's Serum Media.**—There has always been a difficulty in maintaining the virulence of cultures of the pyogenic streptococci, but Marmorek has succeeded in doing so by growing them on the following media, which are arranged in the order of their utility:—

1. Human serum 2 parts, bouillon 1 part.
2. Pleuritic or ascitic serum 1 part, bouillon 2 parts.
3. Asses' or mules' serum 2 parts, bouillon 1 part.
4. Horse serum 2 parts, bouillon 1 part.

Human serum can be obtained from the blood shed in venesection, the usual aseptic precautions being taken. In the case of these media, sterilisation is effected by method B (4), and they are used fluid.

**Serum Media for Gonococcus.**—The two following media will be found suitable. Wertheim's medium consists of one part of sterile human serum (conveniently obtained from placental blood) and two parts of agar. The agar is sterilised, and fluid is allowed to cool to 40° C.; the serum is then added, and the mixture is allowed to solidify in the sloped position.

Gurd's medium is a 2 per cent. agar with acid reaction + 6 to phenol-phthaleine (p. 34), with defibrinated human blood added in the proportion of about 5 drops to 5 c.c. of agar; the blood is added to the melted agar as in Wertheim's medium. W. B. M. Martin recommends the substitution of sodium phosphate (5 per cent.) for sodium chloride in the preparation of the agar, and uses fluid human serum sterilised at 57° C. in



place of blood. He also finds that the same agar medium allowed to solidify and then smeared on the surface with a drop or two of human serum gives excellent results.

Any of these media may be used for plate cultures, the agar being melted and cooled to 40° C. as for agar plates; the serum or blood is then added; the mixture is inoculated in the usual way and poured out in Petri dishes.

**“Nasgar.”**—This is a serum medium introduced by Gordon for the isolation of the meningococcus. It is prepared as follows:—

Ascitic fluid . . . . .	15 c.c.
Distilled water . . . . .	35 c.c.
Nutrose <sup>1</sup> . . . . .	1 gramme.

Put in a flask, bring to boil, constantly shaking till ebullition occurs; filter. Of the resultant fluid take one part and add two parts of ordinary peptone agar. Steam for half an hour and place in tubes.

### *Blood Media.*

**Blood - Smeared Agar.**—This medium was introduced by Pfeiffer for growing the influenza bacillus, and it has been used for the organisms which are not easily grown on the ordinary media, *e.g.* the gonococcus and the pneumococcus. Human blood or the blood of animals may be used. “Sloped tubes” (*vide* p. 53) of agar are employed (glycerin agar is not so suitable). Purify a finger first with 1–1000 corrosive sublimate, dry, and then wash with absolute alcohol to remove the sublimate. Allow the alcohol to evaporate. Prick with a needle sterilised by heat, and, catching a drop of blood in the loop of a sterile platinum wire (*vide* p. 54), smear it on the surface of the agar. The excess of the blood runs down and leaves a film on the surface. Cover the tubes with indiarubber caps, and incubate them for one or two days at 38° C. before use, to make certain that they are sterile. Agar poured out in a thin layer in a Petri dish may be smeared with blood in the same way and used for cultures.

**Serum Agar** is prepared in a similar way by smearing the surface of the agar with blood serum, or by adding a few drops of serum to the tube and then allowing it to flow over the surface.

**Blood Agar.**—For many purposes (*e.g.* the growth of the whooping-cough bacillus, the bacillus of soft sore, the cultivation of trypanosomes and *Leishmaniae*), the use of agar containing defibrinated blood, especially rabbit blood, is desirable. The

<sup>1</sup> Nutrose is an alkaline preparation of casein.



blood may be obtained in several ways, preferably by bleeding from the carotid. For this purpose the vessel is exposed and as long a portion as possible is cleaned. This is ligatured high up, and a ligature is loosely applied round the lower part of the vessel in such a way as not to constrict it. The vessel is clamped above this ligature, and with scissors an oblique opening is made in its side. The clamp being removed, the stream of blood is directed by means of the ligature into the mouth of a stout sterile flask, which ought to contain some fragments of broken glass rod. During the bleeding the flask should be gently agitated, and when filled should be shaken in a bath of water just below blood-heat. We have found that sterile blood can be obtained from the ear vein of the rabbit by the method of bleeding to be subsequently described. The ear is well washed with lysol, the lysol dried off with sterile wool, absolute alcohol dropped on and allowed to evaporate, and the blood withdrawn. The first c.c. or so is rejected.

However the blood is obtained, after defibrination it is warmed to 45° C., and added to agar of the same temperature in the proportion of about one-third of blood and two-thirds of agar. Needless to say, such media must be incubated before use to ensure that bacteria have not gained access during preparation.

**Bordet and Gengou's Medium for *Bacillus* of Whooping-cough.**—An extract of potato is first prepared by adding two parts of water containing 4 per cent. of glycerin to one part of potato chips; the mixture is then boiled and the fluid is separated off. An agar medium is then prepared of the following composition: potato extract, 50 c.c.; 6 per cent. solution of sodium chloride, 150 c.c.; and agar, 5 grms. Of this medium, 2–3 c.c. is placed in each of a series of test-tubes, and then to each there is added, by the method described in the preceding paragraph, an equal part of defibrinated rabbit's (or better, human) blood, obtained by aseptic precautions. The mixture is then allowed to solidify in the sloped position. This medium is also very suitable for the growth of the gonococcus, meningococcus, and influenza bacillus.

**Blood-Alkali-Agar** (Dieudonné).—This medium, introduced for the culture of the cholera spirillum, for which purpose it has been found extremely suitable, has the property of inhibiting the growth of most of the intestinal bacteria; for example, the *b. coli* does not grow on it, or does so very slightly. A blood-alkali solution is prepared by adding equal parts of defibrinated ox blood and of normal caustic soda solution; the solution may then be sterilised in the steam steriliser. Of this solution three parts are added to seven parts of ordinary peptone-agar rendered neutral to litmus, and the mixture is disposed in test-tubes.



**Novy and MacNeal's Medium for Culture of Trypanosomes.**—125 grammes rabbit or ox flesh is treated with 1000 c.c. distilled water, as in making ordinary bouillon, and there are added to the meat extract 20 grms. Witte's peptone, 5 grms. sodium chloride, 20 grms. agar, and 10 c.c. normal sodium carbonate. The medium is placed in tubes and sterilised in the autoclave at 110° C. for thirty minutes. It is cooled to 50° C., and there is added to the medium in each tube twice its volume of defibrinated rabbit blood, which has been prepared with all aseptic precautions; the tubes are allowed to set in the inclined position. In inoculating such tubes they are placed in an upright position for a few minutes, and then the infective material is introduced.

*Potatoes as Culture Material.*

(a) **In Potato Jars.**—The jar consists of a round, shallow, glass vessel with a similar cover (*vide* Fig. 9). It is washed with 1-1000 corrosive sublimate, and a piece of circular filter paper, moistened with the same, is laid in its bottom. On this latter are placed four sterile watch-glasses. Two firm, healthy, small, round potatoes, as free from eyes as possible, and with the skin whole, are scrubbed well with a brush under the tap and steeped for two or three hours in 1-1000 corrosive

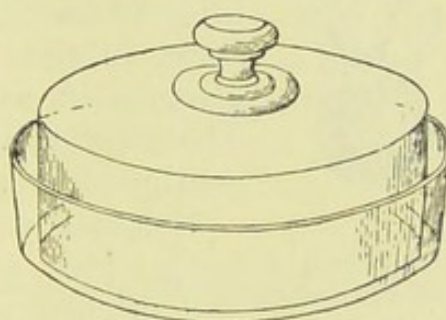


FIG. 9.—Potato jar.

sublimate. They are steamed in the Koch's steriliser for thirty minutes or longer, or in the autoclave for a quarter of an hour. When cold, each is grasped between the left thumb and forefinger (which have been sterilised with sublimate) and cut through the middle with a sterile knife. It is best to have the cover of the jar raised by an assistant, and to perform the cutting beneath it. Each half is put in one of the watch-glasses, the cut surfaces,



FIG. 10.—Cylinder of potato cut obliquely.

which are then ready for inoculation with a bacterial growth, being uppermost. Smaller jars, each of which holds half of a potato, are also used in the same way and are very convenient.

(b) **By Slices in Tubes.**—This method, introduced by Ehrlich, is the best means of utilising potatoes as a medium. A large, long potato is well washed and scrubbed, and peeled with a clean knife. A cylinder is then bored from its interior with an apple corer or a large cork borer, and is cut obliquely, as in Fig 10.



Two wedges are thus obtained, each of which is placed broad end downward in a test-tube of special form (see Fig. 11). In the wide part at the bottom of this tube is placed a piece of



FIG. 11.—  
Ehrlich's  
tube, con-  
taining piece  
of potato.

cotton wool, which catches any condensation water which may form. The wedge rests on the constriction above this bulbous portion. The tubes, washed, dried, and with cotton wool in the bottom and in the mouth, are sterilised before the slices of potato are introduced. After the latter are inserted, the tubes are sterilised in the Koch steam steriliser for one hour, or in the autoclave for fifteen minutes, at  $115^{\circ}\text{C}$ . An ordinary test-tube may be used with a piece of sterile absorbent wool in its bottom, on which the potato may rest.

*Glycerin potato*, suitable for the growth of the tubercle bacillus, may be prepared by covering the slices in the tubes with 6 per cent. solution of glycerin in water, and steaming for half an hour. The fluid is then poured off and the sterilisation continued for another half-hour.

Potatoes ought not to be prepared long before being used, as the surface is apt to become dry and discoloured. It is well to take the reaction of the potato with litmus before sterilisation, as this varies; normally in young potatoes it is weakly acid.

The reaction of the potato may be more accurately estimated by steaming the potato slices for a quarter of an hour in a known quantity of distilled water, and then estimating the reaction of the water by phenol-phthaleine. The required degree of acidity or alkalinity is obtained by adding the necessary quantity of HCl or NaOH solution (p. 35), and steaming for other fifteen minutes. The water is then poured off and sterilisation continued for another half-hour. Potatoes before being inoculated ought always to be incubated at  $37^{\circ}\text{C}$ . for a night, to make sure that their sterilisation has been successful.

#### *Milk as a Culture Medium.*

This is a convenient medium for observing the effects of bacterial growth in changing the reaction, in coagulating the soluble albumin, and in fermenting the lactose. It is prepared as follows: Fresh milk is taken, preferably after having had the cream "separated" by centrifugalisation, as is practised in the best dairies, and is steamed for fifteen minutes in the Koch; it



is then set aside in an ice chest or cool place over night to facilitate further separation of cream. The milk is siphoned off from beneath the cream. The reaction of fresh milk is alkaline. If great accuracy is necessary, any required degree of reaction may be obtained by the titration method. It is then placed in tubes, and sterilised by methods B (2) or B (3).

### *Bread Paste.*

This is useful for growing *torulæ*, moulds, etc. Some ordinary bread is cut into slices, and then dried in an oven till it is so dry that it can be pounded to a fine powder in a mortar, or rubbed down with the fingers and passed through a sieve. Some 100 c.c. flasks are washed, dried, and sterilised, and a layer of the powder half an inch thick placed on the bottom. Distilled water, sufficient to cover the whole of it, is then run in with a pipette held close to the surface of the bread, and, the cotton-wool plugs being replaced, the flasks are sterilised in the Koch's steriliser by method B (2). The reaction is slightly acid.

### *Media used for separating the Members of Bacterial Groups.*

A great number of media have been devised for use in differentiating the members of the coli-typhoid and other bacterial groups. The general feature of these media is that they contain certain substances, often sugars, which tend to bring out the special characters of the organism under investigation. Sometimes also substances are present which inhibit the growth of bacteria other than those belonging to the group. The following are the media which here deserve most attention :—

**Hiss's Serum Water Media.**—These are composed of one part of ox's serum and three parts of distilled water with 1 per cent. litmus ; various sugars in a pure condition are added in the proportion of 1 per cent. The development of acid by fermentation is shown by the alteration of the colour and by coagulation of the medium. These media do not coagulate at 100° C., and thus can be sterilised in the steam steriliser. They have been extensively used by American workers in studying the fermentative properties of the *b. dysenteriae*, *b. coli*, etc.

**Drigalski and Conradi's Medium.**—This is one of the media used for the study of intestinal bacteria, and especially for the isolation of the typhoid group of organisms. (a) Three pounds of meat are treated with two litres of water overnight ; the fluid is separated as usual, boiled for an hour, filtered, and there are added 20 grammes Witte's peptone, 20 grammes nutrose, 10 grammes sodium chloride ; the mixture is then boiled for an hour, 60 grammes finest agar are added, and it is placed in



the autoclave till melted (usually one hour); it is then rendered slightly alkaline to litmus, filtered, and boiled for half an hour. (b) 260 c.c. Kubel-Tiemann litmus<sup>1</sup> solution is boiled for ten minutes, 30 grammes milk sugar (chemically pure) are added, and the mixture is boiled for fifteen minutes; (a) and (b) are then mixed hot, well shaken, and, if necessary, the slightly alkaline reaction restored. There are then added 4 c.c. of a 10 per cent. sterile solution of water-free sodium carbonate and 20 c.c. of a freshly prepared solution made by dissolving .1 gramme crystal-violet B, Hoechst, in 100 c.c. hot sterile distilled water. This is the finished medium, and great care must be taken not to overheat it or to heat it too long, as changes in the lactose may be originated. It is convenient to distribute the medium in 80 c.c. flasks.

The principle of the medium is that while there is a food supply very favourable to the *b. typhosus* and the *b. coli*, the antiseptic action of the crystal-violet tends to inhibit the growth of other bacteria likely to occur in material which has been subjected to intestinal contamination. In examining faeces, a little is rubbed up in from ten to twenty times its volume of sterile bouillon; in the case of urine or water, the fluid is centrifugalised and the deposit or lower portion is used for the inoculation procedures.

For use the medium is distributed in Petri capsules in a rather thicker layer than is customary in an ordinary plate. This sheet of medium must be transparent, but must not be less than 2 mm. in thickness—in fact, ought to be about 4 mm. After being poured, the capsules are left with the covers off for an hour or so, to allow the superficial layers of the medium to become set hard. The effect of this is that during incubation no water of condensation forms on the lid of the capsule, and thus the danger of this fluid dropping on to the developing colonies is avoided. The antiseptic nature of the crystal-violet is sufficient to prevent the growth of any aerial organisms falling on the agar during its exposure to the air. The plates are usually inoculated by means of a glass spatula made by bending 3 inches of a piece of glass rod at right angles to the rest of the rod. This part is dipped in the infective material, and smeared in all directions over the surfaces of three or four plates successively without any intervening sterilisation. The plates are again exposed to the air after inoculation for half an hour, and then incubated for twenty-four hours. At the end of such a period *b. coli* colonies are 2 to 6 mm. in diameter, stained distinctly red, and are non-transparent. Colonies of the *b. typhosus* are seldom larger than 2 mm., they are blue or bluish-violet in colour, are glassy and dew-like in character, and have a single contour. Sometimes in the plates *b. subtilis* and its congeners appear, and colonies of these organisms have a blue colour. Their growth is, however, more exuberant than that of the typhoid bacillus,—being often heaped up in the centre,—and the contour of the colony is often double.

<sup>1</sup> The litmus solution is made as follows: Solid commercial litmus is digested with pure spirit at 30° C. till on adding fresh alcohol the latter becomes only of a light violet colour. A saturated solution of the residue is then made in distilled water and filtered. When this is diluted with a little distilled water it is of a violet colour, which further dilution turns to a pure blue. To such a blue solution very weak sulphuric acid (made by adding two drops of dilute sulphuric acid to 200 c.c. water) is added till the blue colour is turned to a wine-red. Then the saturated solution of the dye is added till the blue colour returns.



**Conradi's Picric Acid Brilliant Green Method.**—Applying his principle of seeking for anilin bodies which while inhibiting the action of ordinary intestinal bacteria rather favour the growth of *b. typhosus* and *b. paratyphosus*, Conradi in 1908 used for this purpose crystalline brilliant green (Hoechst, extra pure), acting along with picric acid (Grübler). The medium is made as follows: 900 c.c. water, 20 grms. Liebig's meat extract and 100 c.c. of a 10 per cent. watery solution of Witte's peptone are mixed and filtered; 30 grms. agar in threads are dissolved in the fluid, and the whole filtered. The reaction is then adjusted with normal sodium hydrate or normal phosphoric acid to an acid content of 3 per cent. (phenol-phthaleine being the indicator), *i.e.* the finished medium is such that to make it neutral would require the addition to each 100 c.c. of 3 c.c. of normal sodium hydrate. The acid medium is then sterilised, and kept in bulk in this form. For use the remaining substances are added in the proportions of 10 c.c. of 1-1000 watery solution of the brilliant green and 10 c.c. of 1 per cent. watery picric acid to 1½ litres of the peptone-agar, and the finished medium is poured in large Petris and allowed to stand at 37° C. till the surface is firm. The capsules are inoculated in the way already described. Typhoid colonies appear sharp-edged, round, flat-surfaced but slightly thicker in the middle, transparent, and of light green colour. Colonies of the paratyphoid bacillus are similar, but tend at the same age to be slightly larger and have a somewhat yellowish green tint.

**Fawcus's Picric Acid and Brilliant Green Medium.**—This is a modification of Conradi's medium which has been used with great success at the Royal Army Medical College in the investigation of typhoid carriers. It is made as follows: To 900 c.c. tap water add 5 grms. sodium taurocholate (which is commercially prepared from ox bile), 30 grms. powdered agar, 30 grms. Witte's peptone, 5 grms. sodium chloride; steam for three hours, clear with white of egg, filter through cotton wool, and bring to a reaction of +15 with normal lactic acid or caustic soda, and sterilise. Dissolve 10 grms. lactose in 100 c.c. sterile distilled water, and add to melted agar. Mix and filter through Chardin paper, sterilise carefully, and store in 100 c.c. flasks. For use, add to each 100 c.c. flask 2 c.c. of a 1-1000 watery solution of brilliant green and 2 c.c. of a 1 per cent. watery solution of picric acid. Pour into large Petri dishes, and leave these to stand inverted at 37° C. till the surface hardens. Inoculate as usual. Colonies of *b. typhosus* of twenty-four hours' growth are of about 1 mm. in diameter, transparent and refracting; those of *b. coli*, on the other hand, have a deep green centre, though later typhoid colonies may also present a pale green centre.

In the case of several of the special media used for the isolation of typhoid bacilli under circumstances where other bacteria are present, a difficulty arises from the fact that the agglutinability of the strains isolated appears to be affected by substances present in the media. The application of this important confirmatory diagnostic method is thus interfered with. This is said not to occur with the Conradi brilliant green method, and we have found with this medium that, if typhoid colonies do not at once clump with typhoid serum, daily sub-culture on ordinary agar yields in a few days a culture to which the agglutination test can be applied.

**Endo's Medium.**—This is another of the modern media introduced for facilitating the separation of the *b. typhosus* from stools, etc. It is made as follows: A litre of 3 per cent. agar is prepared with the usual



constituents, and is boiled, filtered, and rendered neutral. It is then made alkaline by the addition of 10 c.c. of a 10 per cent. solution of sodium hydrate, and there are added 10 grammes of chemically pure milk sugar (free from cane sugar) and 5 c.c. of a filtered saturated alcoholic solution of basic fuchsin. After thorough mixing there is added 25 c.c. of a freshly prepared 10 per cent. solution of sodium sulphite, the effect of this step being to remove the colour of the fuchsin so that the finished medium when cool is quite colourless. Of the medium 15 c.c. are placed in each of a number of tubes, these are steamed for fifteen minutes and must then be kept in the dark. For use the contents of a tube are poured into a sterile Petri capsule, allowed to set in a still, dustless atmosphere, and are then inoculated as in the other methods described. After twenty-four hours' growth colonies of *b. coli* appear red, while those of *b. typhosus* are colourless. Endo also claims for his medium that typhoid bacilli isolated by its means are agglutinable by a typhoid serum. The rationale of the colour reaction appears to be that fuchsin, which is rosanilin hydrochlorate ( $C_{20}H_{19}N_3HCl$ ), is reduced to rosanilin (a colourless substance) by the sodium sulphite. This colourless base produces a red colour with acids, such as the lactic acid formed by the *b. coli* in its fermentation of lactose.

**MacConkey's Bile-Salt Media.**—These media were introduced for the purpose of differentiating the intestinal bacteria, and have been extensively used for the study of the *b. coli*, *b. typhosus*, *b. dysenteriae*, etc. The characteristic ingredients are bile salts and various sugars. The stock solution is the following: Commercial sodium taurocholate, .5 gramme; Witte's peptone, 2.0 grammes; tap water, 100 c.c. (if distilled water be used, .03 per cent. of calcium chloride should be added). The solution is steamed for two hours, filtered when hot, allowed to stand for twenty-four hours or till sedimentation has occurred, and filtered again. For a liquid medium there is added to this .25 per cent. of a freshly prepared 1 per cent. solution of neutral red<sup>1</sup> and the sugar,—when glucose, dulcitol, or adonitol is used, .5 per cent. is added, in the case of other sugars 1 per cent. The fluid is distributed in Durham's fermentation tubes and sterilised in the steamer for ten minutes on two successive days, care being taken not to overheat the medium.

For bile-salt agar 1.5 to 2 per cent. agar is dissolved in the stock solution in the autoclave, if necessary cleared with white of egg and filtered. Neutral red and a sugar are added, as in the case of the liquid medium. As with Drigalski's medium, it is well to sterilise it in flasks containing 80 c.c., this being an amount sufficient for three Petri capsules. When this medium is used for examining urine or faeces, plates are inoculated as with Drigalski's medium (*supra*); for its use in water examinations, see p. 157.

With reference to their behaviour in MacConkey's fluid medium with glucose, organisms are divided into (1) those which produce both acid and gas; (2) those producing acid only; (3) those growing but not producing either acid or gas; (4) those incapable of growing. *B. coli* belongs to the first group and *b. typhosus* to the second, and to these groups also belong most ordinary organisms growing in faeces, practically none of which are found in the third and fourth classes. Further, a number of ordinary non-

<sup>1</sup> The use of neutral red in a bile-salt medium was first introduced by Grünbaum and Hume. It gives a deep crimson with acids and a yellow-brown with alkalis.



pathogenic organisms and also some that are pathogenic have their free growth inhibited in bile-salt media. Thus, if any growth takes place on this medium when inoculated with, say, water, the probability is that the bacteria have been derived from faeces, but of course further procedures for their identification must be undertaken.

When growth of a bacterium producing acid and gas occurs in neutral-red fluid media the latter turns a rose colour, and gas appears in the Durham's tube. Sometimes a fluorescent appearance is also observed, the significance of which will be discussed in the chapter on *b. coli*. With the neutral-red solid media the colonies of any organism giving rise to acid will be of a rose-red colour.

**Petruschky's Litmus Whey.**—The preparation of this medium, which is somewhat difficult, is as follows: Fresh milk is slightly warmed, and sufficient very dilute hydrochloric acid is added to cause precipitation of the casein, which is now filtered off. Dilute sodium carbonate solution is added up to, but not beyond, the point of neutralisation, and the fluid steamed for one to two hours, by which procedure any casein which has been converted into acid albumin by the hydrochloric acid is precipitated. This is filtered off, and a clear, colourless, perfectly neutral fluid should result. Its chief constituent, of course, will be lactose. To this sufficient Kubel-Tiemann solution of litmus is added, the medium is put into tubes and then sterilised. (This is the original method, but it is better, after the casein has been precipitated, to make the medium slightly alkaline with the sodium carbonate and bring to the boiling-point; then filter, neutralise, add the litmus, and sterilise.) After growth has taken place, the amount of acid formed can be estimated by dropping in standardised soda solution till the tint of an uninoculated tube is reached.

**Elsner's Medium.**—This is another of the media introduced in the study of the comparative reactions of the typhoid bacillus and the *b. coli*. The preparation is as follows: 500 grammes potato are grated up in a litre of water, allowed to stand over night, then strained, and added to an equal quantity of ordinary 15 per cent. peptone gelatin which has not been neutralised. Normal sodium hydrate solution is added till the reaction is feebly acid to litmus, the whole boiled together, filtered, and sterilised. Just before use potassium iodide is added so as to constitute 1 per cent. of the medium. Moore has used a similar agar preparation. Here 500 grammes potato are scraped up in one litre of water, allowed to stand for three hours, strained, and put aside over night. The clear fluid is poured off, made up to one litre, rendered slightly alkaline, 20 grammes agar are added, and the whole is treated as in making ordinary agar. The medium is distributed in test-tubes—10 c.c. to each—and immediately before use, to each is added .5 c.c. of a solution of 10 grammes potassium iodide to 50 c.c. water.

Any one of these media in the hands of a worker accustomed to its use will yield good results. MacConkey's medium is that most used by British workers, and it has the merit of being easily prepared. As the result of a considerable experience we have found it most useful and reliable. Next to it we would place Fawcett's modification of Conradi's brilliant green method.



*Media for growing Trichophyta, Moulds, etc.*

1. *Beer Wort Agar*.—Take beer wort as obtainable from the brewery, and dilute it till it has an s.g. of 1100. Add 1·5 per cent. of powdered agar, and heat in the Koch till it is dissolved (usually about two hours are necessary). Filter rapidly and fill into tubes. Sterilise in the Koch for twenty minutes on three successive days. If the medium is heated too long it loses the capacity of solidifying.

2. *Sabouraud's Media*.—Sabouraud recommends the following media :—

(1) Distilled water . . . . .	1000 c.c.
Maltose ("brute de Chanut") . . . . .	40 grms.
Peptone ("granulée de Chassaing") . . . . .	10 "
Agar . . . . .	18 "
(2) Distilled water . . . . .	1000 c.c.
Glucose ("massée de Chanut") . . . . .	40 grms.
Peptone ("granulée de Chassaing") . . . . .	10 "
Agar . . . . .	18 "

In each case the ingredients are mixed and dissolved by gradually raising to 120° C. in an autoclave. The medium is then rapidly filtered through *papier chardin* (Cogit, 36 Boulevard Saint Michel, Paris); when the filtrate begins only to pass in drops the fluid is transferred to another filter, and this is repeated as often as is necessary. The medium is distributed in wide test-tubes or Erlenmeyer's flasks, plugged with non-absorbent cotton wool, and sterilised by slowly raising the temperature to 120° C.

To use these for isolating, say, the *Tinea tonsurans*, pick out an infected hair, wash in absolute alcohol for a few seconds, then wash in changes of sterile water; cut into small pieces and place these on the surface of the medium; incubate at 24° C. Usually, however, it is unnecessary to disinfect hair or skin scales from which dermatophyta are to be isolated.

## THE USE OF THE ORDINARY CULTURE MEDIA.

The culture of bacteria is usually carried on in test-tubes conveniently  $6 \times \frac{5}{8}$  in. These ought to be very thoroughly washed and dripped, and their mouths plugged with plain cotton wool. They are then sterilised for one hour at 170° C. If the tubes be new, the glass, being usually packed in straw, may be contaminated with the extremely resisting spores of the *b. subtilis*. Cotton-wool plugs are universally used for protecting the sterile contents of flasks and tubes from contamination with the bacteria of the air. A medium thus protected will remain sterile for years. Whenever a protecting plug is removed for even a short time, the sterility of the contents may be endangered. It is well to place the bouillon, gelatin, and agar media in the test-tubes directly after filtration. The media can then be sterilised in the test-tubes.



In filling tubes, care must be taken to run the liquid down the centre, so that none of it drops on the inside of the upper part of the tube with which the cotton-wool plug will be in contact, otherwise the latter will subsequently stick to the glass and its removal will be difficult. In the case of liquid media, test-tubes are filled about one-third full. With the solid media the amount varies. In the case of gelatin

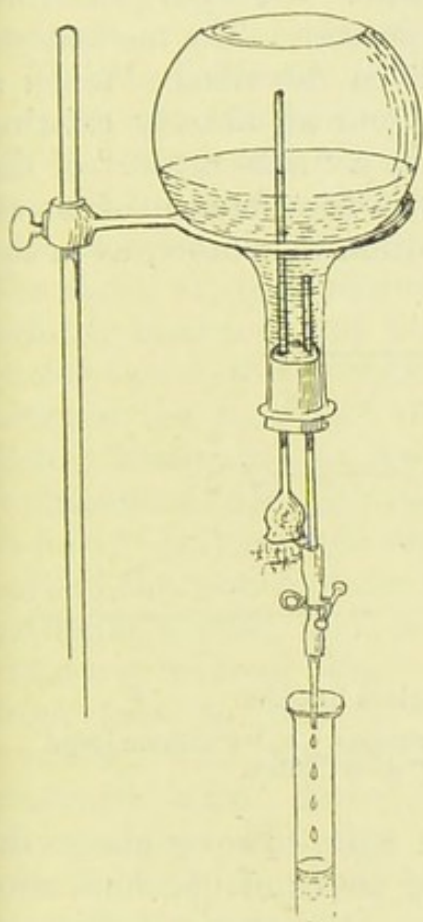


FIG. 12.—Apparatus which may be used for filling tubes. The apparatus explains itself. The indiarubber stopper with its tubes ought to be sterilised before use.

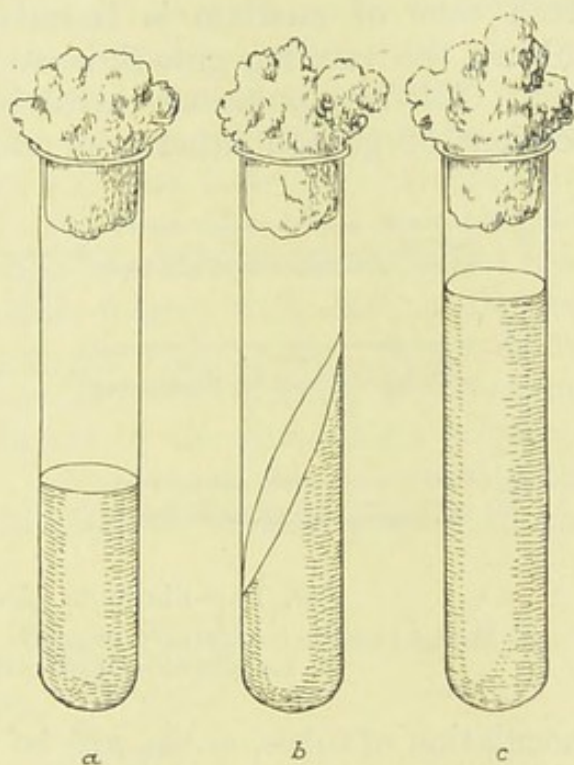


FIG. 13.—Tubes of media.

- a. Ordinary upright tube.
- b. Sloped tube.
- c. "Deep" tube for cultures of anaerobes.

media, tubes filled one-third full and allowed to solidify while standing upright, are those commonly used. With organisms needing an abundant supply of oxygen the best growth takes place on the surface of the medium, and for practical purposes the surface ought thus to be as large as possible. To this end "sloped" agar and gelatin tubes are used. To prepare these, tubes are filled only about one-sixth full, and after sterilisation are allowed to solidify, lying on their sides with their necks supported so that the contents extend



3 to 4 inches up, giving an oblique surface after solidification. Thus agar is commonly used in such tubes (less frequently gelatin is also "sloped"), and this is the position in which blood serum is inspissated. Tubes, especially those of the less commonly used media, should be placed in large jars provided with stoppers, otherwise the contents are apt to evaporate. A tube of medium which has been inoculated with a bacterium, and on which growth has taken place, is called a "culture." A "pure culture" is one in which only one organism is present. The methods of obtaining pure cultures will presently be described. When a fresh tube of medium is inoculated from an already existing culture, the resulting growth is said to be a "sub-culture" of the first. All manipulations involving the transference of small portions of growth either from one medium to another, as in the

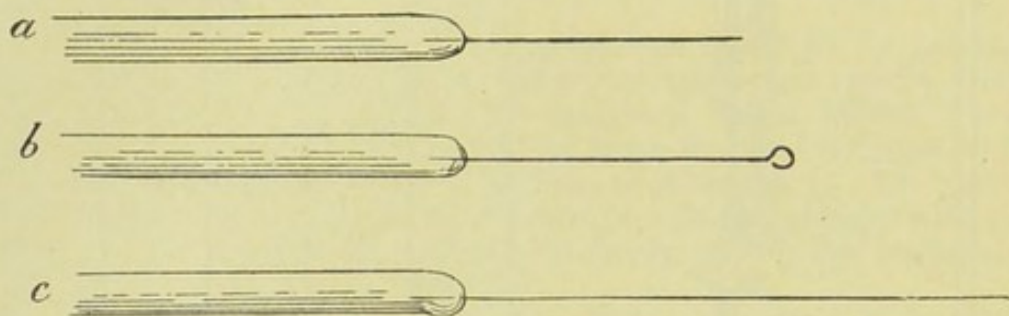


FIG. 14.—Platinum wires in glass handles.

- a.* Straight needle for ordinary puncture inoculations. *b.* "Platinum loop."  
*c.* Long needle for inoculating "deep" tubes.

inoculation of tubes, or, as will be seen later, to cover-glasses for microscopic examination, are effected by pieces of platinum wire (Nos. 24 or 27 Birmingham wire gauge—the former being the thicker) fixed in glass rods 8 inches long.<sup>1</sup> Every worker should have three such wires. Two are  $2\frac{1}{2}$  inches long, one of these being straight (Fig. 14, *a*), and the other having a loop turned upon it (Fig. 14, *b*). The latter is referred to as the platinum "loop" or platinum "eyelet," and is used for many purposes. "Taking a loopful" is a phrase constantly used. The third wire (Fig. 14, *c*) ought to be  $4\frac{1}{2}$  inches long and straight. It is used for making anaerobic cultures. It is also very useful to have at hand a platinum-iridium spud. This consists of a piece of platinum-iridium about  $1\frac{1}{2}$  inches long, 2 mm. broad, and of sufficient thickness to give it a firm consistence; its distal end is

<sup>1</sup> Aluminium rods are made which are very convenient. The end is split with a knife, the platinum wire is inserted and fixed by pinching the aluminium on it in a vice.



expanded into a diamond shape, and its proximal is screwed into an aluminium rod. It is very useful for making scrapings from organs and for disintegrating felted bacterial cultures; in such manipulations the ordinary platinum wire is awkward to work with, as it bends so easily. Cultures on a solid medium are referred to (1) as "puncture" or "stab" cultures (German, *Stichkultur*), or (2) as "stroke" or "slant" cultures (*Strichkultur*), according as they are made (1) on tubes solidified in the upright position, or (2) on sloped tubes.

To inoculate, say, one ordinary upright gelatin tube from another, the two tubes are held in an inverted position between the forefinger and thumb of the left hand with their mouths towards the person holding them; the plugs are twisted round once or twice, to make sure they are not adhering to the glass. The short, straight platinum wire is then heated to redness from point to insertion, and 2 to 3 inches of the glass rod are also passed two or three times through the Bunsen flame. It is held between the right fore and middle fingers, with the needle projecting backwards, *i.e.* away from the right palm. Remove plug from culture tube with right forefinger and thumb, and continue to hold it between the same fingers by the part which projected beyond the mouth of the tube. Now touch the culture with the platinum needle, and, withdrawing it, replace plug. In the same way remove plug from tube to be inoculated, and plunge platinum wire down the centre of the gelatin to within half an inch of the bottom. It must on no account touch the glass above the medium. The wire is then immediately sterilised. A variation in detail of this method is to hold the plug of the tube next the thumb between the fore and middle fingers, and the plug of the other between the middle and ring fingers, then to make the inoculation (Fig. 15). If a tube contain a liquid medium, it must be held in a sloping position between the same fingers, as above. For a stroke culture the platinum loop is

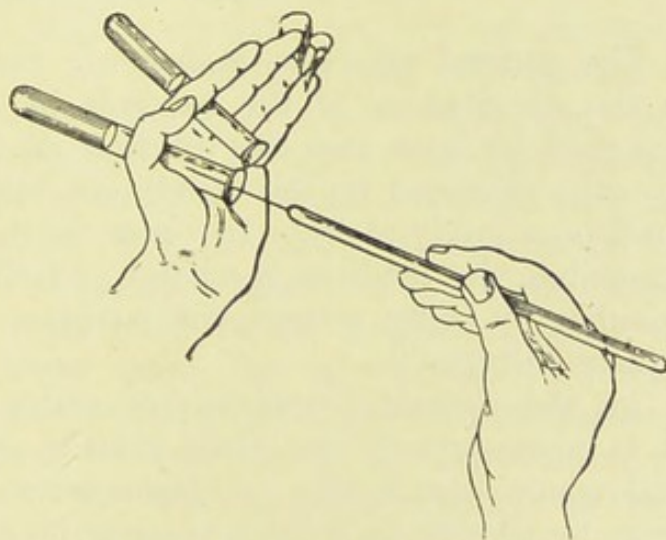


FIG. 15.—Another method of inoculating solid tubes.

the plug of the other between the middle and ring fingers, then to make the inoculation (Fig. 15). If a tube contain a liquid medium, it must be held in a sloping position between the same fingers, as above. For a stroke culture the platinum loop is



used, and a little of the culture is smeared in a line along the surface of the medium from below upwards. In inoculating tubes, it is always well, on removing the plugs, to make sure

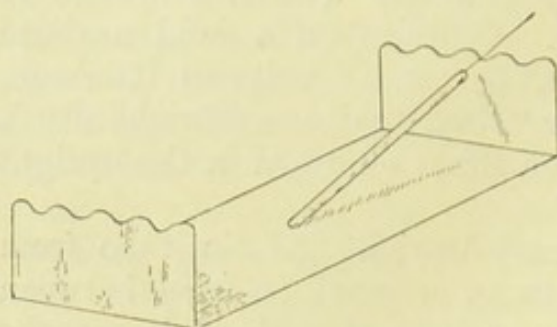


FIG. 16.—Rack for platinum needles.

that no strands of cotton fibre are adhering to the inside of the necks. As these might be touched with the charged needle and the plug thus be contaminated, they must be removed by heating the inoculating needle red-hot and scorching them off with it. When the platinum wires are not in use they may be laid in a rack made by bending up the ends of a piece of tin, as in Fig. 16. To prevent contamination of cultures by bacteria falling on the plugs while these are exposed to the air during inoculation manipulations, some bacteriologists singe the plugs in the flame before replacing. This is, however, in most cases a needless precaution. If the top of a plug be dusty it is best to singe it before extraction.

#### THE METHODS OF THE SEPARATION OF AEROBIC ORGANISMS. PLATE CULTURES.

The general principle underlying the methods of separation is the distribution of the bacteria in one of the solid media liquefied by heat and the dilution of the mixture so that the growths produced by the individual bacteria—called colonies—shall be suitably apart. In order to render the colonies easily accessible, the medium is made to solidify in as thin a layer as possible, by being poured out on glass plates—hence the term “plate cultures.”

As the optimum temperature varies with different bacteria, it is necessary to use both gelatin and agar media. Many pathogenic organisms, *e.g.* pneumococcus, *b. diphtheriæ*, etc., grow too slowly on gelatin to allow its ready use. On the other hand, many organisms, *e.g.* some occurring in water, do not develop on agar incubated at 37° C.

**Separation by Gelatin Media.**—As the naked-eye and microscopic appearances of colonies are often very characteristic, plate cultures, besides use in separation, are often taken advantage of in the description of individual organisms. The plate-culture method can also be used to test whether a tube



culture is or is not pure. The suspected culture is plated (three plates being prepared, as will be described). If all the colonies are the same, then the culture may be held to be pure.

Either simple plates of glass 4 inches by 3 inches are used, or, what are more convenient, circular glass cells with similar overlapping covers. The latter are known as Petri's dishes or capsules (Fig. 17). They are usually 3 inches in diameter and half an inch deep. The advantage of these is that they do not require to be kept level by a special apparatus while the medium is solidifying, and can be readily handled afterwards without admitting impurities. Whether plates or capsules are used, they are washed, dried with a clean cloth, and sterilised for one hour in dry air at  $170^{\circ}$  C., the plates being packed in sheet-iron boxes made for the purpose (see Fig. 18).

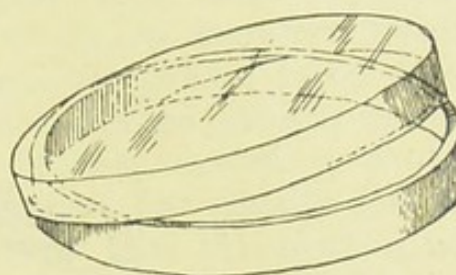


FIG. 17.—Petri's capsule.  
(Cover shown partially raised.)

1. *Glass Capsules*.—While in certain circumstances, as when the number of colonies has to be counted, it is best to use plates of glass, Petri's capsules are to be preferred in the usual laboratory routine for the above reasons.

The contents of three gelatin tubes, marked *a*, *b*, *c*,<sup>1</sup> are liquefied by placing in a beaker of water at any temperature between  $25^{\circ}$  C. and  $38^{\circ}$  C. Inoculate *a* with the bacterial mixture. The amount of the latter to be taken varies, and can only be regulated by experience. If the microscope shows enormous numbers of different kinds of bacteria present, just as much as adheres to the point of a straight platinum needle is sufficient. If the number of bacilli is small, one to three loops of the mixture may be transferred to the medium. Shake *a* well, but not so as to cause many fine air-bubbles to form. Transfer two loops of gelatin from *a* to *b*. Shake *b* and transfer five loops to *c*. The plugs of the tubes are in each case replaced and the tubes returned to the beaker. The contents of the three tubes are then poured out into three capsules. In doing so the plug of each tube is removed and the mouth of the tube passed two or three times through the Bunsen flame, the tube being meantime rotated round a longitudinal axis. Any organisms on its rim are thus killed. The capsules are labelled and set aside till growth takes place.

<sup>1</sup> For marking glass vessels it is convenient to use the red, blue, or yellow oil pencils made for the purpose by Faber.



For accurate work it will be found convenient to carry out the dilutions in definite proportions. The following is the pro-

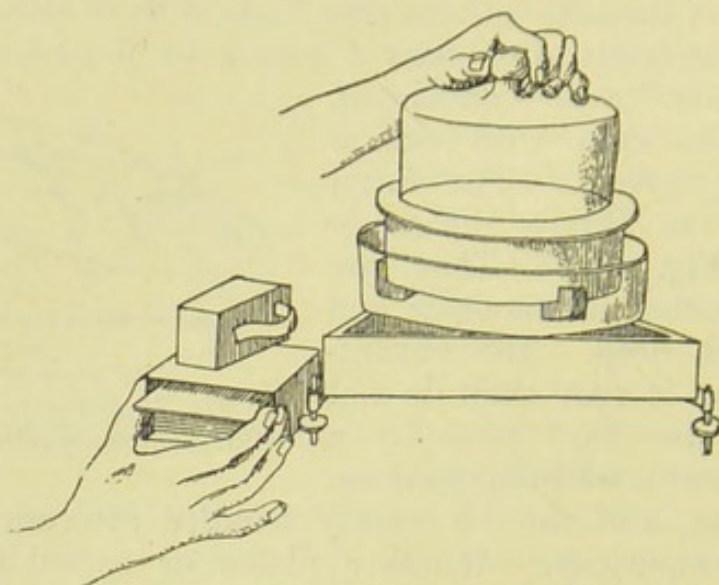


FIG. 18.—Koch's levelling apparatus for use in preparing plates. Hands shown in first position for transferring sterile plate from iron box to beneath bell jar, where it subsequently has the medium poured out upon it.

cedure which we have found very serviceable: In a number of small sterile test-tubes .95 c.c. sterile water is put. To the first

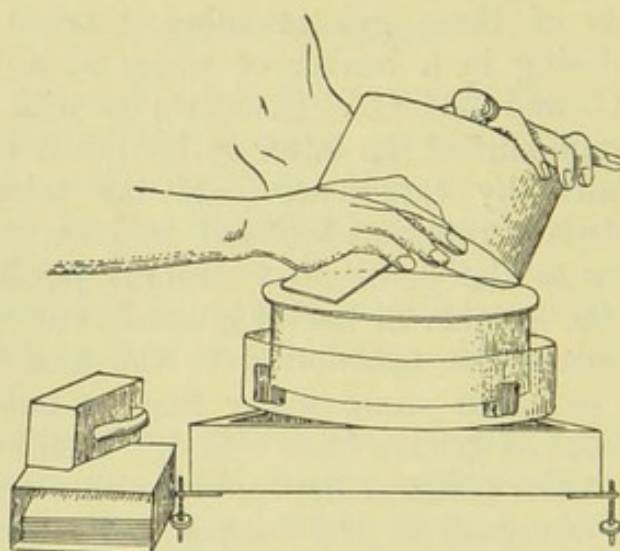


FIG. 19.—Koch's levelling apparatus. Hands shown in second position just as the plate is lowered on to the ground glass surface. By executing the transference of the plate from the box in this way, the surface which was undermost in the latter is uppermost in the leveller, and thus never meets a current of air which might contaminate it.

tube we add .05 c.c. of the bacterial mixture. The contents of the tube are well shaken up, and the pipette is sterilised by



being washed out with boiling water. It is allowed to cool, and .05 c.c. of fluid is transferred from the first tube to the second. By a similar procedure .05 c.c. is transferred from the second to the third, and so on. There is thus effected a twenty-fold dilution in each successive tube. After these steps have been carried out, a definite amount, say, .05 c.c., is transferred from each tube to a tube of melted gelatin,—the gelatin being afterwards plated and the colonies counted when growth occurs. The number of tubes required will vary according to the number of bacteria in the original mixture, but usually four or five will be sufficient. It is quite evident that this method not only enables us to separate bacteria, but if necessary gives us a means of estimating exactly the number in the original mixture.

The colonies appear as minute rounded points, whitish or variously coloured. Their characters can be more minutely studied by means of a hand-lens or by inverting the capsule on the stage of a microscope and examining with a low power through the bottom. From their characters, colour, shape, contour, appearance of surface, liquefaction or non-liquefaction of the gelatin, etc., the colonies can be classified into groups. Further aid in the grouping of the varieties is obtained by making film preparations and examining them microscopically. Gelatin or agar tubes may then be inoculated from a colony of each variety, and the growths obtained are then examined both as to their purity and as to their special characters, with a view to their identification (p. 137).

2. *Glass Plates* (Koch).—When plates of glass are to be used, an apparatus on which they may be kept level while the medium is solidifying is, as has been said, necessary. An apparatus devised by Koch is used (Figs. 18, 19). This consists of a circular plate of glass (with the upper surface ground, the lower polished), on which the plate used for pouring out the medium is placed. The latter is protected from the air during solidification by a bell jar. The circular plate and bell jar rest on the flat rim of a circular glass trough, which is filled quite full with a mixture of ice and water, to facilitate the lowering of the temperature of whatever is placed beneath the bell jar. The glass trough rests on corks on the bottom of a large circular trough, which catches any water that may be spilled. This trough in turn rests on a wooden triangle with a foot at each corner, the height of which can be adjusted, and which thus constitutes the levelling apparatus. A spirit level is placed where the plate is to go, and the level of the ground glass plate thus assured. There is also prepared a "damp chamber," in which the plates are to be stored after being made. This consists of a circular glass trough with a similar cover. It is sterilised by being washed outside and inside with perchloride of mercury 1-1000, and a circle of filter-paper moistened with the same is laid on its bottom. Glass benches on which the plates may be laid are similarly purified.



To separate organisms by this method, three tubes, *a*, *b*, *c*, are inoculated as in using Petri's capsules (p. 57). The hands having been washed in perchloride of mercury 1-1000 and dried, the plate box is opened, and a plate lifted by its opposite edges and transferred to the levelled ground glass (as in Figs. 18, 19). The bell jar of the leveller being now lifted a little, the gelatin in tube *a* is poured out on the surface of the sterile plate, and, while still fluid, is spread by stroking with the rim of the tube. After the medium solidifies, the plate is transferred to the moist chamber as rapidly as possible, so as to avoid atmospheric contamination. In doing this, it is advisable to have an assistant to raise the glass covers. Tubes *b* and *c* are similarly treated, and the resulting plates stacked in series on the top of *a*. The chamber is labelled and set aside for a few days till the colonies appear on the gelatin plates. The further procedure is of the same nature as with Petri's capsules.

3. *Esmarch's Roll Tubes*.—Here the principle is that of dilution as before. In each of three test-tubes  $1\frac{1}{4}$  or  $1\frac{1}{2}$  inch in diameter, gelatin to the depth of three-quarters of an inch is placed.

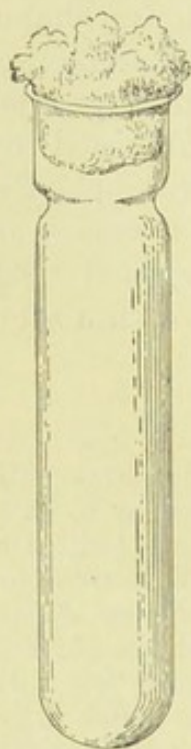


FIG. 20.  
Esmarch's tube  
for roll culture.

These are sterilised. The gelatin is melted and inoculated in series with the bacterial mixture as in making plate cultures, but instead of being poured out it is rolled in a nearly horizontal position under a cold tap or on a block of ice till it solidifies as a uniformly thin layer on the inside of the tube. Practically we deal with a cylindrical sheet of gelatin instead of a flat one. A convenient form of tube for this method is one with a constriction a short distance below the plug of cotton wool (Fig. 20). The great disadvantage of the method is, that if organisms liquefying the gelatin be present, the liquefied gelatin contaminates the rest of the medium.

**Separation by Agar Media.**—1. *Agar Plates*.

—The only difference between the technique here and that with gelatin depends on the difference in the melting-points of the two media. Agar, we have said, melts at  $98^{\circ}$  C., and becomes again solid a little under  $40^{\circ}$  C. As it is dangerous to expose organisms to a temperature much above  $42^{\circ}$  C., it is necessary in preparing tubes

of agar to be used in plate cultures first to melt the agar, by boiling in a vessel of water for a few minutes, and then to cool it to about  $42^{\circ}$  C. before inoculating. The manipulation must be rapidly carried out, as the margin of time, before solidification occurs, is narrow; otherwise the details are the same as for gelatin. Esmarch's tubes are not suitable for use



here, as the agar does not adhere well to the sides. If to the agar 2 per cent. of a strong watery solution of pure gum arabic is added, Esmarch's tubes may, however, be used.

2. *Separation by Stroking Mixture on Surface of Agar Media.*—The bacterial mixture, instead of being mixed in the medium, is spread out on its surface. The method may be used both when the bacteria to be separated are in a fluid, and when contained in a fairly solid tissue or substance, such as a piece of diphtheritic membrane. In the case of a tissue, for example, a small portion entangled in the loop of a platinum needle is stroked in successive parallel longitudinal strokes on sloped agar, the same aspect being brought in contact with the agar in all the strokes. Three strokes may be made in each tube, and three tubes are usually sufficient. In this process the organisms on the surface of the tissue are gradually rubbed off, and when growth has taken place it will be found that in the later strokes the colonies are less numerous than in the earlier, and sufficiently far apart to enable parts of them to be picked off without the needle touching any but one colony. When, as in the case of diphtheritic membrane, putrefactive organisms may be present on the surface of the tissue, these can be in great part removed by washing it well in cold water previously sterilised (*vide* Diphtheria). In the case of liquids, the loop is charged and similarly stroked. Tubes thus inoculated must be put in the incubator in the upright position and must be handled carefully, so that the condensation water, which is always present in incubated agar tubes, may not run over the surface. Agar, poured out in a Petri's capsule and allowed to stand till firm, may be used instead of successive tubes. Here a sufficient number of strokes can be made in one capsule. Sloped blood-serum tubes may be used instead of agar. The method is rapid and easy, and gives good results.

**Separation of Pathogenic Bacteria by Inoculation of Animals.**—It is found difficult and often impossible to separate by ordinary plate methods certain pathogenic organisms, such as *b. tuberculosis*, *b. mallei*, and the pneumococcus, when such occur in conjunction with other bacteria. These grow best on special media, and the first two (especially the tubercle bacillus) grow so slowly that the other organisms present outgrow them, cover the whole plates, and make separation impossible. The method adopted in such cases is to inoculate an animal with the mixture of bacilli, wait until the particular disease develops, kill the animal, and with all aseptic precautions (*vide* p. 145) inoculate tubes of suitable media from characteristic lesions



situated away from the seat of inoculation, *e.g.* from spleen in the case of *b. tuberculosis*, spleen or liver in the case of *b. mallei*, and heart blood in the case of pneumococcus.

**Separation by killing Non-spored Forms by Heat.**—This is a method which has a limited application. As has been said, the spores of a bacterium resist heat more than the vegetative forms. When a mixture contains spores of one bacterium and vegetative forms of this and other bacteria, then if the mixture be boiled for a few minutes all the vegetative forms will be killed, while the spores will remain alive and will develop subsequently. This method can be easily tested in the case of cultivating *b. subtilis* from hay infusion. A little chopped-up hay is placed in a flask of water, which is boiled for about ten minutes. On this being allowed to cool and stand, in a day or two a scum forms on the surface, which is found to be a pure culture of the bacillus subtilis. The method is also often used to aid in the separation of *b. tetani*, *vide infra*.

#### THE PRINCIPLES OF THE CULTURE OF ANAEROBIC ORGANISMS.

All ordinary media, after preparation, may contain traces of free oxygen, and will absorb more from the air on standing. (1) For the growth of anaerobes this oxygen may be expelled by the prolonged passing of an inert gas, such as hydrogen, through the medium (liquefied if necessary). Further, the medium must be kept in an atmosphere of the same gas while growth is going on. (2) Media for anaerobes may be kept in contact with the air, if they contain a reducing agent which does not interfere with bacterial growth. Such an agent takes up any oxygen which may already be in the medium, and prevents further absorption. The reducing body used is generally glucose, though formate of sodium may be similarly employed. The preparation of such media has already been described (pp. 37, 38). In this case the medium ought to be of considerable thickness.

*The Supply of Hydrogen for Anaerobic Cultures.*—The gas is generated in a large Kipp's apparatus from pure sulphuric acid and pure zinc. It is passed through three wash-bottles, as in Fig. 21. In the first is placed a solution of lead acetate (1 in 10 of water) to remove any traces of sulphuretted hydrogen. In the second is placed a 1 in 10 solution of silver nitrate to remove any arseniatted hydrogen which may be present if the zinc is not quite pure. In the third is a 10 per cent. solution of pyrogallie acid in caustic potash solution (1:10) to remove any traces of oxygen. The tube leading from the last bottle to the vessel containing the medium ought to be sterilised by passing through a



Bunsen flame, and should have a small plug of cotton wool in it to filter the hydrogen germ-free.

*Pyrogallate of Potassium for Anaerobic Cultures.*—In arranging for the absorption of oxygen by this substance the proportions used in Bulloch's separation method may be employed. Here 109 grms. solid caustic potash are dissolved in 145 c.c. water, and to this 2-4 grms. pyrogallol are added.

**Separation of Anaerobic Organisms.**—(a) *By Roll-tubes.*—A  $1\frac{1}{4}$  inch test-tube has as much gelatin put into it as would be used in the Esmarch roll-tube method. It is corked with an indiarubber stopper having two tubes passing through it, as in Fig. 22. The ends of the tubes are partly drawn out as shown,

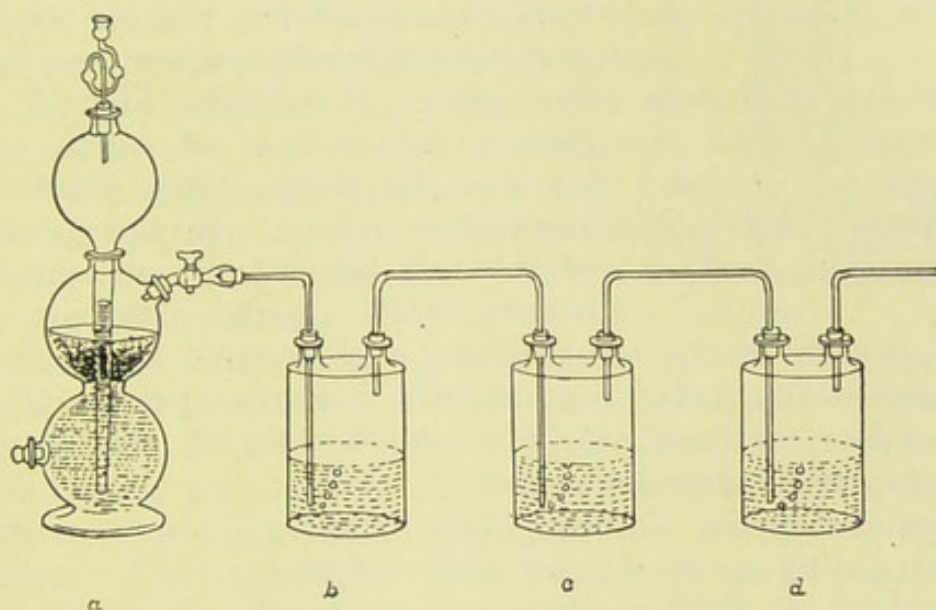


FIG. 21.—Apparatus for supplying hydrogen for anaerobic cultures.

a. Kipp's apparatus for manufacture of hydrogen. b. Wash-bottle containing 1-10 solution of lead acetate. c. Wash-bottle containing 1-10 solution of silver nitrate. d. Wash-bottle containing 1-10 solution of pyrogallous acid. (b, c, and d are intentionally drawn to a larger scale than a to show details.)

and covered with plugs of cotton wool. Three such test-tubes are prepared, and they are sterilised in the steam steriliser (p. 28). After sterilisation the gelatin is melted and one tube inoculated with the mixture containing the anaerobes; the second is inoculated from the first, and the third from the second, as in making ordinary gelatin plates. After inoculation the gelatin is kept liquid by the lower ends of the tubes being placed in water at about  $30^{\circ}\text{C}.$ , and hydrogen is passed in through tube *x* for twenty minutes. The gas-supply tubes are then completely sealed off at *x* and *i*, and each test-tube is rolled as in Esmarch's method till the gelatin solidifies as a thin layer on the internal surface. A little hard paraffin may be run between the rim of the



test-tube and the stopper and round the perforations for the gas-supply tubes, to ensure that the apparatus is airtight. The gelatin is thus in an atmosphere of hydrogen in which the colonies may develop. The latter may be examined and isolated in a way which will be presently described. The method is admirably suited for all anaerobes which grow at the ordinary temperature.

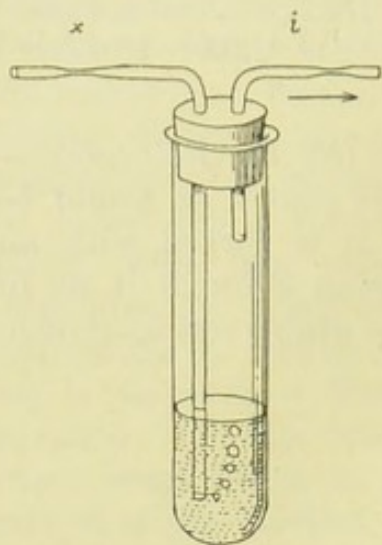


FIG. 22.—Esmarch's roll-tube adapted for culture containing anaerobes.

through each of the latter passes a glass tube on which is a stop-cock. One tube, bent slightly just after passing through the stopper, extends nearly to the bottom of the chamber; the other terminates immediately below the stopper. In using the apparatus there is set on the base-plate a shallow dish, of slightly less diameter than that of the bell jar, and having a little heap of from 2 to 4 grammes of dry pyrogallic acid placed in it towards one side. Culture plates made in the usual way can be stacked on a frame of glass rods resting on the edges of the dish, or a beaker containing culture tubes can be placed in it. The bell jar is then placed in position so that the longer glass tube is situated over that part of the bottom of the shallow dish farthest away from the pyrogallic acid, and the bottom and stoppers are luted. The air in the bell jar is now expelled by passing a current of hydrogen through

(b) **Bulloch's Apparatus for Anaerobic Culture.**—This can be recom-

mended for plating out mixtures containing anaerobes, and for obtaining growths (especially surface growths) of the latter. It consists (Fig. 23) of a glass plate as base on which a bell jar can be firmly luted down with unguentum resinae. In the upper part of the bell jar are two apertures furnished with ground stoppers, and

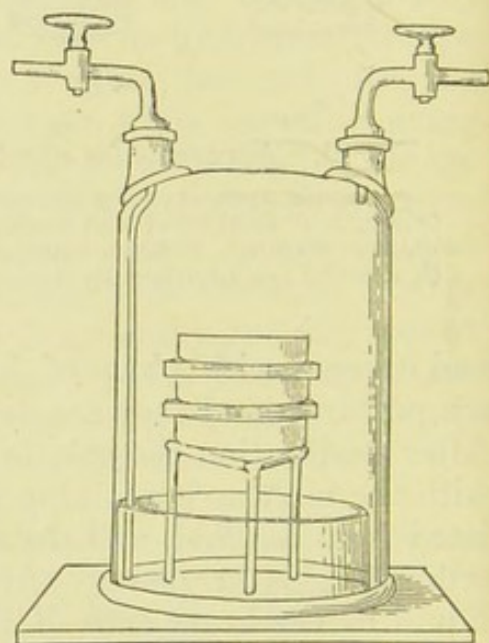


FIG. 23.—Bulloch's apparatus for anaerobic plate cultures.



the short glass tube, and both stoppers are closed. A partial vacuum is then effected in the jar by connecting up the short tube with an air-pump, opening the tap, and giving a few strokes of the latter. A solution of 109 grms. solid caustic potash dissolved in 145 c.c. water is made, and into the vessel containing it a rubber tube connected with the long glass tube is made to dip, and the stopper of the latter being opened, the fluid is forced into the chamber and spreads over the bottom of the shallow dish; potassium pyrogallate is thus formed, which absorbs any free oxygen still present. Before the whole of the fluid is forced in the rubber tube is placed in a little boiled water, and this, passing through the glass tubes, washes out the potash and prevents erosion of the glass. The whole apparatus may be placed in the incubator till growth occurs.

It is often advisable in dealing with material suspected to contain anaerobes to inoculate an ordinary deep glucose agar tube with it, and, incubating for 24 or 48 hours, to then apply an anaerobic separation method to the resultant growth. Sometimes the high powers of resistance of spores to heat may be taken advantage of in aiding the separation (*vide* Tetanus).

**Cultures of Anaerobes.**—When by one or other of the above methods separate colonies have been obtained, growth may be maintained on media in contact with ordinary air. The media generally used are those which contain reducing agents, and the test-tubes containing the medium must be filled to a depth of 4 inches. They are sterilised as usual, and are called “deep” tubes. The long straight platinum wire is used for inoculating, and it is plunged well down into the “deep” tube. A little air gets into the upper part of the needle track, and no growth takes place there, but in the lower part of the needle track growth occurs. From such “deep” cultures growths may be maintained indefinitely by successive sub-cultures in similar tubes. Even ordinary gelatin and agar can be used in the same way if the medium is heated to boiling-point before use to expel any absorbed oxygen.

*Carroll's Method for Anaerobic Cultures.*—This may be used with culture tubes containing any of the media suitable for anaerobes, with Esmarch's roll-tubes, or with fermentation tubes. There is required a dry tube of the same diameter as the culture tube, a short U-shaped glass tube, and two pieces of rubber tubing all of like diameter. The culture tube having been inoculated, the plug is pushed home below the lip of the tube. The ends of the U-tube are smeared with vaseline and a rubber tube slipped over each; the end of the culture tube being similarly



treated, the free end of one of the rubber tubes is pushed over it till the glass of the U-tube is in contact with the glass of the culture tube. In the dry tube 1 or 2 grammes of pyrogallie acid are placed, and the powder is packed down with a layer of filter paper. Ten or twenty cubic centimetres of a 10 per cent. solution of sodium hydrate are then poured in, and the tube is quickly connected up by the rubber tubing with the other end of the U-tube. In this apparatus the oxygen is absorbed by the sodium pyrogallate, and the conditions for anaerobic growth are fulfilled.

*Buchner's Anaerobic Tube.*—This may be used either for maintaining surface growths of anaerobes or for keeping free from oxygen sloped culture media which are being used for separating anaerobes from mixtures. Dry pyrogallol is placed in a cylindrical jar of diameter sufficient to contain the tube or tubes of media. The tubes are then inserted, potassium hydrate solution (p. 65) is poured into the jar, and its mouth quickly stoppered with a rubber or glass stopper. The stopper is made airtight by sealing with paraffin. The pyrogallol absorbs the oxygen in the jar, and thus the cultures are kept in oxygen-free surroundings.

*Growth in Tubes with Pyrogallol-saturated Plug.*—Sloped cultures can be maintained oxygen-free as follows:—The medium is placed in a long test-tube and inoculated. The plug of the tube (which ought to be rather tight) is pushed down into the tube, and a little dry pyrogallol placed on the top of it. A few drops of the potassium hydrate solution are dropped on the crystals, and a second plug is inserted in the mouth of the tube. This is pushed home, and melted paraffin run on to the top to prevent access of outside air.

**Cultures of Anaerobes in Liquid Media.**—It is necessary to employ such in order to obtain the toxic products of the growth of anaerobes. Glucose broth is most convenient. It is placed either (1) in a conical flask with a lateral opening and a perforated indiarubber stopper, through which a bent glass tube passes, as in Fig. 24, *a*, by which hydrogen may be delivered, or (2) in a conical flask with a rubber stopper furnished with two holes, as in Fig. 24, *b*, through a tube in one of which hydrogen is delivered, while through the tube in the other the gas escapes. The inner end of the gas delivery tube must in either case be below the surface of the liquid; the inner end of the lateral nozzle in the one case, and the inner end of the escape tube in the other, must of course be above the surface of the liquid. The single tube in the one case and the two tubes



in the other ought to be partially drawn out in a flame to facilitate subsequent complete sealing. The ends of the tubes through which the gas is to pass are previously protected by pieces of cotton wool tied on them. It is well previously to place in the tube, through which the hydrogen is to be delivered, a little plug of cotton wool. The flask being thus prepared, it is sterilised by methods B (2) or B (3). On cooling it is ready for inoculation. In the case of the flask with the lateral nozzle, the cotton-wool covering having been momentarily removed, a wire charged with the organism is passed down to the bouillon. In the other kind of flask the stopper must be removed for an instant to admit the wire. The flask is then connected with

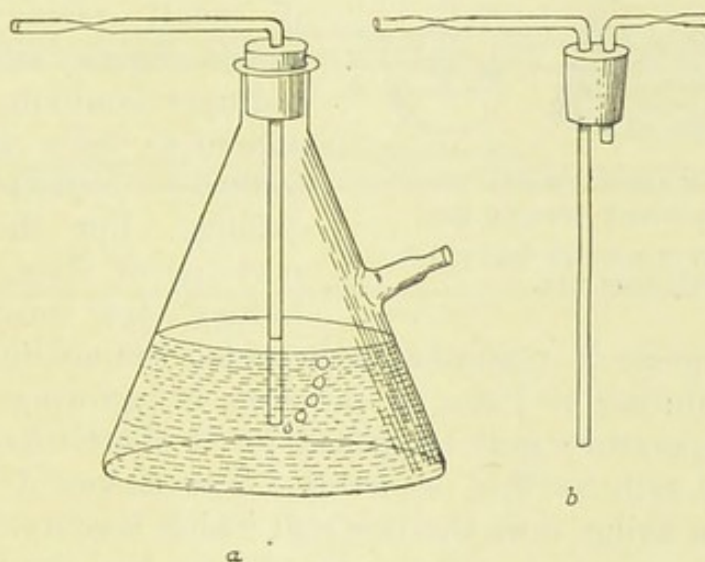


FIG. 24.

*a.* Flask for anaerobes in liquid media. Lateral nozzle and stopper fitted for hydrogen supply. *b.* A stopper arranged for a flask without lateral nozzle.

the hydrogen apparatus by means of a short piece of sterile indiarubber tubing, and hydrogen is passed through for half an hour. In the case of flask (1), the lateral nozzle is plugged with melted paraffin and covered with alternate layers of cotton wool and paraffin, the whole being tightly bound on with string. The entrance tube is now completely drawn off in the flame before being disconnected from the hydrogen apparatus. In the case of flask (2), first the exit tube and then the entrance tube are sealed off in the flame before the flask is disconnected from the hydrogen apparatus. It is well in the case of both flasks to run some melted paraffin all over the rubber stopper. Sometimes much gas is evolved by anaerobes, and in dealing with an organism where this will occur, provision must be made for its escape. This is conveniently done by leading down the



exit tube, and letting the end just dip into a trough of mercury (Fig. 25), or into mercury in a little bottle tied on to the end of the exit tube. The pressure of gas within causes an escape

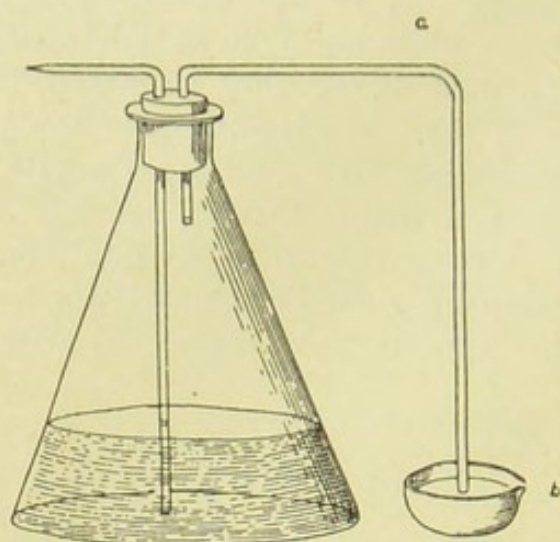


FIG. 25.—Flask arranged for culture of anaerobes which develop gas.

*b* is a trough of mercury into which exit tube dips.

at the mercury contact, which at the same time acts as an efficient valve. The method of culture in fluid media is used to obtain the soluble products of such anaerobes as the tetanus bacillus.

*The Method of Tarozi.*—This observer has found that if small pieces of fresh sterile organs are added to ordinary bouillon, growth of anaerobes takes place under ordinary atmospheric conditions. For this purpose, portions of liver, spleen, or kidney are most suitable.

If after the piece of tissue has been added the medium is boiled for a few minutes it loses its property of growing anaerobes, but the temperature may be raised for a short time almost to boiling-point without this occurring. The tissue of the organs gives off something into the medium which favours the growth of anaerobes, as can be shown by placing the tissue for some time in the medium and then removing it; thereafter the medium is suitable for anaerobic growth.

When it is desired to grow anaerobes on the surface of a

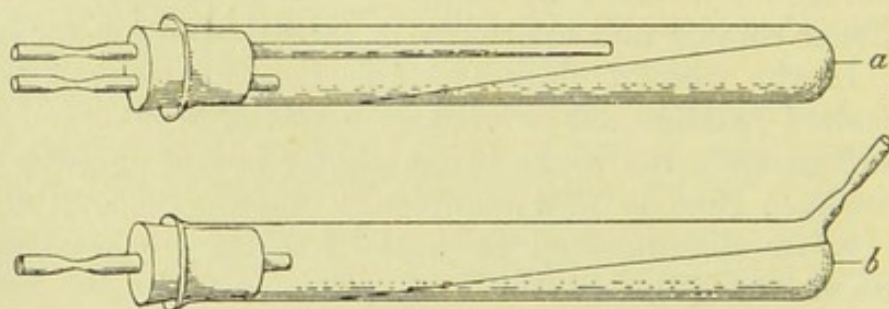


FIG. 26.—Tubes for anaerobic cultures on the surface of solid media.

solid medium such as agar, tubes of the form shown in Fig. 26, *a* and *b*, may be used. A stroke culture having been made, the air is replaced by hydrogen as just described, and the tubes are



fused at the constrictions. Such a method is of great value when it is required to get the bacteria free from admixture of medium, as in the case of staining flagella.

#### MISCELLANEOUS METHODS.

**Hanging-drop Cultures.**—It is often necessary to observe micro-organisms alive, either to watch the method and rate of their multiplication, or to investigate whether or not they are motile. This is effected by making hanging-drop cultures. The method in the form to be described is only suitable for aerobes. For this special slides are necessary. Two forms are in use, and

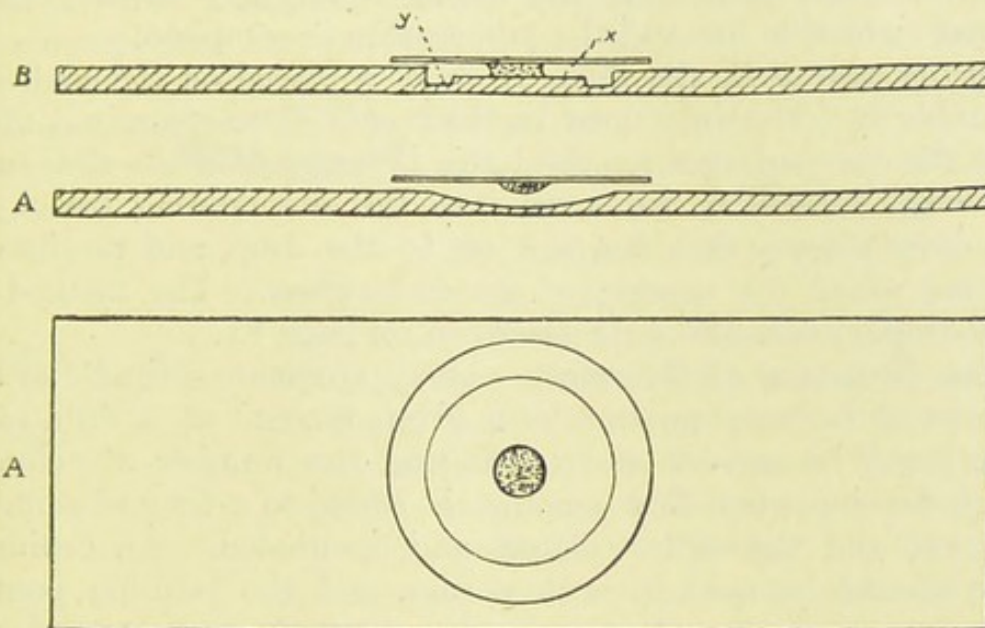


FIG. 27.

- A. Hollow-ground slide for hanging-drop cultures shown in plan and section.  
B. Another form of slide for similar cultures.

are shown in Fig. 27. In A there is ground out on one surface a hollow having a diameter of about half an inch. That shown in B explains itself. The slide to be used and a cover-glass are sterilised by hot air in a Petri's dish, or simply by being heated in a Bunsen and laid in a sterile Petri to cool. In the case of A, one or other of two manipulation methods may be employed. (1) If the organism be growing in a liquid culture, a loop of the liquid is placed on the middle of the under surface of the sterile cover-glass, which is held in forceps, the points of which have been sterilised in a Bunsen flame. If the organism be growing in a solid medium, a loopful of sterile bouillon is placed on the cover-glass in the same position, and a *very* small quantity of the culture (picked up with a platinum needle) is



rubbed up in the bouillon. The cover is then carefully lowered over the cell on the slide, the drop not being allowed to touch the wall or the edge of the cell. The edge of the cover-glass is covered with vaseline, and the preparation is then complete and may be placed under the microscope. If necessary, it may be first incubated and then examined on a warm stage. (2) The sterile cover-glass is placed on a sterile plate (an ordinary glass plate used for plate cultures is convenient). The drop is then placed on its *upper* surface, the details being the same as in the last case. The edge of the cell in the slide is then painted with vaseline, and the slide, held with the hollow surface downwards, is lowered on to the cover-glass, to the rim of which it of course adheres. The slide with the cover attached is then quickly turned right side up, and the preparation is complete.

In the case of B, the drop of fluid is placed on the centre of the table *x*. The drop must be thick enough to come in contact with the cover-glass when the latter is lowered on the slide, and not large enough to run over into the surrounding trench *y*. The cover-glass is then lowered on to the drop, and vaseline is painted along the margin of the cover-glass. The method of microscopic examination is described on page 91.

**The Counting of Colonies.**—An approximate estimate of the number of bacteria present in a given amount of a fluid (say, water) can be arrived at by counting the number of colonies which develop when that amount is added to a tube of suitable medium, and the latter plated and incubated. An ordinary plate should be used in such a case, and the medium poured

out in as rectangular a shape as possible. For the counting, an apparatus such as is shown in Fig. 28 is employed. This consists of a sheet of glass ruled into squares as indicated, and supported by its corners on wooden blocks. The table to which these blocks are attached has a dark surface. The plate-culture

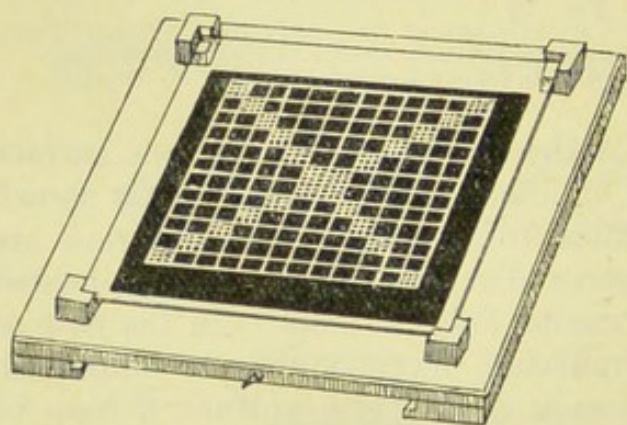


FIG. 28.—Apparatus for counting colonies.

containing the colonies is laid on the top of the ruled glass. The numbers of colonies in, say, twenty of the smaller squares are then counted, and an average struck. The total



number of squares covered by the medium is then taken, and by a simple calculation the total number of colonies present can be obtained. Plate-cultures in Petri's dishes are sometimes employed for purposes of counting. The bottoms of such dishes are, however, never flat, and the thickness of the medium thus varies in different parts. If these dishes are to be used, a circle of the same size as the dish can be drawn with Chinese white on a black card, the circumference divided into equal arcs, and radii drawn. The dish is then laid on the card, the number of colonies in a few of the sectors counted, and an average struck as before. In counting colonies it is always best to aid the eye with a small hand-lens.

**Method of Counting Living Bacteria in a Culture.**—This is accomplished by putting into practice a dilution method

such as that described on p. 58. Measured amounts of high dilutions are plated, and the numbers of colonies which subsequently develop are counted. In applying such a

method it is necessary to have pipettes capable of measuring small quantities of fluid. Those discharging .05 and .1 c.c. will be found convenient, and such pipettes can have subdivisions which enable them to be used for measuring still smaller fractions of a cubic centimetre. Pipettes of this kind can be obtained at the instrument makers. Wright has described a method by which a pipette (Fig. 29) for measuring small quantities of fluid can be made from ordinary quill tubing. The method is as follows:—A piece of quill tubing about 15 cm. long is drawn out to a capillary stem. A standard 5 c.mm. pipette (such as that of the Gower's hæmocytometer), or the pipette described later on p. 118, is filled with mercury and the metal transferred to the capillary stem and run down to near its extremity; the upper and lower limits of the mercury are marked with an oil pencil; the mercury is then displaced up the tube till its previously distal end is at the proximal of the two

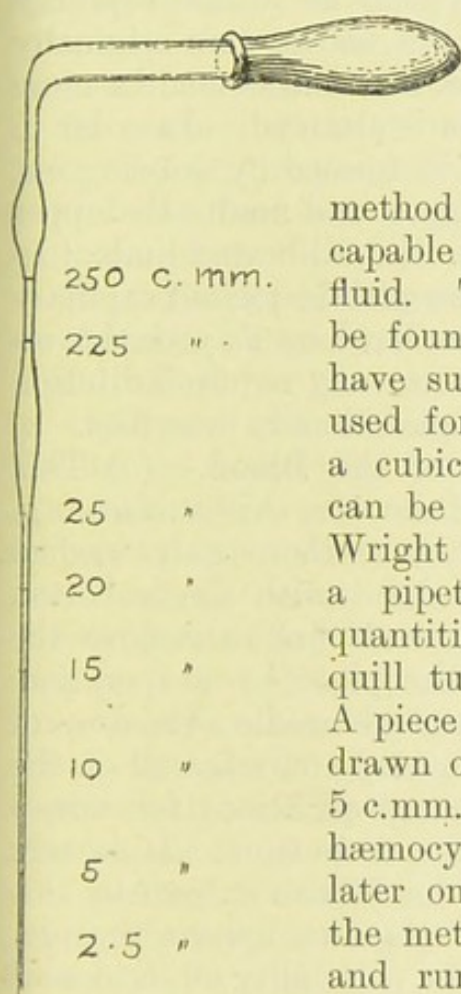


FIG. 29. — Wright's 250 c.mm. pipette fitted with nipple.

marks, and a third mark is made at the new position of the upper end of the droplet; the manipulation is repeated



three more times, and finally the tip of the tube beyond the lowest mark is broken off. Thus on the capillary part of the pipette we have five divisions, each capable of holding 5 c.mm. of fluid. The rest of the pipette is now calibrated so as to determine that part capable of containing 225 c.mm. and 250 c.mm. This is done by placing a rubber nipple on the wide end of the pipette and sucking up some water tinted with, say, methylene-blue till the 25 c.mm. mark is reached; a small air-bubble is then allowed to enter the pipette, then other 25 c.mm. of fluid, then another bubble, and so on till nine volumes each of 25 c.mm. have been sucked up. A mark is then made on the tube at the upper level of this amount, other 25 c.mm. are sucked up, and another mark made. The fluid is expelled, the tube dried, and that part containing the 225 and 250 marks is drawn out into an almost capillary diameter, the manipulation by which the marks were originally arrived at is repeated, and thus in the new marks made a more accurate calibration for these amounts is attained. In order to form a safety chamber a second bulb is formed by drawing out the tube a little higher up, as in the figure, and finally the upper inch or two are bent at right angles to the calibrated limb. In doing this a loop may be thrown on the plastic melted capillary tube exactly in the way in which a similar loop may be thrown on a piece of cord. With such a pipette any required dilution of a culture can be made on the principles already described.

**The Bacteriological Examination of the Blood.**—(a) This may be done by taking a small drop from the skin surface, *e.g.* the lobe of the ear. The part should be thoroughly washed with 1-1000 corrosive sublimate and dried with sterile cotton wool. It is then washed with absolute alcohol to remove the antiseptic, drying being allowed to take place by evaporation. A prick is then made with a sterile surgical needle; the drop of blood is caught with a sterile platinum loop and smeared on the surface of agar or blood serum. Film preparations for microscopic examination may be made at the same time. It is rare to obtain growths from the blood of the human subject by this method (*vide* special chapters), and if colonies appear the procedure should be repeated to exclude the possibility of accidental contamination.

(b) A larger quantity of blood may be obtained by puncture of a vein; this is the only satisfactory method, and should be that followed whenever practicable. The skin over a vein in the forearm or on the dorsum of the foot having been sterilised, the vein is made turgid by pressure, and the needle of a syringe



of 10–15 c.c. capacity, carefully sterilised, is then plunged obliquely through the skin into the lumen of the vessel. Several cubic centimetres of blood can thus be withdrawn into the syringe. Some of the blood (*e.g.* 1 c.c.) should be added to small flasks containing 50 c.c. of bouillon; the rest may be used for smearing the surface of agar tubes, or may be added to melted agar at 42° C., which is then plated. The flasks, etc. are then incubated. By this method cultures can often be obtained where the former method fails, especially in severe conditions such as ulcerative endocarditis, streptococcus infection, etc. Part of the blood may be incubated by itself for twenty-four hours, and cultures then made. Needless to say, the inoculations of media must be done at the bedside, as of course the blood quickly coagulates in the syringe. Coagulation can be prevented by drawing up into the syringe before it is used a quantity of 2 per cent. sterile sodium citrate equivalent to the amount of blood it is intended to withdraw.

In examining the blood of the *spleen* a portion of the skin over the organ is sterilised in the same way, a few drops are withdrawn from the organ by a sterile hypodermic syringe, and cultures made. (For microscopic methods, *vide* p. 94.)

**Bacteriological Examination of the Cerebro-spinal Fluid**  
**—Lumbar Puncture.**—This diagnostic procedure, which is often called for in cases of meningitis, can be carried out with a sterilised “antitoxin needle” as follows:—The patient should lie on the right side, with knees somewhat drawn up and left shoulder tilted somewhat forward, so that the back is fully exposed. The skin over the lumbar region is then carefully sterilised, as above described, and the hands of the operator should be similarly treated. The spines of the lumbar vertebrae having been counted, the left thumb or forefinger is pressed into the space between the third and fourth spines in the middle line; the needle is then inserted about half an inch to the right of the middle line at this level and pushed through the tissues, its course being directed slightly inwards and upwards, till it enters the subdural space. When this occurs, fluid passes along the needle, sometimes actually spurting out, and should be received in a sterile test-tube. Several cubic centimetres of fluid can thus usually be obtained, no suction being required; thereafter it can be examined bacteriologically by the usual methods. The depth of the subdural space from the surface varies from a little over an inch in children to 3 inches, or even more, in adults—the length of the needle must be suited accordingly. In making the puncture it is convenient to have



either a sterile syringe attached, or to have the thick end of the needle covered with a pad of sterile wool, which is of course removed at once when the fluid begins to flow. It is advisable to use the platinum needles which are specially made for the purpose, as a sudden movement of the patient may snap an ordinary steel needle.

**The Bacteriological Examination of Urine.**—In such an examination care must be taken to prevent the contamination of the urine by extraneous organisms. In the male, specimens withdrawn by a sterile catheter into a sterile vessel are preferable, but it is often sufficient to wash thoroughly the glans penis and the meatus with 1-1000 corrosive sublimate—the lips of the meatus being everted for more thorough cleansing; the urine is then passed into a series of sterile flasks, the first of which is rejected in case contamination has occurred. In the female, after similar precautions as regards external cleansing, the catheter must be used. The latter must be boiled for half an hour, and anointed with olive oil sterilised by half an hour's exposure in a plugged flask to a temperature of 120° C. Here, again, it is well to reject the urine first passed. It is often advisable to allow the urine to stand in a cool place for some hours, to then withdraw the lower portion with a sterile pipette, to centrifugalise this, and to use the urine in the lower parts of the centrifuge tubes for microscopic examination or culture.

**Filtration of Cultures.**—For many purposes it is necessary to filter all the organisms from fluids in which they may have been growing. This is done especially in obtaining the soluble toxic products of bacteria. The only filter capable of keeping back such minute bodies as bacteria is that formed from a tube of unglazed earthenware as introduced by Chamberland. The efficiency of such a filter depends on the fineness of the grain of the clay from which it is made; the finest is the Kitasato filter and the Chamberland "B" pattern; the next finest is the Chamberland "F" pattern, which is quite good enough for ordinary work. There are several filters, differing slightly in detail, all possessing the common principle. Sometimes the fluid is forced through the porcelain tube. In one form the filter consists practically of an ordinary tap screwed into the top of a porcelain tube. Through the latter the fluid is forced, and passes into a chamber formed by a metal cylinder which surrounds the porcelain tube. The fluid escapes by an aperture at the bottom. Such a filter is very suitable for domestic use, or for use in surgical operating-theatres. As



considerable pressure is necessary, it is evident it must be put on a pipe leading directly from the main. Sometimes, when fluids to be filtered are very albuminous, they are forced through a porcelain cylinder by compressed carbonic acid gas. The filtration of albuminous fluids may sometimes be facilitated by keeping them near blood-heat during the process. For ordinary bacteriological work, filters of various kinds are in the market (such as those of Klein and others), but the most generally convenient is that in which the fluid is sucked through the porcelain by exhausting the air in the receptacle into which it is to flow. This is conveniently done by means of a Geissler's water-exhaust pump (Fig. 30, *g*), which must be fixed to a tap leading directly from the main. The connection

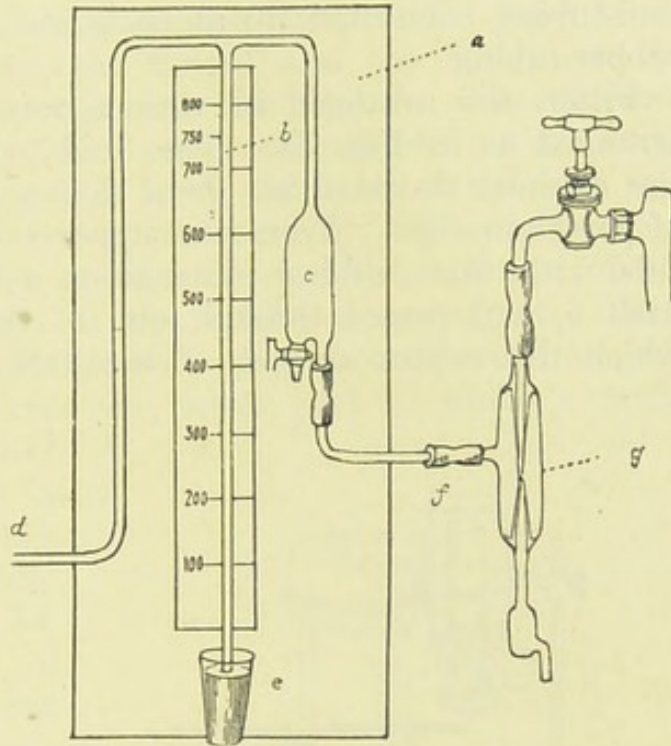


FIG. 30.—Geissler's vacuum pump arranged with manometer for filtering cultures. (The tap and pump are intentionally drawn to a larger scale than the manometer board to show details.)

with the tap must be effected by means of a piece of thick-walled rubber-tubing as short as possible, wired on to tap and pump, and firmly lashed externally with many turns of strong tape. Before lashing with the tape the tube may be strengthened by fixing round it with rubber solution strips of the rubbered canvas used for mending punctures in the outer case of a bicycle tyre. A manometer tube (*b*) and a receptacle (*c*) (the latter to catch any back flow of water from the pump if the filter accidentally breaks) are intercepted between the filter and the

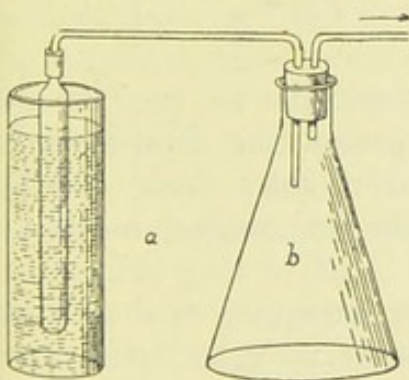


FIG. 31.—Chamberland's candle and flask arranged for filtration.



pump. These are usually arranged on a board *a*, as in Fig. 30. Between the tube *f* and the pump *g*, and between the tube *d* and the filter, it is convenient to insert lengths of flexible lead-tubing connected up at each end with short, stout-walled rubber-tubing.

Filters are arranged in various ways. (*a*) An apparatus is arranged as in Fig. 31. The fluid to be filtered is placed in the cylindrical vessel *a*. Into this a "candle" or "bougie" of porcelain dips. From the upper end of the bougie a glass tube with thick rubber connections, as in Fig. 31, proceeds to flask *b*, and passes through one of the two perforations with which the rubber stopper of the flask is furnished. Through

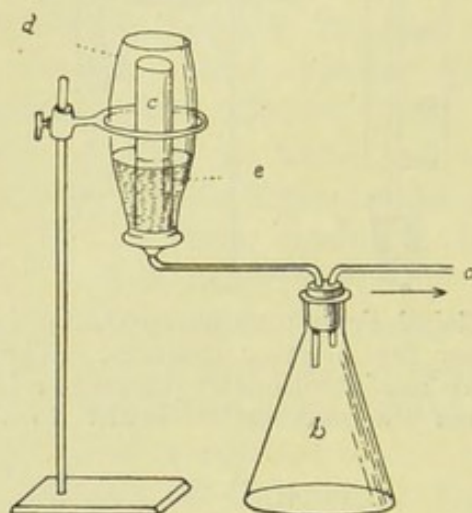


FIG. 32.—Chamberland's bougie arranged with lamp funnel for filtering a small quantity of fluid.

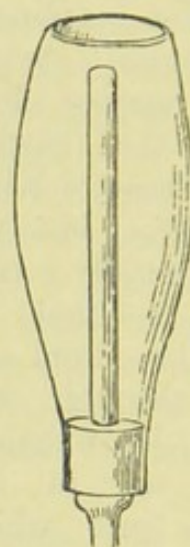


FIG. 33.—Bougie inserted through rubber stopper for same purpose as in Fig. 32.

the other opening a similar tube proceeds to the exhaust-pump. When the latter is put into action the fluid is sucked through the porcelain and passes over into flask *b*. This apparatus is very good, but not suitable for small quantities of fluid.

(*b*) A very good apparatus can be arranged with a lamp funnel and the porcelain bougie. These may be fitted up in two ways. (1) An indiarubber washer is placed round the bougie *c* at its glazed end (*vide* Fig. 32). On this the narrow end of the funnel *d*, which must, of course, be of an appropriate size, rests. A broad band of sheet rubber is then wrapped round the lower end of the funnel, and the projecting part of the bougie. It is firmly wired to the funnel above and to the



bougie below. The extreme point of the latter is left exposed, and the whole apparatus, being supported on a stand, is connected by a glass tube with the lateral tube of the flask *b*; the tube *a* is connected with the exhaust-pump. The fluid to be filtered is placed between the funnel and the bougie in the space *e*, and is sucked through into the flask *b*. The efficiency of such a filter, especially when small amounts of fluid are being dealt with, is much increased if when the level of the fluid falls below the upper end of the candle a closely fitting test-tube is slipped over the latter. By this device the leakage of air through the exposed part of the candle is prevented. There are now in the market candles with glass sheaths cemented into a nickle-plated fitting from the lower part of which a metal tube emerges; the latter can be passed through a rubber stopper into a filter flask. (2) This modification is shown in Fig. 33. Into the narrow part of the funnel an indiarubber bung is fitted, with a perforation in it sufficiently large to receive the candle, which it should grasp tightly.

(c) Muencke's modification of the Chamberland filter is seen in Fig. 34. It consists of a thick-walled flask *a*, the lower

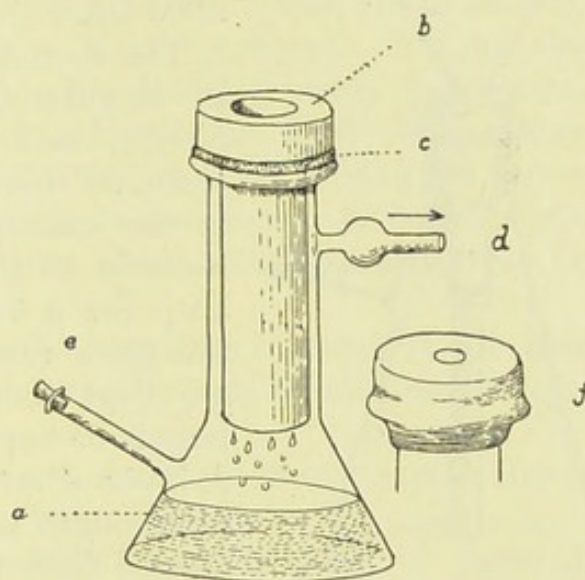


FIG. 34.—Muencke's modification of Chamberland's filter.

part conical, the upper cylindrical, with a strong flange on the lip. There are two lateral tubes, one horizontal to connect with exhaust-pipe, and one sloping, by which the contents may be poured out. Passing into the upper cylindrical part of the flask is a hollow porcelain cylinder *b*, of less diameter than the cylindrical part of flask *a*. It is closed below, open above, and rests by a projecting rim on the flange of the flask, an asbestos washer, *c*, being interposed. The fluid to be filtered is placed in the porcelain cylinder, and the whole top covered, as shown at *f*, with an indiarubber cap with a central perforation; the tube *d* is connected with the exhaust-pump, and the tube *e* plugged with a rubber stopper. For filtering small quantities of fluid the apparatus shown in Fig. 35 may be used. It consists of a small Chamberland bougie fitted by a rubber tube



to a funnel, the stem of which has been passed through a rubber cork; this cork fits into a conical flask with side arm for connection with exhaust.

Before any one of the above apparatus is used it ought to be connected up as far as possible and sterilised in the Koch's steriliser. The ends of any important unconnected parts ought to have pieces of cotton wool tied over them. After use the bougie is to be sterilised in the autoclave, and after being dried is to be passed carefully through a Bunsen flame to burn off all organic matter. If the latter is allowed to accumulate, the pores become filled up.

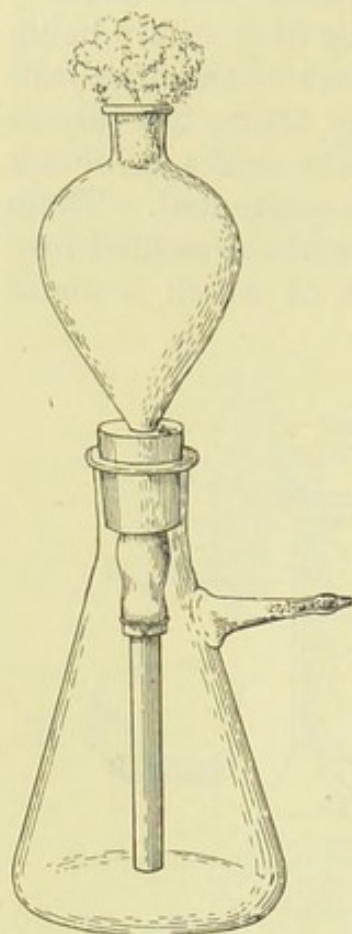


FIG. 35. — Flask for filtering small quantities of fluid.

The success of filtration must be tested by inoculating tubes of media from the filtrate, and observing if growth takes place, as there may be minute perforations in the candles sufficiently large to allow bacteria to pass through. Filtered fluids keep for a long time if the openings of the glass vessels in which they are placed are kept thoroughly closed, and if these vessels be kept in a cool place in the dark. A layer of sterile toluol about half an inch thick ought to be run on to the top of the filtered fluid to protect the latter from the atmospheric oxygen.

Instead of being filtered off, the bacteria may be killed by various antiseptics, chiefly volatile oils, such as oil of mustard

(Roux). These oils are stated to have no injurious effect on the chemical substances in the fluid, and they may be subsequently removed by evaporation. It is not practicable to kill the bacteria by heat when their soluble products are to be studied, as many of the latter are destroyed by a lower temperature than is required to kill the bacteria themselves.

Bacteria can be almost entirely removed from fluid cultures by spinning the latter in a centrifuge of very high speed (*e.g.* C. J. Martin's turbine centrifuge), and this method is sometimes adopted in practice.

**The Observation of Bacterial Fermentation of Sugars, etc.**  
—The capacity of certain species of bacteria to originate fermentations in sugars constitutes an important biological factor. It



is well to consider this factor in relation to the chemical constitution of the sugars. These bodies are now known to be (to use the definition of Holleman) aldehyde or ketone alcohols containing one or more hydroxyl groups, one of which is directly linked to a carbon atom in union with carbonyl. The group characteristic of a sugar is thus  $-\text{CHOH}-\text{CO}-$ . The sugars are divided into monosaccharides or monoses, disaccharides (dioses), and polysaccharides (polyoses). The members of the last two groups may be looked on as derived from the combination of two or more molecules of a monosaccharide with the elimination of water (*e.g.*  $2\text{C}_6\text{H}_{12}\text{O}_6 = \text{C}_{12}\text{H}_{22}\text{O}_{11} + \text{H}_2\text{O}$ ).

*Monosaccharides.* — These are classified according to the number of C atoms they contain. The pentoses ordinarily used are arabinose (obtained from gum arabic), xylose (from wood), and rhamnose (which is really a methylpentose). Among the hexoses are glucose (dextrose) with dextro-rotatory properties. Glucose is an aldehyde alcohol (aldose). In fruit there is also a ketone alcohol (ketose) called fructose, which from its lævoro-rotatory properties is also known as lævulose. Other hexoses are mannose (from the vegetable ivory nut) and galactose (a hydrolytic derivative of lactose).

*Disaccharides* ( $\text{C}_{12}\text{H}_{22}\text{O}_{11}$ ). — The ordinary members of this group are maltose (derived from starch), lactose, and cane sugar (sucrose, saccharose).

*Polysaccharides.* — Examples are starch, raffinose, inulin (from dahlia roots), dextrin, arabin, glycogen, cellulose.

If we consider sugars generally from the point of view of the capacity of yeast to originate alcoholic fermentation in them, we may say that the simpler the constitution of the sugar the more easily is it fermented. Thus the monosaccharides are more easily acted on by yeast than the di- or poly-saccharides. Usually an independent process resulting in the splitting of the higher into the lower is preliminary to the alcoholic fermentation. Thus yeast first inverts cane sugar into glucose and fructose, and then acts on these products. From what is known it is probable that similar facts hold with regard to the action of bacteria.

Besides sugars, other alcohols with large molecules may be broken down by bacterial action, and these bodies have been used for differentiating the properties of allied bacteria. Among these substances may be mentioned the trihydric alcohol glycerol (glycerin), the tetrahydric erythritol and the hexahydric dulcitol (dulcite), mannitol (mannite), and sorbitol (sorbite).

Similarly certain glucosides, such as salicin, coniferin, etc., have been used for testing the fermentative properties of



bacteria. Other substances allied to sugars (*e.g.* inosite) have also been used.

The end products of bacterial fermentations may be various. They differ according to the sugar employed and according to the species of bacterium under observation, and frequently a species which will ferment one sugar has no effect on another. The substances finally produced, speaking roughly, may be alcohols, acids, or gaseous bodies (chiefly carbon dioxide, hydrogen, and methane). For the estimation of the first groups complicated chemical procedure may be necessary. The tests usually employed for the detection of ordinary fermentative processes depend on two kinds of changes, namely, (*a*) the evolution of gases and (*b*) the formation of acids. Generally speaking, we may say that such tests are reliable, and the methods to be pursued are simple. Besides such gases as those named, some organisms give rise to sulphuretted hydrogen by breaking up the proteid. The formation of this gas can be detected by the blackening of lead acetate when it is added to the gas-containing medium.

In testing the effect of a bacterium on a given sugar it is essential that this sugar alone be present; the basis of the medium ought therefore to be either peptone solution (*vide p. 39*) or a dextrose-free bouillon (*vide infra*). The sugar or other substance is added in the proportion of from a half to one per cent., and care is taken not to overheat during sterilisation.

It is preferable that the addition should be made in the form of a sterile solution. If the sugar in solid form be placed in the bouillon and this then sterilised, there is danger that chemical changes may take place in the sugar, in consequence of its being heated in the presence of substances (such as alkalies) which may act deleteriously upon it; in any case sterilisation should not be at a temperature above 100° C.

To obtain a "dextrose-free" bouillon it is usual to inoculate ordinary bouillon with some organism, such as *b. coli*, which is known to ferment dextrose, and allow it to act for forty-eight hours. The bouillon is then filtered and re-sterilised. A sample is tested for another period of forty-eight hours with *b. coli*, to make certain that all the dextrose has been removed. If no fresh gas-formation is observed, then to the remainder of the bouillon the sugar to be investigated may be added.

For the observation of gas-formation either of the following methods may be employed:—

(1) *Durham's Tubes* (Fig. 36, *b*).—The plug of a tube which contains about one-third more than usual of a liquid medium is removed, and a small test-tube is slipped into the latter, mouth downwards. The plug is replaced and the tube sterilised thrice for ten minutes at 100° C. The air remaining in the smaller



tube is thereby expelled. The tube is then inoculated with the bacterium to be tested. Any gas developed collects in the upper part of the inner tube. As some of the sugars now used for fermentation tests are rather expensive, it is well to arrange the Durham apparatus with very small tubes; with these a satisfactory result can be obtained with only 1 c.c. of medium.

(2) *The Fermentation Tube* (Fig. 36, c).—This consists of a tube of the form shown, and the figure also indicates the extent to which it ought to be filled. It is inoculated in the bend with the gas-forming organism, and when growth occurs the gas

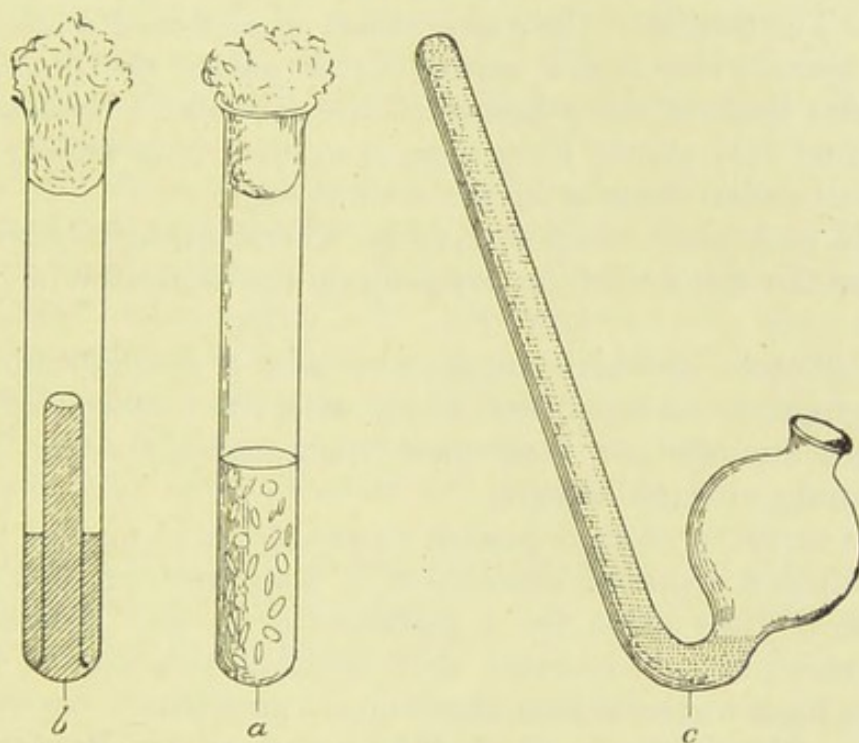


FIG. 36.—Tubes for demonstrating gas-formation by bacteria.

*a*, tube with "shake" culture.

*b*, Durham's fermentation tube.

*c*, ordinary form of fermentation tube.

collects in the upper part of the closed limit, the medium being displaced into the bulb.

For the observation of the effect of an organism on glucose, the following method may be employed:—

*Gelatin Shake Cultures* (Fig. 36, *a*).—The gelatin in the tube is melted as for making plates; while liquid it is inoculated with the growth to be observed, and shaken to distribute the organisms throughout the jelly. It is then allowed to solidify, and is set aside at a suitable temperature. If the bacterium used is a gas-forming one, then, as growth occurs, little bubbles appear round the colonies.



In this method the gas-formation results from fermentation of the glucose naturally present in the medium from transformation of the carbo-hydrates of muscle. The amount of glucose naturally present, however, varies much, and therefore glucose should be added to the medium if the effects on this sugar are to be observed with certainty. The shake culture method may be utilised for observing fermentation in other sugars by adding to peptone solution containing the sugar 10 to 15 per cent. of gelatin.

The *development of an acid reaction* is demonstrated by the addition of an indicator to the medium, litmus being generally used. The details of the composition of such media have already been given. In Hiss's serum water media the production of acid also leads to coagulation of the medium. Sometimes acid is formed very slowly from sugars, so that it is well to keep the cultures under observation for several days.

Acid and gas-formation may be simultaneously tested for, by placing the fluid medium containing the indicator in Durham's tubes.

In all tests in which sugars are used, a control uninoculated tube ought to be incubated along with the bacterial cultures, as changes in reaction sometimes spontaneously occur in media containing unstable sugars.

The capacity of an organism to produce acid may be measured by taking a standard amount of a fluid medium and allowing growth to take place for a standard time, and then adding an amount of, say, decinormal soda solution sufficient to bring the litmus back to the tint of the original medium.

**The Observation of Indol-formation by Bacteria.**—The formation of indol from albumin by a bacterium sometimes constitutes an important specific characteristic. To observe indol production the bacterium is grown, preferably at incubation temperature, in a fluid medium containing peptone. The latter may either be sugar-free bouillon or preferably peptone solution (see p. 39). Any medium containing sugars must be avoided, as the presence of these substances may inhibit the production of indol. Two methods are in use for the detection of this body.

(1) *The Nitroso-indol Method.*—Indol is here recognised by the fact that when it is acted on by nitric acid *in the presence of nitrites*, a nitroso-indol compound is produced, which has a rosy red colour. Some bacteria (*e.g.* the cholera vibrio) produce nitrites as well as indol, but usually in making the test (*e.g.* in the case of *b. coli*) the nitrites must be added. This is effected by adding to an ordinary tube of medium 1 c.c. of a .02 per



cent. solution of potassium nitrite, and testing with pure nitric or sulphuric acid. In any case only a drop of the acid need be added to, say, 10 c.c. of medium. If no result be obtained at once it is well to allow the tube to stand for an hour, as sometimes the reaction is very slowly produced. In many instances incubation at 37° C. for several days may be necessary before the presence of indol is demonstrable. The amount of indol produced by a bacterium seems to vary very much with certain unknown qualities of the peptone. It is well, therefore, to test a series of peptones with an organism (such as the *b. coli*) known to produce indol, and, noting the sample with which the best reaction is obtained, to reserve it for making media to be used for the detection of this product. This method has for long been felt not to be satisfactory, and the following at present bids fair to replace it :—

(2) *Ehrlich's Rosindol Reaction*.—The adaptation of this to bacteriological purposes was brought forward by Böhme in 1906. For ease of application and delicacy of effect the reaction possesses great advantages. It depends on the fact that paradimethylamidobenzaldehyde unites with indol to form a rosindol body whose colour is readily developed, especially in presence of an oxidising substance such as potassium persulphate ( $K_2S_2O_8$ ). Two solutions are required :—

- |   |                            |
|---|----------------------------|
| (1) Paradimethylamidobenzaldehyde (Grübler) | 4 grms.                    |
| Absolute alcohol (96 per cent.)             | 380 c.c.                   |
| Concentrated hydrochloric acid              | 80 c.c.                    |
| (2) Potassium persulphate                   | Saturated watery solution. |

To a 10 c.c. bouillon culture of the organism add 5 c.c. of (1) and then 5 c.c. of (2), and shake well (according to MacConkey 1 c.c. of each solution is sufficient); if indol be present a rose-red colour will appear in a few minutes. Sometimes the rose colour appears on the addition of solution (1), and the addition of a special oxidising agent is unnecessary. The rosindol compound can be separated from the culture by shaking the latter up with amyl alcohol, and MacConkey recommends that this should be done in cases of a doubtful reaction, as sometimes when a faint pink colour appears in the culture tube the extracting alcohol remains colourless, showing that no real reaction has occurred. Marshall has pointed out that by means of the reaction a quantitative estimate of the amount of indol formation can be obtained. To do this a large culture, say 100 c.c., is distilled, and the colour obtained by applying the



test to the distillate in a Nessler's tube is matched against that obtained with different amounts of a standard solution of indol (prepared by dissolving 1 gr. indol in 5 c.c. absolute alcohol, and making up to 500 c.c. with distilled water).

There is no doubt that the Ehrlich test is from five to ten times more delicate than the ordinary nitroso-indol reaction, and it is of especial value in dealing with organisms of the colityphoid group. With strains of *b. coli* it can often be obtained in from twenty-four to forty-eight hours, but in the case of a negative result a culture of from six to seven days ought to be used. The reaction is also obtainable with the cholera vibrio, but further investigation is here necessary, as Marshall states that under certain circumstances the nitrites formed by this bacterium may have an inhibitory effect on the production of the rose colour.

**The Drying of Substances *in vacuo*.**—As many substances, for example toxins and antitoxins, with which bacteriology is concerned would be destroyed by drying with heat as is done in ordinary chemical work, it is necessary to remove the water at the ordinary room temperature. This is most quickly effected by drying *in vacuo* in the presence of some substance such as strong sulphuric acid, which readily takes up water vapour. The vacuum produced by a water-pump is here not available, as in such a vacuum there must always be water vapour present. An air-pump is therefore to be employed. Here we have found the Geryk pump most efficient, and it has this further advantage, that its internal parts are lubricated with an oil of very low vapour density, so that almost a perfect vacuum is obtainable. The apparatus is shown in Fig. 37. The vacuum chamber consists of a bell-jar set on a brass plate. A perforation in the centre of the latter leads into the pipe *a*, which can be connected by strong-walled rubber-tubing with the air-pump, and which can be cut off from the latter by a stop-cock *b*. In using the apparatus the substance to be dried is poured out in flat dishes (one-half of a Petri capsule does very well), and these are stacked alternately with similar dishes of strong sulphuric acid on a stand which rests on the brass plate. The edge of the bell-jar is well luted with unguentum resinæ and placed in position and the chamber exhausted. In a few hours, if, as is always advisable, each dish have contained only a thin layer of fluid, the drying will be complete. The vacuum is then broken by admitting air very slowly through a by-pass *c*, and the bell-jar is removed. In such an apparatus it is always advisable, as is shown in the figure, to have interposed between the pump and the vacuum chamber a Wolff's bottle containing sulphuric acid.



This protects the oil of the pump from contamination with water vapour. Whenever the vacuum is produced, the rubber-tube should be at once disconnected from *a*, the cock *b* being shut. It is advisable when the apparatus is exhausted to cover the vacuum chamber and the Wolff's bottle with wire guards covered with strong cloth, in case, under the external pressure, the glass vessels give way. The connecting and disconnecting of rubber-tubing of sufficient thickness to withstand collapse when exhausted is difficult. Ordinary stout rubber-tubing can be used if through it there is passed a length of narrow flexible

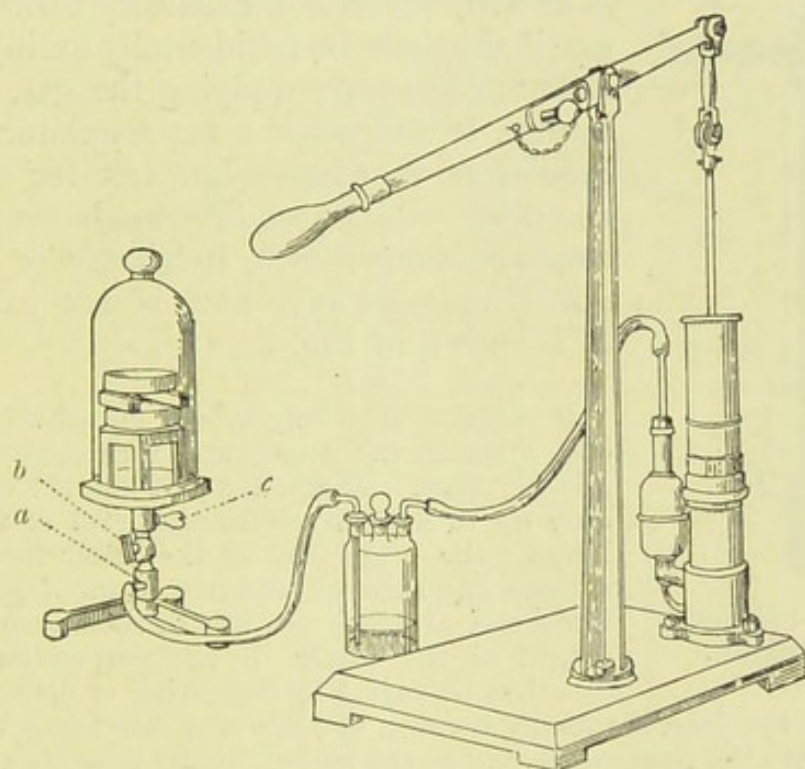


FIG. 37.—Geryk air-pump for drying *in vacuo*.

metal-tubing, the ends of which project beyond the rubber-tubing so as to enter the parts of the apparatus to which the latter is fitted.

**The Storing and Incubation of Cultures.**—Gelatin cultures must be grown at a temperature below their melting-point, *i.e.* for 10 per cent. gelatin, below 22° C. They are usually kept in ordinary rooms, which vary, of course, in temperature at different times, but which have usually a range of from about 12° C. to 18° C. Agar and serum media are employed to grow bacteria at a higher temperature, corresponding to that at which the organisms grow best, usually 37° C. in the case of pathogenic organisms. For the purpose of maintaining a uniform temperature incubators are used. These vary much in the



details of their structure, but all consist of a chamber with double walls between which some fluid (water or glycerin and water) is placed, which, when raised to a certain temperature,

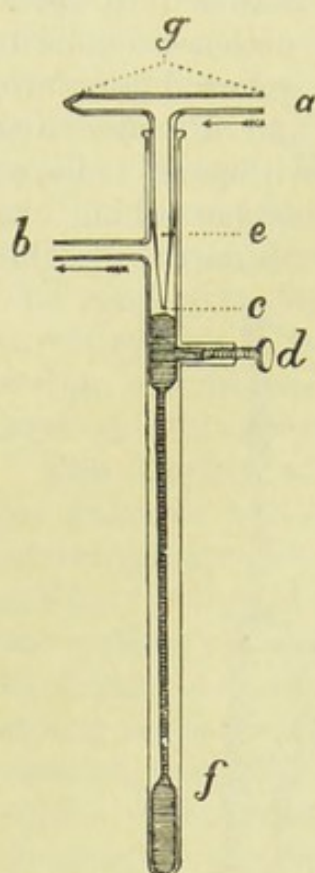


FIG. 38.—Reichert's gas regulator.

ensures a fairly constant distribution of the heat round the chamber. The latter is also furnished with double doors, the inner being usually of glass. Heat is supplied from a burner fixed below. These burners vary much in design. Sometimes a mechanism devised in Koch's laboratory is affixed, which automatically turns off the gas if the light be accidentally extinguished. Between the tap supplying the gas, and the burner, is interposed a gas regulator. Such regulators vary in design, but for ordinary chambers which require to be kept at a constant temperature, Reichert's is as good and simple as any, and is not expensive. It is shown in Fig. 38.

It consists of a long tube *f* closed at the lower end, open at the upper, and furnished with two lateral tubes. The lower part is filled with mercury up to a point above the level of the lower lateral tube. The end of the latter is closed by a brass cap through which a screw *d* passes, the inner end of which lies free in the mercury. The height of the latter in the perpendicular tube can thus be varied by increasing or decreasing the capacity of the lateral tube by turning the screw a few turns out of or into it. Into the upper open end of the perpendicular tube fits accurately a bent tube *g*, drawn out below to a comparatively small open point *c*, and having in its side a little above the point a minute needle-hole called the peephole or by-pass *e*. To fix the apparatus the long mercury bulb is placed in the jacket of the chamber to be controlled, tube *a* is connected to gas supply, tube *b* with the burner. The upper level of the mercury should be some distance below the lower open end of tube *c*. The burner is now lit. The gas passes in at *a* through *c* and *e* and out at *b* to the burner. When the thermometer in the interior of the chamber indicates that the desired temperature has been reached, the screw *d* is turned till the mercury reaches the end of the tube *c*. Gas can only now pass through the peephole *e*, and the flame goes down. The contents of the jacket cool, the mercury contracts off the end of tube *c*, and the flame rises. This alternation going on, the temperature of the chamber is kept very nearly constant. If the mercury cuts off the gas supply before the desired temperature is reached, and the screw *d* is as far out as it will go, then some of the mercury must be removed. Similarly, if when the desired temperature is reached and the screw *d* is as far in as it can go, the mercury does not reach *c*, some more must be introduced. If the amount of gas which passes through the



peephole is sufficient still to raise the temperature of the chamber when *c* is closed by the rise of the mercury, then the peephole is too large. Tube *g* must be unshipped and *e* plastered over with sealing-wax, which is pricked, while still soft, with a very fine needle. The gas flame, when only the peephole is supplying gas, ought to be sufficiently large not to be blown out by small currents of air. If the pressure of gas supplied to a regulator varies much in the twenty-four hours, a pressure regulator ought to be interposed between the gas tap and the instrument. Several varieties of these can be obtained. In all cases *g* ought to be fixed to *b* with a turn of wire.

The varieties of incubators are, as we have said, numerous. The most complicated and expensive are made by German

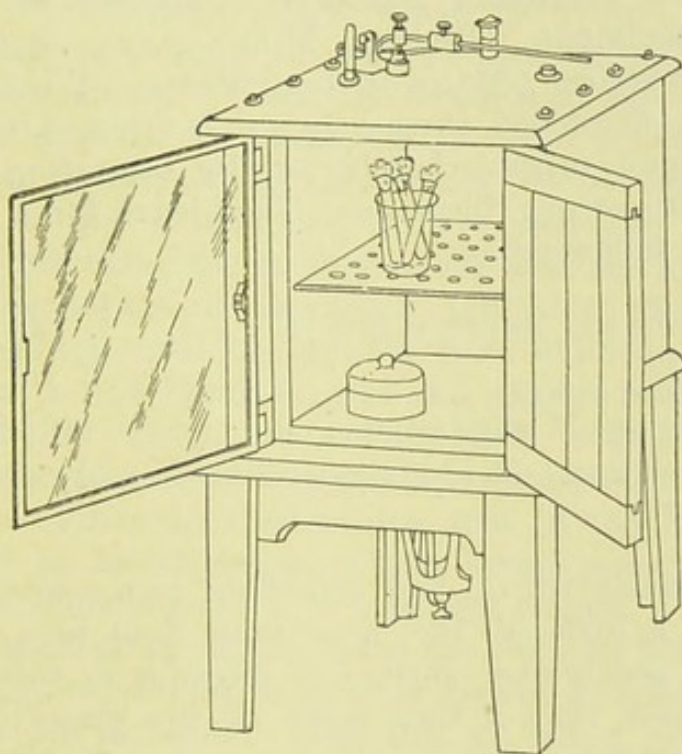


FIG. 39.—Hearson's incubator for use at 37° C.

manufacturers. Many of these are unsatisfactory, as they easily get out of order and are difficult to repair. We have found those of Hearson of London extremely good, and in proportion to their size much cheaper than the German articles. They are fitted with an admirable regulator. It is preferable in using an incubator to connect the regulator with the gas supply and with the Bunsen by flexible metal-tubing. It is necessary to see that there is not too much evaporation from the surface of cultures placed within incubators, otherwise they may quickly dry up. It is thus advisable to raise the amount of water vapour in the interior by having in the bottom of the incubator a flat dish full of water from which evaporation may take place. With tubes



which will require to be long in the incubator, the plugs should be pushed a little way into the tube and a few drops of melted paraffin dropped on the top of the wool, or the plugs should be covered either by indiarubber caps or by pieces of sheet rubber tied over them. These caps should be previously sterilised in 1-1000 corrosive sublimate and then dried. Before they are placed on the tube the cotton-wool plug ought to be well singed in a flame. "Cool" incubators are often used for incubating gelatin at  $21^{\circ}$  to  $22^{\circ}$  C. An incubator of this kind fitted with a low-temperature Hearson's regulator is in the market.

**Method of Mounting Bacterial Cultures as Permanent Museum Specimens** (Richard Muir). — (a) *Stab or Stroke Cultures in Nutrient Gelatin or Agar Media*.—When the culture shows typical characters, further growth is arrested by placing the tube in a formol vapour chamber, or by saturating the cotton-wool plug with strong formalin. Then leave for a day or two. Make up the following:—

(1) Thymol water (saturated in cold) . . . . .	100 c.c.
Glycerin . . . . .	20 c.c.
Acetate of potash . . . . .	5 grms.
Coignet's (gold label) gelatin . . . . .	10 grms.

Render the mixture acid to litmus with acetic acid; clear with white of egg and filter.

Warm to about  $40^{\circ}$  C., and removing cotton-wool plug from culture take a little of the preserving fluid in a pipette and allow to run gently over surface of medium in tube. Place in such a position that a thin layer of the preserving medium remains completely covering the growth and the surface of culture medium. The gelatin is now allowed to solidify. Add three or four drops of strong formalin to the tube, and fill up to within a quarter of an inch of the top of the tube with the following fluid:—

(2) Thymol water (saturated in cold) . . . . .	100 c.c.
Glycerin . . . . .	20 c.c.
Acetate of potash . . . . .	5 grms.

Cover top of tube with a small piece of paper so as to keep out dust, allow to stand for a day or two so that small air-bells may rise to the surface.

To seal tube, pour melted paraffin gently on to the surface of fluid to near the top of tube; allow to solidify. Cover paraffin with layer of alcoholic orange shellac cement; allow



this to set, and repeat until the cement becomes level with top of test-tube. When set, a few drops of black lacquer are put on, and a circular cover-glass of about the same diameter as the mouth of tube is placed so as completely to seal it.

(b) The following method is useful for preparing *plate cultures*: Instead of making the cultures in Petri's capsules, use ordinary watch-glasses. The watch-glass is sterilised in a Petri's capsule, and the inoculated medium is poured out into the watch-glass, allowed to solidify in the usual way, and left in the Petri's capsule until the colonies of growth have developed. The watch-glass is now removed from capsule, and a layer of the preserving gelatin medium (1), to which have been added a few drops of strong formalin, is allowed to spread over the surface of the culture medium. When the layer is solidified the watch-glass is filled up with the same, and a clean square or oblong piece of glass (which of course should be of slightly larger diameter than the watch-glass) is now carefully placed over watch-glass, care being taken that no air-bells are formed. The edge of watch-glass should be closely applied to the glass cover, and left in position until the gelatin has solidified. The superfluous gelatin is now removed, and the glasses sealed first with the orange shellac cement, then with black lacquer. It is now finished off by using a circular mask of suitable size.

The various kinds of solid media used in the cultivation of bacteria, such as blood serum, potato, bread paste, etc., can be treated in the same manner with excellent results.

**General Laboratory Rules.**—On the working bench of every bacteriologist there should be a large dish of 1–1000 solution of mercuric chloride in water. Into this all tubes, vessels, plates, hanging-drop cultures, etc., which have contained bacteria and with which he has finished, ought to be at once plunged (in the case of tubes, the tube and plug should be put in separately). On no account whatever are such infected articles to be left lying about the laboratory. The basin is to be repeatedly cleaned out. All the glass is carefully washed in repeated changes of tap water to remove the last trace of perchloride of mercury, a very minute quantity of which is sufficient to inhibit growth. Old cultures which have been stored for a time, and from which fresh sub-cultures have been made, ought to be steamed in the Koch's steriliser for two or three hours, or in the autoclave for a shorter period, and the tubes thoroughly washed out. Besides a basin of mercuric chloride solution for infected apparatus, etc., there ought to be a second reserved for the worker's hands in case of any accidental contamination. When,



as in public-health work, a large number of tubes are being daily put out of use, they may be placed in an enamelled slop-pail, and this when full is placed in the steam steriliser.

A white glazed tile on which a bell-jar can be set is very convenient to have on a bench. Infective material in watch-glasses can be placed thus under cover while investigation is going on, and if anything is spilled the whole can be easily disinfected. In making examinations of organs containing virulent bacteria, the hands should be previously dipped in 1-1000 mercuric chloride and allowed to remain wet with this solution. No food ought to be partaken of in the laboratory, and pipes, etc., are not to be laid with their mouth-pieces on the bench. No label is to be licked with the tongue. Before leaving the laboratory the bacteriologist ought to wash the hands and forearms with 1-1000 mercuric chloride and then with yellow soap. In the case of any fluid containing bacteria being accidentally spilt on the bench or floor, 1-1000 mercuric chloride is to be at once poured on the spot. The air of the laboratory ought to be kept as quiet as possible.



## CHAPTER III.

### MICROSCOPIC METHODS.

**The Microscope.**—For ordinary bacteriological work a good microscope is essential. It ought to have a heavy stand, with rack and pinion and fine adjustment, a double mirror (flat on one side, concave on the other), a good condenser, with an iris diaphragm, and a triple nose-piece. It is best to have three objectives, either Zeiss A, D, and  $\frac{1}{12}$ -inch oil immersion, or the lenses of other makers corresponding to these. The oil immersion lens is essential. It is well to have two eye-pieces, say Nos. 2 and 4 of Zeiss or lenses of corresponding strengths. The student must be thoroughly familiar with the focussing of the light on the lens by means of the condenser, and also with the use of the immersion lens. It may here be remarked that when it is desired to bring out in sharp relief the margins of unstained objects, *e.g.* living bacteria in a fluid, a narrow aperture of the diaphragm should be used, whereas, in the case of stained bacteria, when a pure coloured picture is desired, the diaphragm ought to be widely opened. The flat side of the mirror ought to be used along with the condenser. When the observer has finished for the time being with the immersion lens he ought to wipe off the oil with a piece of silk or very fine washed linen. If the oil has dried on the lens it may be moistened with xylol—never with alcohol, which will dissolve the material by which the lens is fixed in its metal carrier.

**Microscopic Examination of Bacteria.**—1. **Hanging-drop Preparations.**—Micro-organisms may be examined: (1) alive or dead in fluids; (2) in film preparations; (3) in sections of tissues. In the two last cases advantage is always taken of the affinity of bacteria for certain stains. When they are to be examined in fluids a drop of the liquid may be placed on a slide



and covered with a cover-glass.<sup>1</sup> It is more usual, however, to employ hanging-drop preparations. The technique of making these has already been described (p. 69). In examining them microscopically, it is necessary to use a very small diaphragm. It is best to focus the edge of the drop with a low-power objective, and, arranging the slide so that part of the edge crosses the centre of the field, to clamp the preparation in this position. A high-power lens is then turned into position, and lowered by the coarse adjustment to a short distance above its focal distance; it is now carefully screwed down by the fine adjustment, the eye being kept at the tube meanwhile. The shadow of the edge will be first recognised, and then the bacteria must be carefully looked for. Often a dry lens is sufficient, but for some purposes the oil immersion is required. If the bacteria are small and motile, a beginner may have great difficulty in seeing them, and it is well to practise at first on some large non-motile form, such as anthrax. In fluid preparations the natural appearance of bacteria may be studied, and their rate of growth determined. The great use of such preparations, however, is to find whether or not the bacteria are motile, and for determining this point it is advisable to use either broth or agar cultures not more than twenty-four hours old. In the latter case a small fragment of growth is broken down in broth or in sterile water. Sometimes it is an advantage to colour the solution in which the hanging-drop is made up with a minute quantity of an aniline dye, say a small crystal of gentian violet to 100 c.c. of bouillon. Such a degree of dilution will not have any effect on the vitality of the bacteria. Ordinarily, living bacteria will not take up a stain, but even though they do not, the contrast between the unstained bacteria and the tinted fluid will enable the observer more easily to recognise them. In determining whether or not a bacterium is motile, great difficulty is often experienced in distinguishing between true motion and Brownian movement, especially if the organism be small. The essential criterion to be fulfilled is that the bacteria shall be moving in all directions, the observation of individuals lying close together starting to move in opposite directions being important. The observation of hanging-drop preparations must be correlated with the results of staining for the presence of flagella which, so far as is known, are present in all motile forms.

Within recent years the method of observing living micro-

<sup>1</sup> In bacteriological work it is essential that cover-glasses of No. 1 thickness (*i.e.* .14 mm. thick) should be used, as those of greater thickness are not suitable for a  $\frac{1}{2}$ -inch lens.



organisms by oblique illumination has been much practised, and a number of substage condensers are in the market, by means of which this is effected. The general principle involved in these instruments is to stop out the rays passing directly towards the tube of the microscope, and to arrange for light being thrown obliquely on bacteria mounted in a drop of fluid between a slide and cover-glass. The bacteria disperse these rays in all directions, and some passing up through the lens are focussed by it. The organisms thus appear as brightly illumined objects on a dark background. The method has been employed for bacteria in general, and especially for the demonstration of the *spirochæte pallida* in secretions as a means of diagnosis. Generally speaking, the internal structure of the organisms under observation is well brought out.

2. **Film Preparations.**—(a) *Dry Method.*—This is the most extensively applicable method of microscopically examining bacteria. Fluids containing bacteria, such as blood, pus, scrapings of organs, can be thus investigated, as also cultures in fluid and solid media. The first requisite is a perfectly clean cover-glass. Many methods are recommended for obtaining such. The test of this being accomplished is that, when the drop of fluid containing the bacteria is placed upon the glass, it can be uniformly spread with the platinum needle all over the surface without showing any tendency to retract into droplets. The best method is that recommended by Van Ermengem. The cover-glasses are placed for some time in a mixture of concentrated sulphuric acid 6 parts, potassium bichromate 6 parts, water 100 parts, then washed thoroughly in water and stored in absolute alcohol. For use, a cover-glass is either dried by wiping with a clean duster or is simply allowed to dry. This method will amply repay the trouble, and really saves time in the end. A clean cover having been obtained, the film preparation can now be made. If a fluid is to be examined a loopful may be placed on the cover-glass, and either spread out over the surface with the needle, or another clean cover may be placed on the top of the first, the drop thus spread out between them and the two then drawn apart. When a culture on a solid medium is to be examined, a loopful of distilled water is placed on the cover-glass, and a minute particle of growth rubbed up in it and spread over the glass. The great mistake made by beginners is to take too much of the growth. The point of the straight needle should just touch the surface of the culture, and when this is rubbed up in the droplet of water and the film dried, there should be an opaque cloud just



visible on the cover-glass. When the film has been spread, it must next be dried by being waved backwards and forwards at arm's-length above a Bunsen flame. The film must then be fixed on the glass by being passed three or four times slowly through the flame. In doing this a good plan is to hold the cover-glass

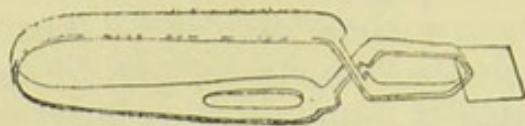


FIG. 40.—Cornet's forceps for holding cover-glasses.

between the right forefinger and thumb; if the fingers just escape being burned no harm will accrue to the bacteria in the film.

In making films of a thick fluid such as *pus* it is best to spread it out on one cover with the needle. The result will be a film of irregular thickness, but sufficiently thin at many parts for proper examination. Scrapings of organs may be smeared directly on the cover-glasses.

In the case of *blood*, a fairly large drop should be allowed to spread itself between two clean cover-glasses, which are then to be slipped apart, and being held between the forefinger and thumb are to be dried by a rapid to-and-fro movement in the air. A film prepared in this way may be too thick at one edge, but at the other is beautifully thin. If it is desired to preserve the red blood corpuscles in such a film it may be fixed by one of the following methods: by being placed (*a*) in a hot-air chamber at 120° C. for half an hour; (*b*) in a mixture of equal parts of alcohol and ether for half an hour, then washed and dried; (*c*) in formol-alcohol (Gulland) (formalin 1 part, absolute alcohol 9 parts) for five minutes, then washed and dried; or (*d*) in a saturated solution of corrosive sublimate for two or three minutes, then washed well in running water and dried. (Fig. 69 shows a film prepared by the last method.) In using the Romanowsky stains no previous fixation is necessary (*vide infra*). In the case of *urine*, the specimen must be allowed to stand, and films made from any deposit which occurs; or, what is still better, the urine is centrifugalised, and films made from the deposit which forms. After dried films are thus made from urine it is an advantage to place a drop of distilled water on the film and heat gently to dissolve the deposit of salts; then wash in water and dry. In this way a much clearer picture is obtained when the preparation is stained.

Within recent years it has become common to make blood films on ordinary microscopic slides instead of upon cover-glasses. Here the slides must be clean. This can be effected by washing thoroughly first with weak alkali and then with water



and storing in alcohol. For use, a slide is taken from the alcohol and the fluid adhering to it set on fire and allowed to burn off, a dry clean slide being thus obtained. To make a film on such, a small drop of blood is placed near one end, the edge of a second clean slide is lowered through the drop on to the surface of the glass on which the blood has been placed. This second slide is held at an angle to the first, and the droplet of blood by capillarity spreads itself in the angle between the two slides. The edge of the second slide is then stroked along the surface of the first slide, and the blood is spread out in a film whose thickness can be regulated by the angle formed by the second slide. Large-sized films can thus be obtained, and when these are stained they are often examined without any cover-glass being placed upon them. A drop of cedar oil is placed on the preparation, and after use this can be removed by the careful application of xylol.

Films dried and fixed by the above methods are now ready to be stained by the methods to be described below.

(b) *Wet Method*.—If it is desired to examine the fine histological structure of the cells of a discharge as well as to investigate the bacteria present, it is advisable to substitute “wet” films for the “dried” films, the preparation of which has been described. The nuclear structure, mitotic figures, etc., are by this method well preserved, whereas these are considerably distorted in dried films. The initial stages in the preparation of wet films are the same as above, but instead of being dried in air they are placed, while still wet, film downwards in the fixative. The following are some of the best fixing methods:—

(a) A saturated solution of perchloride of mercury in .75 per cent. sodium chloride; fix for five minutes. Then place the films for half an hour, with occasional gentle shaking, in .75 per cent. sodium chloride solution to wash out the corrosive sublimate; they are thereafter washed in successive strengths of methylated spirit. After this treatment the films are stained and treated as if they were sections.

(b) Formol-alcohol—formalin 1 part, absolute alcohol 9. Fix films for three minutes; then wash well in methylated spirit. This is an excellent and very rapid method.

(c) Another excellent method of fixing has been devised by Gulland. The fixing solution has the composition—absolute alcohol 25 c.c., pure ether 25 c.c., alcoholic solution of corrosive sublimate (2 grms. in 10 c.c. of alcohol) about 5 drops. The films are placed in this solution for five minutes or longer. They are then washed well in water, and are ready for staining. A contrast stain can be applied at the same time as the fixing solution, by saturating the 25 c.c. of alcohol with eosin before mixing. Thereafter the bacteria, etc., may be stained with methylene-blue or other stain, as described below. This method has the advantage



over (a) that, as a small amount of corrosive sublimate is used, less washing is necessary to remove it from the preparation, and deposits are less liable to occur.

**3. Examination of Bacteria in Tissues.**—For the examination of bacteria in the tissues, the latter must be fixed and hardened, in preparation for being cut with a microtome. Fixation consists in so treating a tissue that it shall permanently maintain, as far as possible, the condition it was in when removed from the body. Hardening consists in giving such a fixed tissue sufficient consistence to enable a thin section of it to be cut. A tissue, after being hardened, may be cut in a freezing microtome (*e.g.* Cathcart's or one of the newer instruments in which the freezing is accomplished by compressed carbonic acid gas), but far finer results can be obtained by embedding the tissue in solid paraffin and cutting with some of the more delicate microtomes of which, for pathological purposes, the small Cambridge rocker is by far the best. For bacteriological purposes embedding in celloidin is not advisable, as the celloidin takes on the aniline dyes which are used for staining bacteria, and is apt thus to spoil the preparation, and besides, thinner sections can be obtained by the paraffin method.

**The Fixation and Hardening of Tissues.**—The following are amongst the best methods for bacteriological purposes :—

(a) *Absolute alcohol* may be used for the double purpose of fixing and hardening. If the piece of tissue is not more than  $\frac{1}{8}$  inch in thickness, it is sufficient to keep it in this reagent for a few hours. If the pieces are thicker a longer exposure is necessary, and in such cases it is better to change the alcohol at the end of the first twenty-four hours. The tissue must be tough without being hard, and the necessary consistence, as estimated by feeling with the fingers, can only be judged of after some experience. If the tissues are not to be cut at once, they may be preserved in 50 per cent. spirit.

(b) *Formol-alcohol*—formalin 1, absolute alcohol 9. Fix for not more than twenty-four hours; then place in absolute alcohol if the tissue is to be embedded at once, in 50 per cent. spirit if it is to be kept for some time. For small pieces of tissue fixation for twelve hours or even less is sufficient. The method is a rapid and very satisfactory one.

(c) *Corrosive sublimate* is an excellent fixing agent. It is best used as a saturated solution in .75 per cent. sodium chloride solution. Dissolve the sublimate in the salt solution by heat; the separation of crystals on cooling shows that the solution is saturated. For small pieces of tissue  $\frac{1}{8}$  inch in thickness, twelve hours' immersion is sufficient. If the pieces are larger, twenty-four hours is necessary. They should then be tied up in a piece of gauze, and placed in a stream of running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate. They are then placed for twenty-four hours in each of the following strengths of methylated



spirit (free from naphtha<sup>1</sup>): 30 per cent., 60 per cent., and 90 per cent. Finally they are placed in absolute alcohol for twenty-four hours and are then ready to be prepared for cutting.

If the tissue is very small, as in the case of minute pieces removed for diagnosis, the stages may be all compressed into twenty-four hours. In fact, after fixation in corrosive the tissue may be transferred directly to absolute alcohol, the perchloride of mercury being removed after the sections are cut, as will be afterwards described.

(d) *Methylated spirit*.—Small pieces of tissue may be placed in methylated spirit, which is to be changed after the first day. In from six to seven days they will be hardened. If the pieces are large, a longer time is necessary.

**The Cutting of Sections.**—1. *By Means of the Freezing Microtome*.—Pieces of tissue hardened by any of the above methods must have all the alcohol removed from them by washing in running water for twenty-four hours. They are then placed for from twelve to twenty-four hours (according to their size) in a thick syrupy solution containing two parts of gum arabic and one part of sugar. They are then cut on a freezing microtome and placed for a few hours in a bowl of water so that the gum and syrup may dissolve out. They are then stained, or they may be stored in methylated spirit.

2. *Embedding and Cutting in Solid Paraffin*.—This method gives by far the finest results, and should always be adopted when practicable. The principle is the impregnation of the tissue with paraffin in the melted state. This paraffin when it solidifies gives support to all the tissue elements. The method involves that, after hardening, the tissue shall be thoroughly dehydrated, and then thoroughly permeated by some solvent of paraffin which will expel the dehydrating fluid and prepare for the entrance of the paraffin. The solvents most in use are chloroform, cedar oil, xylol, and turpentine; of these, chloroform and cedar oil are the best, the former being preferred, as it permeates the tissue more rapidly. The more gradually the tissues are changed from reagent to reagent in the processes to be gone through, the more successful is the result. A necessity of the process is an oven with hot-water jacket, in which the paraffin can be kept at a constant temperature just above its melting-point, a gas regulator, *e.g.* Reichert's, being of course necessary. The tissues occurring in pathological work have a tendency to

<sup>1</sup> In Britain ordinary commercial methylated spirit has mineral naphtha added to it to discourage its being used as a beverage. The naphtha being insoluble in water a milky fluid results from the dilution of the spirit. By law, chemists can only sell 8 ounces of pure spirit at a time. Most pathological laboratories are, however, permitted by the Excise to buy "industrial spirit," which contains only one-nineteenth of naphtha.



become brittle if overheated, and therefore the best results are obtained by using paraffin melting at a somewhat low temperature. We have used for some years a mixture of one part of paraffin, melting at  $48^{\circ}$ , and two parts of paraffin melting at  $54^{\circ}$  C. This mixture has a melting-point between  $52^{\circ}$  and  $53^{\circ}$  C., and it serves all ordinary purposes well. An excellent quality of paraffin is that known as the "Cambridge paraffin," but many scientific-instrument makers supply paraffins which, for ordinary purposes, are quite as good, and much cheaper. The successive steps in the process of paraffin embedding are as follows: <sup>1</sup>—

1. Pieces of tissue, however hardened, are placed in fresh absolute alcohol for twenty-four hours in order to their complete dehydration.

2. Transfer now to a mixture of equal parts of absolute alcohol and chloroform for twenty-four hours.

3. Transfer to pure chloroform for twenty-four hours or longer. At the end of this time the tissues should sink or float heavily.

4. Transfer now to a mixture of equal parts of chloroform and paraffin and place on the top of the oven for from twelve to twenty-four hours. If the temperature there is not sufficient to keep the mixture melted then they must be put inside.

5. Place in pure melted paraffin in the oven for twenty-four hours. For holding the paraffin containing the tissues, small tin dishes such as are used by pastry-cooks will be found very suitable. There must be a considerable excess of paraffin over the bulk of tissue present, otherwise sufficient chloroform will be present to vitiate the final result and not give the perfectly hard block obtained with pure paraffin. With experience, the persistence of the slightest trace of chloroform can be recognised by smell.

In the case of very small pieces of tissue the time given for each stage may be much shortened, and where haste is desirable Nos. 2 and 4 may be omitted. Otherwise it is better to carry out the process as described.

6. Cast the tissues in blocks of paraffin as follows: Pairs of L-shaped pieces of metal made for the purpose by instrument makers must be at hand. By laying two of these together on a glass plate, a rectangular trough is formed. This is filled with melted paraffin taken from a stock in a separate dish. In it is immersed the piece of tissue, which is lifted out of its pure paraffin bath with heated forceps. The direction in which it is to be cut must be noted before the paraffin becomes opaque. When the paraffin has begun to set, the glass plate and trough have cold water run over them. When the block is cold, the metal L's are broken off, and, its edges having been pared, it is stored in a pill-box.

*The Cutting of Paraffin Sections.*—Sections must be cut as

<sup>1</sup> While the method given is sufficient for ordinary purposes, a more elaborate technique is necessary if it is desired that no changes shall take place in the tissue. Thus after fixation the tissue must be taken up to absolute alcohol through successive dilutions of spirit, but differing from each other by more than 10 per cent. Again, when alcohol has been replaced by chloroform the latter must be saturated with chips of paraffin, first at even temperature, then at  $37^{\circ}$  C., and must be kept at  $55^{\circ}$  C. as short a time as possible.



thin as possible, the Cambridge rocking microtome being, on the whole, most suitable. They should not exceed  $8\ \mu$  in thickness, and ought, if possible, to be about  $4\ \mu$ . For their manipulation it is best to have two needles on handles, two camel's-hair brushes on handles, and a needle with a rectangle of stiff

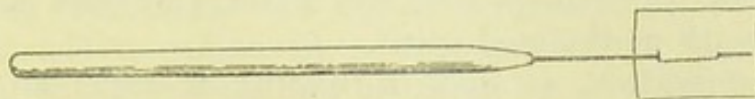


FIG. 41.—Needle with square of paper on end for manipulating paraffin sections.

writing paper fixed on it as in the diagram (Fig. 41). When cut, sections are floated on the surface of a beaker of water kept at a temperature about  $10^{\circ}\text{C}$ . below the melting-point of the paraffin. On the surface of the warm water they become perfectly flat.

*Fixation on Ordinary Slides.* (a) *Gulland's Method.*—A supply of slides well cleaned being at hand, one of them is thrust obliquely into the water below the section, a corner of the section is fixed on it with a needle and the slide withdrawn. The surplus of water being wiped off with a cloth, the slide is placed on a support, with the section downwards, and allowed to remain on the top of the paraffin oven or in a bacteriological incubator for from twelve to twenty-four hours. It will then be sufficiently fixed on the slide to withstand all the manipulations necessary during staining and mounting.

(b) *Fixation by Mann's Method.*—This has the advantage of being more rapid than the previous one. A solution of albumin is prepared by mixing the white of a fresh egg with ten parts of distilled water and filtering. Slides are made perfectly clean with alcohol. One is dipped into the solution and its edge is then drawn over one surface of another slide so as to leave on it a thin film of albumin. This is repeated with the others. As each is thus coated it is leant, with the film downwards, on a ledge till dry, and then the slides are stored in a wide stoppered jar till needed. The floating out is performed as before. The albuminised side of the slide is easily recognised by the fact that if it is breathed on, the breath does not condense on it. The great advantage of this method is that the section is fixed after twenty to thirty minutes' drying at  $37^{\circ}\text{C}$ . If the tissue has been hardened in any of the bichromate solutions and embedded in paraffin, this or some corresponding method of fixing the sections on the slide must be used.

*Preparation of Paraffin Sections for Staining.*—Before staining, the paraffin must be removed from the section. This is best done by dropping on xylol out of a drop bottle. When the paraffin is dissolved out, the superfluous xylol is wiped off with a cloth and a little absolute alcohol dropped on. When the xylol is removed, the superfluous alcohol is wiped off and a little 50 per cent. methylated spirit dropped on. During these



procedures sections must on no account be allowed to dry. The sections are now ready to be stained. Deposits of crystals of corrosive sublimate often occur in sections which have been fixed by this reagent. These can be removed by placing the sections, before staining, for a few minutes in equal parts of Gram's iodine solution (p. 106) and water, and then washing out the iodine with methylated spirit.

To save repetition, we shall in treating of stains suppose that, with paraffin sections, the above preliminary steps have already been taken, and further, that sections cut by a freezing microtome are also in spirit and water.

**Dehydration and Clearing.**—It is convenient, first of all, to indicate the final steps to be taken after a specimen is stained. *Dry films* after being stained are washed in water, dried and mounted in xylol balsam; *wet films* and *sections* must be dehydrated, cleared, and then mounted in xylol balsam.

*Dehydration* is most commonly effected with absolute alcohol. Alcohol, however, sometimes decolorises the stained organisms more than is desirable, and therefore Weigert devised the following method of dehydrating and clearing by aniline oil, which, though it may decolorise somewhat, does not do so to the same extent as alcohol. As much as possible of the water being removed, the section placed on a slide is partially dried by draining with fine blotting-paper. Some aniline oil is placed on the section and the slide moved to and fro. The section is dehydrated and becomes clear. The process may be accelerated by heating gently. The preparation is then treated with a mixture of two parts of aniline oil and one part of xylol, and then with xylol alone, after which it is mounted in xylol balsam. Balsam as ordinarily supplied has often an acid reaction, and preparations stained with aniline dyes are apt to fade when mounted in it. It is accordingly a great advantage to use the acid-free balsam supplied by Grübler. Paraffin sections can usually be dehydrated and cleared by the mixture of aniline oil and xylol alone.

Sections stained for bacteria should always be *cleared*, at least finally, in xylol, as it dissolves out aniline dyes less readily than such clearing reagents as clove oil, etc. Xylol, however, requires the previous dehydration to have been more complete than clove oil, which will clear a section readily when the dehydration has been only partially effected by, say, methylated spirit. If a little decolorisation of a section is still required before mounting, clove oil may be used to commence the clearing, the process being finished with xylol. With a little



experience the process of decolorisation can be judged of by observing the appearances under a low objective.

### THE STAINING OF BACTERIA.

**Staining Principles.**—To speak generally, the protoplasm of bacteria reacts to stains in a manner similar to the nuclear chromatin, though sometimes more and sometimes less actively. The bacterial stains *par excellence* are the basic aniline dyes. These dyes are more or less complicated compounds derived from the coal-tar product aniline ( $C_6H_5 \cdot NH_2$ ). Many of them have the constitution of salts. Such compounds are divided into two groups according as the staining action depends on the basic or the acid portion of the molecule. Thus the acetate of rosaniline derives its staining action from the rosaniline. It is therefore called a basic aniline dye. On the other hand, ammonium picrate owes its action to the picric acid part of the molecule. It is therefore termed an acid aniline dye. These two groups have affinities for different parts of the animal cell. The basic stains have a special affinity for the nuclear chromatin, the acid for the protoplasm and various formed elements. Thus it is that the former—the basic aniline dyes—are especially the bacterial stains.

The number of basic aniline stains is very large. The following are the most commonly used :—

*Violet Stains.*—Methyl-violet, R-5R (synonyms : Hoffmann's violet, dahlia).

Gentian-violet (synonyms : benzyl-violet, Pyoktanin).

Crystal violet.

*Blue Stains.*—Methylene-blue<sup>1</sup> (synonym : phenylene-blue).

Victoria-blue.

Thionin-blue.

*Red Stains.*—Basic fuchsin (synonyms : basic rubin, magenta).

Safranin (synonyms : fuchsia, Giroflé).

*Brown Stain.*—Bismarck-brown (synonyms : vesuvin, phenylene-brown).

It is of the greatest importance that the stains used by the bacteriologist should be good, and therefore it is advisable to obtain those prepared by Grüber of Leipzig.

Of the stains specified, the violets and reds are the most intense in action, especially the former. It is thus easy in using them to overstain a specimen. Of the blues, methylene-blue probably gives the best differentiation of structure, and it is

<sup>1</sup> This is to be distinguished from methyl-blue, which is a different compound.



difficult to overstain with it. Thionin-blue also gives good differentiation and does not readily overstain. Its tone is deeper than that of methylene-blue, and it approaches the violets in tint. Bismarck-brown is a weak stain, but is useful for some purposes. Formerly it was much used in photomicrographic work, as it was less actinic than the other stains. It is not, however, needed now, on account of the improved sensitiveness of plates.

It is most convenient to keep saturated alcoholic solutions of the stains made up, and for use to filter a little into about ten times its bulk of distilled water in a watch-glass. A solution of good body is thus obtained. Most bacteria (except those of tubercle, leprosy, and a few others) will stain in a short time in such a fluid. Watery solutions may also be made up, *e.g.* a saturated watery solution of methylene-blue or a 1 per cent. solution of gentian-violet. Stains must always be filtered before use; otherwise there may be deposited on the preparation granules which it is impossible to wash off. The violet stains in solution in water have a great tendency to decompose. Only small quantities should therefore be prepared at a time.

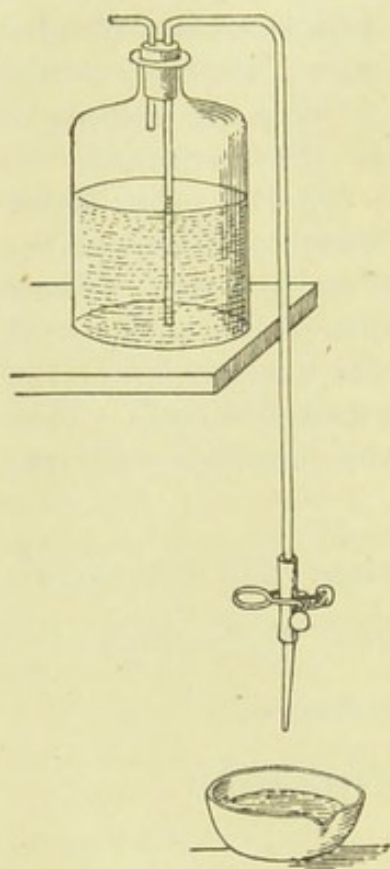


FIG. 42. — Siphon wash-bottle for distilled water used in washing preparations.

*The Staining of Cover-glass Films.*

—Films are made from *cultures* as described above. The cover-glass may be floated on the surface of the stain in a watch-glass, or the cover-glass held in Cornet's forceps with film side uppermost may have as much stain poured on it as it will hold. When the preparation has been exposed for the requisite time, usually a few minutes, it is well washed in tap water

in a bowl, or with distilled water with such a simple siphon arrangement as that figured (Fig. 42). The figure explains itself. When the film has been washed the surplus of water is drawn off with a piece of filter-paper, the preparation is carefully dried high over a flame, a drop of xylol balsam is applied, and the cover-glass mounted on a slide. It is sometimes advantageous to examine films in a drop of water in place of balsam. The films can be subsequently dried and mounted permanently.



Films of *fluids from the body* (blood, pus, etc.) can be generally stained in the same way, and this is often quite sufficient for diagnostic purposes. The blue dyes are here preferable, as they do not readily overstain. In the case of such fluids, if the histological elements also claim attention it is best first to stain the cellular protoplasm with 1-2 per cent. watery solution of eosin (which is an acid dye), and then to use a blue which will stain the bacteria and the nuclei of the cells. The Romanowsky stains (*vide* p. 113) are here most useful, as by these the preparations are fixed as well as stained. Fixation by heat, which is apt to injure delicate cellular structures, is thus avoided. In the case of films made from urine, where there is little or no albuminous matter present, the bacteria may be imperfectly fixed on the slide, and are thus apt to be washed off. In such a case it is well to modify the staining method. A drop of stain is placed on a slide, and the cover-glass, film-side down, lowered upon it. After the lapse of the time necessary for staining, a drop of water is placed at one side of the cover-glass and a little piece of filter-paper at the other side. The result is that the stain is sucked out by the filter-paper. By adding fresh drops of water and using fresh pieces of filter-paper, the specimen is washed without any violent application of water, and the bacteria are not displaced.

For the general staining of films a saturated watery solution of methylene-blue will be found to be the best stain to commence with, the Gram method (*vide infra*) is also used, and subsequently any special stains which may appear advisable.

**The Use of Mordants and Decolorising Agents.**—In films of blood and pus, and still more so in sections of tissues, if the above methods are used, the tissue elements may be stained to such an extent as to quite obscure the bacteria. Hence many methods have been devised in which the general principle may be said to be (*a*) the use of substances which, while increasing the staining power, tend to fix the stain in the bacteria, and (*b*) the subsequent treatment by substances which decolorise the overstained tissues to a greater or less extent, while they leave the bacteria coloured. The staining capacity of a solution may be increased—

(*a*) By the addition of substances such as carbolic acid, aniline oil, or metallic salts.

(*b*) By the addition of alkalies, such as caustic potash or ammonium carbonate, in weak solution.

(*c*) By the employment of heat.

(*d*) By long duration of the staining process.



As decolorising agents we use chiefly mineral acids (hydrochloric, nitric, sulphuric), vegetable acids (especially acetic acid), alcohol (either methylated spirit or absolute alcohol), or a combination of spirit and acid, *e.g.* methylated spirit with a drop or two of hydrochloric acid added, also various oils, *e.g.* aniline, clove, etc. In most cases about thirty drops of acetic acid in a bowl of water will be sufficient to remove the excess of stain from over-stained films and sections. More of the acid may, of course, be added if necessary.

Hot water also decolorises to a certain extent; over-stained films can often be readily decolorised by placing a drop of water on the film and heating gently over a flame.

When preparations have been sufficiently decolorised by an acid, they should be well washed in tap water, or in distilled water with a little lithium carbonate added.

Different organisms take up and retain the stains with various degrees of intensity, and thus duration of staining and decolorising must be modified accordingly. We sometimes have to deal with bacteria which show a special tendency to be decolorised. This tendency can be obviated by adding a little of the stain to the alcohol, or aniline oil, employed in dehydration. In the latter case a little of the stain is rubbed down in the oil. The mixture is allowed to stand. After a little time a clear layer forms on the top with stain in solution, and this can be drawn off with a pipette.

When methylene-blue, methyl-violet, or gentian-violet is used, the stain can, after the proper degree of decolorisation has been reached, be fixed in the tissues by treating for a minute with ammonium molybdate ( $2\frac{1}{2}$  per cent. in water).

#### The Formulæ of some of the more commonly used Stain Combinations.

##### 1. *Löffler's Methylene-blue.*

Saturated solution of methylene-blue in alcohol	30 c.c.
Solution of potassium hydrate in distilled water (1-10,000)	100 „

(This dilute solution may be conveniently made by adding 1 c.c. of a 1 per cent. solution to 99 c.c. of water.)

*Sections* may be stained in this mixture for from a quarter of an hour to several hours. They do not readily overstain. The tissue containing the bacteria is then decolorised if necessary with  $\frac{1}{2}$ -1 per cent. acetic acid, till it is a pale blue-green. The section is washed in water, rapidly dehydrated with alcohol or aniline oil, cleared in xylol, and mounted.

The tissue may be contrast-stained with eosin. If this is desired, after decolorisation wash with water, place for a few seconds in 1 per cent. solution of eosin in absolute alcohol, rapidly complete dehydration with pure absolute alcohol, and proceed as before.



*Films* may be stained with Löffler's blue by five minutes' exposure or longer in the cold. They usually do not require decolorisation, as the tissue elements are not overstained.

2. *Kühne's Methylene-blue.*

Methylene-blue	. . . . .	1.5 grm.
Absolute alcohol	. . . . .	10 c.c.
Carbolic acid solution (1-20)	. . . . .	100 ,,

Stain and decolorise as with Löffler's blue, or decolorise with very weak hydrochloric acid (a few drops in a bowl of water).

3. *Carbol-Thionin-blue.*—Make up a stock solution consisting of 1 gramme of thionin-blue dissolved in 100 c.c. carbolic acid solution (1-40). For use, dilute one volume with three of water, and filter. Stain sections for five minutes or upwards. Wash very thoroughly with water, otherwise a deposit of crystals may occur in the subsequent stages. Decolorise with very weak acetic acid. A few drops of the acid added to a bowl of water are quite sufficient. Wash again thoroughly with water. Dehydrate with absolute alcohol. Thionin-blue stains more deeply than methylene-blue, and gives equally good differentiation. It is very suitable for staining typhoid and glanders bacilli in sections. Cover-glass preparations stained by this method do not usually require decolorisation. As a contrast stain, 1 per cent. watery solution of eosin may be used before staining with the thionin.

4. *Gentian-violet in Aniline Oil Water.*—Two solutions have here to be made up. (a) Aniline oil water. Add about 5 c.c. aniline oil to 100 c.c. distilled water in a flask, and shake violently till as much as possible of the oil has dissolved. Filter and keep in a covered bottle to prevent access of light. (b) Make a saturated solution of gentian-violet in alcohol. When the stain is to be used, 1 part of (b) is added to 10 parts of (a), and the mixture filtered. The mixture should be made not more than twenty-four hours before use. Stain sections for a few minutes; then decolorise with methylated spirit. Sometimes it is advantageous to add to the methylated spirit a little hydrochloric acid (2-3 minims to 100 c.c.). This staining solution is not so much used by itself as in Gram's method, which is presently to be described.

5. *Carbol-Gentian-Violet.*—1 part of saturated alcoholic solution of gentian-violet is mixed with 10 parts of 5 per cent. solution of carbolic acid. It is used as No. 4.

6. *Carbol-Fuchsin* (see p. 108).—This is a very powerful stain, and, when used in the undiluted condition,  $\frac{1}{2}$ -1 minute's staining is usually sufficient. It is better, however, to dilute with from five to ten times its volume of water and stain for a few minutes. In this form it has a very wide application. Methylated spirit with or without a few drops of acetic acid is the most convenient decolorising agent. Then dehydrate thoroughly, clear, and mount.

**Gram's Method and its Modifications.**—In the methods already described, the tissues, and more especially the nuclei, retain some stain when decolorisation has reached the point to which it can safely go without the bacteria themselves being affected. In the method of Gram, now to be detailed, this does not occur, for the stain can here be removed completely from



the ordinary tissues, and left only in the bacteria. All kinds of bacteria, however, do not retain the stain in this method, and therefore in the systematic description of any species it is customary to state whether it is, or is not, stained by Gram's method—by this is meant, as will be understood from what has been said, *whether the particular organism retains the colour after the latter has been completely removed from the tissues*. It must, however, be remarked that some tissue elements may retain the stain as firmly as any bacteria, *e.g.* keratinised epithelium, calcified particles, the granules of mast cells, and sometimes altered red blood corpuscles, etc.

In Gram's method the essential feature is the treating of the tissue, after staining, with a solution of iodine. This solution is spoken of as Gram's solution, and has the following composition :—

Iodine	.	.	.	.	.	1 part.
Potassium iodide	.	.	.	.	.	2 parts.
Distilled water	.	.	.	.	.	300 „

The following is the method :—

1. Stain in aniline oil gentian-violet or in carbol-gentian-violet (*vide supra*), for about five minutes.
2. Without washing in water, now treat the section or film with repeated doses of Gram's solution till its colour becomes a purplish black, and allow the solution to act for one minute.
3. Again without washing with water, decolorise with absolute alcohol or methylated spirit till the colour has almost entirely disappeared, the tissues having only a faint violet tint. The period of time for which the alcohol is allowed to act varies in different laboratories. The best period is probably about three minutes.
4. Dehydrate completely, clear with xylol, and mount. In the case of film preparations, the specimen is simply washed in water, dried, and mounted.

In stage (3) the process of decolorisation is more satisfactorily performed by using clove oil after sufficient dehydration with alcohol, the clove oil being afterwards removed by xylol.

As a *contrast* stain for the tissues, carmalum or lithia carmine is used before staining with gentian-violet (1). As a contrast stain for other bacteria which are decolorised by Gram's method, carbol-fuchsin diluted with twenty volumes of water or a saturated watery solution of Bismark-brown may be used before stage (4); the former should not be applied for longer than a few seconds.

The following modifications of Gram's method may be given :—

1. *Weigert's Modification*.—The contrast staining of the tissues and stages (1) and (2) are performed as above.
- (3) After using the iodine solution the preparation is dried by blotting and then decolorised by aniline-xylol (aniline-oil 2, xylol 1).



(4) Wash well in xylol, and mount in xylol balsam. Film preparations after being washed in xylol may be dried, and thereafter dilute carbol-fuchsin may be used to stain bacteria which have been decolorised.

This modification probably gives the most uniformly successful results.

2. *Nicoll's Modification*.—Carbol-gentian-violet is used as the stain. Treatment with iodine is carried out as above, and decolorisation is effected with a mixture of acetone (1 part) and alcohol (2 parts).

3. *Kühne's Modification*.—(1) Stain for five minutes in a solution made up of equal parts of saturated alcoholic solution of crystal-violet ("Krystall-violet") and 1 per cent. solution of ammonium carbonate.

(2) Wash in water.

(3) Place for two to three minutes in Gram's iodine solution, or in the following modification by Kühne :—

Iodine	.	.	.	.	.	2 parts.
Potassium iodide	.	.	.	.	.	4 "
Distilled water	.	.	.	.	.	100 "

For use, dilute with water to make a sherry-coloured solution.

(4) Wash in water.

(5) Decolorise in a saturated alcoholic solution of fluorescein (a saturated solution in methylated spirit does equally well).

(6) Dehydrate, clear, and mount.

There is great variability in the avidity with which organisms stained by Gram retain the dye when washed with alcohol, and sometimes difficulty is experienced in saying whether an organism does or does not stain by this method.

Most bacteria are either frankly Gram-positive or frankly Gram-negative, but cases occur when an organism, usually Gram-positive or Gram-negative, tends when grown on certain media to show an opposite tendency, and sometimes an organism is met with in which the individuals in a film show slightly different reactions to the Gram stains.

**Stain for Tubercle and other Acid-fast Bacilli.**—These bacilli cannot be well stained with a simple watery solution of a basic aniline dye. This fact can easily be tested by attempting to stain a film of a tubercle culture with such a solution; with the Gram method, however, a partial staining is sometimes effected. Such bacteria require a powerful stain containing a mordant, and must be exposed to the stain for a long time, or its action may be aided by a short application of heat. When once stained, however, they resist decolorising even with very powerful acids; they are therefore called "acid-fast." The smegma bacillus also resists decolorising with strong acids (p. 280), and a considerable number of other acid-fast bacilli are now known (p. 278). Any combination of gentian-violet or fuchsin with aniline oil or carbolic acid or



other mordant will stain the bacilli named, but the following methods are most commonly used :—

*Ziehl-Neelsen Carbol-Fuchsin Stain.*

Basic fuchsin . . . . .	1 part.
Absolute alcohol . . . . .	10 parts.
Solution of carbolic acid (1 : 20) . . . . .	100 „

1. Place the specimen in this fluid, and having heated it till steam rises, allow it to remain there for five minutes, or allow it to remain in the cold stain for from twelve to twenty-four hours. (Films and paraffin sections are usually stained with hot stain, loose sections with cold ; in hot stain the latter shrink.)

2. Decolorise with 20 per cent. solution of strong sulphuric acid, nitric acid, or hydrochloric acid, in water. In this the tissues become yellow.

3. Wash well with water. The tissues will regain a faint pink tint. If the colour is distinctly red, the decolorisation is insufficient, and the specimen must be returned to the acid. As a matter of practice, it is best to remove the preparation from the acid every few seconds and wash in water, replacing the specimen in the acid and re-washing till the proper pale pink tint is obtained. Then wash in alcohol for half a minute, and replace in water.

4. Contrast stain with a saturated watery solution of methylene-blue for half a minute, or with saturated watery Bismarck-brown for from two to three minutes.

5. Wash well with water. In the case of films, dry and mount. In the case of sections, dehydrate, clear, and mount.

*Fraenkel's Modification of the Ziehl-Neelsen Stain.*

Here the process is shortened by using a mixture containing both the decolorising agent and the contrast stain.

The sections or films are stained with the carbol-fuchsin as above described, and then placed in the following solution :—

Distilled water . . . . .	50 parts.
Absolute alcohol . . . . .	30 „
Nitric acid . . . . .	20 „
Methylene-blue in crystals to saturation.	

They are treated with this till the red colour has quite disappeared and been replaced by blue. The subsequent stages are the same as in No. 5, *supra*.

Leprosy bacilli are stained in the same way, but are rather more easily decolorised than tubercle bacilli, and it is better to use only 5 per cent. sulphuric acid in decolorising.

In the case of specimens stained either by the original Ziehl-Neelsen method, or by Fraenkel's modification, the tubercle or



leprosy bacilli ought to be bright red, and the tissue blue or brown, according to the contrast stain used. Other bacteria which may be present are also coloured with the contrast stain.

**The Staining of Spores.**—If bacilli containing spores are stained with a watery solution of a basic aniline dye the spores remain unstained. The spores either take up the stain less readily than the protoplasm of the bacilli, or they have a resisting envelope which prevents the stain penetrating to the protoplasm. Like the tubercle bacilli, when once stained they retain the colour with considerable tenacity. The following is the simplest method for staining spores :—

1. Stain cover-glass films as for tubercle bacilli.
2. Decolorise with 1 per cent. sulphuric acid in water or with methylated spirit. This removes the stain from the bacilli.
3. Wash in water.
4. Stain with saturated watery methylene-blue for half a minute.
5. Wash in water, dry, and mount in balsam.

The result is that the spores are stained red, the protoplasm of the bacilli blue.

The spores of some organisms lose the stain more readily than those of others, and for some, methylated spirit is a sufficiently strong decolorising agent for use. If sulphuric acid stronger than 1 per cent. is used, the spores of many bacilli are readily decolorised.

*Möller's Method.*—The following method, recommended by Möller, is much more satisfactory than the previous. Before being stained, the films are placed in chloroform for two minutes, and then in a 5 per cent. solution of chromic acid for  $\frac{1}{2}$ –2 minutes, the preparation being well washed after each reagent. Thereafter they are stained and decolorised as above.

**The Staining of Capsules.**—The two following methods may be recommended in the case of capsulated bacteria :—

(a) *Welch's Method.*—This depends on the fact that in many cases the capsules can be fixed with glacial acetic acid.

Films when still wet are placed in this acid for a few seconds.

The superfluous acid is removed with filter-paper, and the preparation is treated with gentian-violet in aniline oil water repeatedly till all the acetic acid is removed.

Then wash with 1–2 per cent. solution of sodium chloride, and examine in the same solution.

The capsule appears as a pale violet halo around the deeply stained bacterium.

(b) *Hiss's Method.*—The staining solution consists of 1 part of a saturated alcoholic solution of fuchsin or gentian-violet and 19 parts of distilled water. A few drops of the stain are placed on a film, previously dried and fixed by heat, and the preparation is steamed for a few seconds over a flame. The staining solution is washed off with a 20 per cent. solution of copper sulphate, the preparation (without being washed in water) is dried between filter-papers, and when thoroughly dry is mounted in balsam. The capsules of pneumococci growing in a fluid serum medium can be readily



demonstrated by this method; in the case of solid cultures films should be made without any diluent, or a drop of fluid serum should be used. The method is easily applied, and gives excellent results.

(c) *Richard Muir's Method* (as recently modified).

1. The film containing the bacteria must be very thin. It is dried and stained in filtered carbol-fuchsin for half a minute, the preparation being gently heated.

2. Wash slightly with spirit and then well in water.

3. Place in following mordant for a few seconds:—

Saturated solution of corrosive sublimate.	.	.	.	2 parts.
Tannic acid solution—20 per cent.	.	.	.	2 „
Saturated solution of potash alum	.	.	.	5 „

4. Wash well in water.

5. Treat with methylated spirit for about a minute.

The preparation has a pale reddish appearance.

6. Wash well in water.

7. Counterstain with watery solution of ordinary methylene-blue for half a minute.

8. Dehydrate in alcohol, clear in xylol, and mount in balsam.

The bacteria are a deep crimson, and the capsules of a blue tint. The capsules of bacteria in certain culture media may be demonstrated by this method.

**The Staining of Flagella.**—The staining of the flagella of bacteria is the most difficult of all bacteriological procedures, and it requires considerable practice to ensure that good results shall be obtained. Many methods have been introduced, of which the two following are the most satisfactory:—

*Preparation of Films.*—In all the methods of staining flagella, young cultures on agar should be used, say a culture incubated for from ten to eighteen hours at 37° C. A very small portion of the growth is taken on the point of a platinum needle, and carefully mixed in a little water in a watch-glass; the amount should be such as to produce scarcely any turbidity in the water. A film is then made by placing a drop on a clean cover-glass and carefully spreading it out with the needle. It is allowed to dry in the air, and is then passed twice or thrice through a flame, care being taken not to over-heat it. The cover-glasses used should always be cleaned in the mixture of sulphuric acid and potassium bichromate described on page 93.

#### 1. *Pitfield's Method as modified by Richard Muir.*

Prepare the following solutions:—

##### A. *The Mordant.*

Tannic acid, 10 per cent. watery solution, filtered	.	.	.	10 c.c.
Corrosive sublimate, saturated watery solution	.	.	.	5 „
Alum, saturated watery solution	.	.	.	5 „
Carbol-fuchsin ( <i>vide</i> p. 108)	.	.	.	5 „



Mix thoroughly. A precipitate forms, which must be allowed to deposit, either by centrifugalising or simply by allowing to stand. Remove the clear fluid with a pipette, and transfer to a clean bottle. The mordant keeps well for one or two weeks.

B. *The Stain.*

Alum, saturated watery solution . . . . .	10 c.c.
Gentian-violet, saturated alcoholic solution . . . . .	2 „

The stain should not be more than two or three days old when used. It may be substituted in the mordant in place of the carbol-fuchsin.

The film having been prepared as above described, pour over it as much of the mordant as the cover-glass will hold. Heat gently over a flame till steam begins to rise, allow to steam for about a minute, and then wash well in a stream of running water for about two minutes. Then dry carefully over the flame, and when thoroughly dry pour on some of the stain. Heat as before, allowing to steam for about a minute, wash well in water, dry, and mount in a drop of xylol balsam.

This method has yielded the best results in our hands.

2. *Van Ermengem's Method for Staining Flagella.*

The films are prepared as above described. Three solutions are here necessary :—

Solution A. (*Bain fixateur*)—

Osmic acid, 2 per cent. solution . . . . .	1 part.
Tannin, 10-25 per cent. solution . . . . .	2 parts.

Place the films in this for one hour at room temperature, or heat over a flame till steam rises and keep in the hot stain for five minutes. Wash with distilled water, then with absolute alcohol for three to four minutes, and again in distilled water, and treat with

Solution B. (*Bain sensibilisateur*)—

5 per cent. solution of nitrate of silver in distilled water. Allow films to be in this a few seconds. Then without washing transfer to

Solution C. (*Bain reducteur et renforçateur*)—

Gallic acid . . . . .	5 grms.
Tannin . . . . .	3 „
Fused potassium acetate . . . . .	10 „
Distilled water . . . . .	350 c.c.

Keep in this for a few seconds. Then treat again with Solution B till the preparation begins to turn black. Wash, dry, and mount.

It is better, as Mervyn Gordon recommends, to leave the specimen in B for two minutes, and then to transfer to C for one and a half to two minutes, and not to transfer again to B. It will also be found an advantage to use a fresh supply of C for each preparation, a small quantity being sufficient. The beginner will find the typhoid bacillus or the bacillus coli communis very suitable organisms to stain by this method.



Although the results obtained by this method are sometimes excellent, they vary considerably. Frequently both the organisms and flagella appear of abnormal thickness. This is due to the fact that the process on which the method depends is a precipitation rather than a true staining. The pictures on the whole are less faithful than in the first method.

**Staining of Spirochætes in Sections.**—The following impregnation methods have been applied for this purpose by Levaditi, and give excellent results:—

*(a) Levaditi's Original Method.*

(1) The tissues, which ought to be in thin slices, about 1 mm. in thickness, are best fixed in 10 per cent. formalin solution for twenty-four hours.

(2) They are washed for an hour in water, and then brought into 96 per cent. alcohol for twenty-four hours.

(3) They are then placed in 1.5 per cent. solution of nitrate of silver in a dark bottle, and are kept in an incubator at 37° C. for three days.

(4) They are washed in water for about twenty minutes, and are thereafter placed in the following mixture, namely:—

Pyrogallic acid, 4 parts.

Formalin, 5 parts.

Distilled water up to 100 parts.

They are kept in this mixture in a dark bottle for forty-eight hours at room temperature.

(5) They are then washed in water for a few minutes, taken through increasing strengths of alcohol, and embedded in paraffin in the usual way. The sections ought to be as thin as possible. In satisfactory preparations the spirochætes appear of an almost black colour against the pale yellow background of the tissues. The latter can be contrast-stained by weak carbol-fuchsin or by toluidin blue.

*(b) Levaditi's Newer Pyridin Method.*

(1) The tissues are fixed in formalin as in the previous method, are hardened in alcohol for twelve to sixteen hours, and then washed in water.

(2) They are then impregnated with a 1 per cent. solution of silver nitrate, to which 10 per cent. of pyridin puriss. is added at the time of use. The tissues are placed in the solution in a well-stoppered bottle, and are kept for two to three hours at room temperature and four to six hours at about 50° C. They are thereafter washed quickly in 10 per cent. pyridin solution.

(3) Reduction is then carried out in the following mixture, namely, a 4 per cent. solution of pyrogallic acid to which are added, at the time of use, 10 per cent. pure acetone and 15 per cent. pyridin.

(4) The tissues are then put through alcohol and xylol, and embedded in paraffin. The sections can be stained with toluidin blue or Unna's polychrome blue.

(For the staining of spirochætes in films, see p. 115.)



**The Romanowsky Stains.**—Within recent years the numerous modifications of the Romanowsky stain have been extensively used. The dye concerned is the compound which is formed when watery solutions of medicinal methylene-blue and water-soluble eosin are brought together. This compound is insoluble in water but soluble in alcohol—the alcohol employed being methyl alcohol. The stain was originally used by Romanowsky for the malarial parasite, and its special quality is that it imparts to certain elements, such as the chromatin of this organism, a reddish-purple hue. This was at first thought to be simply due to the combination of the methylene-blue and the eosin, but it is now recognised that certain changes, such as occur in methylene-blue solutions with age, are necessary. In the modern formulæ these changes are brought about by treatment with alkalies, especially alkaline carbonates, as was first practised by Unna in the preparation of his polychrome methylene-blue. The stains in use thus contain a mixture of methylene-blue and its derivatives in combination with eosin; the differences in these bodies and the different proportions in which they occur in individual stains account for the different effects produced on the various constituents of a cell. The underlying chemical reactions are complicated and as yet not fully understood. Thus it is not certainly known to what particular new body the reddish hue produced in chromatin is due, but the active constituent may be methyl-violet or methyl-azure or thionin, all of which result from the action of alkali on methylene-blue. The stains are much used in staining blood-films (in which the characters of both nucleus and cytoplasm in leucocytes are beautifully brought out), in staining bacteria in tissues or exudates, the malaria parasite, trypanosomes, the pathogenic spirochætes (such as the spirochæte pallida), and protozoa generally.

The following are the chief formulæ in use :—

1. *Jenner's Stain.*—This is an excellent blood stain, but is not so good for the study of parasites as the others to be mentioned. In its preparation no alkali is used. It is made by mixing equal parts of (a) a 1·2 to 1·25 per cent. solution of Grüber's water-soluble eosin (yellow shade) in distilled water and (b) 1 per cent. Grüber's medicinal methylene-blue (also a watery solution). The mixture is allowed to stand twenty-four hours, is filtered, and the residue is dried at 55° C.; the powder is shaken up in distilled water, filtered, washed with distilled water, and dried. Of the powder, 5 grm. is dissolved in 100 c.c. Merck's methyl alcohol. For use a few drops are placed on the dried unfixed film for one to three minutes, the dye is poured off, and the preparation washed with distilled water till it presents a pink colour; it is then dried between filter-paper and mounted in xylol balsam.



2. *Leishman's Stain*.—The following solutions are prepared: (a) to a 1 per cent. solution of medicinal methylene-blue is added .5 per cent. sodium carbonate; the mixture is kept at 65° C. for twelve hours, and then for ten days at room temperature (.25 per cent. formalin may be added as a preservative); (b) 1-1000 solution of eosin, extra B.A., in distilled water. Equal volumes of the two solutions are mixed and allowed to stand for six to twelve hours with occasional stirring, the precipitate is collected, filtered, washed with distilled water, and dried. For use, .15 per cent. is dissolved in Merck's methyl alcohol ("for analysis, acetone free") as follows: The powder is placed in a clean mortar, a little of the alcohol is added and well rubbed up with a pestle; the undissolved powder is allowed to settle and the fluid decanted into a dry bottle; the process is repeated with fresh fractions of the solvent till practically all the stain is dissolved, and the bottle is well stoppered. The stain will keep for a long period. For the staining of films a few drops of the stain are placed on the unfixed preparation for fifteen to thirty seconds so as to cover it with a shallow layer (the stain may be conveniently spread over the film with a glass rod), and the film is tilted to and fro so as to prevent drying. This treatment efficiently fixes the film by the action of the methyl alcohol. About double the quantity of distilled water is now dropped on the film, and the stain and diluent are quickly mixed with the rod. Five minutes are now allowed for staining, and the stain is then gently washed off with distilled water. A little of the water is kept on the film for half a minute to intensify the colour contrasts in the various cells. For certain special structures, such as Schüffner's dots or Maurer's dots in the malarial parasite, a longer staining (up to one hour) may be necessary, and in any case it is well to practise being able to control the depth of the staining effect by observation with a low-power objective. If a preparation is to be stained for a long time it must be kept covered, and if in such cases a granular deposit is formed this may be got rid of by a quick wash with absolute alcohol. If in blood films the red corpuscles appear bluish instead of pink, the colour may be restored by washing the film with acetic acid, 1-1500. The film is dried between filter-paper and mounted.

For staining sections a little modification is necessary. A paraffin section is taken into distilled water as usual, the excess of water is drained off, and a mixture of one part of stain and two parts of distilled water is placed on it. The stain is allowed to act for five to ten minutes till the tissue appears a deep Oxford blue; it is then decolorised with 1-1500 acetic acid—the effect being watched under a low-power lens. The blue begins to come out, and the process is allowed to go on till only the nuclei remain blue. The section is then washed with distilled water, rapidly dehydrated with alcohol, cleared, and mounted. If, as sometimes happens, the eosin tint be too well marked, it can be lightened by the action of 1-7000 solution of caustic soda, this being washed off whenever the desired colour has been attained.

In certain cases, *e.g.* for the staining of old films or of trypanosomes or Leishmaniae in sections, Leishman recommends an initial treatment of the preparation with serum. This modification is described in Appendix E.

3. *J. H. Wright's Stain*.—In this modification 1 per cent. methylene-blue (BX or Ehrlich's rectified) and  $\frac{1}{2}$  per cent. sodium carbonate (both in water) are mixed and placed in a Koch's steriliser for an hour. When



the fluid is cold, 1-1000 solution of extra 'B.A. eosin is added till the mixture becomes purplish and a finely granular black precipitate appears in suspension (about 500 c.c. eosin to 100 c.c. methylene-blue solution are required); the precipitate is filtered off and dried without being washed. A saturated solution of this is made in the pure methyl alcohol; this is filtered and diluted by adding to 80 c.c. of the saturated solution 20 c.c. of methyl alcohol. The application of the stain is almost the same as with Leishman's. A few drops are placed on the preparation for a minute for fixation; water is then dropped on till a green iridescent scum appears on the top of the fluid, and staining goes on for about two minutes; the stain is then washed off with distilled water, and a little is allowed to remain on the film till differentiation is complete; the preparation is carefully dried with filter-paper, and mounted.

4. *Giemsa's Stain*.—Giemsa believes that the reddish-blue hue characteristic of the Romanowsky stain is due to the formation of methyl-azure, and he has prepared this by a method of his own under the name "Azur I." From this, by the addition of equal parts of medicinal methylene-blue, he prepares what he calls "Azur II.," and from this again by the addition of eosin he prepares "Azur II.-eosin." The latest formula for the finished stain is as follows: Azur II.-eosin, 3 gr.; Azur II., 8 gr.; glycerin (Merck, chemically pure), 250 gr.; methyl alcohol (Kahlbaum, I.), 250 gr. This stain has been extensively used for demonstrating the spirochæte pallida, but it can be used for any other purpose to which the Romanowsky stains are applicable. For the spirochæte the following are Giemsa's directions:—

(1) Fix films in absolute alcohol for fifteen to twenty minutes, dry with filter-paper. (2) Dilute stain with distilled water—one drop of stain to 1 c.c. water (the mixture being well shaken). (Sometimes the water is made alkaline by the addition of one drop of 1 per cent. potassium carbonate to 10 c.c. water.) (3) Stain for fifteen minutes. (4) Wash in brisk stream of distilled water. (5) Drain with filter-paper, dry, and mount in Canada balsam.

With regard to the Jenner and Giemsa stains it is best to obtain the solutions from Grüber ready for use; the powder for Leishman's stain may be obtained from the same source, and the solution made up by the worker himself. Cabot states that Wright's stain can be obtained from the Harvard Co-operative Society, Boylston Street, Boston, U.S.A.

*Neisser's Stain*.—(a) The following is the original method introduced by Neisser as an aid to the diagnosis of the diphtheria bacillus. Two solutions are used as follows: (a) 1 grm. methylene-blue (Grüber) is dissolved in 20 c.c. of 96 per cent. alcohol, and to the solution are added 950 c.c. of distilled water and 50 c.c. of glacial acetic acid; (b) 2 grms. Bismarck-brown (vesuvin) dissolved in a litre of distilled water. Films are stained in (a) for 1-3 seconds or a little longer, washed in water, stained for 3-5 seconds in (b), dried, and mounted. The protoplasm of the diphtheria bacillus is stained a faint brown colour, the granules a blue colour. Neisser considers that this reaction is characteristic of the organism, provided that cultures on Löffler's serum are used and examined after 9-24 hours' incubation at 34° to 35° C. Satisfactory results are not always obtained in the case of films prepared from membrane, etc., but there is no doubt that here also the method is one of considerable value.



(b) The following is Neisser's modified cresoidin method :—

1. Stain films for a few seconds in a mixture of solutions A and B, two parts of the former to one of the latter.

A. Methylene-blue . . . . .	1 part.
Absolute alcohol . . . . .	50 parts.
Glacial acetic acid . . . . .	50 „
Distilled water . . . . .	1000 „
B. Crystal-violet (Höchst) . . . . .	1 part.
Absolute alcohol . . . . .	10 parts.
Distilled water . . . . .	300 „

2. Wash in water, and
3. Stain in cresoidin solution (1 : 300) for a few seconds (the cresoidin should be dissolved in warm water and the solution then filtered).
4. Wash in water, dry, and mount.

**Sabouraud's Method for Staining Trichophyta.**—Remove the fat from the hair or epithelial squames with chloroform. Place in a test-tube with 10 per cent. formol, and warm for two or three minutes till ebullition commences. Wash well in distilled water, and stain for one minute in Sahli's blue, which is made up as follows :—

Distilled water . . . . .	40 parts.
Saturated watery methylene blue . . . . .	24 „
5 per cent. solution of borax in water . . . . .	16 „

Mix the constituents. Allow to stand for a day, and filter. After staining, wash in water, dehydrate with absolute alcohol, clear in xylol, and mount in balsam.



## CHAPTER IV.

### METHODS OF EXAMINING THE PROPERTIES OF SERUM—PREPARATION OF VACCINES— GENERAL BACTERIOLOGICAL DIAGNOSIS—IN- OCULATION OF ANIMALS.

#### THE TESTING OF AGGLUTINATIVE AND SEDIMENTING PROPERTIES OF SERUM.

##### **Wright's Method of measuring Small Amounts of Fluids.—**

It is convenient here to describe this method. In ordinary work fine calibrated pipettes may be used for measuring small quantities of fluids, but such pipettes are not always available, and by Wright's technique if a Gower's 5 c.mm. hæmocytometer pipette be at hand any measurements may be undertaken—in fact, once the pipette now to be described (see Fig. 43) is made we are independent of other means of measurement. A piece of quill tubing is drawn out to capillary dimensions, and the extreme tip of it is heated in a peep flame and then drawn out till it is of the thickness of a hair, though still possessing a bore. If the point be broken off this hair, and mercury be run into the tube, the metal will be caught where the tube narrows and will pass no further—in fact, though air will pass, mercury will not. Into the wide end of this tube 5 c.mm. of mercury, measured from a Gower's pipette, is run down till it will go no further. A mark is made on the tube at the proximal end of the mercury, which is now allowed to run out, and the tube is carefully cut through at the mark. A piece of ordinary quill tubing is drawn out and broken off just below where its narrowing has begun, the hair end of the capillary tube is slipped through the broken-off end, and the tube is fixed in position with wax as shown in the figure. A rubber nipple placed on the end of the pipette completes the apparatus. If by pressing the nipple the air be expelled from the pipette, and the end dipped under mercury, exactly 5 c.mm. will be taken up. Thus, when pressure on the



nipple is relaxed, other tubes can be very readily calibrated by the mercury being expelled into them, and its limits marked on their bores.

For measuring equal parts of different fluids, the pipette shown in Fig. 44, *d*, in connection with agglutination is very useful.

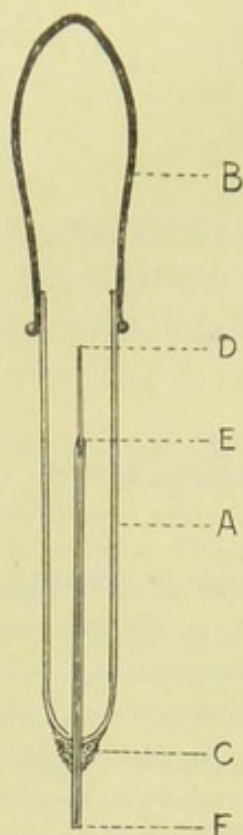


FIG. 43. — Wright's 5 c.mm. pipette. A, Casing of quill tubing; B, rubber nipple; C, wax luting; E to F, capillary tube of 5 c.mm. capacity; D to E, hair capillary.

**Methods of testing for Simple Agglutination.**—By *agglutination* is meant the aggregation into clumps of uniformly disposed bacteria in a fluid; by *sedimentation* the formation of a deposit composed of such clumps when the fluid is allowed to stand. Sedimentation is thus the naked-eye evidence of agglutination. The blood serum may acquire this clumping power towards a particular organism under certain conditions; these being chiefly met with when the individual is suffering from the disease produced by the organism, or has recovered from it, or when a certain degree of immunity has been produced artificially by injections of the organism. The nature of this property will be discussed later. Here we shall only give the technique by which the presence or absence of the property may be tested. There are two chief methods, a microscopic and a naked-eye, corresponding to the effects mentioned above. In both, the essential process is the bringing of the diluted serum into contact with the bacteria uniformly disposed in a fluid. In the

former this is done on a glass slide, and the result is watched under the microscope; the occurrence of the phenomenon is shown by the aggregation of the bacteria into clumps, and if the organism is motile this change is preceded or accompanied by more or less complete loss of motility. In the latter method the mixture is placed in an upright thin glass tube; sedimentation is shown by the formation within a given time (say from two hours at 37° C. to twenty-four hours at room temperature) of a somewhat flocculent layer at the bottom, the fluid above being clear. Two points should be attended to: (*a*) controls should always be made with normal serum, and (*b*) the serum to be tested should never be brought in the undiluted condition into



contact with the bacteria. The stages of procedure are the following:—

1. Blood is conveniently obtained by pricking the lobe of the ear, which should previously have been washed with a mixture of alcohol and ether, and allowed to dry. The blood is drawn up into a Wright's blood-capsule (Fig. 45) or into the bulbous portion of a capillary pipette, such as in Fig. 44, *a*. (These pipettes can be readily made by drawing out quill glass-tubing in a flame. It is convenient always to have several ready for use.) The pipette is kept in the upright position, one end being closed. For purposes of transit, break off the bulb at the constriction and seal the ends. After the serum has separated from the coagulum the bulb is broken through near its upper end, and the serum removed by means of another capillary pipette. The serum is then to be diluted.

2. The serum may be diluted (*a*) by means of a graduated pipette—either a leucocytometer pipette (Fig. 44, *b*) or some corresponding form. In this way successive dilutions of 1:10, 1:20, 1:100, etc. can be rapidly made. This is the best method. (*b*) By means of a capillary pipette with a mark on the tube, the serum is drawn up to the mark and then blown out into a glass capsule; equal quantities of bouillon are successively measured in the same way, and added till the requisite dilution is obtained. (*c*) By means of a platinum needle with a loop at the end (Delépine's method). A loopful of serum is placed on a slide, and the desired number of similar loopfuls of bouillon are separately placed around on the slide. The drops are then mixed.

A very convenient and rapid method of combining the steps 1 and 2

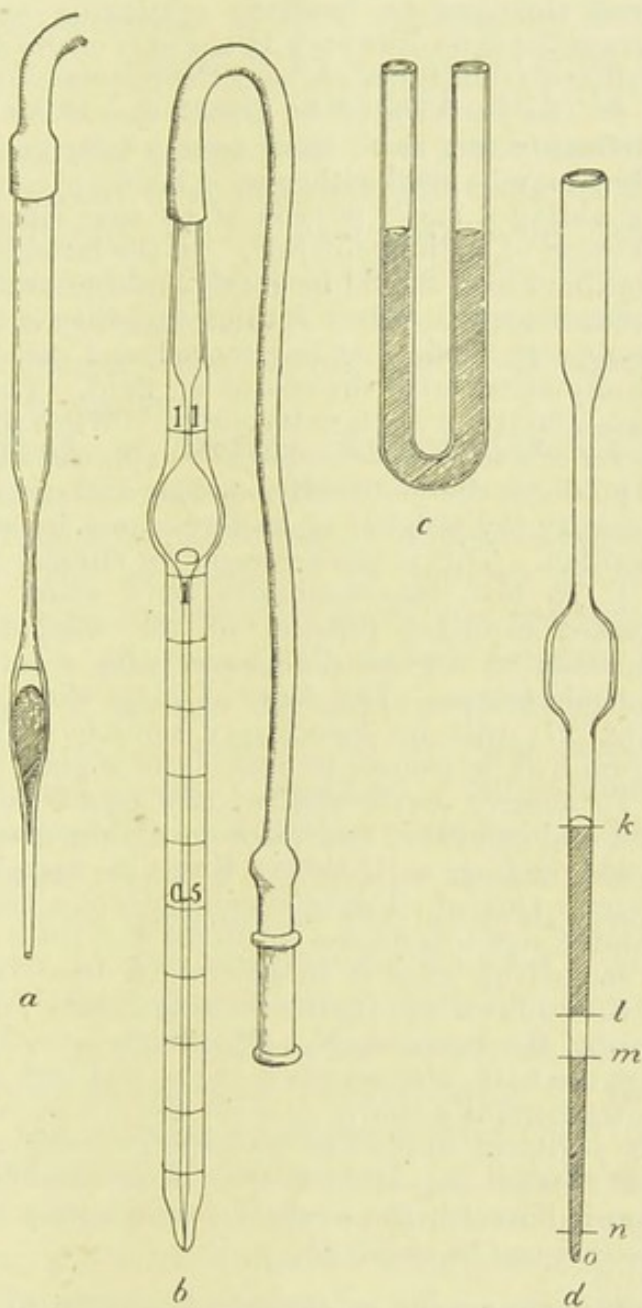


FIG. 44.—Tubes used in testing agglutinating and sedimenting properties of serum.



is to draw a drop of *blood* up to the mark 1 or .5 on a leucocytometer pipette, and draw the bouillon after it till the bulb is filled. A dilution of 10 or 20 times is thus obtained. Then blow the mixture into a U-shaped tube (Fig. 44, c), and centrifugalise or simply allow the red corpuscles to separate by standing. (In this method, of course, the dilution is really greater than if pure serum were used, and allowance must therefore be made in comparing results.) The presence of red corpuscles is no drawback in the case of the microscopic method, but when sedimentation tubes are used the corpuscles should be separated first.

3. The bacteria to be tested should be taken from young cultures, preferably not more than twenty-four hours old, incubated at 37° C. They may be used either as a bouillon culture or as an emulsion made by adding a small portion of an agar culture to bouillon or .8 per cent. solution of sodium chloride. In the latter case the mass of bacteria on a platinum loop should be gently broken down at the margin of the fluid in a watch-glass. When a thick turbidity is thus obtained, any remaining fragments should first be removed, and then the organisms should be uniformly mixed with the rest of the fluid. The bacterial emulsion ought to have a faint but distinct turbidity. (When the exact degree of sedimenting power of a serum is to be tested—expressed as the highest dilution in which it produces complete sedimentation within twenty-four hours—a standard quantity (by weight) of bacteria must be added to a given quantity of bouillon. This is not necessary for clinical diagnosis.)

4. To test *microscopically*, mix equal quantities (measured by a marked capillary pipette) of the diluted serum and the bacterial emulsion on a glass slide, cover with a cover-glass, and examine under the microscope. The form of glass slide used for hang-drop cultures (Fig. 27) will be found very suitable. The ultimate dilution of the serum will, of course, be double the original dilution.

To observe *sedimentation*, mix equal parts of diluted serum and of bacterial emulsion, and place in a thin glass tube—a simple tube with closed end or a U-tube. Keep in upright position for twenty-four hours. One of Wright's sedimentation tubes is shown in Fig. 44, d. Diluted serum is drawn up to fill the space *mn*, a small quantity of air is sucked up after it to separate it from the bacterial emulsion, which is then drawn up in the same quantity; the diluted serum will then occupy the position *kl*. The fluids are then drawn several times up into the bulb, and returned to the capillary tube so as to mix, and finally blown carefully down close to the lower end, which is then sealed off. The sediment collects at the lower extremity.

It is often important to observe not merely the fact that agglutination occurs, but also the weakest concentration of the serum with which the reaction can be obtained.

**Measurement of Group Agglutinins.**—In the case of certain groups of allied organisms,—notably the *b. coli* and its allies,—it has been found that when a serum clumps one member of the group it frequently also clumps the allied forms. If the greatest dilution with which agglutination is obtained be estimated, the end-points for the different strains affected are usually found to differ. The determination of the end-point is important, as the disease condition from which the serum is derived is generally caused by the organism which is clumped in highest dilution.



In comparing the effect of a serum on different bacteria, the sedimentation method is usually employed. A series of emulsions of the different bacteria to be tested is prepared by scraping off the growth on an agar tube, and suspending in bouillon. Each of these should approximately contain the same number of bacteria per unit volume. This is attained by using emulsions of equal opacity, as judged of by noting the point at which transparency to some arbitrary standard such as a particular type or set of parallel lines ceases. A given amount of each emulsion is now mixed with different dilutions of the serum to be tested, the mixtures are all made up to the same volume, say 1 c.c., and the tubes placed at 37° C. for two or three hours. The results are then read, the tubes are set aside at room temperature for twenty-four hours, and read again; usually the two readings correspond.

**The Absorption Method of testing Agglutinins.**—This method is applied under circumstances similar to those of the last, namely, when several agglutinins acting on allied organisms are present in a serum. The principle is to remove all the agglutinins acting on one organism, and to study the properties of those which remain. In practice the method consists in adding to the serum a mass of one of the bacteria of the group under study (the organisms being scraped off an agar slope), allowing the mixture to stand at 37° C. for two or three hours, and then separating the bacteria with the centrifuge. The supernatant clear fluid is now pipetted off, and its agglutinating properties studied on the other members of the bacterial group either by sedimentation or by the microscopic method. The use of the method is to aid in differentiating which member of a bacterial group is causally related to the condition from which the serum is obtained, and an example of its application for this purpose will be found in the chapter on typhoid fever (p. 375). It has also been used by Park and Collins and by Bainbridge for identifying strains of organisms of the typhoid-coli group. Here the principle is that, when an unknown strain belonging to such a bacterial group is under investigation, if its capacities for absorbing agglutinins from a serum containing a mixture of such are the same as those of an already recognised strain, then the two are probably identical.

#### OPSONIC METHODS.

**Method of measuring the Phagocytic Capacity of the Leucocytes.**—This was first done by Leishman by a very



simple method, as follows: A piece of quill tubing is drawn out to a capillary diameter so as to make a pipette about 6 inches long. The point is broken off, and a rubber nipple adjusted to the wide end; a mark is made with an oil pencil about three-quarters of an inch above the orifice. Blood is drawn from the finger up to the mark, then an air-bubble is allowed to pass in. A thin emulsion of the bacterium to be tested having been prepared, a quantity of this is also drawn up to the mark. The two fluids are thoroughly mixed by being first blown out on to a sterile slide and then being drawn back into the pipette and expelled,—this being repeated several times. A cover-glass is placed over the drop, and the slide is placed in the incubator at 37° C. for fifteen minutes. The cover-glass is then slipped off so as to make a film preparation, which in the case of ordinary bacteria may be stained by Leishman's method. The number of bacteria present in, say, fifty polymorphonuclear cells successively examined is determined, and an average struck. The method was first used for showing that in cases of staphylococcus infection the average number of bacteria taken up was less than in a control in which the same bacterial emulsion was exposed to the blood of a healthy individual. In making such an observation, drops from the two mixtures are placed on the same slide under separate cover-glasses, and the preparation incubated. One cover is then slipped to one end of the slide, and the other to the other,—the two films being then stained as one.

Leishman's method gives what may be called the total phagocytic capacity of the blood, but according to Wright's view the process of phagocytosis in blood outside the body is not a simple one, and before a leucocyte takes up a bacterium the latter must be acted on in some way by substances present in the serum, which Wright calls *opsonins* (*see* Immunity). The technique by which the actions of these opsonins is studied has been elaborated by Wright and his co-workers in connection with bacterial vaccines, especially in relation to infection by the pyogenic cocci and the tubercle bacillus. This technique involves (1) the preparation of the bacterial emulsion, (2) the preparation of the leucocytes, (3) the preparation of samples of (a) serum from a normal person, and (b) serum from the infected person.

(1) *Preparation of Bacterial Emulsion*.—In the case of the pyogenic cocci, a little of a twenty-four hour living culture off a sloped agar tube is taken and rubbed up in a watch-glass with .85 per cent. saline. The mixture is placed in a tube and centrifuged, so as to deposit any masses of bacteria which may be



present. Only by experience can a knowledge be gained of the amount of culture to be used in the first instance, but the resultant emulsion usually should exhibit only the merest trace of cloudiness to the naked eye. Wright states it will then contain from 7000 to 10,000 million bacteria per c.cm. If too strong an emulsion be used, the leucocytes may take up so many organisms that these cannot be accurately enumerated. In the case of the tubercle bacillus, as short a variety of the organism as possible should be selected, and a mass of growth off a solid medium is taken (bacilli in mass can be obtained in the market from wholesale chemists) and is well washed with changes of distilled water, drained on filter paper in a Petri dish, and thoroughly rubbed up with a little 1·5 per cent. saline in an agate mortar, so as to disintegrate the bacterial masses and get an emulsion composed as far as possible of individual bacilli. It is extremely difficult to obtain a good emulsion of tubercle bacilli, *i.e.* one that shall consist as far as possible of separate bacilli—on the one hand without clumps, and on the other without portions of disintegrated bacilli. The rubbing-up in the mortar, which usually occupies many hours, must be done very slowly and gently with a very light pestle, and the manipulation must be frequently controlled by microscopic observation. A thick cream should be obtained, and this should be sterilised by steaming for half an hour on three successive days. Before sterilisation it is convenient to seal up the stock emulsion in small quantities in a number of pieces of quill tubing, so that in the subsequent procedures only small portions of the emulsion are exposed to aerial contamination at one time. For actual use, one of those tubes is opened, a little is withdrawn with a sterile pipette, and a weak emulsion made in the same way as with the staphylococcus, except that 1·5 per cent. saline is used. The stock tube may be sealed with wax and kept for use again. A fresh emulsion ought to be made up for each day's work.

(2) *Preparation of Leucocytes.*—Here the observer uses his own blood cells. A 1·5 per cent. solution of sodium citrate in 85 per cent. sodium chloride is prepared. This is placed in a glass tube 3 inches long, made by drawing out a piece of half-inch tubing to a point, the tube being filled nearly to the brim. A handkerchief being bound round the finger, this is now pricked, and the blood allowed to flow directly into the fluid, to the bottom of which it sinks. The tube ought to be inverted between the addition of every few drops of blood, so as to bring the blood in contact with the citrate and prevent coagulation. The equivalent of about ten to twenty drops of



blood should be obtained. The diluted blood is then centrifuged, and when the corpuscles are separated the supernatant fluid is removed, .85 per cent. saline is substituted, and the centrifugalisation repeated. The fluid is again removed, care being taken not to disturb the layer of white cells lying on the top of the red corpuscles. This layer is then pipetted off into a watch-glass or tube, and the leucocytes required are thus obtained.

(3) *Preparation of the Sera.*—The serum whose sensitising effect on the bacteria it is desired to test is obtained by Wright as follows: A "blood-capsule" is made by drawing a piece of No. 3 quill tubing into the shape shown in Fig. 45, the part not drawn out being about 1 inch in length. It is convenient to

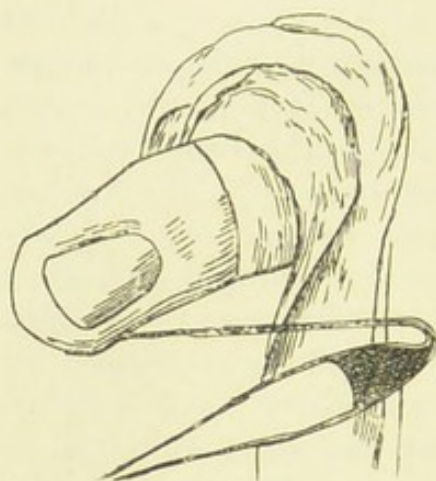


FIG. 45.—Wright's blood-capsule, and method of filling same.

make a number of these capsules at one time, and to draw off their extremities and seal them in the flame. For use, the tips of both extremities are broken off, the finger is pricked, and blood allowed to pass into the capsule through the bent limb till the capsule is about half full. The air remaining in the capsule is rarefied by passing the straight end through a flame and then sealing it off. By this manipulation the blood is sucked over the bend into the straight part of the tube, and the bent end is now also sealed off or closed with wax. It is

well to shake the blood down towards the closed straight end, care being taken to previously allow the glass to cool sufficiently. The capsule is now hung by the bend on the edge of a centrifuge tube, and the serum separated by spinning the instrument. In any particular case a capsule of serum from the infected person and one from a normal individual are prepared.

The emulsion, corpuscles, and serum being thus prepared, the next step is to mix them. This is done by taking a piece of quill tubing and drawing it out to a capillary point so as to make a pipette about 8 inches long; on the thick end of this a rubber teat is fixed, and about 1 inch from the capillary point a mark is made with an oil pencil. From the watch-glass containing the separated leucocytes a portion is sucked up to the mark, and then an air-bubble is allowed to pass in. A similar portion of the serum is drawn up, and then another



air-bubble, and finally a similar portion of the bacterial emulsion. The three droplets are carefully blown on to a slide, and are thoroughly mixed with one another by being alternately drawn up into the tube and expelled ten times. The mixture is then drawn into the tube, and the end sealed off in the flame. The rubber nipple is removed, and the tube placed in the incubator at  $37^{\circ}$  for fifteen minutes. A slide is now prepared by rubbing it once or twice with very fine emery paper (No. 000) and thoroughly wiping it. This is a procedure adopted by Wright to cause an evenly distributed film to be made. The tube being removed from the incubator and the end broken off, its contents are again mixed by expelling and drawing up into the tube. A minute droplet is placed on the prepared slide, and by means of the edge of the end of another slide a film is made, which is then dried and is ready for staining. The spreader should be slightly narrower than the slide on which the film is made; in this way the film has two definite edges—a fact of importance, as the leucocytes are usually in greatest abundance near these edges. Films containing staphylococci are stained either by Leishman's stain (*q.v.*) or with carbolthionin blue. In the former case no fixation is necessary, in the latter it is usual to fix in saturated perchloride of mercury for one and a half minutes, wash in water, and then stain. With tubercle films the following is the procedure: The film is fixed for two minutes in perchloride of mercury, washed thoroughly, stained with carbol-fuchsin as usual, decolorised with 2.5 per cent. sulphuric acid, cleared with 4 per cent. acetic acid, counter-stained with watery solution of methylene-blue, and dried.

In applying the technique two preparations are made, in both of which the same emulsion and the same leucocytes are employed; but in one the bacteria have been exposed to the serum of the infected individual under observation, and in the other to that of a normal person,—usually the observer himself,—or better still, to a mixture of sera from several normal persons. Each of these preparations is now examined microscopically with a movable stage, the number of bacteria in the protoplasm of at least fifty polymorphonucleated leucocytes is counted, and an average per leucocyte struck (this is often called the “phagocytic index”); the proportion which this average in the case of the abnormal serum bears to the average in the preparation in which the healthy serum was used constitutes the *opsonic index*—that of healthy serum being reckoned as unity.

The reliability of the opsonic method, of course, depends on whether or not the phagocytic activity of the cells counted



represents the phagocytic activity of the cells in the preparation. Considerable controversy has arisen on this point. The general result may be said to be that where such organisms as the pyogenic cocci are concerned, the ordinary opsonic technique gives on the whole reliable results. In the case of the tubercle bacillus, there is considerable difference of opinion. Generally speaking, it may be said that indices varying between .8 and 1.2 are to be reckoned as unity—that is to say, that no deduction can be drawn from indices falling between these limits. In the case of such organisms as those of the coli-typhoid group and cholera, which are susceptible to bacteriolytic influences in the serum, it may be necessary to heat the sera of the patient and observer for half an hour at 55° C. This destroys any complement present and prevents bacteriolysis occurring. In the case of the *b. typhosus* the virulence of the strain employed has been shown to be an important factor.

Several modifications of Wright's technique have been suggested. Thus Klien, instead of enumerating the bacteria ingested, takes a series of dilutions of the serum and estimates the dilution with which capacity for opsonising bacteria disappears (or at any rate the dilution with which the phagocytic index falls below .5). The content of the patient's serum and of that of the observer may be thus compared, or the course of an immunisation may be followed by making daily observations of the content in opsonin. In another modification of Wright's technique Simon compares not the numbers of bacteria ingested, but the percentages of cells containing bacteria to those not containing bacteria. This he calls the "percentage index," and he states that the figure thus obtained corresponds very closely to the ordinary opsonic index; he claims that the method eliminates some of the errors which may arise in the use of the ordinary technique if only a relatively small number of phagocytting cells, such as 50, be examined.

#### BACTERICIDAL METHODS—DEVIATION OF COMPLEMENT.

**The Estimation of the Bactericidal Action of Serum.**—This may be carried out by various methods, of which those of Neisser and Wechsberg and of Wright may be given as examples. In the former, the effects of varying amounts of serum on the same amounts of bacteria are observed by means of plate cultures; in the latter, the number of bacteria which can be completely killed off by a given quantity of serum is ascertained. In carrying out experiments of this kind it is convenient to have



a number of small test-tubes sterilised and plugged with cotton-wool. We can then make any required dilution of a young bacterial culture in bouillon as follows: To each of a number of tubes we add .9 c.c. of .8 per cent. solution of sodium chloride. To the first tube (*a*) we add .1 c.c. of the bacterial culture, and thoroughly shake up the mixture; to the second (*b*) we add .1 c.c. of the contents of (*a*), and shake up; to the third tube (*c*) we add .1 c.c. of the contents of (*b*), and so on. It is thus evident that .1 c.c. of the contents of (*a*) will correspond to .01 c.c., and .1 c.c. of (*b*) to .001 c.c. of the original culture; any required fraction can thus be readily obtained. In the making of all mixtures of serum and bacteria it is essential that none of the latter shall escape the action of the former, *e.g.* by remaining on a part of the mixing vessel with which the serum does not come in contact.

(*a*) *Method of Neisser and Wechsberg*.—A series of small plugged sterile tubes is taken, and to each we add .5 c.c. of .8 per cent. sodium chloride solution, and a given quantity, say  $\frac{1}{5000}$  c.c., of a young bouillon culture to be tested. To the several tubes in series we then add varying amounts of the fresh serum whose action is to be observed, *e.g.* .2 c.c., .1 c.c., .05 c.c., .025 c.c., etc. The contents of each tube are then made up to 1 c.c. with salt solution, and a few drops of sterile bouillon are added to each tube. The tubes are then well shaken and placed in the incubator at 37° C. for three hours, to allow the serum to act. (Of course several series of such tubes may be prepared and placed in the incubator for varying periods of time; we can thus observe when the bactericidal effect reaches the maximum.) At the end of the given period of time a small quantity, say .05 c.c., of the contents of each tube is added to a tube of melted agar (cooled to about 40° C.); each agar tube is then shaken, and the contents are poured out into a sterile Petri capsule. The other tubes are similarly treated, and the Petri capsules are placed in the incubator for a suitable period of time. The number of colonies in each can then be noted. Of course gelatine can be substituted for the agar in the plates if desired.

(*b*) *Wright's Method*.—A twenty-four hours' bouillon culture is used, and various dilutions with sterile bouillon are made according to the method described on p. 58: thus 5-, 10-, 20-, 50-, 100-, 1000-, etc., fold dilutions may be prepared. A small quantity, say 1 c.mm., of the fresh serum to be tested is mixed with an equal amount of the bacterial culture, and the mixture is placed in a small capillary tube which is sealed at the ends; similar



mixtures of equal parts of serum and of each of the dilutions of culture are prepared and treated in the same way. The tubes are then placed in the incubator for eighteen to twenty-four hours at 37° C., and at the end of that time the contents of each are tested as regards sterility by means of cultures. In this way the greatest dilution in which the bacteria are completely killed off is ascertained. The number of bacteria in the original culture per c.mm. can be counted by the method given on p. 70, and thus the total number of bacteria killed off by the quantity of serum used can readily be calculated.

As will afterwards (*see* chapter on Immunity) be described in greater detail, when an animal is immunised against a particular bacterium the bactericidal action of its serum may be greatly increased, and this depends on the development of a particular substance called an immune-body, which is comparatively thermostable and is not destroyed at 55° C. To analyse the bactericidal properties of such a serum, it should in the first place be heated in order to destroy the normal complement. Then to each of a series of sterile tubes we add (*a*) a quantity of normal unheated serum insufficient of itself to destroy the bacteria, (*b*) a given amount of the bacterial culture, and (*c*) varying amounts of the heated immune-serum—.1, .01, .001, etc. c.c. In this way we can find the quantity of the immune-serum which gives the maximum bactericidal action.

In some cases, however, when an animal is immunised against a given bacterium, or when a patient is infected with the organism, the serum may not have increased bactericidal action, but nevertheless contains an immune-body which leads to the absorption or fixation of complement. In other words, the immune-body is a substance which, along with the corresponding or homologous bacterium, binds complement (p. 130). In order, however, to explain the methods by which the fixation of complement may be demonstrated, we must first of all give some facts with regard to hæmolytic sera.

**Methods of Hæmolytic Tests.**—A hæmolytic serum is usually prepared by injecting the red corpuscles of an animal into the peritoneum of an animal of different species—the corpuscles of the ox are most frequently used, and the rabbit is the most suitable animal for injection. The corpuscles ought to be completely freed of serum by repeatedly washing them in sterile salt solution, and centrifugalising. An injection of the corpuscles of 5 c.c. of ox's blood, followed by two injections, each of 10 c.c., at intervals of eight days, will usually give an active serum. The animal should be killed by bleeding it, aseptically as far as poss-



ible, seven to ten days after the last injection; the serum which separates may be collected in suitable lengths of quill glass-tubing drawn out at the ends, which are afterwards sealed in the flame. To ensure sterility when the serum is to be kept some time, it is advisable to heat it for an hour at  $55^{\circ}$  C. on three successive days; we have always found that serum treated in this way remains sterile. It is, of course, devoid of complement. The test amount of corpuscles is usually 1 c.c. of a 5 per cent. suspension of corpuscles in .8 per cent. sodium chloride solution: that is, the corpuscles of 5 c.c. blood are completely freed of serum by repeatedly washing in salt solution, and then salt solution is added to make up 100 c.c. In any investigation it is necessary to obtain the minimum hæmolytic dose (M.H.D.) of the immune-body and of the complement to be used. (It is to be noted that as complement does not increase during immunisation, the hæmolytic dose of the fresh serum will come far short of representing the amount of immune-body present.) In testing the dose of immune-body, the fresh serum to be used as complement must be devoid of hæmolytic action (in the present instance rabbit's serum will be found suitable), and more than sufficient to produce lysis with immune-body is added to each of a series of tubes. Varying amounts of immune-body are added to the tubes, the contents are shaken, made up to 1.5 c.c., and incubated for two hours. The amount of lysis is then noted, and the tubes are placed in a cool chamber till next morning, when a final reading is taken. The smallest amount of immune-body which gives complete lysis is, of course, the M.H.D.: sometimes this may be as low as .001 c.c. for the test amount of corpuscles. When further observations are to be continued on the same day, the reading after incubation must be taken as the working standard. To estimate the M.H.D. of complement, proceed in a corresponding manner; to each of a series of tubes add several doses of immune-body, and then to the several tubes different amounts of complement. The activity of a serum as complement varies considerably, and each sample must be separately tested.<sup>1</sup> The above will serve as an indication of the fundamental methods; for further details, special papers on the subject must be consulted. Corpuscles treated with sufficient immune-body to

<sup>1</sup> Complement is a substance which rapidly (often within twenty-four hours) loses its strength when kept at room temperature. It can, however, be preserved for a considerable time at or near its original strength if it be kept frozen. Even if this be done, however, the strength of the complementary serum must be titred at the commencement of every experiment in which it is employed.



produce complete lysis on the addition of complement are usually spoken of as *sensitised corpuscles*.

**The Removal of Blood-Samples from Rabbits, etc.**—In such work as that just described, it is often convenient to watch the progress of an immunisation procedure by removing a sample of blood without the animal being killed. With proper care any amount of blood up to one-third of that contained in the body can be removed from the ear vein of a rabbit. The animal, which must not be flurried, is placed on a bench, and its body kept warm by being covered with a cloth. The root of the ear should be shaved over the marginal vein, the hairs on the edge of the ear should also be clipped short. It is best to have the ear dry, as the evaporation of a fluid causes contraction of the vessels. In a great deal of hæmolytic work absolute sterility of the sample is not necessary, so that washing the ear is not required. When sterile blood is desired, the precautions detailed on p. 44 may be applied. A frosted incandescent electric lamp, such as is used for microscopic illumination, is placed lighted an inch or two from the ear. The left hand of the operator should cover the animal's head in front of the ears, the thumb and index finger being left free to compress the vein at the root of the ear. In this way not only is the animal's eye protected from the glare of the lamp, but the distance of the latter from the ear can be regulated so as to keep it at what to the operator's hand is a pleasant warmth. In a minute or two the ear vessels will dilate, and the vein, being compressed at the root, a lateral opening is made with a bayonet-pointed surgical needle (the triangular-pointed needles supplied with the Gowers-Haldane hæmoglobinometer are also very suitable), and the blood allowed to drop into a sterile test-tube. Usually waves of contraction of the ear vessels will be observed to occur, the passing off of which must be waited for, and from time to time the clot must be gently squeezed out of the opening in the vein with the flat side of the needle, or it may be necessary slightly to enlarge the opening. The blood should be allowed to clot completely, and then, by means of a sterile platinum needle, the clot should be loosened from the sides of the tube in order that it may freely contract. The tube should be placed in the ice-chest till the following morning, when the serum can be pipetted off with a sterile, nipped pipette.

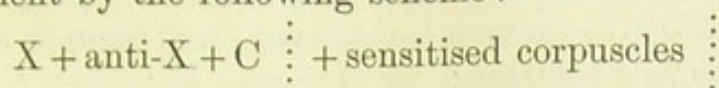
Daily samples can thus be obtained from an animal. If care be taken not to make ragged openings in the vein, often the simple removal of the previous scab will be followed by a free blood flow.

A worker associated with one of us has shown that this method can be applied in guinea-pigs, provided these be of fair size. Here successive samples of 2 c.c. can be obtained from the ear veins.

**Fixation of Complement or Complement Deviation.**—From the facts given above it follows that sensitised corpuscles, *i.e.* corpuscles treated with immune-body, may be made to serve as an indicator for the presence of complement. If an immune-body is present in a serum heated at 55° C., the serum when added to the corresponding bacterium leads to the fixation of complement, and thus prevents hæmolysis when the sensitised corpuscles are added. If we represent the bacteria, or rather the receptors in the bacteria, by X, the immune-body by anti-X, and the complement by C



(normal serum, say of a guinea-pig), we may represent the method of experiment by the following scheme:—



(The vertical dotted line represents a period of incubation for one and a half hours at 37° C.)

If lysis of the sensitised corpuscles does not occur after incubation at 37° C., then the complement has been fixed and an immune-body has been shown to be present, provided that a suitable control shows that the bacteria alone, without immune-body, do not fix sufficient complement to interfere with lysis.

This method has now been extensively used for demonstrating the presence of immune-bodies in the blood of patients suffering from a particular bacterial infection. It has also been applied to determine whether a suspected bacterium is really the cause of a disease, for if the bacterium gives with the serum of the patient deviation of complement, then there is a strong presumption that it is the infective agent (*vide* Immunity).

#### **The Serum Diagnosis of Syphilis, Wassermann Reaction.**—

Wassermann, Neisser and Bruck, proceeding in accordance with the facts established with regard to the deviation of complement, tested whether a similar phenomenon might not be obtained in the case of syphilis. For this purpose they mixed together a watery extract of syphilitic liver, rich in spirochaetes (antigen), and serum from a syphilitic case (supposed to contain anti-substances), and found that a relatively large amount of complement was fixed. On the other hand, when the serum from a non-syphilitic case was substituted for the syphilitic serum, little or no fixation of complement occurred. The result was thus in accordance with expectations on theoretical grounds. Marie and Levaditi, however, found that an extract of normal guinea-pig's liver along with syphilitic serum fixed complement, and subsequent observations showed that extracts of other tissues are also more or less efficient, as are also certain definite substances, such as sodium oleate, sodium glycocholate, lecithin, mixtures of such and especially mixtures of lecithin and cholesterin, etc. Although abundant observations have established the validity of the test as a means of diagnosis, the reaction which led to its discovery is no longer sufficient to explain it, and the nature of the reaction is not yet understood.

In order to carry out the test, we require (a) serum from the suspected case, (b) an extract of liver or other organ, and (c) the fresh serum of an animal to act as complement. The following are the details, arranged in two stages:—



1. We add to a small test-tube—

(a) .05 c.c. of serum from the suspected case, heated for half an hour at 55° C. to destroy the human complement, and .5 c.c. of .8 per cent. salt solution ;

(b) .1 c.c. of an alcoholic extract of guinea-pig's or ox's liver (this can be prepared by extracting finely minced liver with four volumes of alcohol for 3 to 4 days and then filtering) ;

(c) A certain amount of guinea-pig's serum, usually .1 c.c., to act as complement.

The mixture is then placed in the incubator for one and a half hours, to allow fixation of complement to occur.

2. We then add to the tube 1 c.c. of a 5 per cent. suspension of sensitised corpuscles (usually sheep's or ox's), *i.e.* corpuscles to which there has been added a sufficient quantity of immune serum to produce lysis on the addition of complement.

The mixture is then placed in the incubator for another hour. If lysis of the corpuscles does not occur, the complement has been fixed in the first stage by the mixture of serum and liver extract. This is a positive result, and indicates the presence of syphilis. If the corpuscles undergo lysis, all the complement has not been fixed—the result is negative. When the amount of serum to be tested is small, the amounts given may all be proportionately reduced.

Such is the test as usually performed, and in this form it usually gives satisfactory results. It is to be noted, however, on the one hand, that the liver extract alone may fix a certain amount of complement, rarely more than three doses, and, on the other hand, that the hæmolytic value of fresh serum varies, *i.e.* the amount of complement is not always indicated by the volume of serum. It is accordingly better, and in a laboratory this can be readily done, to estimate the hæmolytic dose of the guinea-pig's serum, and to prepare a series of tubes, each containing the same amounts of serum and of liver extract, but with a different number of doses of complement in each tube. In this way we can find the number of doses of complement deviated in each case. As controls, the effect of the extract alone and of the serum alone can be tested at the same time. With the amounts of extract and serum mentioned, a positive result indicating the presence of syphilis may be accepted when five or more doses of complement are deviated in addition to the amount absorbed in the controls. Some observers use the same amount of complement in each tube, but vary the amounts of suspected serum, and in this way some idea of the deviating power of the serum is obtained, but we consider that the method given is to be preferred.



## THE PREPARATION OF VACCINES.

During recent years, in consequence of the work of Sir Almroth Wright, the principle of treating bacterial disease by vaccines has been very much developed. The general principle is to inject into the infected individual an emulsion of dead bacteria. In certain cases the bacteria are subjected to disintegrating processes before being used, but most frequently the vaccines simply contain killed bacterial cells, and the preparation is comparatively simple.

In the case of *pyogenic cocci*, either bouillon cultures or a growth off sloped agar emulsified in normal saline is taken and killed by heat. The temperature employed should be the minimum at which death occurs, say  $65^{\circ}\text{C}$ ., applied for half an hour. In the case of certain staphylococci, we have found, however, that a higher temperature is necessary. After any sterilisation procedure, tubes of agar must be inoculated from the presumably dead vaccine, and incubated for twenty-four hours in order to ascertain if the sterilisation has been effective. As the dosage of a vaccine is of great importance, it is necessary to count the bacteria present. This is done by one of the methods given below. Appropriate doses (*see* Chapter VII.) are then with all aseptic precautions measured by means of a sterile graduated pipette, and placed, along with an equal volume of .5 per cent. lysol, in little glass bulbs drawn out to a capillary tube at one end. These when charged are sealed off, and for use the sealed end is broken off, the contents are sucked up into a sterile hypodermic needle, and injected fairly deeply into the skin, usually in the region of the flank.

In the case of the *typhoid bacillus*, organisms are used of such virulence that a quarter of a twenty-four hours' old sloped agar culture, when administered hypodermically, will kill a guinea-pig of from 350 to 400 grams. Flasks of bouillon are inoculated with such a culture for forty-two hours at  $37^{\circ}\text{C}$ . The bacteria are then killed by the flask being put into a water bath at  $62^{\circ}\text{C}$ . for fifteen minutes; .5 per cent. lysol is added, and the bacteria in the vaccine are counted. By such methods, vaccines against any of the pyogenic cocci and against any members of the colityphoid group can be made.

The vaccines used in tuberculosis, cholera, and plague will be described in the chapters on these diseases.

**Wright's Method of counting the Bacteria in Dead Cultures.**—In the making of vaccines it is, as indicated above, necessary to know the total number of bacterial cells, whether



dead or living, present in a culture, for the dead as well as the living contain the toxins which may stimulate the therapeutic capacities of the body. The method consists in making a mixture of blood (whose content in red blood corpuscles is known) with the bacterial culture, and comparing the number of bacteria with the number of corpuscles. The observer first estimates the red cells in his blood; a capillary pipette with a rubber nipple and with a mark near its capillary extremity is then taken, blood is sucked up to the mark, then an air-bubble, and then an equal volume of the bacterial emulsion diluted according to the empirical estimate the observer forms of its strength. The blood and bacterial emulsion are then thoroughly mixed by being drawn backwards and forwards in the wide part of the pipette, a drop is blown out on to a slide, and a blood film is spread which may be stained by Leishman's method. The bacteria and blood corpuscles are now separately enumerated in a series of fields in different parts of the preparation. If a dilution has been taken in which a large number of bacteria are present, an artificial field may be used, made by drawing with the oil pencil a small square on a circular cover-glass, and dropping the latter on to the diaphragm of the microscope eye-piece. Suppose, now, that the observer's blood contained 5,000,000 red cells per c.mm., that to the bacterial emulsion three volumes of diluent had been added, and that in the fields examined there were 500 red cells and 600 bacteria. It is evident that in the undiluted culture for 500 red cells there would have been 2400 bacteria. Now  $500 : 2400 :: 5,000,000 : 24,000,000$ , which last figure is the number of bacteria per c.mm. of the emulsion.

It has been found in the case of certain bacteria, *e.g.* the members of the coli-typhoid and cholera groups, that when an emulsion of these is mixed with whole blood, the serum of the latter may have a bacteriolytic or an agglutinating action on the organisms, which interferes with the counting. To obviate the inaccuracies or difficulties thus introduced, Harrison has modified Wright's method by substituting, in a given quantity of blood, normal saline for the serum. The method is as follows:—A capillary pipette has a mark made upon it, to which blood is sucked up and quickly expelled into a small tube containing a little .75 per cent. sodium citrate solution; any remaining blood is washed out of the pipette with the same fluid. The tube is then centrifuged to deposit the corpuscles, the supernatant fluid carefully removed, and the corpuscles are washed by centrifuging twice or thrice with normal saline, care being always taken not



to lose any of the corpuscles in the successive washings. After the last washing the corpuscles are sucked up into the pipette, and then saline up to the mark which indicated the volume of the original blood. Such a mixture is taken, and, to prevent loss of corpuscles, the pipette and tubes are washed with a definite number of equal volumes of broth or saline. Thus there can be obtained in a watch-glass a mixture of, say, one volume of corpuscles and saline, and two volumes of the diluting fluid. To this mixture is now added an appropriate number of volumes, again measured in the same pipette, of the bacterial emulsion to be counted, the amount, of course, depending upon a rough judgment which with experience can be made of the probable numbers present. A drop of the mixture is put under a cover-glass, and the numbers of corpuscles on the one hand and of bacteria on the other present in a number of fields are counted. It is not necessary to stain the bacteria, but in the case of motile organisms it is recommended that they be rendered motionless by using as a diluent saline to which formol has been added in the proportion of two or three drops to 10 c.c. If the number of red blood corpuscles in the observer's blood be known, it is evident that the amount of blood corresponding to a certain number of blood corpuscles in a microscopic field can be calculated, and the number of bacteria present in the same amount of the mixture will be the number corresponding to the number of corpuscles. Thus it is now only necessary to allow for the dilution to obtain the number of bacteria in the original emulsion.

#### GENERAL BACTERIOLOGICAL DIAGNOSIS.

Under this heading we have to consider the general routine which is to be observed by the bacteriologist when any material is submitted to him for examination. The object of such examination may be to determine whether any organisms are present, and if so, what organisms; or the bacteriologist may simply be asked whether a particular organism is or is not present. In any case, his inquiry must consist (1) of a microscopic examination of the material submitted; (2) of an attempt to isolate the organisms present; and (3) of the identification of the organisms isolated. We must, however, before considering these points, look at a matter often neglected by those who seek a bacteriological opinion, namely, the *proper methods of obtaining and transferring to the bacteriologist the material which he is to be asked to examine*. The general principles here are



(1) that every precaution must be adopted to prevent the material from being contaminated with extraneous organisms; (2) that nothing be done which may kill any organisms which may be proper to the inquiry; and (3) that the bacteriologist obtain the material as soon as possible after it has been removed from its natural surroundings.

The sources of materials to be examined, even in pathological bacteriology alone, are, of course, so varied that we can but mention a few examples. It is, for instance, often necessary to examine the contents of an abscess. Here the skin must be carefully purified by the usual surgical methods; the knife used for the incision is preferably to be sterilised by boiling; the first part of the pus which escapes is allowed to flow away (as it might be spoilt by containing some of the antiseptics used in the purification), and a little of what subsequently escapes allowed to flow into a sterile test-tube. If test-tubes sterilised in a



FIG. 46.—Test-tube and pipette arranged for obtaining fluids containing bacteria.

laboratory are not at hand, an ordinary test-tube may be quarter-filled with water and vigorously boiled over a spirit-lamp. The tube is then emptied and plugged with a plug of cotton wool, the outside of which has been singed in a flame. Small stoppered bottles may be sterilised and used in the same way. A discharge to be examined may be so small in quantity as to make the procedure described impracticable. It may be caught on a piece of sterile plain gauze, or of plain absorbent wool, which is then placed in a sterile vessel. Wool or gauze used for this purpose, or for swabbing out, say the throat, to obtain shreds of suspicious matter, must have no antiseptic impregnated in it, as the latter may kill the bacteria present and make the obtaining of cultures impossible.

Fluids from the body cavities, urine, etc., may be secured with sterile pipettes. To make one of these, take 9 inches of ordinary quill glass-tubing, draw out one end to a capillary diameter, and place a little plug of cotton wool in the other end. Insert this tube through the cotton plug of an ordinary test-tube, and sterilise by heat. To use it, remove test-tube plug with the quill tube in its centre, suck up some of the fluid into the latter, and replace in its former position in the test-tube (Fig. 46). Another method



very convenient for transport is to make two constrictions on the glass tube at suitable distances, according to the amount of fluid to be taken. The fluid is drawn up into the part between the constrictions, but so as not to fill it completely. The tube is then broken through at both constrictions, and the thin ends are sealed by heating in a flame.

Solid organs to be examined should, if possible, be obtained whole. They may be treated in one of two ways. (1) The surface over one part about an inch broad is seared with a cautery heated to dull red heat. All superficial organisms are thus killed. An incision is made in this seared zone with a sterile scalpel, and small quantities of the juice are removed by a platinum spud to make cover-glass preparations and plate or smear cultures. (2) An alternative method is as follows:—The surface is sterilised by soaking it well with 1 to 1000 corrosive sublimate for half an hour. It is then dried, and the capsule of the organ is cut through with a sterile knife, the incision being further deepened by tearing. In this way a perfectly uncontaminated surface is obtained. Hints are often obtained from the clinical history of the case as to what the procedure ought to be in examination. Thus, as a matter of practice, cultures of tubercle and often of glanders bacilli can be easily obtained only by inoculation experiments. Typhoid bacilli need hardly be looked for in the fæces after the first ten days of the disease, and so on.

**Routine Procedure in Bacteriological Examination of Material.**—In the case of a discharge regarding which nothing is known, the following procedure should be adopted:—(1) Several cover-glass preparations should be made. One ought to be stained with saturated watery methylene-blue, one with a stain containing a mordant such as Ziehl-Neelsen carbol-fuchsin, one by Gram's method. (2) *a.* Gelatin plates should be made and kept at room temperature; *b.* a series of agar plates or successive strokes on agar tubes (p. 60) should be made and incubated at 37° C. Method *b* of course gives results more quickly. In every case when an unknown disease is being investigated, some of the material should be subjected to methods suitable to the growth of anaerobic bacteria. If microscopic investigation reveals the presence of bacteria, it is well to keep the material in a cool place till next day, when, if no growth has appeared in the incubated agar, some other culture medium (*e.g.* blood serum or agar smeared with blood) may be employed. If growth has taken place, say in the agar plates, one with about two hundred or fewer colonies should be made



the chief basis for research. In such a plate the first question to be cleared up is: Do all the colonies present consist of the same bacterium? The shape of the colony, its size, the appearance of the margin, the graining of the substance, its colour, etc., are all to be noted. One precaution is necessary, namely, it must be noted whether the colony is on the surface of the medium or in its substance, as colonies of the same bacterium may exhibit differences according to their position. The arrangement of the bacteria in a surface colony may be still more minutely studied by means of *impression preparations*. A cover-glass is carefully cleaned and sterilised by passing quickly several times through a Bunsen flame. It is then placed on the surface of the medium, and gently pressed down on the colony. The edge is then raised by a sterile needle, it is seized with forceps, dried high over the flame, and treated as an ordinary cover-glass preparation. In this way very characteristic appearances may sometimes be noted and preserved, as in the case of the anthrax bacillus. The colonies on a plate having been classified, a microscopic examination of each group may be made by means of cover-glass preparations, and tubes of gelatin and agar are inoculated from each representative colony. Each of the colonies used must be marked for future reference, preferably by drawing a circle round it on the under surface of the plate or capsule with one of Faber's pencils for marking on glass, a number or letter being added for easy reference.

The general lines along which observation is to be made in the case of a particular bacterium may be indicated as follows:—

1. *Microscopic Appearances*.—For ordinary descriptive purposes, young cultures, say of twenty-four hours' growth, on agar should be used, though appearances in older cultures, such as involution forms, etc., may also require attention. Note,—(1) the form; (2) the size; (3) the appearance of the protoplasmic contents, especially as regards uniformity or irregularity of staining; (4) the method of grouping; (5) the staining reactions. Has it a capsule? Does the bacterium stain with simple watery solutions? Does it require the use of stains containing mordants? How does it behave towards Gram's method? It is important to investigate the first four points, both when the organism is in the fluids or tissues of the body and when growing in artificial media, as slight variations occur. It must also be borne in mind that slight variations are observed according to the kind and consistence of the medium in which the organism is growing. (6) Is it motile, and has it flagella? If so, how



are they arranged? (7) Does it form spores, and if so, under what conditions as to temperature, etc.?

2. *Growth Characteristics*.—Here the most important points on which information is to be asked are, What are the characters of growth and what are the relations of growth (1) to temperature; (2) to oxygen? These can be answered from some of the following experiments:—

A. Growth on gelatin. (1) Stab culture. Note,—(a) rate of growth; (b) form of growth, ( $\alpha$ ) on surface, ( $\beta$ ) in substance; (c) presence or absence of liquefaction; (d) colour; (e) presence or absence of gas formation and of characteristic smell; (f) relation to reaction of medium. (2) Streak culture. (3) Shake culture. (4) Plate cultures. Note appearances of colonies, ( $\alpha$ ) superficial, ( $\beta$ ) deep. (5) Growth in fluid gelatin at 37° C.

B. Growth on agar at 37° C. (1) Stab. (2) Streak. Also on glycerin-agar, blood-agar, etc. Appearances of colonies in agar plates.

C. Growth in bouillon, ( $\alpha$ ) character of growth, ( $\beta$ ) smell, ( $\gamma$ ) reaction.

D. Growth on special media. (1) Solidified blood serum. (2) Potatoes. (3) Lactose and other sugar media. Does fermentation occur, and is gas formed? (4) Milk. Is it curdled or turned sour? (5) Litmus media. Note changes in colour. (6) Peptone solution. Is indol formed?

E. What is the viability of organism on artificial media?

3. *Results of inoculation experiments on animals*.

By attention to such points as these a considerable knowledge is attained regarding the bacterium, which will lead to its identification. In the case of many well-known organisms, however, a few of the above points taken together will often be sufficient for the recognition of the species, and experience teaches what are the essential points as regards any individual organism. In the course of the systematic description of the pathogenic organisms, it will be found that all the above points will be referred to, though not in every case.

The methods by which the morphological and biological characteristics of any growth may be observed have already been fully described. It need only be pointed out here that in giving descriptions of bacteria the greatest care must be taken to state every detail of investigation. Thus in any description of microscopic appearances the age of the growth from which the preparation was made, the medium employed, the temperature at which development took place, must be noted, along with the stain which was used; and with regard to the latter it is always preferable to employ one of the well-known staining combinations, such as Löffler's methylene-blue. Especial care is necessary in stating the size of a



bacterium. The apparent size often shows slight variations dependent on the stain used and the growth conditions of the culture. Accurate measurements of bacteria can only be made by preparing microphotographs of a definite magnification, and measuring the sizes on the negatives. From these the actual sizes can easily be calculated. A rough method of estimating the size of an organism is to mix a little with a drop of the observer's blood and make a blood film. As the size of a normal red blood corpuscle is about  $7.5 \mu$ , an idea of the size of a bacterium can be obtained by comparing it with this as a standard. In describing bacterial cultures it must be borne in mind that the appearances often vary with the age. It is suggested that in the case of cultures grown at from  $36^{\circ}$  to  $37^{\circ}$  C. the appearances between twenty-four and forty-eight hours should be made the basis of description, and in the case of cultures grown between  $18^{\circ}$  and  $22^{\circ}$  C. the appearances between forty-eight and seventy-two hours should be employed. The culture fluids used must be made up and neutralised by the precise methods already described. The investigator must give every detail of the methods he has employed, in order that his observations may be capable of repetition.

In the case of a number of pathogenic organisms, identification is a comparatively easy matter. In some cases, however, great difficulties arise in consequence of the existence of groups of organisms presenting closely allied characters, and the difficulty and importance of identification is enhanced by the fact that the same group may include both harmful and innocent members. Examples of this occurrence are found in the pyogenic cocci and their allies, in the coli-typhoid group of bacilli, and in the group of cholera vibrios. In such cases it is usually necessary to take into account all the morphological and cultural reactions of an organism before it can be adequately classified. Within recent years attempts have been made to apply the statistical method to the solution of the difficulties of the situation, and here the results appear to be promising. The method has been applied to the coccaceæ by Winslow and Rogers, who have investigated 500 strains of cocci isolated from the tissues in disease, from the outer surfaces of the normal human body, from water, earth, and air. A great variety of properties was studied, and while in each test applied wide variation was exhibited in such bacteria, there usually emerged a type property to which individual strains tended to approach. Thus, while the size varies from  $.1$  to  $2.0 \mu$ , out of about 350 strains examined about 115 measured  $.3 \mu$ , and the remaining strains tended to be a little below or a little above this figure. When similar lines of inquiry were pursued with regard to other characteristics of the organisms, it was found that important correlations could be noted. Thus capacity for staining by Gram's method was found especially amongst the staphylococci and streptococci as



contrasted with forms tending to grow in sarcinal packets, and the Gram-staining forms were chiefly parasitic in habitat. Looking at their results as a whole, Winslow and Rogers divide the cocci into two great groups, the *Paracoccaceæ* and the *Metacoccaceæ*. The former comprise most of the forms derived from the body, show a staphylococcal or streptococcal tendency, stain by Gram, yield only moderate surface growths, form acid in carbohydrates, and produce no pigment or a white or orange colour. The latter come chiefly from air and water, often are sarciniform, decolorise by Gram, grow well on the surface of media, do not ferment carbohydrates, and produce red or yellow pigment. On similar lines, further subdivision of the groups could be effected. It is manifest that important means of differentiating allied bacteria may be available by the extended application of this method.

#### INOCULATION OF ANIMALS.<sup>1</sup>

The animals generally chosen for inoculation are the mouse, the rat, the guinea-pig, the rabbit, and the pigeon. Great caution must be shown in drawing conclusions from isolated experiments on rabbits, as these animals often manifest exceptional symptoms, and are very easily killed. Dogs are, as a rule, rather insusceptible to microbic disease, and the larger animals are too expensive for ordinary laboratory purposes. In the case of the mouse and rat the variety must be carefully noted, as there are differences in susceptibility between the wild and tame varieties, and between the white and brown varieties of the latter. In the case of the wild varieties, these must be kept in the laboratory for a week or two before use, as in captivity they are apt to die from very slight causes; and, further, each individual should be kept in a separate cage, as they show great tendencies to cannibalism. Of all the ordinary animals the most susceptible to microbic disease is the guinea-pig. Practically all inoculations are performed by means of the hypodermic syringe. The best variety is made on the ordinary model with metal mountings, asbestos washers, and preferably furnished with platinum-iridium needles. Before use, the syringe and the needle are sterilised by boiling for five minutes. The materials used for inoculation are cultures, animal exudations, or the juice of organs. If the bacteria already exist in a fluid there is no difficulty. The syringe is most conveniently filled out of a shallow conical test-glass, which ought previously

<sup>1</sup> Experiments on animals, of course, cannot, in Britain, be performed without a licence granted by the Home Secretary.



to have been covered with a cover of filter paper and sterilised. If an inoculation is to be made from organisms growing on the surface of a solid medium, either a little ought to be scraped off and shaken up in sterile bouillon or .85 per cent. salt solution to make an emulsion, or a little sterile fluid is poured on the growth, and the latter scraped off into it. This fluid is then filtered into the test-glass through a plug of sterile glass wool. This is easily effected by taking a piece of  $\frac{5}{8}$ -inch glass-tubing 3 inches long, drawing one end out to a fairly narrow point, plugging the tube with glass wool above the point where the narrowing commences, and sterilising by heat. By filtering an emulsion through such a pipette, flocculi which might block the needle are removed. If a solid organ or an old culture is used for inoculation, it ought to be rubbed up in a sterile porcelain or metal crucible with a little sterile distilled water, by means of a sterile glass rod, and the emulsion filtered as in the last case.

The methods of inoculation generally used are: (1) by scarification of the skin; (2) by subcutaneous injection; (3) by intraperitoneal injection; (4) by intravenous injection; (5) by injections into special regions, such as the anterior chamber of the eye, the substance of the lung, etc. Of these (2) and (3) are most frequently used. When an anæsthetic is to be administered, this is conveniently done by placing the animal, along with a piece of cotton wool or sponge soaked in chloroform, under a bell-jar or inverted glass beaker of suitable size.

1. *Scarification*.—A few parallel scratches are made in the skin of the abdomen previously cleansed, just sufficiently deep to draw blood, and the infective material is rubbed in with a platinum eyelet. The disadvantage of this method is that the inoculation is easily contaminated. The method is only occasionally used.

2. *Subcutaneous Injection*.—A hypodermic syringe is charged with the fluid to be inoculated. The hair is cut off the part to be inoculated, and the skin purified with 1 to 1000 corrosive sublimate, or by dropping upon it some strong solution of iodine. The skin is then pinched up, and, the needle being inserted, the requisite dose is administered. The wound is then sealed with a little collodion.

3. *Intraperitoneal Injection*.—This may be performed by means of a special form of needle. The needle is curved, and has its opening not at the point, but in the side in the middle of the arch (Fig. 47). The hair over the lower part of the abdomen is cut, and the skin purified with an antiseptic. The whole thickness of the abdominal walls is then pinched up



between the forefingers and thumbs of the two hands, and the needle is plunged through the fold thus formed. The result is that the hole in the side of the needle is within the abdominal cavity, and the inoculation can thus be made. Intraperitoneal inoculation can also be practised with an ordinary needle. The mode of procedure is similar, but, after the needle is plunged through the abdominal fold, it is partially withdrawn till the point is felt to be free in the peritoneal cavity, when the injection is made. There is little risk of injuring the intestines by either method.

4. *Intravenous Injection*.—The vein most usually chosen is one of the auricular veins. The part has the hair removed, the skin is purified, and the vein made prominent by pressing on it between the point of inoculation and the heart. The needle is then plunged into the vein, and the fluid injected. That it has perforated the vessel will be shown by the escape of a little blood; and that the injection has taken place into the lumen of the vessel will be known by the absence of the small swelling which occurs in subcutaneous injections. If preferred, the vein may be first laid bare by snipping the skin over it. The needle is then introduced.

5. *Inoculation into the Anterior Chamber of the Eye*.—Local anæsthesia is established by applying a few drops of 2 per cent. solution of hydrochlorate of cocaine. The eye is fixed by pinching up the orbital conjunctiva with a pair of fine forceps, and, the edge of the cornea being perforated by the hypodermic needle, the injection is easily accomplished.

Sometimes inoculations are made by planting small pieces of pathological tissues in the subcutaneous tissue. This is especially done in the case of glanders and tubercle. The skin over the back is purified, and the hair cut. A small incision is made with a sterile knife, and the skin being separated from the subjacent tissues by means of the ends of a blunt pair of forceps, a little pocket is formed into which a piece of the suspected tissue is inserted. The wound is then closed with a suture, and collodion is applied. In the case of guinea-pigs, the abdominal wall is to be preferred as the site of inoculation, as the skin over the back is extremely thick.

Injectons are sometimes made into other parts of the body, e.g. the pleuræ, the cranium, the spinal canal. With regard to

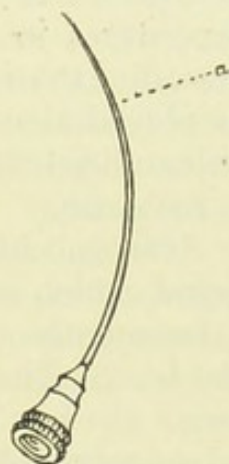


FIG. 47.—Hollow needle with lateral aperture (at *a*) for intra-peritoneal inoculations.



the last, Ford-Robertson has pointed out that in the rabbit it can be easily practised through the space between the seventh lumbar and first sacral vertebræ. The spine of the former lies in a line with the iliac crests. With regard to operative procedures in special regions of the body, it is unnecessary to describe these, as the application of the general principles employed above, together with those of modern aseptic surgery, will sufficiently guide the investigator as to the technique which is requisite.

After inoculation, the animals ought to be kept in comfortable cages, which must be capable of easy and thorough disinfection subsequently. For this purpose galvanised iron wire cages are the best. They can easily be sterilised by boiling them in the large fish-kettle which it is useful to have in a bacteriological laboratory for such a purpose. It is preferable to have the cages opening from above. Otherwise material which may be infective may be scratched out of the cage by the animal. The general condition of the animal is to be observed, how far it differs from the normal, whether there is increased rapidity of breathing, etc. The temperature is usually to be taken. This is generally done *per rectum*. The thermometer (the ordinary clinical variety) is smeared with vaselin, and the bulb inserted just within the sphincter, where it is allowed to remain for a minute; it is then pushed well into the rectum for five minutes. If this precaution be not adopted a reflex contraction of the vessels may take place, which is likely to vitiate the result by giving too low a reading.

**Collodion Capsules.**—These have been used to allow the sojourn of bacteria within the animal body without their coming into contact with the cells of the tissues. Various substances in solution can pass in either direction through the wall by diffusion, but the wall is impermeable alike to bacteria and leucocytes. The following method of preparing such capsules is that of M'Rae modified by Harris:—A gelatin capsule, such as is used by veterinary surgeons, is taken, and in one end there is fixed a small piece of thin glass tubing by gently heating the glass and inserting it. The tube becomes fixed when quite cold, and the junction is then painted round with collodion, which is allowed to dry thoroughly. The bore of the tubing is cleared of any obstructing gelatin, and the whole capsule is dipped into a solution of collodion so as to coat it completely. The collodion is allowed to dry, and the coating is repeated; it is also advisable to strengthen the layer by further painting it at the extremity and at the junction. The interior of the capsule is



then filled with water by a fine capillary pipette, and the capsule is placed in hot water in order to liquefy the gelatin, which can be removed from the interior by means of the fine pipette. The sac is filled with bouillon and is placed in a tube of bouillon. It is then sterilised in the autoclave. A small quantity of the bouillon is removed, and the contents are inoculated with the particular bacterium to be studied, or an emulsion of the bacterium is added. The glass tubing is seized in sterile forceps, and is sealed off in a small flame a short distance above the junction. The closed sac ought then to be placed in a tube of sterile bouillon to test its impermeability. The result is satisfactory if no growth occurs in the surrounding medium. The sac with its contents can now be transferred to the peritoneal cavity of an animal.

**Autopsies on Animals dead or killed after Inoculation.—**

These should be made as soon as possible after death—in fact, it is preferable to kill the animal when it shows serious signs of illness. It is necessary to have some shallow troughs, constructed either of metal or of wood covered with metal, conveniently with sheet lead, and having a perforation at each corner to admit a tape or strong cord. The animal is tightly stretched out in the trough and tied in position. The size of the trough will therefore have to vary with the size of the outstretched body of the animal to be examined. In certain cases it is well to soak the surface of the animal in carbolic acid solution (1 to 20) or in corrosive sublimate (1 to 1000) before it is tied out. This not only to a certain extent disinfects the skin, but, what is more important, prevents hairs which might be affected with pathogenic products from getting into the air of the laboratory. The instruments necessary are scalpels (preferably with metal handles), dissecting forceps, and scissors. They are to be sterilised by boiling for five minutes. This is conveniently done in one of the small portable sterilisers used by surgeons. Two sets at least ought to be used in an autopsy, and they may be placed, after boiling, on a sterile glass plate covered by a bell-jar. It is also necessary to have a medium-sized hatchet-shaped cautery, or other similar piece of metal. It is well to have prepared a few freshly-drawn-out capillary tubes stored in a sterile cylindrical glass vessel, and also some larger sterile glass pipettes. The hair of the abdomen of the animal is removed. If some of the peritoneal fluid is wanted, a band should be cauterised down the linea alba from the sternum to the pubes, and another at right angles to the upper end of this; an incision should be made in the middle of these bands, and the abdominal



walls thrown to each side. One or more capillary tubes should then be filled with the fluid collected in the flanks, the fluid being allowed to run up the tube and the point sealed off; or a larger quantity, if desired, is taken in a sterile pipette. If peritoneal fluid be not wanted, then an incision may be made from the episternum to the pubes, and the thorax and abdomen opened in the usual way. The organs ought to be removed with another set of instruments, and it is convenient to place them pending examination in deep Petri's capsules (sterile). It is generally advisable to make cultures and film preparations from the heart's blood. To do this, open the pericardium, sear the front of the right ventricle with a cautery, make an incision in the middle of the part seared, and remove some of the blood with a capillary tube for future examination; or, introducing a platinum eyelet, inoculate tubes and make cover-glass preparations at once. To examine any organ, sear the surface with a cautery, cut into it, and inoculate tubes and make film preparations with a platinum loop. For removing small parts of organs for making inoculations on tubes, a small platinum spud is very useful, as the ordinary wires are apt to become bent. Place pieces of the organs in some preservative fluid for microscopic examination. The organs ought not to be touched with the fingers. When the examination is concluded, the body should have corrosive sublimate or carbolic acid solution poured over it, and be forthwith burned. The dissecting trough and all the instruments ought to be boiled for half an hour. The amount of precaution to be taken will, of course, depend on the character of the bacterium under investigation, but as a general rule every care should be used.



## CHAPTER V.

### BACTERIA IN AIR, SOIL, AND WATER. ANTISEPTICS.

It is impossible here to do more than indicate the chief methods which are employed by bacteriologists in the investigation of the bacteria present in air, soil, and water, and to add an outline of the chief results obtained. In dealing with the latter, the subject has been approached mainly from the standpoint of the bearings which the results have towards human pathology. In dealing with antiseptics, so far as possible the effects of the various agents on the chief pathogenic bacteria have been given, though in many cases our information is very imperfect.

#### AIR.

Very little information of value can be obtained from the examination of the air, but the following are the chief methods used, along with the results obtained. More can be learned from the examination of atmospheres experimentally contaminated than by the investigation of the air as it exists under natural conditions.

**Methods of Examination.**—The methods employed vary with the objects in view. If it be sought to compare the relative richness of different atmospheres in organisms, and if the atmospheres in question be fairly quiescent, then it is sufficient to expose gelatin plates for definite times in the rooms to be examined. Bacteria, or the particles of dust carrying them, fall on the plates, and from the number of colonies which develop a rough idea of the richness of the air in bacteria can be obtained. Petri states that in five minutes the bacteria present in 10 litres of air are deposited on 100 square centimetres of a gelatin plate.

More complete results are available when some method is employed by which the bacteria in a given quantity of air are examined. The oldest method employed, and one which is still used, is that of Hesse. The apparatus is shown in Fig. 48. It consists of a cylindrical tube *a* about 20 inches long and 2 inches in diameter. At one end this is closed by a rubber cork having a piece of quill tubing, *f*, passing through it and



projecting some distance into the interior. For use the tube is sterilised in a tall "Koch," and then a quantity of peptone gelatin, sufficient to cover the whole interior to the thickness of an ordinary gelatin plate, is poured in. This gelatin is kept from escaping by the projection of the quill tubing into the lumen of the large tube. A plug of cotton wool is now placed in the outer end of the quill tubing. Over the other end of the large tube is tied a sheet of rubber having a hole about a quarter of an inch in diameter in its centre, and over this again is tied a piece of similar but unperforated sheet rubber. The tube is then sterilised

in the tall "Koch." On removal from this it is rolled, after the manner of an Esmarch's tube (*q.v.*), till the gelatin is set as a layer over its interior, and it is then placed horizontally on the tripod as shown. The other part of the apparatus is an aspirator, by means of which a known quantity of air can be brought in contact with the gelatin. It consists of two conical glass flasks connected by means of a tube which passes through the cork of each down to the bottom of the flask. When this tube is filled with water it, of course, can act as a syphon tube between volumes of water in the flasks. Such a syphon system being established, the levels of the water are marked on the flasks, and to one a litre of water is added; by depressing flask *b* the whole litre can be got into it, and the connect-

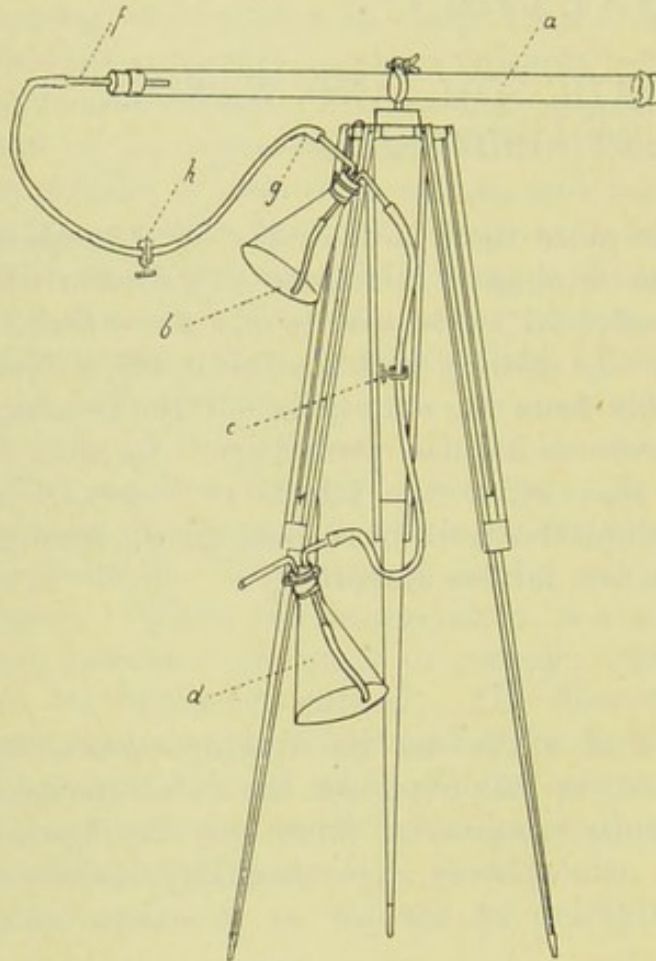


FIG. 48.—Hesse's tube, mounted for use.

ing tube *c* is then clamped. The two flasks are now connected by a rubber tube with the tube *f*, the clamp on *c* is opened, and the passing of a litre of water into *d* will draw a litre of air through the gelatin tube, when the outer rubber sheet is removed from the end and the clamp *h* opened. By disconnecting at *g* and reversing the syphon flasks, another litre can be sucked through, and so any desired quantity of air can be brought in contact with the gelatin. The speed ought not to be more than one litre in two minutes, and in such a case practically all the organisms will be found to have fallen out of the air on to the gelatin in the course of their transit. This fact can be tested by interposing between the tube *a* and the aspirator a second tube prepared in the same way, which ought, of course, to show no growth. When forty-eight hours at 20° C. or four days at lower temperature have elapsed, the



colonies which develop in *a* may be counted. The disadvantage of the method is that if particles of dust carrying more than one bacterium alight on the gelatin, these bacteria develop in one colony, and thus the numeration results may be too low; difficulties may also arise from liquefying colonies developing in the upper parts of the tube and running over the gelatin.

**Petri's Sand-Filter Method.**—A glass tube open at both ends, and about  $3\frac{1}{2}$  inches long and half an inch wide, is taken, and in its centre is placed a transverse diaphragm of very fine iron gauze (Fig. 49, *e*); on each side of this is placed some fine quartz sand which has been well washed, dried, and burned to remove all impurities, and this is kept in position by cotton plugs. The whole is sterilised by dry heat. One plug is removed, and a sterile rubber cork, *c*, inserted, through which a tube, *d*, passes to an exhausting apparatus. The tube is then clamped in an upright position in the atmosphere to be examined, with the remaining plug, *f*, uppermost. The latter is removed and the air sucked through. Difficulty may be experienced from the resistance of the sand if quick filtration be attempted. The best means to adopt is to use an air-pump—the amount of air drawn per stroke of which is accurately known—and to have a manometer (as in Fig. 30) interposed between the tube and the pump. Between each two strokes of the air-pump the mercury is allowed to return to zero. After the required amount of air has passed, the sand *a* is removed, and is distributed among a number of sterile gelatin tubes which are well shaken; plate cultures are then made, and when growth has occurred the colonies are enumerated; the sand *b* is similarly treated, and acts as a control.

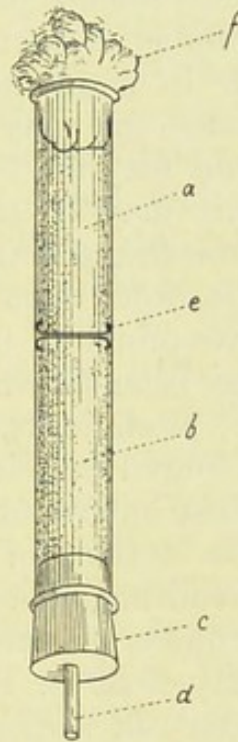


FIG. 49.—Petri's sand filter.

When it is necessary to examine air for particular organisms, special methods must often be adopted. Thus in the case of the suspected presence of tubercle bacilli a given quantity of air is drawn through a small quantity of water and then injected into a guinea-pig.

It must be admitted that comparatively little information bearing on the harmlessness or harmfulness of the air is obtainable by the mere enumeration of the living organisms present, for under certain conditions the number may be increased by the presence of many individuals of a purely non-pathogenic character. The organisms found in the air belong to two groups—firstly, a great variety of bacteria; secondly, yeasts and the spores of moulds and of the lower fungi. With regard to the spores, the organisms from which they are derived often consist of felted masses of threads, from which are thrust into the air special filaments, and in connection with these the spores are formed. By currents of air these latter can easily be detached,



and may float about in a free condition. With the bacteria, on the other hand, the case is different. Usually these are growing together in little masses on organic materials, or in fluids, and it is very much by the detachment of minute particles of the substratum that the organisms become free. The entrance of bacteria into the air, therefore, is associated with conditions which favour the presence of dust, minute droplets of fluid, etc. The presence of dust, in particular, would specially favour a large number of bacteria being observed, and this is the case with the air in many industrial conditions, where the bacteria, though numerous, may be quite innocuous. Great numbers of bacteria thus may not indicate any condition likely to injure health, and this may be true also even when the bacteria come from the crowding together of a number of healthy human beings. On the other hand, there is no doubt that disease germs can be disseminated by means of the air. The possibility of this has been shown experimentally by infecting the mouth with the *b. prodigiosus*, which is easily recognised by its brilliantly coloured colonies, and then studying its subsequent distribution. Most important here is the infection of the air from sick persons. The actions of coughing, sneezing, speaking, and even of deep breathing, distribute, often to a considerable distance, minute droplets of secretions from the mouth, throat, and nose, and these may float in the air for a considerable time. Even five hours after an atmosphere has been thus infected evidence may be found of bacteria still floating free. Before this time, however, most of the bacteria have settled upon various objects, where they rapidly dry, and are no longer displaceable by ordinary air currents. The diseases of known etiology where infection can thus take place are diphtheria, influenza, pneumonia, and phthisis; and here also possibly typhus fever and measles are to be added, though the morbid agents are unknown. In the case of phthisis, the deposition of tubercle bacilli has been demonstrated on cover-glasses held before the mouths of patients while talking, and animals made to breathe directly in front of the mouths of such patients have become infected with tuberculosis. Apart from direct infection from individuals, however, pathogenic bacteria may be spread in some cases from the splashing of infected water, as from a sewage outfall. This possibility has to be recognised especially in the cases of typhoid and cholera. Besides infection through fluid particles, infection can be caused in the air by dust coming from infected skin or clothes, etc. Flügge, in dealing with this subject in an experimental inquiry, distinguishes between large particles of dust which



require an air current moving at the rate of 1 centimetre per second to keep them suspended, and the finer dust which can be kept in suspension by currents moving at from 1 to 4 millimetres per second. In the former case, when once the particles settle they cannot be displaced by currents of air except when these are moving at, at least, 5 metres per second, but the brushing, shaking, or beating of objects may, of course, distribute them. In the case of the finer dust the particles will remain for long suspended, and when they have settled can be more easily displaced, as by the waving of an arm, breathing, etc. With regard to infection by dust, a most important factor, however, is whether or not the infecting agent can preserve its vitality in a dry condition. In the case of a sporing organism such as anthrax, vitality is preserved for long periods of time, and great resistance to drying is also possessed by the tubercle and diphtheria bacilli; but apart from such cases there is little doubt that infection is usually necessarily associated with the transport of moist particles, and is thus confined to a limited area around a sick person. Among diseases which may occasionally be thus spread, cholera and typhoid have been classed. Considerable controversy has arisen with regard to certain outbreaks of the latter disease, which have apparently been spread by dusty winds, although we have the fact that the typhoid bacillus does not survive being dried even for a short time. It appears, however, that in such epidemics the transport of infection by means of insects carried by the wind has not been entirely excluded.

As in the cases of the soil and of water, presently to be described, attempts have been made to obtain indirect evidence of the contamination of the air from human sources. Thus Gordon has shown that certain streptococci are common in the saliva; these usually correspond to the *streptococcus salivarius* of Andrewes and Horder (*q.v.*) in that they grow at 37° C., form acid and clot in litmus milk, reduce neutral-red, and ferment saccharose, lactose, and raffinose. Andrewes and Horder also describe another group,—*str. equinus*,—as common in London air, which they think is there derived from horse dung. Thus the finding of streptococci of the first group in plates exposed to air would indicate that a human source was probable, and, if the observation were made on air from the neighbourhood of a sick person, that risk of the dissemination of disease germs was present. The value of this as a practical method has yet to be determined.

#### SOIL.

The investigation of the bacteria which may be found in the soil is undertaken from various points of view. Information



may be desired as to the change its composition undergoes by a bacterial action, the result of which may be an increase in fertility and thus in economic value. Under this head may be grouped inquiries relating to the bacteria which convert ammonia and its salts into nitrates and nitrites, and to the organisms concerned in the fixation of the free nitrogen of the air. The discussion of the questions involved in such inquiries is outside the scope of the present chapter, which is more concerned with the relation of the bacteriology of the soil to questions of public health. So far as this narrower view is concerned, soil bacteria are chiefly of importance in so far as they can be washed out of the soils into potable water supplies. An important aspect of this question thus is as to the significance of certain bacteriological appearances in a water in relation to the soil from which it has come or over which it has flowed. In this country these questions have been chiefly investigated by Houston, and it is from his papers that the following account is largely taken.

**Methods of Examination.**—For examination of soil on surface or not far from surface, Houston recommends tin troughs 10 in. by 3 in., and pointed at one extremity, to be wrapped in layers of paper and sterilised by dry heat. If several of these be provided, then the soil can be well rubbed up and a sample secured and placed in a sterile test-tube for examination as soon as convenient after collection. If samples are to be taken at some depth beneath the surface, then a special instrument of which many varieties have been devised must be used. The general form of these is that of a gigantic gimlet stoutly made of steel. Just above the point of the instrument the shaft has in it a hollow chamber, and a sliding lateral door in this can be opened and shut by a mechanism controlled at the handle. The chamber being sterilised and closed, the instrument is bored to the required depth, the door is slid back, and by varying devices it is effected that the chamber is filled with earth; the door is reclosed and the instrument withdrawn.

In any soil the two important lines of inquiry are first, as to the total number of organisms (usually reckoned per gramme of the fresh sample); and secondly, as to the varieties of organisms present. The number of organisms present in a soil is often, however, so enormous that it is convenient to submit only a fraction of a gramme to examination. The method employed is to weigh the tube containing the soil, shake out an amount of about the size of a bean into a litre of distilled water, and reweigh the tube. The amount placed in the water is distributed as thoroughly as possible by shaking, and, if necessary, by rubbing down with a sterile glass rod, and small quantities measured from a graduated pipette are used for the investigation. For estimating the total number of organisms present in the portion of soil used, small quantities, say .1 c.c. and 1 c.c., of the fluid are added to melted tubes of ordinary alkaline peptone gelatin; after being shaken, the gelatin is plated, incubated at 22° C., and the colonies are counted as late as the liquefaction, which always occurs round some of them, will allow. From these numbers the total number of organisms, which grow in gelatin, in a given amount of soil can be calculated.



The numbers of bacteria in the soil vary very much. According to Houston's results, fewest occur in uncultivated sandy soils, these containing on an average 100,000 per gramme. Peaty soils, though rich in organic matter, also give low results, it being possible that the acidity of such soils inhibits free bacterial growth. Garden soils yield usually about 1,500,000 bacteria per gramme, but the greatest numbers are found in soils which have been polluted by sewage, when the figures may rise to several millions. In addition to the enumeration of the numbers of bacteria present, it is a question whether something may not be gained from a knowledge of the number of spores present in a soil relative to the total number of bacteria. This is a point which demands further inquiry, especially by the periodic investigation of examples of different classes of soils. The method is to take 1 c.c. of such a soil emulsion as that just described, add it to 10 c.c. of gelatin, heat for ten minutes at 80° C. to destroy the non-spored bacteria, plate, incubate, and count as before.

Besides the enumeration of the numbers of bacteria present in a soil, an important question in its bacteriological examination lies in inquiring what kinds of bacteria are present in any particular case. Practically this resolves itself into studying the most common bacteria present, for the complete examination of the bacterial flora of any one sample would occupy far too much time. Of these common bacteria the most important are those from whose presence indications can be gathered of the contamination of the soil by sewage, for from the public health standpoint this is by far the most important question on which bacteriology can shed light.

*Bacillus mycoides*.—This bacillus is 1.6 to 2.4  $\mu$  in length, and about .9  $\mu$  in breadth. It grows in long threads which often show motility. It can be readily stained by such a combination as carbol-thionin, and retains the dye in Gram's method. All ordinary media will support its growth, and, in surface growths on agar or potato, spore formation is readily produced. Its optimum temperature is about 18° C. On gelatin plates it shows a very characteristic appearance. At first under a low power it shows a felted mass of filaments throwing out irregular shoots from the centre, and later to the naked eye these appear to be in the form of thick threads like the growth of a mould. They rapidly spread over the surface of the medium, and the whole resembles a piece of wet teased-out cotton wool. The gelatin is liquefied.

*Cladothrices*.—Of these several kinds are common in the soil. The ordinary *cladothrix dichotoma* is among them. This organism appears as a colourless flocculent growth with an opaque centre, and can be seen under the microscope to send out into the medium apparently branched threads which vary in thickness, being sometimes 2  $\mu$  across. They consist of rods enclosed in a sheath. These rods may divide at any point, and thus the terminal elements may be pushed along the sheath.



Sometimes the sheath ruptures, and thus by the extrusion of these dividing cells and their further division the branching appearance is originated. Reproduction takes place by the formation of gonidia in the interior of the terminal cells. These gonidia acquire at one end a bundle of flagella, and for some time swim free before becoming attached and forming a new colony. Houston describes as occurring in the soil another variety, which with similar microscopic characters appears as a brownish growth with a pitted surface and diffuses a Bismarck-brown pigment into the gelatin which it liquefies.

A few experiments made with an ordinary field soil will, however, familiarise the worker with the non-pathogenic bacteria usually present. We have referred to these two because of their importance. In regard to pathogenic organisms, especially in relation to possible sewage contamination, attention is to be directed to three groups of organisms, those resembling the *b. coli*, the *bacillus enteritidis sporogenes*, and the *streptococcus pyogenes*. The characters of the first two of these will be found in the chapter on Typhoid Fever; of the third in Chapter VII. For the detection of these bacteria Houston recommends the following procedure:—

(a) *The B. coli Group*.—A third of a gramme of soil is added to 10 c.c. broth containing .2 per cent. of phenol and incubated at 37° C. In this medium very few if any other bacteria except those of the *b. coli* group will grow, so that if after twenty-four hours a turbidity appears, some of the latter may be suspected to be present. In such a case a loopful of the broth is shaken up in 5 c.c. sterile distilled water, and of this one or two loopfuls are spread over the surface of a solid plate of phenol gelatin in a Petri capsule either by means of the loop or of a small platinum spatula, and the plate is incubated at 20° C. Any colonies which resemble *b. coli* are then examined by the culture methods detailed under that organism. Further, all organisms having the microscopic appearances of *b. coli*, and which generally conform to its culture reactions, are to be reckoned in the *coli* group. The media of MacConkey and Drigalski are very useful in connection with the plating and separation of such soil organisms (*vide* pp. 50, 47).

(b) *The Bacillus enteritidis sporogenes*.—To search for this organism 1 gramme of the soil is thoroughly distributed in 100 c.c. sterile distilled water, and of this 1 c.c., .1 c.c., and .01 c.c. is added to each of three sterile milk tubes. These are heated to 80° C. for ten minutes, and then cultivated anaerobically at 37° C. for twenty-four hours. If the characteristic appearances seen in such cultures of the *b. enteritidis* (*q.v.*) are developed, then it may fairly safely be deduced that it is this organism which has produced them.

(c) *Faecal Streptococci*.—The method here is to pour out a tube of agar into a Petri capsule, and when it has solidified to spread out .1 c.c. of the emulsion of soil over it and incubate at 37° C. for twenty-four hours. At this temperature many of the non-pathogenic bacteria grow with difficulty, and thus the number of colonies which develop is relatively small. Colonies having appearances resembling those of the *streptococcus pyogenes* (*q.v.*) can thus be investigated.

Another method is that of Prescott and Winslow modified by Mair. This depends on the fact that when *b. coli* and streptococci are growing together in glucose broth, as the medium becomes acid the streptococci tend to outgrow the *b. coli*. If lactose agar plates be made at this stage, the colonies of streptococci, being small and intensely red, can be distinguished from the larger and less acid colonies of the *b. coli*. They can



then be picked off for investigation. It is evident that here the method must be adopted of taking as a measure of the number of streptococci present the least quantity of the original fluid in which evidence of their presence can be detected.

We may now give in brief the results at which Houston has arrived by the application of these methods. First of all, uncultivated soils contain very few, if any, representatives of the *b. mycoides*, and this is also true to a less extent of the cladothrices. Cultivated soils, on the other hand, do practically always contain these organisms. With regard to the *b. coli*, its presence in a soil must be looked on as indicative of recent pollution with excremental matter. The presence of *b. enteritidis* is also evidence of such pollution, but from the fact that this is a sporing organism the pollution may not have been recent. With regard to the streptococci, on the other hand, the opinion is advanced that their presence is, on account of their feeble viability outside the animal body, to be looked on as evidence of extremely recent excremental pollution. The very great importance of these results in relation to the bacteriological examination of water supplies will be at once apparent, and will be referred to again in connection with this subject.

While such means have been advanced for the obtaining of indirect evidence of excremental pollution of soil, and therefore of a pollution dangerous to health from the possible presence of pathogenic organisms in excreta, investigations have also been conducted with regard to the viability in the soil of pathogenic bacteria, especially of those likely to be present in excreta, namely, the typhoid and cholera organisms. The solution of this problem is attended with difficulty, as it is not easy to identify these organisms when they are present in such bacterial mixtures as naturally occur in the soil. Now there is evidence that bacteria when growing together often influence each other's growth in an unfavourable way, so that it is only by studying the organisms in question when growing in unsterilised soils that information can be obtained as to what occurs in nature. For instance, it has been found that the *b. typhosus*, when grown in an organically polluted soil which has been sterilised, can maintain its vitality for fifteen weeks, but if the conditions occurring naturally be so far imitated by growing it in soil in the presence of a pure culture of a soil bacterium, it is found that sometimes the typhoid bacillus, sometimes the soil bacterium in the course of a few weeks, or even in a few days, disappears. Further, the character of the soil exercises an important effect on the results; for instance, the typhoid bacillus soon dies out in a virgin sandy



soil, even when it is the only organism present. In experiments made by sowing cultures of cholera and diphtheria in plots in a field, it was found that after, at the longest, forty days they were no longer recognisable. Further, it is a question whether ordinary disease organisms, even if they remain alive, can multiply to any great extent in soil under natural conditions. If we are dealing with a sporing organism such as the *b. anthracis*, the capacity for remaining in a quiescent condition of potential pathogenicity is, of course, much greater. The most important principle to be deduced from these experiments is that the ordinary conditions of soil rather tend to be unfavourable to the continued existence of pathogenic bacteria, so that by natural processes soil tends to purify itself. It must, however, be noted that such an organism as the typhoid bacillus can exist long enough in soil to be a serious source of danger.

#### WATER.

In the bacteriological examination of water three lines of inquiry may have to be followed. First, the number of bacteria per cubic centimetre may be estimated. Second, the kinds of bacteria present may be investigated. Third, it may be necessary to ask if a particular organism is present, and, if so, in what number per c.c. it occurs.

**Methods.**—*Collection of Samples.*—In all water examinations it is preferable that the primary culture media (*i.e.* those to which the water is actually to be added) should be inoculated at the spot at which the sample is collected. When this is not possible, the samples should be packed in sawdust and ice and the primary inoculations made as soon as possible. Otherwise the bacteria will multiply, and an erroneous idea of the number present will be obtained. Immediately after collection a slight diminution in numbers may be observed, but at any rate after six hours an increase over the initial numbers is manifest.

When samples have to be taken for transport to the laboratory, these are best collected in 8-ounce, wide-mouthed, stoppered bottles, which are to be sterilised by dry heat (the stopper must be sterilised separately from the bottle and not inserted in the latter till both are cold, otherwise it will be so tightly held as to make removal very difficult).

In the case of water taken from a house tap, the water should be allowed to run for some time before the sample is taken, as water standing in pipes in a house is under very favourable conditions for multiplication of bacteria taking place, and if this precaution be not adopted an altogether erroneous idea of the number present may be obtained.

With river waters it is best to immerse the sampling bottle and then remove the stopper with forceps. Care must be taken not to touch the river bed, as the vegetable matter covering it contains many organisms. When water has to be taken from below the surface of a well or lake, a weighted sample bottle must be used. Several special bottles have been



devised for such a purpose. Quite good results are obtained by tying two short lengths of string to the neck and stopper of an ordinary bottle respectively, winding them round the neck and enveloping in cotton wool; any required length of string can afterwards be knotted on these. A piece of lead can be attached to the bottom of the bottle by wires passing round the neck. The whole is then wrapped in paper and sterilised. For use the bottle is carefully lowered to the required depth by the string attached to the neck, the stopper is jerked out, and the bottle filled. If the bottle and stopper be rapidly jerked through the topmost layers, contamination with surface bacteria does not appear as a serious factor.

*Counting of Bacteria in Water.*—This is done by adding a given quantity of water to 10 c.c. of liquefied gelatin or agar, plating, and counting the colonies which develop. The amount of water added depends on its source, and varies from .1 c.c. of a water likely to have a high bacterial content to 5 c.c. of a purer water. It is usual to inoculate both gelatin and agar tubes. The former, incubated at 20° C., gives an idea of the numbers of bacteria present which grow at summer heat; the latter, incubated at 37° C., those which grow at blood-heat. As the pathogenic and intestinal bacteria grow at this temperature, the determination of the numbers of blood-heat bacteria is important. The counts on the two media usually differ as each is favourable to the growth of its own group of organisms. With regard to the summer-heat bacteria it is important to note that gelatine of a slightly greater alkalinity than that ordinarily prepared—such an increased degree as is caused by the addition of .01 grm. of  $\text{Na}_2\text{CO}_3$  to 10 c.c. peptone gelatin—will give a greater yield of colonies. In the case of both gelatin and agar plates usually forty-eight hours' incubation is allowed before the colonies are counted, but, with the former, difficulties may arise in consequence of the presence of rapidly liquefying colonies, and it may thus be necessary to count after twenty-four hours.

Probably no one medium will support the growth of all the organisms present in a given sample of water, and under certain circumstances special media must therefore be used. Thus Hansen found that in testing waters to be used in brewing it was advisable to have in the medium employed some sterile wort or beer, so that the organisms in the test experiments should be provided with the food materials which would be present in the commercial use of the water. Manifestly this principle applies generally in the bacteriological examination of waters to be used for industrial purposes.

*Detection of the Presence of Special Organisms.*—(a) *The B. coli Group.*—In ordinary public health work, it may be taken that the most frequent and important inquiry with regard to a water is directed to the investigation of the presence or absence of the *b. coli* and its congeners. Here the method adopted is to determine the smallest quantity of a water which gives evidence of containing organisms of this type. In applying any method with this object in view it is, we consider, absolutely necessary that it shall be carried out at the spot at which samples are collected.

The usual method is to use as the primary culture medium one of the bile-salt preparations, of which the best is MacConkey's bile-salt glucose bouillon to which litmus has been added—glucose being used in preference to lactose in order to bring out *b. enteritidis* of Gaertner if this be present. In this medium the members of the *b. coli* group cause changes resulting in the formation of acid and gas. It is thus convenient to put the medium into Durham's fermentation tubes. In practice we employ



2-ounce cylindrical medicine bottles,  $4\frac{1}{2}$  in. high by  $1\frac{1}{2}$  in. in diameter. The medium, along with the inverted test-tube, is placed in these; rubber stoppers are inserted in the mouths, and they are sterilised. It is customary to test for the presence of the organisms in any sample by adding to a series of such tubes the following quantities of the water—50 c.c., 20 c.c., 10 c.c., 5 c.c., 1 c.c., and, it may be, in specially suspicious waters, .5 c.c., .1 c.c., and even .01 c.c. The result is estimated in terms of the smallest amount of water with which the occurrence of acid and gas formation is observed. By starting with a concentrated MacConkey's mixture, it is arranged that, when the sample is added, the resulting fluid shall be of the concentration of MacConkey's medium as ordinarily prepared. Thus, in the bottle to which the 50 c.c. sample is to be added, there are placed 10 c.c. of a sixfold concentration of MacConkey's medium. In the 20 c.c. tube, there are present 20 c.c. of a medium of double strength; in the 10 c.c. tube, 10 c.c. of a mixture of double strength; and in the 5 c.c. tube, 5 c.c. of a mixture of double strength. With smaller samples, we simply use the ordinary MacConkey's medium.

For the taking of the samples, sterile 8-ounce stoppered bottles are convenient, and for each sample it is necessary to have sterile 25 c.c., 10 c.c. (graduated to tenths), and 1 c.c. (graduated to hundredths) pipettes. The armamentarium being thus simple, there is no difficulty in carrying out the necessary manipulations at the spot where the sample is collected.

The tubes are incubated for forty-eight hours, and it is well to read the results at the end of the first twenty-four hours also. The formation of acid and gas in the tube is usually recognised as "presumptive evidence" of the presence of members of the *b. coli* group, but it is necessary to further investigate the bacteria giving rise to this change to determine whether they are "typical" or "atypical" *b. coli*. With this end in view, each bottle in which acid and gas is present is well shaken up, two or three loopfuls are placed on a plate of MacConkey's neutral-red bile-salt lactose agar. These loopfuls are spread over the surface by means of a sterile spreader, made by taking a piece of glass rod and turning a portion about 2 inches long at right angles to the shaft. The plates are incubated for twenty-four hours. As typical *b. coli* produces acid in lactose, any colonies of such an organism are of a rosy red colour. These are then picked off, sloped agar tubes are inoculated and used for the further investigation of the properties of the bacterium isolated.

The media inoculated should be gelatin stab, litmus milk, neutral-red lactose bouillon, glucose broth, peptone water, dulcete peptone water, adonite peptone water, inuline peptone water, saccharose peptone water, and potato.

It is well in dealing with the neutral-red lactose agar plates to inoculate a lactose peptone water tube from all the kinds of colonies present, whether these are red or not, as MacConkey rightly points out that sometimes an organism which is really a lactose fermenter does not produce a red colour on the solid medium. There is another point to be noted here, namely, that the naked-eye appearances of colonies on lactose agar are not of value in identifying the kind of organism present.

The object of growing suspicious colonies on a range of media such as that given, is to enable typical *b. coli* to be recognised when present. At the present time it cannot be said that bacteriologists are in agreement as to what characters determine the type of organism most frequently found in the human intestine—this, of course, being the important point



in judging of the contamination of a water supply. The subject will be more fully discussed in the chapter on Typhoid Fever. Here it may be said that for work on water two attitudes are taken up in this country. First, that of Houston, who recognises as typical qualities the following: fluorescence in neutral red broth, production of acid and gas in lactose peptone water, production of indol, production of acid and clot in litmus milk (so-called "flaginac" reaction). Secondly, that of the English Committee of 1904, which, on the one hand, laid stress on the additional factor of non-liquefaction of gelatin, and on the other, attached less importance to the production of indol and the occurrence of fluorescence (see p. 355).

With regard to saccharose fermentation, different strains of coli of undoubted intestinal origin behave differently towards saccharose, but when saccharose is fermented the occurrence is significant, as indicating a great probability that the organism is intestinal in origin.

(b) *B. enteritidis sporogenes* and *streptococci*.—As in the case of sewage, the presence of these in a water may be sought for. The methods are those which have already been given (p. 154).

Much work has been devoted to the question of these faecal streptococci presenting specific characters by which they could be differentiated from other streptococci. Houston has found that the prevailing type of organism here is one which produces acid and clot in milk, reduces neutral-red, and ferments saccharose, lactose, and salicin. It corresponds to the *streptococcus faecalis* of Andrewes and Horder. The important point in this connection is to recognise that streptococci of such a type exist in great numbers in human faeces, and that when in any circumstances faecal contamination is suspected, the isolation of streptococci strengthens the suspicion.

With regard to the objects with which the bacteriological examination of water may be undertaken, though these may be of a purely scientific character, they usually aim at contributing to the settlement of questions relating to the potability of waters, to their use in commerce, and to the efficiency of processes undertaken for the purification of waters which have undergone pollution. The last of these objects is often closely associated with the first two, as the question so often arises whether a purification process is so efficient as to make the water again fit for use.

Water derived from any natural source contains bacteria, though, as in the case of some artesian wells and some springs, the numbers may be very small, *e.g.* 4 to 100 per c.c. In rain, snow, and ice there are often great numbers, those in the first two being derived from the air. Great attention has been paid to the bacterial content of wells and rivers. With regard to the former, precautions are necessary in arriving at a judgment. If the water in a well has been standing for some time, multiplication of bacteria may give a high value. To meet this difficulty the well ought, if practicable, to be pumped dry and



then allowed to fill, in order to get at what is really the important point, namely, the bacterial content of the water entering the well. Again, if the sediment of the well has been stirred up, a high value is obtained. Ordinary wells of medium depth contain from 100 to 2000 per c.c. With regard to rivers very varied results are obtained. Moorland streams are usually fairly pure. In an ordinary river the numbers present vary at different seasons of the year, whilst the prevailing temperature, the presence or absence of decaying vegetation, or of washings from land, and dilution with large quantities of pure spring water, are other important features. Thus the Franklands found the rivers Thames and Lea purest in summer, and this they attributed to the fact that in this season there is most spring water entering, and very little water as washings off land. In the case of other rivers the bacteria have been found to be fewest in winter. A great many circumstances must therefore be taken into account in dealing with mere enumerations of water bacteria, and such enumerations are only useful when they are taken simultaneously over a stretch of river, with special reference to the sources of the water entering the river. Thus it is usually found that immediately below a sewage effluent the bacterial content rises, though in a comparatively short distance the numbers may markedly decrease, and it may be that the river as far as numbers are concerned may appear to return to its previous bacterial content. The numbers of bacteria present in rivers vary so greatly that there is little use in quoting figures, most information being obtainable by comparative enumerations before and after a given event has occurred to a particular water. Such a method is thus of great use in estimating the efficacy of the filter-beds of a town water supply. These usually remove from 95 to 98 per cent. of the bacteria present, and a town supply as it issues from the filter-beds should not contain more than 100 bacteria per c.c. Again, it is found that the storage of water effects a very marked bacterial purification. Thus Houston has shown in one series of observations that while 93 per cent. of samples of raw river Lea water contained *b. coli.* in 1 c.c. or less, in the stored water 62 per cent. of the samples showed no *b. coli.* to be present in 100 c.c. The highest counts of bacteria per c.c. are observed with sewage; for example, in the London sewage the numbers range from six to twelve millions.

Much more important than the mere enumeration of the bacteria present in a water is the question whether these include



forms pathogenic to man. The chief interest here, so far as Europe is concerned, lies in the fact that typhoid fever is so frequently water-borne, but cholera and certain other intestinal diseases have a similar source. The search in waters for the organisms concerned in these diseases is a matter of the greatest difficulty, for each belongs to a group of organisms morphologically similar, very widespread in nature, and many of which have little or no pathogenic action. The biological characters of these organisms will be given in the chapters devoted to the diseases in question, but here it may be said that from the public health standpoint the making of their being found a criterion for the condemning of a water is impracticable. There is no doubt that the typhoid and cholera bacteria can exist for some time in water—at least this has been found to be the case when sterile water has been inoculated with these bacteria. But to what extent the same is true when they are placed in natural conditions, which involve their living in the presence of other organisms, is unknown, for by no known method can the presence of either be with certainty demonstrated in the complex mixtures which occur in nature. With regard to the typhoid bacillus, of late the tendency has been to seek for the presence of indirect bacteriological evidence which might point in the direction of the possibility of the presence of this organism. The whole question turns on the possibility of recognising bacteriologically the contamination of water with sewage. Klein and Houston here insist on the fact that in crude sewage the *b. coli* or the members of the coli group are practically never fewer than 100,000 per c.c., and therefore if in a water this organism forms a considerable proportion of the total number of organisms present, then there is great reason for suspecting sewage pollution. In these circumstances, all modern work tends to taking the presence of *b. coli* in a water as the best indirect evidence of the possibility of disease organisms of intestinal origin being likely to gain access to that water. It must, however, be at once clearly recognised that the presence of members of the coli group is only an indication, and so far as the potability of any water is concerned, there is no evidence that these organisms, however undesirable, are under ordinary circumstances actually harmful to man. In all inquiries there is the difficulty that at present no means exist of differentiating between *b. coli* as derived from the human intestine on the one hand, and from the intestine of animals on the other. It is thus necessary in reporting upon a water to have had an opportunity of inspecting the locality. We have



known cases where a moorland water had a very high content in *b. coli*, without there being the remotest possibility that such came from man. With this proviso, we must inquire as to what criteria are to be adopted in determining the significance of the presence of different members of the group in a water, and here reliance is chiefly to be placed on the presence of the typical forms of *b. coli*.

If a sufficient quantity of practically any water be taken, except, perhaps, that coming from artesian wells, organisms of the coli group will be found to be present. Therefore, the question resolves itself into setting up some standards of relative purity which may be followed in dealing with waters coming from different sources. These standards are at present empirical, and different bacteriologists have different views on the subject. There is, however, a general agreement that deep well water and the filtered water supplied to urban communities should be entirely free from *b. coli* in quantities of 100 c.c. or less. The great difficulty lies in dealing with river water and water from shallow and surface wells. Here the usual view is that the presence of *b. coli* in 10 c.c. or less is sufficient to condemn the water. It may be said that under ordinary circumstances an inspection of the surroundings and an unfavourable chemical analysis are sufficient to condemn such a water, for even if a bacteriological examination showed the absence of *b. coli* in large samples, yet the water ought to be condemned; and further, if in a suspicious locality the bacteriological analysis yielded a bad result, the water ought to be condemned even if from the chemical analysis it could be passed. The difficult cases are those where the inspection of the locality is satisfactory, and yet *b. coli* is present in large numbers. Here contamination is often of animal origin, and the water can after careful inquiry be passed.

Great care is often necessary in interpreting bacteriological analysis in consequence of the delicacy of the method. Thus in examining raw waters, especially those derived from moorland catchment areas to be used for urban supplies, bacteriological examinations are relatively of little value, as storage and filtration will completely alter the bacterial content. Bacteriological methods are, however, of the greatest value—much more than mere chemical analysis—in determining the efficiency of filtration processes.

As the *b. coli* is fairly widespread in nature, Klein and Houston hold that valuable supporting evidence is found in the presence of the *b. enteritidis sporogenes* and of strepto-



cocci, both of which are probably constant inhabitants of the human intestine. The spores of the former usually number 100 per c.c. in sewage, and the presence of the latter can always be recognised in .001 grm. of human faeces. The deductions to be drawn from the presence of these in water are the same as those to be drawn from their presence in soil.

It may be said that in water artificially polluted with sewage containing intestinal bacteria, these can be detected by bacteriological methods in mixtures from ten to a hundred times more dilute than those in which the pollution can be detected by purely chemical methods.

**Bacteriology of Sewage.**—It is sometimes necessary to examine the bacterial content of sewage, especially in connection with the efficiency of purification works. The main lines of inquiry are here the same as for water, and the general methods are identical, the only modification necessary being that, in consequence of the high bacterial content, much smaller quantities of the raw material must be worked with. With regard to the numbers of bacteria in sewage, these may run from a million to ten millions or even more per c.c., and here of course the question of the presence of intestinal organisms of the coli group is of great importance. The numbers of these are large, and members of the group may be detected in a .000001 c.c. or less. The numbers present are frequently considerably reduced by purification methods, but it is to be noted that, even when such methods are most successful, *b. coli* may yet be present in considerable quantities. This is especially true in Britain, where sewage is much more concentrated than it apparently is in America. In the latter country, purification may yield effluents in which *b. coli* can be detected in only .001 c.c. By no purification method has the production of a potable water been attained, and the high content of effluents in *b. coli* makes the passage of typhoid bacilli through a purification system possible although the organism has perhaps never been certainly demonstrated.

The part which bacteria play in the purification of sewage constitutes a question of great interest, to which much attention has been directed. The methods adopted for sewage purification may be divided into two groups. In the first of these, the sewage coming from the mains is run on to a bed of gravel, clinker, or coke, on which it is allowed to stand for some hours. The effluent is then run out through the bottom of the bed, which is then allowed to rest for some hours before being recharged. In a modification of this method the sewage is



allowed to percolate slowly through a bed consisting of large porous objects, such as broken bricks or large pieces of coke, and here the percolation may be constant, no interval of rest being given. The bacterial processes which take place in these two methods are, however, probably closely similar. In the second, the essential feature is a preliminary treatment of the sewage in more or less closed tanks ("septic tanks"), where the conditions are supposed to be largely anaerobic. This method has been adopted at Exeter, Sutton, and Yeovil in this country, and very fully worked at in America by the State Board of Health of Massachusetts. In the explanation given of the rationale of this process, sewage is looked on as existing in three stages. (1) First of all, *fresh sewage*—the newly mixed and very varied material as it enters the main sewers. (2) Secondly, *stale sewage*—the ordinary contents of the main sewers. Here there is abundant oxygen, and as the sewage flows along there occurs by bacterial action a certain formation of carbon dioxide and ammonia, which combine to form ammonium carbonate. This is the sewage as it reaches the purification works. Here a preliminary mechanical screening may be adopted, after which it is run into an airtight tank—the septic tank. (3) It remains there for from twenty-four to thirty-six hours, and becomes a foul-smelling fluid—the *septic sewage*. The chemical changes which take place in the septic tank are of a most complex nature. The sewage entering it contains little free oxygen, and therefore the bacteria in the tank are probably largely anaerobic, and the changes which they originate consist of the formation of comparatively simple compounds of hydrogen with carbon, sulphur, and phosphorus. As a result, there is a great reduction in the amount of organic nitrogen, of albuminoid ammonia, and of carbonaceous matter. The last is important, as the clogging of ordinary filter-beds is largely due to the accumulation of such material, and of matters generally consisting of cellulose. One further important effect is that the size of the particles of the deposited matter is decreased, and therefore it is more easily broken up in the next stage of the process. This consists of running the effluent from the septic tank on to filter-beds, preferably of coke, where a further purification process takes place. By this method there is first an anaerobic treatment, succeeded by an aerobic; in the latter the process of nitrification occurs by means of the special bacteria concerned. The results are of a satisfactory nature, there being often a marked diminution in the number of coli organisms present.

In the earlier stages of any sewage purification, there is little



doubt that the albuminous material present is being split up by ordinary putrefactive bacteria. In the mains and where open systems of purification are at work, aerobic forms play the chief part, while in the closed methods anaerobic organisms are those chiefly concerned. In contact and percolating systems there is evidence that at first the purifying action of bacteria is materially furthered by physical processes. Thus Dunbar has shown that when such a substance as coke is used in a sewage filter-bed a considerable amount of the albuminous material is removed in a very few minutes by adsorption, for, albumin, being of a colloidal nature, is readily deposited under such circumstances in the pores of the coke in the form of films. After a time such a filter-bed becomes clogged, but on access of oxygen being allowed, it regains its adsorptive properties—probably from the oxidation of the material adsorbed.

During this stage, as in the whole purification process, four, and it may be five, processes are at work:—First, the action of ordinary bacteria splitting up the higher albuminous molecules; secondly, the action of nitrifying bacteria building up nitrites and nitrates from ammoniacal products; thirdly, the action of denitrifying bacteria which reduce nitrates to lower gaseous oxides and to free nitrogen (the presence of which in filter-beds can be demonstrated); fourthly, the action of higher forms of vegetable and animal life; fifthly, it is possible that direct chemical oxidation of the earlier products of bacterial action may occur, and in any case the access of an abundant oxygen supply to adsorbed material hastens its destruction. It is possible, as is indicated by the work of Lorrain Smith and of Mair, that perhaps too little weight has been attached to the parts played by the two last processes specified, for in the later stages of the purification process there is a very marked diminution in the number of bacteria present in the filter. Much further work, however, is necessary before the part to be assigned to each factor in operation can be properly estimated.

Further, the details of the essentially bacterial part of the process are obscure, and the relative parts played, even in an open purification process, by aerobes on the one hand, and anaerobes on the other, is little understood. When sewage is drained off to rest a filter-bed, great quantities of oxygen are sucked in, but as to how long the bed thus remains aerated, authorities differ—some maintaining that oxidation processes persist even after the bed has been recharged, while others state that soon the oxygen in the resting bed is consumed, and its place taken by carbon dioxide and nitrogen. Certainly, at certain



stages of the purification process, large amounts of free nitrogen come off the bed, but whether at such periods anaerobic bacteria are or are not in the ascendant, is not known. It is probable that, from the practical standpoint, the later stages of purification should take place with free oxidation, as when anaerobic bacteria are active at this point a very offensive effluent is produced.

Often the effluent from a sewage purification system contains as many bacteria as the sewage entering, but there is often a marked diminution. It is said by some that pathogenic bacteria do not live in sewage. The typhoid bacillus has been found to die out when placed in sewage, but it certainly can live in this fluid for a much longer period than that embraced by any purification method. Thus the constant presence of *b. coli*, *b. enteritidis*, and streptococci which has been observed in sewage effluents must here still be looked on as indicating a possible infection with the typhoid bacillus, and it is only by great dilution and prolonged exposure to the conditions present in running water that such an effluent can become suitable for forming a part of a potable water.

#### ANTISEPTICS.

The death of bacteria is judged of by the fact that, when they are placed on a suitable food medium, no development takes place. Microscopically it would be observed that division no longer occurred, and that in the case of motile species movement would have ceased, but such an observation has only scientific interest. From the importance of being able to kill bacteria, an enormous amount of work has been done in the way of investigating the means of doing so by chemical means, and the bodies having such a capacity are called antiseptics. It is now known that the activity of these agents is limited to the killing of bacteria outside the animal body, but still even this is of high importance.

**Methods.**—These vary very much. In early inquiries a great point was made of the prevention of putrefaction, and work was done in the way of finding how much of an agent must be added to a given solution such as beef extract, urine, etc., in order that the bacteria accidentally present might not develop; but as bacteria vary in their powers of resistance, the method was unsatisfactory, and now an antiseptic is usually judged of by its effects on pure cultures of definite pathogenic microbes, and in the case of a sporing bacterium, the effect on both the vegetative and spore forms is investigated. The organisms most used are the staphylococcus pyogenes, streptococcus pyogenes, and the organisms of typhoid, cholera, diphtheria, and anthrax—the latter being most used



for testing the action on spores. The best method to employ is to take sloped agar cultures of the test organism, scrape off the growth, and mix it up with a small amount of distilled water, and filter this emulsion through a plug of sterile glass wool held in a small sterile glass funnel, add a measured quantity of this fluid to a given quantity of a solution of the antiseptic in distilled water, then after the lapse of the period of observation to remove one or two loopfuls of the mixture and place them in a great excess of culture medium. Here it is preferable to use fluid agar, which is then plated and incubated; such a procedure is preferable to the use of bouillon tubes, as any colonies developing can easily be recognised as belonging to the species of bacterium used. In dealing with strong solutions of chemical agents it is necessary to be sure that the culture fluid is in great excess, so that the small amount of the antiseptic which is transferred with the bacteria may be diluted far beyond the strength at which it still can have any noxious influence. Sometimes it is possible at the end of the period of observation to change the antiseptic into inert bodies by the addition of some other substance, and then test the condition of the bacteria, and if the inert substances are fluid there is no objection to this proceeding; but if in the process a precipitate results, then it is better not to have recourse to such a method, as sometimes the bacteria are carried down with the precipitate and may escape the culture test. The advisability of, when possible, thus chemically changing the antiseptic was first brought to notice by the criticism of Koch's statements as to the efficacy of mercuric chloride in killing the spores of the *b. anthracis*. The method he employed in his experiments was to soak silk threads in an emulsion of anthrax spores and dry them. These were then subjected to the action of the antiseptic, well washed in water, and laid on the surface of agar. It was found, however, that, with threads exposed to a far higher concentration of the corrosive sublimate than Koch had stated was sufficient to prevent growth, if the salt were broken up by the action of ammonium sulphide and this washed off, growth of anthrax still occurred when the threads were laid on agar. The explanation given was that the antiseptic had formed an albuminate with the case of each spore, and that this prevented the antiseptic from acting upon the contained protoplasm. Such an occurrence only takes place with spores, and the method given above, in which the small amount of antiseptic adhering to the bacteria is swamped in an excess of culture fluid, can safely be followed, especially when a series of antiseptics is being compared. Krönig and Paul introduced what is known as the Garnet method for testing antiseptics. In this, small garnets of equal size are carefully cleaned, dipped in an emulsion of anthrax spores, and allowed to dry. They are then placed in mercuric chloride, and from time to time some are removed, gently washed, and treated with ammonium sulphide to decompose the chloride. They are then well shaken in a measured quantity of water. This is plated, and the number of anthrax colonies developing is counted.

Ponder and Woodhead have introduced an ingenious apparatus by which the effects of different concentrations of an antiseptic on the vitality of such an organism as the *b. coli* can be automatically recorded.

Much attention has been paid to the standardisation of antiseptics, and a watery solution of carbolic acid is now generally taken as the standard with which other antiseptics are compared. Rideal and Walker point out that 110 parts by weight of B.P. carbolic acid equal



100 parts by weight of phenol, and they recommend the following method of standardising: To 5 c.c. of a particular dilution of the disinfectant add 5 drops of a 24-hour-old bouillon culture of the organism (usually *b. typhosus*), which has been incubated at 37° C. Shake the mixture and make subcultures every 2½ minutes to 15 minutes. Perform a parallel series of experiments with carbolic acid, and express the comparative result in multiples of the carbolic acid doing the same work.

**The Action of Antiseptics.**—In inquiries into the actions of antiseptics attention to a great variety of factors is necessary, especially when the object is not to compare different antiseptics with one another, but when the absolute value of any body is being investigated. Thus the medium in which the bacteria to be killed are situated is important; the more albuminous it is, the greater degree of concentration is required. Again, the higher the temperature at which the action is to take place, the more dilute may the antiseptic be, or the shorter the exposure necessary for a given effect to take place. The most important factor, however, to be considered is the chemical nature of the substances employed. Chick has shown that the action of a disinfectant upon a bacterium presents close analogies with the interaction of simple chemical substances, such as an acid and an alkali. In the case of anthrax spores, during the first few minutes a great fatality occurs, after which the action of the antiseptic gradually tails off. With certain other organisms, however, such as the paratyphoid bacillus, the presence in a culture—especially in a young culture—of highly resistant forms renders the initial action of an antiseptic less marked. The action of an antiseptic, like the action of an acid and an alkali, is very much increased by raising the temperature; from which follows the practical conclusion that, in any disinfection, the use of warm solutions is advisable. Chick and C. J. Martin have further investigated the fact that the presence of albuminous material in a mixture of disinfectant and bacteria decreases the action of the disinfectant, and consider that the latter is adsorbed by the albumin. They have shown grounds for believing that a disinfectant in an emulsionised form is more efficient than a similar disinfectant in actual solution, because of a similar phenomenon occurring; for, just as a disinfectant may be put out of action by being adsorbed by organic particles, so when these organic particles happen to be bacteria, the adsorption process causes a greater concentration of the antiseptic round the bacterial protoplasm, and thus hastens its death.

Though nearly every substance which is not a food to the



animal or vegetable body is more or less harmful to bacterial life, yet certain bodies have a more marked action than others. Thus it may be said that the most important antiseptics are the salts of the heavy metals, certain acids, especially mineral acids, certain oxidising and reducing agents, a great variety of substances belonging to the aromatic series, and volatile oils generally. In comparing different bodies belonging to any one of these groups the chemical composition or constitution is very important, and if such comparisons are to be made, the solutions compared must be equimolecular; in other words, the action of a molecule of one body must be compared with the action of a molecule of another body. This can be done by dissolving the molecular weight in grammes in, say, a litre of water (see p. 34). When this is done, important facts emerge. Thus, generally speaking, the compounds of a metal of high atomic weight are more powerful antiseptics than those of one belonging to the same series, but of a lower atomic weight. Among organic bodies, again, substances with high molecular weight are more powerful than those of low molecular weight—thus butyric alcohol is more powerful than ethylic alcohol—and important differences among the aromatic bodies are associated with their chemical constitution. Thus among the cresols the ortho- and para-bodies resemble each other in general chemical properties, and stand apart from metacresol; they also are similar in antiseptic action, and are much stronger than the meta-body. The same may be observed in other groups of ortho-, meta-, and para-bodies. Again, such a property as acidity is important in the action of a substance, and, generally speaking, the greater the avidity of an acid to combine with an alkali, the more powerful an antiseptic it is. With regard to oxidising agents and reducing agents, probably the possession of such properties has been overrated as increasing bactericidal potency. Thus in the case of such reducers as sulphurous acid and formic acid, the effect is apparently chiefly due to the fact that these substances are acids. Formic acid is much more efficient than formate of sodium. In the case of permanganate of potassium, which is usually taken as the type of oxidising agents in this connection, it can be shown that the greater amount of the oxidation which takes place when this agent is brought into contact with bacteria occurs after the organisms are killed. Such an observation is, however, not conclusive as to the non-efficiency of the oxidation process, for the death of the bacteria might be due to the oxidation of a very small part of the bacterial protoplasm. Apart from the chemical nature of antiseptic agents, the physical factors con-



cerned in their solution, especially when they are electrolytes, probably play a part in their action. The part played by such factors is exemplified in the important fact that a strong solution acting for a short time will have the same effect as a weaker solution acting for a longer time. From what has been said it will be realised that the real causes of a material being an antiseptic are very obscure, and at present we can only have a remote idea of the factors at work.

**The Effects of certain Antiseptics.**—Here we can only briefly indicate certain results obtained with the more common members of the group.

*Chlorine.*—All the halogens have been found to be powerful antiseptics, but from the cheapness with which it can be produced chlorine has been most used; not only is it the chief active agent in the somewhat complex action of bleaching powder, but it is also the chief constituent of several proprietary substances, of which "Electrozone" is a good example. This last substance is made from electrolysing sea-water, when magnesia, and chlorine being liberated, magnesium hypochlorite and magnesium chloride are formed. In the action of this substance free hypochlorous acid is formed, and the effect produced is thus similar to that of bleaching powder. Nissen, investigating the action of the latter, found that  $1\frac{1}{2}$  per cent. killed typhoid bacilli in fæces; and Rideal found that 1 part to 400–500 disinfected sewage in fourteen minutes, and Delépine's results show that 1 part to 50 (equal to .66 per cent. of chlorine) rapidly kills the tubercle bacillus, and 1 part to 10 (equal to 3.3 per cent.) killed anthrax spores. Klein found that .05 per cent. of chlorine killed most bacterial spores in five minutes.

*Iodine Terchloride.*—This is a very unstable compound of iodine and chlorine, and, seeing that the substance only remains as  $\text{ICl}_3$  in an atmosphere of chlorine gas, it is open to doubt whether the antiseptic effects attributed to it are not due to a very complicated action of free hydrochloric acid, hydriodic acid, of oxyacids of chlorine and iodine produced by its decomposition, and also, in certain cases, of organic iodine compounds formed from its contact with albuminous material. It is stated that the action is very potent: a 1 per cent. solution is said instantly to kill even anthrax spores, but if the spores be in bouillon, death occurs after from ten to twelve minutes. In serum the necessary exposure is from thirty to forty minutes. A solution of 1–1000 will kill the typhoid, cholera, and diphtheria organisms in five minutes.

*Nascent Oxygen.*—This is chiefly available in two ways—firstly,



when in the breaking up of ozone the free third atom of the ozone molecule is seeking to unite with another similar atom; secondly, when peroxide of hydrogen is broken up into water and an oxygen atom is thereby liberated. In commerce the activity of "Sanitas" compounds is due to the formation of ozone by the slow oxidation of the resin, camphor, and thymol they contain.

*Perchloride of Mercury.*—Of all the salts of the heavy metals this has been most widely employed, and must be regarded as one of the most powerful and useful of known antiseptics. In testing its action on anthrax spores there is no doubt that in the earlier results its potency was overrated from a neglect of the fact already alluded to, that in the spore-case an albuminate of mercury was formed which prevented the contained protoplasm from developing, while not depriving it of life. It has been found, however, that this salt in a strength of 1-100 will kill the spores in twenty minutes, although an hour's exposure to 1-1000 has no effect. The best results are obtained by the addition to the corrosive sublimate solution of .5 per cent. of sulphuric acid or hydrochloric acid; the spores will then be killed by a seventy-minute exposure to a 1-200 solution. When, however, organisms in the vegetative condition are being dealt with, much weaker solutions are sufficient; thus anthrax bacilli in blood will be killed in a few minutes by 1-2000, in bouillon by 1-40,000, and in water by 1-500,000. Plague bacilli are killed by one to two minutes' exposure to 1-3000. Generally speaking, it may be said that a 1-2000 solution must be used for the practically instantaneous killing of vegetative organisms.

Perchloride of mercury is one of the substances which have been used for disinfecting rooms by distributing it from a spray producer, of which the Equifex may be taken as a type. With such a machine it is calculated that 1 oz. of perchloride of mercury used in a solution of 1-1000 will probably disinfect 3000 square feet of surface. Such a procedure has been extensively used in the disinfection of plague houses, but the use of a stronger solution (1-500 acidulated) is probably preferable.

*Formalin* as a commercial article is a 40 per cent. solution of formaldehyde in water. This is a substance which of late years has come much into vogue, and it is undoubtedly a valuable antiseptic. A disadvantage, however, to its use is that, when diluted and exposed to air, amongst other changes which it undergoes it may be transformed, under little understood conditions, into trioxymethylene and paraformaldehyde, these being polymers of formaldehyde. The bactericidal values of these



mixtures are thus indefinite. Formalin may be used either by applying it in its liquid form or as a spray, or the gas which evaporates at ordinary temperatures from the solution may be utilised. To disinfect such an organic mixture as pus containing pyogenic organisms, a 10 per cent. solution acting for half an hour is necessary. In the case of pure cultures, a 5 per cent. solution will kill the cholera organism in three minutes, anthrax bacilli in a quarter of an hour, and the spores in five hours. When such organisms as pyogenic cocci, cholera spirillum, and anthrax bacillus infect clothing, an exposure to the full strength of formalin for two hours is necessary, and in the case of anthrax spores, for twenty-four hours. Silk threads impregnated with the plague bacillus were found to be sterile after two minutes' exposure to formalin.

The action of formalin vapour has been much studied, as its use constitutes a cheap method of treating infected rooms, in which case some spray-producing machine is employed. It is stated that a mixture of 8 c.c. of formalin with 48 c.c. of water is sufficient when vaporised to disinfect one cubic metre, so far as non-sporing organisms are concerned. It is also stated that 1 part formalin in 10,000 of air will kill the cholera vibrio in one hour, diphtheria bacillus in three hours, the staphylococcus pyogenes in six hours, and anthrax spores in thirteen hours. In the case of organisms which have become dry it is probable, however, that much longer exposures are necessary, but on this point we have not definite information.

Formalin gas has only a limited application; it has little effect on dry organisms, and in the case of wet organisms, in order to be effective, probably must become dissolved so as to give the moisture a proportion analogous to the strengths stated above with regard to the vapour.

*Sulphurous Acid.*—This substance has long been in use, largely from the cheapness with which it can be produced by burning sulphur in the air. An atmosphere containing .98 per cent. will kill the pyogenic cocci in two minutes if they are wet, and in twenty minutes if they are dry; and anthrax bacilli are killed by thirty minutes' exposure, but to kill anthrax spores an exposure of from one to two hours to an atmosphere containing 11 per cent. is necessary. For a small room the burning of about a pound and a half (most easily accomplished by moistening the sulphur with methylated spirit) is usually considered sufficient. It has been found that if bacteria are protected, *e.g.* when they are in the middle of small bundles of clothes, no effect is produced even by an atmosphere containing a large proportion



of the sulphurous acid gas. The practical applications of this agent are therefore limited.

*Potassium Permanganate.*—The action of this agent very much depends on whether it can obtain free access to the bacteria to be killed or whether these are present in a solution containing much organic matter. In the latter case the oxidation of the organic material throws so much of the salt out of action that there may be little left to attack the organisms. Koch found that to kill anthrax spores a 5 per cent. solution required to act for about a day; for most organisms a similar solution acting for shorter periods has been found sufficient, and in the cases of the pyogenic cocci a 1 per cent. solution will kill in ten minutes. There is little doubt that such weaker solutions are of value in disinfecting the throat on account of their non-irritating properties, and good results in this connection have been obtained in cases of diphtheria. A solution of 1 in 10,000 has been found to kill plague bacilli in five minutes.

*Carbolic Acid.*—Of all the aromatic series this is the most extensively employed antiseptic. All ordinary bacteria in the vegetative condition, and of these the staphylococcus pyogenes is the most resistant, are killed in less than five minutes by a 2-3 per cent. solution in water, so that the 5 per cent. solution usually employed in surgery leaves a margin of safety. But for the killing of such organisms as anthrax spores a very much longer exposure is necessary; thus Koch found it necessary to expose these spores for four days to ensure disinfection. The risk of such spores being present in ordinary surgical procedure may be overlooked, but there might be risk of tetanus spores not being killed, as these will withstand fifteen hours' exposure to a 5 per cent. solution.

In the products of the distillation of coal there occur, besides carbolic acid, many bodies of a similar chemical constitution, and many mixtures of these are in the market—the chief being cyllin, izal, and lysol, all of which are agents of value. Of these lysol is perhaps the most noticeable, as from its nature it acts as a soap, and thus can remove fat and dirt from the hands. A one-third per cent. solution is said to destroy the typhoid and cholera organisms in twenty minutes. A one per cent. solution is sufficient for ordinary surgical procedures.

*Iodoform.*—This is an agent regarding the efficacy of which there has been much dispute. There is little doubt that it owes its efficiency to its capacity for being broken up by bacterial action in such a way as to set free iodine, which acts as a powerful disinfectant. The substance is therefore of value in the



treatment of foul wounds, such as those of the mouth and rectum, where reducing bacteria are abundantly present. It acts more slightly where there are only pyogenic cocci, and it seems to have a specially beneficial effect in tubercular affections. In certain cases its action may apparently be aided by the presence of the products of tissue degeneration.

From the results which have been given it will easily be recognised that the choice of an antiseptic and the precise manner in which it is to be employed depend entirely on the environment of the bacteria which are to be killed. In many cases it will be quite impossible, without original inquiry, to say what course is likely to be attended with most success.



## CHAPTER VI.

### RELATIONS OF BACTERIA TO DISEASE—THE PRODUCTION OF TOXINS BY BACTERIA.

**Introductory.**—It has already been stated that a strict division of micro-organisms into *saprophytes* and true *parasites* cannot be made. No doubt there are organisms, such as the bacillus of leprosy, which as yet have not been cultivated outside the animal body, and others, such as the gonococcus, which are in natural conditions always parasites associated with disease. But these latter can lead a saprophytic existence in specially prepared conditions, and there are many of the disease-producing organisms, such as the organisms of typhoid and cholera, which can flourish readily outside the body, even in ordinary conditions. The conditions of growth are, however, of very great importance in the study of the modes of infection in the various diseases, though they do not form the basis of a scientific division.

A similar statement applies to the terms *pathogenic* and *saprophytic*, and even to the terms *pathogenic* and *non-pathogenic*. By the term pathogenic is meant the power which an organism has of producing morbid changes or effects in the animal body, either under natural conditions or in conditions artificially arranged, as in direct experiment. Now we know of no organisms which will in all circumstances produce disease in all animals, and, on the other hand, many bacteria described as harmless saprophytes will produce pathological changes if introduced in sufficient quantity. When, therefore, we speak of a pathogenic organism, the term is merely a relative one, and indicates that in certain circumstances the organism will produce disease, though in the science of human pathology it is often used for convenience as implying that the organism produces disease in man in *natural* conditions.

**Modifying Conditions.**—In studying the pathogenic effects in any instance, both the micro-organisms and the animal affected



must be considered, and not only the species of each, but also its exact condition at the time of infection. In other words, the resulting disease is the product of the sum-total of the characters of the infecting agent, on the one hand, and of the subject of infection, on the other. We may, therefore, state some of the chief circumstances which modify each of these two factors involved, and, consequently, the diseased condition produced.

1. *The Infecting Agent*.—In the case of a particular species of bacterium its effect will depend chiefly upon (a) its virulence, and (b) the number introduced into the body. To these may be added (c) the path of infection.

The *virulence*, *i.e.* the power of multiplying in the body and producing disease, varies greatly in different conditions, and the methods by which it can be diminished or increased will be afterwards described (*vide* Chapter XXI.). One important point is that when a bacterium has been enabled to invade and multiply in the tissues of an animal, its virulence for that species is often increased. This is well seen in the case of certain bacteria which are normally present on the skin or mucous surfaces. Thus it has been repeatedly proved that the bacillus coli cultivated from a septic peritonitis is much more virulent than that taken from the bowel of the same animal. The virulence may be still more increased by inoculating from one animal to another in series—the method of *passage*. Widely different effects are, of course, produced on the virulence being altered. For example, a streptococcus which produces merely a local inflammation or suppuration, may produce a rapidly fatal septicaemia when its virulence is raised. Virulence also has a relation to the animal employed, as occasionally on being increased for one species of animal it is diminished for another. For example, streptococci, on being inoculated in series through a number of mice, acquire increased virulence for these animals, but become less virulent for rabbits (Knorr). The theoretical consideration of virulence must be reserved for a later chapter (see Immunity).

The *number* of the organisms introduced, *i.e.* the dose of the infecting agent, is another point of importance. The healthy tissues can usually resist a certain number of pathogenic organisms of given virulence, and it is only in a few instances that one or two organisms introduced will produce a fatal disease, *e.g.* the case of anthrax in white mice. The healthy peritoneum of a rabbit can resist and destroy a considerable number of pyogenic micrococci without any serious result, but



if a larger dose be introduced, a fatal peritonitis may follow. Again, a certain quantity of a particular organism injected subcutaneously may produce only a local inflammatory change, but in the case of a larger dose the organisms may gain entrance to the blood stream and produce septicæmia. There is, therefore, for a particular animal, a minimum lethal dose which can be determined by experiment only; a dose, moreover, which is modified by various circumstances difficult to control.

*The path of infection* may alter the result, serious effects often following a direct entrance into the blood stream. Staphylococci injected subcutaneously in a rabbit may produce only a local abscess, whilst on intravenous injection multiple abscesses in certain organs may result and death may follow. Local inflammatory reaction with subsequent destruction of the organisms may be restricted to the site of infection or may occur also in the related lymphatic glands. The latter therefore act as a second barrier of defence, or as a filtering mechanism which aids in protecting against blood infection. This is well illustrated in the case of "poisoned wounds." In some other cases, however, the organisms are very rapidly destroyed in the blood stream, and Klemperer has found that, in the dog, subcutaneous injection of the pneumococcus produces death more readily than intravenous injection.

2. *The Subject of Infection*.—Amongst healthy individuals susceptibility and, in inverse ratio, resistance to a particular microbe may vary according to (a) species, (b) race and individual peculiarities, (c) age. Different species of the lower animals show the widest variation in this respect, some being extremely susceptible, others highly resistant. Then there are diseases, such as leprosy, gonorrhœa, etc., which appear to be peculiar to the human subject and have not yet been transmitted to animals. And further, there are others, such as cholera and typhoid, which do not naturally affect animals, and the typical lesions of which cannot be experimentally reproduced in them, or appear only imperfectly, although pathogenic effects follow inoculation with the organisms. In the case of the human subject, differences in susceptibility to a certain disease are found amongst different races, and also amongst individuals of the same race, as is well seen in the case of tubercle and other diseases. Age also plays an important part, young subjects being more liable to certain diseases, *e.g.* to diphtheria. Further, at different periods of life certain parts of the body are more susceptible, for example, in early life, the bones and joints to tubercular and acute suppurative affections.



In increasing the susceptibility of a given individual, conditions of *local or general diminished vitality* play the most important part. It has been experimentally proved that conditions such as exposure to cold, fatigue, starvation, etc., all diminish the natural resistance to bacterial infection. Rats naturally immune to glanders can be rendered susceptible by being fed with phloridzin, which produces a sort of diabetes, a large amount of sugar being excreted in the urine (Leo). Guinea-pigs may resist subcutaneous injection of a certain dose of the typhoid bacillus, but if at the same time a sterilised culture of the bacillus coli be injected into the peritoneum, they quickly die of a general infection. Also a local susceptibility may be produced by injuring or diminishing the vitality of a part. If, for example, previous to an intravenous injection of staphylococci, the aortic cusps of a rabbit be injured, the organisms may settle there and set up an ulcerative endocarditis; or if a bone be injured, they may produce suppuration at the part, whereas in ordinary circumstances these lesions would not take place.

Such facts, established by experiment (and many others might be given), illustrate the important part which local or general conditions of diminished vitality may play in the production of disease in the human subject. This has long been known by clinical observation. In normal conditions the blood and tissues of the body, with the exception of the skin and certain of the mucous surfaces, are bacterium-free, and if a few organisms gain entrance, they are destroyed. But if the vitality becomes lowered, their entrance becomes easier and the possibility of their multiplying and producing disease greatly increased. In this way the favouring part played by fatigue, cold, etc., in the production of diseases of which the direct cause is a bacterium, may be understood. It is important to keep in view in this connection that many of the inflammation-producing and pyogenic organisms are normally present on the skin and various mucous surfaces. The action of a certain organism may devitalise the tissues to such an extent as to pave the way for the entrance of other bacteria; we may mention the liability of the occurrence of pneumonia, erysipelas, and various suppurative conditions in the course of or following infective fevers. In some cases the specific organism may produce lesions through which the other organisms gain entrance, *e.g.* in typhoid, diphtheria, etc. A notable example of diminished resistance to bacterial infection is seen in the case of diabetes; tuberculosis and infection with pyogenic organisms are prone to occur in



this disease, and are of a severe character. It is not uncommon to find in the bodies of those who have died from chronic wasting disease, collections of micrococci or bacilli in the capillaries of various organs, which have entered in the later hours of life; that is to say, the bacterium-free condition of the blood has been lost in the period of prostration preceding death.

The methods by which the natural resistance may be specifically increased belong to the subject of immunity, and are described in the chapter on that subject.

**Modes of Bacterial Action.**—In the production of disease by micro-organisms there are two main factors involved, namely, (a) the multiplication of the living organisms after they have entered the body, and (b) the production by them of poisons which may act both upon the tissues around and upon the body generally. The former corresponds to *infection*, the latter is of the nature of *intoxication* or poisoning. In different diseases one of these is usually the more prominent feature, but both are always more or less concerned.

1. *Infection and Distribution of the Bacteria in the Body.*—After pathogenic bacteria have invaded the tissues, or in other words, after infection by bacteria has taken place, their further behaviour varies greatly in different cases. In certain cases they may reach and multiply in the blood stream, producing a fatal septicæmia. In the lower animals this multiplication of the organisms in the blood throughout the body may be very extensive (for example, the septicæmia produced by the pneumococcus in rabbits); but in septicæmia in man it very seldom, if ever, occurs to so great a degree, the organisms rarely remain in large numbers in the circulating blood, and their detection in it during life by microscopic examination is rare, and even culture methods may give negative results unless a large amount of blood is used. In such cases, however, the organisms may be found *post mortem* lying in large numbers within the capillaries of various organs, *e.g.* in cases of septicæmia produced by streptococci. In the human subject more frequently one of two things happens. In the first place, the organisms may remain local, producing little reaction around them, as in tetanus, or a well-marked lesion, as in diphtheria, etc. Or in the second place, they may pass by the lymph or blood stream to other parts or organs in which they settle, multiply, and produce lesions, as in tubercle.

2. *Production of Chemical Poisons.*—In all these cases the growth of the organisms is accompanied by the formation of



*chemical products*, which act generally or locally in varying degree as toxic substances. The toxic substances become diffused throughout the system, and their effects are manifested chiefly by symptoms such as the occurrence of fever, disturbances of the circulatory, respiratory, and nervous systems, etc. In some cases corresponding changes in the tissues are found, for example, the changes in the nervous system in diphtheria, to be afterwards described. The general toxic effects may be so slight as to be of no importance, as in the case of a local suppuration; or they may be very intense, as in tetanus; or again, less severe but producing cachexia by their long continuance, as in tuberculosis.

The occurrence of *local tissue changes or lesions* produced in the neighbourhood of the bacteria, as already mentioned, is one of the most striking results of bacterial action, but these also must be traced to chemical substances formed in or around the bacteria, and either directly or through the medium of ferments. In this case it is more difficult to demonstrate the mode of action, for in the tissues the chemical products are formed by the bacteria slowly, continuously, and in a certain degree of concentration, and these conditions cannot be exactly reproduced by experiment. It is also to be noted that more than one poison may be produced by a given bacterium, *e.g.* the tetanus bacillus (p. 424). Further, it is very doubtful whether all the chemical substances formed by a certain bacillus growing in the tissues are also formed by it in cultures outside the body (*vide* p. 191). The separated toxin of diphtheria, like various vegetable and animal toxins, however, possesses a local toxic action of very intense character, evidenced often by extensive necrotic change.

The injection of large quantities of many different pathogenic organisms in the *dead* condition results in the production of a local inflammatory change which may be followed by suppuration, this effect being possibly brought about by certain substances in the bacterial protoplasm common to various species, or at least possessing a common physiological action (Buchner and others). When dead tubercle bacilli, however, are introduced into the blood stream, nodules do result in certain parts which have a resemblance to ordinary tubercles. In this case the bodies of the bacilli evidently contain a highly resistant and slowly acting substance which gradually diffuses around and produces effects (*vide* Tuberculosis).

*Summary.*—We may say, then, that the action of bacteria as disease-producers, as in fact their power to exist and multiply in the living body, depends upon the chemical products formed



directly or indirectly by them. This action is shown by *tissue changes* produced in the vicinity of the bacteria or throughout the system, and by *toxic symptoms* of great variety of degree and character.

We shall first consider the effects of bacteria on the body generally, and afterwards the nature of the chemical products.

### EFFECTS OF BACTERIAL ACTION.

These may be for convenience arranged in a tabular form as follows :—

#### A. *Tissue Changes.*

- (1) Local changes, *i.e.* changes produced in the neighbourhood of the bacteria.

Position (a) At primary lesion.

(b) At secondary foci.

Character (a) Tissue reactions } Acute or  
(b) Degeneration and necrosis } chronic.

- (2) Produced at a distance from the bacteria, directly or indirectly, by the absorption of toxins.

(a) In special tissues—

(α) as the result of damage, *e.g.* nerve cells and fibres, secreting cells, vessel walls, or

(β) changes of a reactive nature in the blood-forming organs.

(b) General anatomical changes, the effects of malnutrition or of increased waste.

#### B. *Symptoms and Changes in Metabolism.*

The occurrence of fever, of errors of assimilation and elimination, etc.

**A. Tissue Changes produced by Bacteria.**—The effects of bacterial action are so various as to include almost all known pathological changes. However varied in character, they may be classified under two main headings: (a) those of a degenerative or necrotic nature, the direct result of damage; and (b) those of reactive nature, defensive or reparative. The former are the expression of the necessary vulnerability of the tissues, the latter of protective powers evolved for the benefit of the organism. In the means of defence both leucocytes and the fixed cells of the



tissues are concerned. Both show phagocytic properties, *i.e.* have the power of taking up bacteria into their protoplasm. The cells are guided towards the focus of infection by chemiotaxis, and thus we find that different bacteria attract different cells. The most rapid and abundant supply of phagocytes is seen in the case of suppurative conditions where the neutrophile leucocytes of the blood are chiefly concerned. When the local lesion is of some extent there is usually an increase of these cells in the blood—a neutrophile leucocytosis. And further, observation has shown that associated with this there is in the bone-marrow an increased number of the mother-cells of these leucocytes—the neutrophile myelocytes. The passage of the neutrophile leucocytes from the marrow into the blood, with the resulting leucocytosis, is also apparently due to the absorbed bacterial toxins acting chemiotactically on the marrow. These facts abundantly show that the means of defence is not a mere local mechanism, but that increased proliferative activity in distant tissues is called into play. In addition to direct phagocytosis by these leucocytes, there is now abundant evidence that an important function is the production in the body of bactericidal and other antagonistic substances. In other cases the cells chiefly involved are the mononuclear hyaline leucocytes, and with them the endothelial cells, *e.g.* of serous membranes, often play an important part in the defence; this is well seen in typhoid fever, where the specific bacillus appears to have little or no action on the neutrophile leucocytes. In other cases, again, the reaction is chiefly on the part of the connective cells, though their proliferation is always associated with some variety of leucocytic infiltration and usually also with the formation of new blood vessels. Such a connective tissue reaction occurs especially in slow infections or in the later stages of an acute infection. The tissue changes resulting from cellular activity in the presence of bacterial invasion are naturally very varied,—examples of this will be found in subsequent chapters,—but they may be said to be manifestations of the two fundamental processes of (*a*) increased functional activity—movement, phagocytosis, secretion, etc.—and (*b*) increased formative activity—cell growth and division. The exudation from the blood vessels has been variously interpreted. There is no doubt that the exudate has bactericidal properties and also acts as a diluting agent, but it must still be held as uncertain whether the process of exudation ought to be regarded as primarily defensive or as the direct result of damage to the endothelium of the vessels. It may also be pointed out that the various changes referred to



are none of them peculiar to bacterial invasion ; they are examples of the general laws of tissue change under abnormal conditions, and they can all be reproduced by chemical substances in solution or in a particulate state. What constitutes their special feature is their progressive or spreading nature, due to the bacterial multiplication.

(1) *Local Lesions*.—In some diseases the lesion has a special site ; for example, the lesion of typhoid fever and, to a less extent, that of diphtheria. In other cases it depends entirely upon the point of entrance, *e.g.* malignant pustule and the conditions known as wound infections. In others again, there is a special tendency for certain parts to be affected, as the upper parts of the lungs in tubercle. In some cases the site has a mechanical explanation.

When organisms gain an entrance to the blood from a primary lesion, the organs specially liable to be affected vary greatly in different diseases. Pyogenic cocci show a special tendency to settle in the capillaries of the kidneys and produce miliary abscesses, whilst these lesions rarely occur in the spleen. On the other hand, the nodules in disseminated tubercle or glanders are much more numerous in the spleen than in the kidneys, which in the latter disease are usually free from them. The important point is that the position of the disseminated lesions is not to be explained by a mechanical process, such as embolism, but depends upon a special relation between the organisms and the tissues, which may be spoken of either as a selective power on the part of the organisms or a special susceptibility of tissues, possibly in part due to their affording to the organisms more suitable conditions of nutriment. Even in the case of the lesions produced by dead tubercle bacilli, a certain selective character is observed.

*Acute Local Lesions*.—The local inflammatory reaction presents different characters in different conditions. It may be accompanied by abundant fibrinous exudation, or by great catarrh (in the case of an epithelial surface), or by hæmorrhage, or by œdema ; it may be localised or spreading in character ; it may be followed by suppuration, and may lead up to necrosis of the tissues of the part, a good example of the latter event being found in a boil. Examples will be given in subsequent chapters. The necrotic or degenerative changes affecting especially the more highly developed elements of tissues are chiefly produced by the direct action of the bacterial poisons, though aided by the disturbances of nutrition involved in the vascular phenomena. It may here be pointed out that a well-



marked inflammatory reaction is often found in animals which occupy a medium position in the scale of susceptibility, and that an organism which causes a general infection in a certain animal may produce only a local inflammation when its virulence is lessened.

*Chronic Local Lesions.*—In a considerable number of diseases produced by bacteria the local tissue reaction is a more chronic process than those described; there is less vascular disturbance and a greater preponderance of the proliferative processes, leading to new formation of connective tissue. This formation may occur in foci here and there, so that nodules result, or it may be more diffuse. Such changes especially occur in the diseases often known as the *infective granulomata*, of which tubercle, leprosy, glanders, actinomycosis, syphilis, etc., are examples. A hard-and-fast line, however, cannot be drawn between such conditions and those described above as acute. In glanders, for example, especially in the human subject, the lesion often approaches very nearly to an acute suppurative change, and sometimes actually is of this nature. Whilst in these diseases the fundamental change is the same—namely, a reaction to an irritant of minor intensity—the exact structural characters and arrangement vary in different diseases. In some cases the disease may be identified by the histological changes alone, but on the other hand, this is often impossible.

(2) *General Lesions produced by Toxins.*—In the various infective conditions produced by bacteria, changes commonly occur in certain organs unassociated with the presence of the bacteria; these are produced by the action of bacterial products circulating in the blood. Many such lesions can be produced experimentally. The secreting cells of various organs, especially the kidney and liver, are specially liable to change of this kind. Cloudy swelling, which may be followed by fatty change or by actual necrosis with granular disintegration, is common. Hyaline change in the walls of arterioles may occur, and in certain chronic conditions amyloid change is brought about in a similar manner. The latter has been produced in animals by repeated injections of the staphylococcus aureus. Capillary hæmorrhages are not uncommon, and are in many cases due to an increased permeability of the vessel walls, aided by changes in the blood plasma, as evidenced sometimes by diminished coagulability. Similar hæmorrhages may follow the injection of some bacterial toxins, *e.g.* of diphtheria, and also of vegetable poisons, *e.g.* ricin and abrin. Skin eruptions occurring in the exanthemata are probably produced in the same way, though in



many of these diseases the causal organism has not yet been isolated. We have, however, the important fact that corresponding skin eruptions may be produced by poisoning with certain drugs. In the nervous system degenerative changes have been found in diphtheria, both in the spinal cord and in the peripheral nerves, and have been reproduced experimentally by the products of the diphtheria bacilli. There is also experimental evidence that the bacillus coli communis and the streptococcus pyogenes may, by means of their products, produce areas of softening in the spinal cord, and this may furnish an explanation of some of the lesions found clinically. It is also possible that some serous inflammations may be produced in the same way.

**B. Disturbances of Metabolism, etc.**—It will easily be realised that such profound tissue changes as have been detailed cannot occur without great interference with the normal bodily metabolism. General malnutrition and cachexia are of common occurrence, and it is a striking fact found by experiment that after injection of bacterial products, *e.g.* of the diphtheria bacillus, a marked loss of body weight often occurs which may be progressive, leading to the death of the animal. In bacterial disease assimilation is often imperfect, for the digestive glands are affected, it may be, by actual poisoning by bacterial products, it may be by the occurrence of fever, and excretion is interfered with by the damage done to the excretory cells. But of all the changes in metabolism the most difficult to understand is the occurrence of that interference with the heat-regulating mechanism which results in fever. The degree and course of the latter vary, sometimes conforming to a more or less definite type, where the bacilli are selective in their field of operation, as in croupous pneumonia or typhoid, sometimes being of a very irregular kind, especially when the bacteria from time to time invade fresh areas of the body, as in pyæmic affections. The main point of interest regarding the development of fever is as to whether it is a direct effect of the circulation of bacterial toxins, or if it is to be looked on as part of the reaction of the body against the irritant. This question has still to be settled, and all that we can do is to adduce certain facts bearing on it. Thus in diphtheria and tetanus, where toxic action leading to degeneration plays such an important part, fever may be a very subsidiary feature, except in the terminal stage of the latter disease; and in fact in diphtheria profoundly toxic effects may be produced with little or no interference with heat regulation. On the other hand, in bacterial disease, where defensive and reparative



processes predominate, fever is rarely absent, and it is nearly always present when there is an active leucocytosis going on. In this connection it may be remarked that several observers have found that, when a relatively small amount of the dead bodies of certain bacteria are injected into an animal, fever occurs; while the injection of a large amount of the same is followed by subnormal temperatures and rapidly fatal collapse. It might appear as if this indicated that the occurrence of fever had a beneficial effect, but this is one of the points at issue. Certainly such an effect is not due to the bacteria being unable to multiply at the higher degrees of temperature occurring in fever, for this has been shown not to be the case. Whether the increase of bodily temperature indicates the occurrence of changes resulting in the production of bactericidal bodies, etc., is very doubtful; a production of antagonistic substances may be effected without the occurrence of fever or of any apparent disturbance of health. If we consider the site of the heat production in fever we again are in difficulties. It might appear as if the tissue destruction, indicated by the occurrence of fatty degeneration, would lead to heat development, but frequently excessive heat production with increased proteid metabolism occurs without any discoverable changes in the tissues; and further, in phosphorus poisoning there is little fever with great tissue destruction. The increased work performed by the heart in most bacterial infections no doubt contributes to the rise of bodily temperature. But we must bear in mind that in fever there is more than mere increase of heat production—there is also a diminished loss of heat from interference with the nervous mechanism of the sweat apparatus. The known facts would indicate that in fever there is a factor involving the nervous system to be taken into account. The whole subject is thus very obscure.

*Symptoms.*—Many of the symptoms occurring in bacterial infections are produced by the histological changes mentioned, as can be readily understood; whilst in the case of others, corresponding changes have not yet been discovered. Of the latter those associated with fever, with its disturbances of metabolism and manifold affections of the various systems, are the most important. The nervous system is especially liable to be affected—convulsions, spasms, coma, paralysis, etc., being common. The symptoms due to disturbance or abolition of the functions of secretory glands also constitute an important group, forming, as they do, a striking analogy to what is found in the action of various drugs.



These tissue changes and symptoms are given only as illustrative examples, and the list might easily be greatly amplified. The important fact, however, is that *nearly all, if not quite all, the changes found throughout the organs (without the actual presence of bacteria), and also the symptoms occurring in infective diseases, can either be experimentally reproduced by the injection of bacterial poisons or have an analogy in the action of drugs.*

#### THE TOXINS PRODUCED BY BACTERIA.

**Early Work on Toxins.**—We know that bacteria are capable of giving rise to poisonous bodies within the animal body and also in artificial media. We know, however, comparatively little of the actual nature of such bodies, and therefore we apply to them as a class the general term *toxins*. The necessity for accounting for the general pathogenic effects of certain bacteria, which in the corresponding diseases were not distributed throughout the body, directed attention to the probable existence of such toxins; and the first to systematically study the production of such poisonous bodies was Brieger. This observer isolated from putrefying substances, and also from bacterial cultures, nitrogen-containing bodies, which he called *ptomaines*. Similar bodies occurring in the ordinary metabolic processes of the body had previously been described and called *leucomaines*. Ptomaines isolated from pathogenic bacteria in no case reproduced the symptoms of the disease, except perhaps in tetanus, and this only owing to their impurity. The methods by which they were isolated were faulty, and they have therefore only a historic interest.

The introduction of the principle of rendering fluid cultures bacteria-free by filtration through unglazed porcelain, and its application by Roux and Yersin to obtain, in the case of the *b. diphtheriæ*, a solution containing a toxin which reproduced the symptoms of this disease (*vide* Chapter XVI.),—encouraged the further inquiry as to the nature of this toxin. An attempt on the part of Brieger and Fraenkel to obtain a purified diphtheria toxin by precipitating bouillon cultures by alcohol (the product being denominated a toxalbumin) did not greatly advance knowledge on the subject, and further investigation soon showed that specific toxins can be isolated from but few bacteria.

**General Facts regarding Bacterial Toxins.**—The following may be regarded as the chief facts regarding bacterial toxins which have been revealed by the study, partly of the bodily tissues of animals infected by the bacteria concerned, partly of



artificial cultures of these bacteria. In dealing with these it is necessary to distinguish between the effects produced by the actual constituents of the bacterial protoplasm (intracellular toxins) and those which in a few bacteria are traceable to soluble substances passing out into the media in which these bacteria may be growing (extracellular toxins). The former are concerned in the action of by far the greater number of pathogenic bacteria; the latter account for the pathogenic processes originated in a limited number of cases of which diphtheria and tetanus are the most important. This distinction is important as, in consequence of these last two diseases having had much attention directed towards them early in the history of research on the subject, there has hitherto been too much tendency to take for granted that poisons of a similar constitution are concerned in all cases of bacterial intoxication. At present such an assumption is not justified by facts, and we do not even know whether the intracellular and extracellular toxins belong to the same group of chemical bodies. At present, however, the terms are used as a convenient means of accentuating a difference in solubility between the two groups of toxic bodies.

The dead bodies of certain bacteria have been found to be very toxic. When, for instance, tubercle bacilli are killed by heat and injected into the body tissues of a susceptible animal, tubercular nodules are found to develop round the sites where they have lodged. From this it is inferred that they must have contained characteristic toxins, seeing that characteristic lesions result. The bodies of such organisms as the pyogenic cocci, the *b. typhosus*, and the *v. cholerae* likewise give rise to pathogenic effects. Such *intracellular* toxins may appear in the fluids in which the bacteria are living (1) by excretion in an unaltered or altered condition, (2) by the disintegration of the bodies of the organisms which we know are always dying in any bacterial growth. The death of bacteria occurs also in the body of an infected animal, and the disintegration of these dead bacteria constitutes an important means by which the poisons they contain are absorbed. There is some evidence that often bacteria originate during growth poisons which are hurtful to their own vitality, and also that ferments are produced by them which have a solvent effect on the poisoned members of the colony. Such a process of *autolysis*, as it has been called, may have an important effect in liberating intracellular toxins. It is impossible, at present, to obtain intracellular toxins apart from other derivatives of the bacterial protoplasm, and thus



our chief knowledge concerning their effects is derived from the study of what happens when the bodies of bacteria killed by chloroform vapour or by heat are injected into animals. When effects are produced by such injections they do not present in any particular case specific characters. They are of the nature of general disturbances of metabolism, as manifested by fever, or by depression of temperature, loss of weight, etc., often of such serious degree as to result in death. It is important to note that when pathogenic effects are produced these usually appear very soon, it may be in a few hours after injection of the toxic material; there is not the definite period of incubation which with other toxins often elapses before symptoms appear.

In certain cases there is difficulty in understanding the action of bacteria which neither form soluble toxins in a fluid medium nor possess a highly toxic protoplasm, and yet with which we often see effects produced at a distance from the focus of infection, *e.g.* b. anthracis. To explain such occurrences it has long been regarded as a possibility that some bacteria are only capable of producing toxins within the animal tissues, and it has further been thought possible that bacteria, such as, for example, the typhoid bacillus, which do distribute into media intracellular toxins, might either produce these toxins more readily in the tissues or might produce in addition other toxins of a different nature. During recent years such toxins have been much studied, and the name *aggressins* has been given to them. The evidence adduced for the existence of these aggressins as a separate group of bacterial poisons is of the following kind: An animal is killed by a dose of the typhoid, dysentery, cholera, or tubercle bacillus, or by a staphylococcus, the organism being introduced into one of the serous cavities. After death the serous exudation, which in all these cases is present, is taken, and centrifugalised to remove the bacteria so far as this can be done by such a procedure; the bacteria which are left are killed by shaking the fluid up with toluol and leaving it to stand for some days. It is stated that such a fluid is of itself without pathogenic effect, but has the property of transforming a non-lethal dose of the bacterium used into one having fatal effect. Further, the effects of the combined actions of the bacteria and aggressins are often of a much more acute character than can be obtained with toxic products developed *in vitro*. Thus, in the case of the action of a non-lethal dose of the tubercle bacillus plus its aggressin, it is said that death may occur in twenty hours, a result never obtained with artificial cultures of the organism. The effects



produced by aggressins are attributed to a paralysing action on the phagocytic functions of the leucocytes. The subject is full of difficulties, and in the case of certain of the organisms employed it is stated that results similar to those attributed to aggressin action have been observed with macerated cultures,—the deduction being that in the aggressins we are merely dealing with concentrated intracellular toxins. On the other hand, as evidence of the existence of a special group of toxins, it has been stated that a special type of immunity against the aggressins can be originated. Perhaps the most important aspect of the controversy is the recognition of the existence of toxins having an action on the leucocytes. A poison causing death of these cells in connection with the pus-forming action of the pyogenic cocci has been described under the name of leucocidin, and Eisenberg records that in *in vitro* mixtures of leucocytes and cultures of the bacillus of symptomatic anthrax loss of motility and degeneration of the cells may be observed. The investigation of such poisons must be of the highest importance in view of the part played by the blood-cells in the protection of the body against infection, and it is possible that toxins having a fatal effect in strong concentrations may, when dilute, be responsible for the phenomena of attraction or repulsion of leucocytes which we know occur round a focus of bacterial growth in the body.

Sometimes the media in which bacteria are growing become extremely toxic. This is more marked in some cases than in others. The two best examples of bacteria thus producing soluble toxins are the diphtheria and tetanus bacilli. In these and similar cases when bouillon cultures are filtered bacterium-free by means of a porcelain filter, toxic fluids are obtained, which on injection into animals reproduce the highly characteristic symptoms of the corresponding diseases. In the case of the b. anthracis and of many others, at any rate when growing in artificial media, such toxin production is much less marked, a filtered bouillon culture being relatively non-toxic. Poisons appearing in culture media have been called *extracellular* toxins, but we cannot as yet say whether they are excreted by the bacteria or whether they are produced by the bacteria acting on the constituents of the media. The extracellular toxins are easily obtainable in large quantities, and it is their nature and effects which are best known. No method has been discovered of obtaining them in a pure form, and our knowledge of their properties is exclusively derived from the study of the toxic filtrates of bouillon cultures—these filtrates being usually re-



ferred to simply as the toxins. These toxins differ in their effects from the intracellular poisons in that specific actions on certain tissues are often manifested. Thus the toxins of the diphtheria, the tetanus, and the botulismus bacilli all act on the nervous system; with some of the pyogenic bacteria, on the other hand, poisons, probably of similar nature, produce solution of red blood corpuscles (this last might be thought to explain the anæmias so common in the associated diseases, but it is to be noted that, in cultures at least, these hæmolytic toxins are developed in very small amounts). In the action of many of these toxins the occurrence of a period of incubation between the introduction of the poison into the animal tissues and the appearance of symptoms is often a feature.

The whole question of the parts played by toxins in bacterial action is manifestly very complex. On the one hand, we have a few processes, for example, diphtheria and tetanus, in which very characteristic effects are produced on special tissues, these being accounted for by the formation of soluble toxins which are capable of being separated from the bacterial growths *in vitro*. On the other hand, we have the great mass of bacterial infections. With regard to these, the distribution of the bacteria in the bodies of infected animals makes it necessary for us to take for granted that a toxic action is at work. All that we know, however, regarding a possible explanation of such toxicity is that the bodies of the bacteria or substances directly derived from them are capable of producing pathogenic effects. These effects are of a non-specific character in the sense that they are not the result of an action on any particular tissue in the body, but on the vital processes of the organism as a whole. We are at present entirely ignorant of the interpretation to be put, for instance, on the lowering of bodily temperature on the one hand and of the occurrence of fever on the other, both of which may be produced by the injection of the so-called intracellular toxins in varying doses, and we are ignorant of the relations which either event may have to the bringing into play of the defensive mechanisms of the body. At the same time we must admit the possibility that with any one species of organism different effects may be produced by, it may be, different elements in the protoplasm of the invading bacterial cell. Some of these elements may act on certain groups of specialised cells of the body, such as those of the nervous system, liver, or kidneys, giving rise to what we are forced to describe in general terms as disturbances of metabolism. Other poisonous elements may mainly act on the defensive cells of the body, of which the



leucocytes may be taken as the type. Here a small dose of toxin may stimulate these cells to an activity which results in the infection being thrown off, either by the poison being neutralised, or by the supply of toxin being cut off by the killing of the bacterium producing it. A large dose of such a toxin, may, on the other hand, altogether break down the defensive mechanism of the invaded body. A possible complexity in toxic action may occur even in such an apparently simple case as diphtheria. As will be seen later, the special neuro-toxin excreted by the diphtheria bacillus can be neutralised by an antitoxic substance, but the action of this does not necessarily cause the death of the bacteria in the throat whose capacity for multiplication may be dependent on a vital activity of the protoplasm distinct from neurotoxin production, and therefore requiring another mechanism for its neutralisation. The complexity of the toxic process is also illustrated by the facts known regarding the cholera vibrio. In man, this organism is confined in its habitat to the intestinal tract, and its serious effects are attributed to the absorption of toxins therefrom. On the other hand, in animals, not susceptible to such intestinal infection, death can be readily produced by the injection intraperitoneally of a comparatively small amount of dead cholera vibrios, and it will be seen in the chapter on Cholera that the possibility has to be faced of the toxins acting in the two conditions being different. Thus it is possible that the toxic element in an organism which enables it to effect its initial multiplication in or on the tissues is not necessarily bound up with the toxicity which is responsible for the manifestation of specific disease effects. This is borne out by the work of Grassberger and Schattenfroh on the bacillus of symptomatic anthrax. In this case an organism, which *in vitro* has lost to a large extent its capacity of producing soluble toxins, may show great capacity for multiplying when introduced into a susceptible animal.

There is another point which must be kept in view, namely, that some of the phenomena which have been regarded as dependent upon the activity of bacterial toxins may possibly be related to the little-understood process of anaphylaxis (see Immunity). Anaphylaxis essentially consists in the development under certain circumstances in an animal of a hypersensitiveness to foreign albuminous materials which in themselves are not essentially toxic. Effects of the gravest kind may be produced during this period of hypersensitiveness, and it has been thought that some of the phenomena of an infectious disease, *e.g.* the occurrence of an incubation period, may be



accounted for by the development of hypersensitiveness to the protoplasm of the invading bacteria. It may be said here that the effect seen when horse serum is injected into a rabbit during its hypersensitive stage to this substance bears a striking resemblance to what is seen in natural infection in man by the cholera vibrio.

The phenomena of any bacterial disease may thus in reality be due to very different and complex causes.

**The Nature of Toxins.**—There is still comparatively little known regarding this subject, and it chiefly relates to the extra-cellular toxins. The earlier investigations upon toxins suggested that analogies exist between the modes of bacterial action and what takes place in ordinary gastric digestion, and the idea was worked out for anthrax, diphtheria, tetanus, and ulcerative endocarditis by Sidney Martin. This observer found that albumoses<sup>1</sup> and peptones were formed by the action of the pathogenic bacteria studied, and further, that the precipitate containing these albumoses was toxic. A similar digestive action has been traced in the case of the tubercle bacillus by Kühne.

Further evidence that bacterial toxins are either albumoses or bodies having a still smaller molecule is adduced by C. J. Martin. This worker, by filling the pores of a Chamberland bougie with gelatin, has obtained what is practically a strongly supported colloid membrane through which dialysis can be made to take place under great pressure, say, of compressed oxygen. He finds that in such an apparatus toxins—at least two kinds tried—will pass through just as an albumose will.

Brieger and Boer, working with bouillon cultures of diphtheria and tetanus, separated, by precipitation with zinc chloride,

<sup>1</sup> In the digestion of albumins by the gastric and pancreatic juices, the albumoses are a group of bodies formed preliminarily to the production of peptone. Like the latter they differ from the albumins in their not being coagulated by heat, and in being slightly dialysable. They differ from the peptones in being precipitated by dilute acetic acid in presence of much sodium chloride, and also by neutral saturated sulphate of ammonia. Both are precipitated by alcohol. The first albumoses formed in digestion are proto-albumose and hetero-albumose, which differ in the insolubility of the latter in hot and cold water (insolubility and coagulability are quite different properties). They have been called the primary albumoses. By further digestion both pass into the secondary albumose, deuterio-albumose, which differs slightly in chemical reactions from the parent bodies, *e.g.* it cannot be precipitated from watery solutions by saturated sodium chloride unless a trace of acetic acid be present. Dysalbumose is probably merely a temporary modification of hetero-albumose. Further digestion of deuterio-albumose results in the formation of peptone.



bodies which show characteristic toxic properties, but which had the reactions neither of peptone, albumose, nor albuminate, and the nature of which is unknown. It has also been found that the bacteria of tubercle, tetanus, diphtheria, and cholera can produce toxins when growing in proteid-free fluids. In the case of diphtheria, when the toxin is produced in such a fluid a proteid reaction appears. Of course this need not necessarily be caused by the toxin. Further investigation is here required, for Uschinsky, applying Brieger and Boer's method to a toxin so produced, states that the toxic body is not precipitated by zinc salts, but remains free in the medium. If the toxins are really non-proteid they may, on the one hand, be the final product of a digestive action, or they may be the manifestation of a separate vital activity on the part of the bacteria. On the latter theory the toxicity of the toxic albumoses of Sidney Martin may be due to the precipitation of the true toxins along with these other bodies. From the chemical standpoint this is quite possible. When we take into account the extraordinary potency of these poisons (in the case of tetanus the fatal dose of the pure poison for a guinea-pig must often be less than '000001 gm.), we can understand how attempts by present chemical methods to isolate them in a pure condition are not likely to be successful, and of their real nature we know nothing. Friedberger and Moreschi have shown that the intravenous injection in the human subject of a fraction of a loopful of a dead typhoid culture gives rise to toxic symptoms, including marked febrile reaction. Such injections are followed by the appearance of agglutinating and bacteriolytic substances in the serum. These results show that intracellular toxins may be comparable with extracellular toxins so far as concerns the extremely small dose sufficient to produce toxic effects.

Amongst the properties of the extracellular toxins are the following: They are apparently all uncrystallisable; they are soluble in water and they are dialysable; they are precipitated along with proteids by concentrated alcohol, and also by ammonium sulphate; if they are proteids they are either albumoses or allied to the albumoses; they are often relatively unstable, having their toxicity diminished or destroyed by heat (the degree of heat which is destructive varies much in different cases), light, and by certain chemical agents. Their potency is often altered in the precipitations practised to obtain them in a pure or concentrated condition, but among the precipitants ammonium sulphate has little if any harmful effect. Regarding the toxins which are more intimately associated with the bacterial proto-



plasm we know much less, but it is probable that, chemically, their nature is similar, though some of them at least are not so easily injured by heat, *e.g.* those of the tubercle bacillus, already mentioned. In the case of all toxins the fatal dose for an animal varies with the species, body weight, age, and previous conditions as to food, temperature, etc. In estimating the minimal lethal dose of a toxin these factors must be carefully considered.

The following is the best method of obtaining concentrated extracellular toxins: The toxic fluid is placed in a shallow dish, and ammonium sulphate crystals are well stirred in till no more dissolve. Fresh crystals to form a bulk nearly equal to that of the whole fluid are added, and the dish set in an incubator at 37° C. over night. Next day a brown scum of precipitate will be found floating on the surface. This contains the toxin. It is skimmed off with a spoon, placed in watch-glasses; these are dried *in vacuo* and stored in the dark, also *in vacuo*, or in an exsiccator containing strong sulphuric acid. For use the contents of one are dissolved up in a little normal saline solution.

The comparison of the action of bacteria in the tissues in the production of these toxins to what takes place in the gastric digestion, has raised the question of the possibility of the elaboration by these bacteria of *ferments* by which the process may be started. Thus Sidney Martin puts forward the view that ferments may be produced which we may look on as the primary toxic agents, and which act by digesting surrounding material and producing albumoses—these poisons being, as it were, secondary poisons. Hitherto all attempts at the isolation of bacterial ferments of such a nature have failed.

But apart from the fact that with such bacteria as those of tetanus and diphtheria, a digestive action may occur, analogies have been drawn between ferment and toxic action. The chief facts upon which such analogies have been founded are as follows: Thus the toxic products of these and other bacteria lose their toxicity by exposure to a temperature which puts an end to the activity of such an undoubted ferment as that of the gastric juice. If a bouillon containing diphtheria toxin be heated at 65° C. for one hour, it is found to have lost much of its toxic effect, and in the case of *b. tetani* all the toxicity is lost by exposure at this temperature. In both diseases there is a still further fact which is adduced in favour of the toxic substances being of the nature of ferments, namely, the existence of a definite period of incubation between the injection of the toxic bodies and the appearance of symptoms. This may be interpreted as showing that after the introduction of, say, a filtered



bouillon culture, further chemical substances are formed in the body before the actual toxic effect is produced. Too much reliance must not be placed on such an argument, for in the case of tetanus, at least, the delay may be explained by the fact that the poison apparently has to travel up the nerve trunks before the real poisonous action is developed. Further, with some poisons presently to be mentioned which are closely allied to the bacterial toxins, an incubation period may not exist. It would not be prudent to dogmatise as to whether the toxins do or do not belong to such an ill-defined group of substances as the ferments. It may be pointed out, however, that the essential concept of a ferment is that of a body which can originate change without itself being changed, and no evidence has been adduced that toxins fulfil this condition. Another property of ferments is that so long as the products of fermentation are removed, the action of a given amount of ferment is indefinite. Again, in the case of toxins no evidence of such an occurrence has been found. A certain amount of a toxin is always associated with a given amount of disease effect, though a process of elimination of waste products must be all the time going on in the animal's body. Again, too much importance must not be attached to loss of toxicity by toxins at relatively low temperatures. This is not true of all toxins, and furthermore many proteids show a tendency to change at such temperatures; for instance, if egg albumin be kept long enough at 55° C. nearly the whole of it will be coagulated. We must therefore maintain an open mind on this subject.

**Similar Vegetable and Animal Poisons.**—It has been found that the bacterial poisons belong to a group of toxic bodies all presenting very similar properties, other members of which occur widely in the vegetable and animal kingdoms. Among plants the best-known examples are the ricin and abrin poisons obtained by making watery emulsions of the seeds of the *Ricinus communis* and the *Abrus precatorius* (jequirity) respectively. From the *Robinia pseudacacia* another poison—robin—belonging to the same group is obtained. The chemical reactions of ricin and abrin correspond to those of the bacterial toxins. They are soluble in water, they are precipitable by alcohol, but being less easily dialysable than the albumoses they have been called toxalbumins. Their toxicity is seriously impaired by boiling, and they also gradually become less toxic on being kept. Both are among the most active poisons known—ricin being the more powerful. When they are injected subcutaneously a period of twenty-four hours usually elapses—whatever be the dose—before symptoms set in. Both tend to produce great inflammation at the seat of inoculation, which in the case of ricin may end in an acute necrosis; in fatal cases hæmorrhagic enteritis and nephritis may be found. Both act as irritants to mucous membranes, abrin especially being capable of setting up most acute conjunctivitis.



In the action of a poisonous fungus, *Amanita phalloides*, a similar toxin is at work. After an incubation period of some hours, symptoms of abdominal pain, diarrhoea with bloody stools, and later jaundice occur. *In vitro* the toxin has a hæmolytic action. Like other poisons of this class, an antitoxin can be produced towards the fungus poison.

It is also certain that the poisons of scorpions and of poisonous snakes belong to the same group. The poisons derived from the latter are usually called venins, and a very representative group of such venins derived from different species has been studied. To speak generally, there is derivable from the natural secretions of the poison glands a series of venins which have all the reactions of the bodies previously considered. Like ricin and abrin, they are not so easily dialysable as bacterial toxins, and therefore have also been classed as toxalbumins. Their properties are also similar; many of them are destroyed by heat, but the degree necessary here also varies much, and some will stand boiling. There is also evidence that in a crude venom there may be several poisons differently sensitive to heat. All the venins are very powerful poisons, but here there is practically no period of incubation—the effects are almost immediate. An outstanding feature of the venins is the complexity of the crude poison secreted by any particular species of snake. C. J. Martin, in summing up the results of many observers, has pointed out that different venoms have been found to contain one or more of the following poisons: a neurotoxin acting on the respiratory centre, a neurotoxin acting on the nerve-endings in muscle, a toxin causing hæmolysis, toxins acting on other cells, *e.g.* the endothelium of blood vessels (this from its effects has been named hæmorrhagin), leucocytes, nerve-cells, a toxin causing thrombosis, a toxin having an opposite effect and preventing coagulation, a toxin neutralising the bactericidal qualities of the body fluids and thus favouring putrefaction, a toxin causing agglutination of the red blood corpuscles, a proteolytic ferment, a toxin causing systolic standstill of the excited heart. Any particular venom contains a mixture in varying proportions of such toxins, and the different effects produced by the bites of different snakes largely depend on this variability of composition. The neurotoxic, the thrombotic, and the hæmolytic toxins are very important constituents of any venom. The toxicity of different venoms varies much, and no general statement can be made with regard to the toxicity of different poisons towards man. Lamb has calculated that the fatal dose of crude cobra venom for man is probably about  $\cdot 015$  of a gramme, and that if such a snake bites with full glands many times this dose would probably be injected, but, of course, the amount emitted depends largely on the period which has elapsed since the animal last emptied its glands. When a dose of a venom not sufficient to cause immediate death from general effects be given, very rapid and widespread necrosis often may occur in a few hours round the site of inoculation.

An extremely important fact was discovered by Flexner and Noguchi, namely, that the hæmolytic toxin of cobra venom in certain cases has no action by itself, but produces rapid solution of red corpuscles when some normal serum is added, the latter containing a labile complement-like body, which activates the venom. In this there is a close analogy to what holds in the case of a hæmolytic serum deprived of complement by heat at  $55^{\circ}$  C. (p. 130). Kyes and Sachs further showed that in addition to serum-complement a substance with definitely known constitution, namely lecithin, had the property of activating the hæmolytic substance in cobra venom, the two apparently uniting to form an actively toxic



substance. So far no example of the activation of a bacterial toxin is known, but the results mentioned point to the possibility of this occurring in some cases in the tissues of the body.

There is another group of toxic manifestations which present some analogies to those of the bacterial toxins, but concerning which very little is known. The best example of these is found in the toxic properties of the serum of the eel. If a small quantity of such serum, say .25 of a c.c., be injected into a rabbit subcutaneously, death occurs in a few minutes. Although nothing is known of the substances giving rise to such effects, the phenomenon is to be considered in relation, on the one hand, to the action of bacterial toxins, and on the other to the phenomenon of anaphylaxis. (See Chapter on Immunity.)

**The Theory of Toxic Action.**—While we know little of the chemical nature of any toxins, we may, from our knowledge of their properties, group together the tetanus and diphtheria poisons, ricin, abrin, snake poisons, and scorpion poisons. Besides the points of agreement already noted, all possess the further property that, as will be afterwards described, when introduced into the bodies of susceptible animals they stimulate the production of substances called antitoxins. The nature of the antagonism between toxin and antitoxin will be discussed later. Here, to explain what follows, it may be stated (1) that the molecule of toxin forms directly a combination with the molecule of antitoxin, and (2) that it has been shown that toxin molecules may lose much of their toxic power and still be capable of uniting with exactly the same proportion of antitoxin molecules. From these and other circumstances Ehrlich has advanced the view that the toxin molecule has a very complicated structure, and contains two atom groups. One of these, the *haptophorous* ( $\alpha\pi\tau\epsilon\upsilon\nu$ , to bind to), is that by which combination takes place with the antitoxin molecule, and also with presumably corresponding molecules naturally existing in the tissues. The other atom group he calls the *toxophorous*, and it is to this that the toxic effects are due. This atom group is bound to the cell elements, *e.g.* the nerve cells in tetanus, by the *haptophorous* group. Ehrlich explains the loss of toxicity which with time occurs in, say, diphtheria toxin, on the theory that the *toxophorous* group undergoes disintegration. And if we suppose that the *haptophorous* group remains unaffected we can then understand how a toxin may have its toxicity diminished and still require the same proportion of antitoxin molecules for its neutralisation. To the bodies whose *toxophorous* atom groups have become degenerated, Ehrlich gives the name *toxoids*. The theory may afford an explanation of what has been suspected, namely, that in some instances toxins derived from



different sources may be related to one another. For example, Ehrlich has pointed out that ricin produces in a susceptible animal body an antitoxin which corresponds almost completely with that produced by another vegetable poison, robin (*vide supra*), though ricin and robin are certainly different. This may be explained on the supposition that robin is a toxoid of ricin, *i.e.* their haptophorous groups correspond, while their toxophorous differ. The evidence on which Ehrlich's deductions are based is of a very weighty character, but another view of toxic action is that the relation between a toxin and the cell on which it acts is an example of the physical phenomenon of adsorption. The whole subject will be again referred to in the chapter on Immunity.

With regard to the intracellular toxins we shall see it is difficult to determine whether or not they share with the extracellular poisons the property of stimulating antitoxin formation,—if they do not, then they may belong to an entirely different class of substances. It is certain that a tolerance against such poisons is difficult to establish and is not of a lasting character. We thus cannot say what the mechanism is by which these poisons act. It may be said that Macfadyen, by grinding up typhoid bacilli frozen by liquid air, claimed that on thawing he obtained the intracellular toxins in liquid form, and he further stated that by using this fluid he could immunise animals not only against the toxins but also against the living bacteria.

We have already pointed out that those who claim for the aggressins a special character hold that the activity of these bodies has as its effect the interference with the phagocytic functions of the leucocytes. They also hold that a special type of immunity can be developed against the aggressins.



## CHAPTER VII.

### INFLAMMATORY AND SUPPURATIVE CONDITIONS.

THIS subject is an exceedingly wide one, and embraces a great many pathological conditions which in their general characters and results are widely different. Thus, in addition to suppuration, various inflammations, ulcerative endocarditis, septicæmia and pyæmia, will come up for consideration. With regard to these, the two following general statements, established by bacteriological research, may be made in introducing the subject. In the first place, there is no one specific organism for any one of these conditions; various organisms may produce them, and not infrequently more than one organism may be present. In the second place, the same organism may produce widely varying results under different circumstances,—at one time a local inflammation or abscess, at another multiple suppurations or a general septicæmia. The principles on which this diversity in results depends have already been explained (p. 177). Furthermore, there are conditions like acute pneumonia, epidemic meningitis, acute rheumatism, etc., which have practically the character of specific diseases, and yet which, as regards their essential pathology, belong to the same class. The arrangement followed is to a certain extent one of convenience.

It may be well to emphasise some of the chief points in the pathology of these conditions. In *suppuration* the two main phenomena are—(a) a progressive immigration of leucocytes, chiefly of the polymorpho-nuclear (neutrophile) variety, and (b) a liquefaction or digestion of the supporting elements of the tissue along with necrosis of the cells of the part. The result is that the tissue affected becomes replaced by the cream-like fluid called pus. A suppurative inflammation is thus to be distinguished on the one hand from an inflammation without destruction of tissue, and on the other from necrosis or death *en masse*, where the tissue is not liquefied, and leucocyte



accumulation may be slight. When, however, suppuration is taking place in a very dense fibrous tissue, liquefaction may be incomplete, and a portion of dead tissue or slough may remain in the centre, as is the case in boils. In the case of suppuration in a serous cavity the two chief factors are the progressive leucocytic accumulation and the disappearance of any fibrin which may be present.

Many experiments have been performed to determine whether suppuration can be produced in the absence of micro-organisms by various chemical substances, such as croton oil, nitrate of silver, turpentine, etc.—care, of course, being taken to ensure the absence of bacteria. The general result obtained by independent observers is that as a rule suppuration does not follow, but that in certain animals and with certain substances it may, the pus being free from bacteria. Buchner showed that suppuration may be produced by the injection of dead bacteria, *e.g.* sterilised cultures of *bacillus pyocyaneus*, etc. The subject has now more a scientific than a practical interest, and the general statement may be made that practically all cases of true suppuration met with clinically are due to the action of living micro-organisms.

The term *septicæmia* is applied to conditions in which the organisms multiply within the blood and give rise to symptoms of general poisoning, without, however, producing abscesses in the organs. The organisms are usually more numerous in the capillaries of internal organs than in the peripheral circulation, but the application of the newer methods of cultivation has shown that they can be detected in the peripheral blood much more frequently than was formerly supposed to be the case. The essential fact in *pyæmia*, on the other hand, is the occurrence of multiple abscesses in internal organs and other parts of the body. In most of the cases of typical pyæmia, common in pre-antiseptic days, the starting-point of the disease was a septic wound with bacterial invasion of a vein leading to thrombosis and secondary embolism. Multiple foci of suppuration may be produced, however, in other ways, as will be described below (p. 213). If the term “pyæmia” be used to embrace all such conditions, their method of production should always be distinguished.

#### BACTERIA AS CAUSES OF INFLAMMATION AND SUPPURATION.

A considerable number of species of bacteria have been found in acute inflammatory and suppurative conditions, and of these



many have been proved to be causally related, whilst of some others the exact action has not yet been fully determined.

Ogston, who was one of the first to study this question (in 1881), found that the organisms most frequently present were micrococci, of which some were arranged irregularly in clusters (staphylococci), whilst others formed chains (streptococci). He found that the former were more common in circumscribed acute abscesses, the latter in spreading suppurative conditions. Rosenbach shortly afterwards (1884), by means of cultures, differentiated several varieties of micrococci, to which he gave the following special names: *staphylococcus pyogenes aureus*, *staphylococcus pyogenes albus*, *streptococcus pyogenes*, *micrococcus pyogenes tenuis*. Other organisms are met with in suppuration, such as *staphylococcus pyogenes citreus*, *staphylococcus cereus albus*, *staphylococcus cereus flavus*, *pneumococcus*, *pneumobacillus*, (Friedländer), *bacillus pyogenes foetidus* (Passet), *bacillus coli communis*, *bacillus lactis aerogenes*, *bacillus aerogenes encapsulatus*, *bacillus pyocyaneus*, *micrococcus tetragenus*, *pneumococcus*, *pneumobacillus*, *diplococcus intracellularis meningitidis*, and others. Various anaerobic bacteria are also concerned in the production of inflammation, which is often associated with œdema, hæmorrhage, or necrosis (*vide* Chap. XVII.).

In secondary inflammations and suppurations following acute diseases, the corresponding organisms have been found in some cases, such as gonococcus, typhoid bacillus, influenza bacillus, etc. Suppuration is also produced by the actinomyces and the glanders bacillus, and sometimes chronic tubercular lesions have a suppurative character.

**Staphylococcus Pyogenes Aureus.**—*Microscopical Characters.*

—This organism is a spherical coccus about  $\cdot 9 \mu$  in diameter, which grows irregularly in clusters or masses (Fig. 50). It stains readily with all the basic aniline dyes, and retains the colour in Gram's method (Plate I., Fig. 1).

*Cultivation.*—It grows readily in all the ordinary media at the room temperature, though much more rapidly at the temperature of the body. In stab cultures in *peptone gelatin* a streak of growth is visible on the day after inoculation, and on the second or third day liquefaction commences at the top. As liquefaction proceeds, the growth falls to the bottom as a flocculent deposit, which soon assumes a bright yellow colour, while a yellowish film may form on the surface, the fluid portion still remaining turbid. Ultimately liquefaction extends out to the wall of the tube (Fig. 51). In *gelatin plates* colonies may be seen with the low power of the microscope in twenty-four



hours, as little balls somewhat granular on the surface and of brownish colour. On the second day they are visible to the naked eye as whitish yellow points, which afterwards become



FIG. 50.—*Staphylococcus pyogenes aureus*, young culture on agar, showing clumps of cocci. Stained with weak carbol-fuchsin.  $\times 1000$ .

more distinctly yellow. Liquefaction occurs around these, and little cups are formed, at the bottom of which the colonies form little yellowish masses. On *agar*, a stroke culture forms a line of abundant yellowish growth, with smooth, shining surface, well formed after twenty-four hours at  $37^{\circ}\text{C}$ . Later it becomes bright orange in colour, and resembles a streak of oil paint. Single colonies on the surface of agar are circular discs of similar appearance, which may reach 2 mm. or more in diameter. On *potatoes* it grows well at ordinary temperature, forming a somewhat abundant layer of orange colour. In *bouillon* it produces a uniform turbidity, which afterwards settles to the bottom as an abundant layer and assumes a brownish yellow tint. In the various media it renders the reaction acid, and it coagulates milk, in which it readily grows. The cultures have a somewhat sour odour. It has considerable tenacity of life

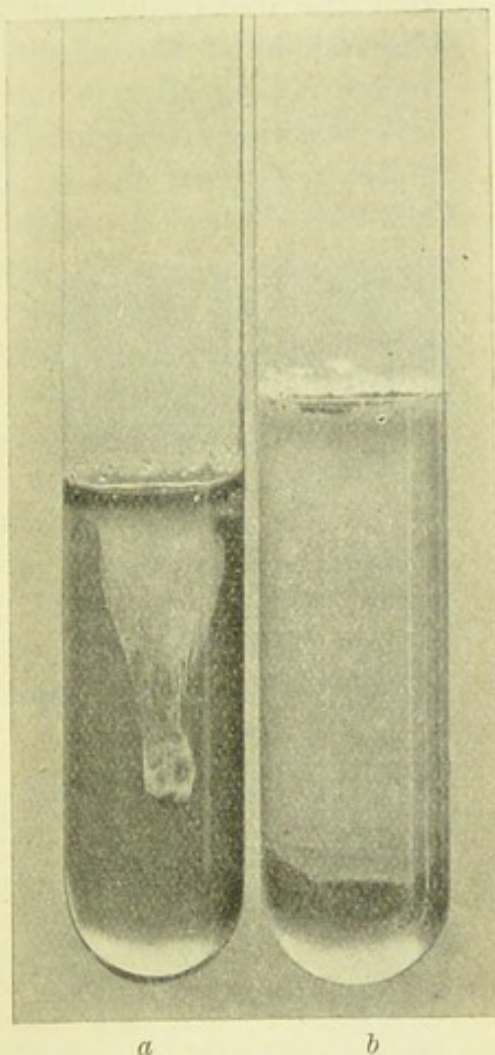


FIG. 51.—Two stab cultures of *staphylococcus pyogenes aureus* in gelatin, (a) 10 days old, (b) 3 weeks old, showing liquefaction of the medium and characters of growth. Natural size.



outside the body, cultures<sup>56</sup> in gelatin often being alive after having been kept for several months.

The *staphylococcus pyogenes albus* is similar in character, with the exception that its growth on all the media is white. The colour of the *staphylococcus aureus* may become less distinctly yellow after being kept for some time in culture, but it never assumes the white colour of the *staphylococcus albus*, and it has not been found possible to transform the one organism into the other. A micrococcus called by Welch *staphylococcus epidermidis albus* is practically always present in the skin epithelium; it is distinguished by its relatively non-pathogenic properties and by liquefying gelatin somewhat slowly. It is probably an attenuated variety of the *staphylococcus albus*.

The *staphylococcus pyogenes citreus*, which is less frequently met with, differs in the colour of the cultures, being a lemon yellow, and is less virulent than the other two.

The *staphylococcus cereus albus* and *staphylococcus cereus flavus* are of much less importance. They produce a wax-like growth on gelatin without liquefaction; hence their name.

**Streptococcus pyogenes.**—This organism (Plate I., Fig. 1) is a

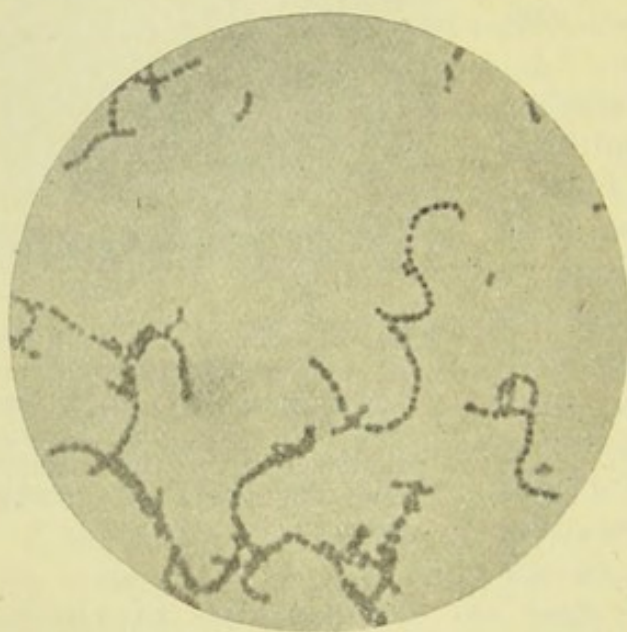


FIG. 52.—*Streptococcus pyogenes*, young culture on agar, showing chains of cocci. Stained with weak carbol-fuchsin.  $\times 1000$ .

coccus of slightly larger size than the *staphylococcus aureus*, about  $1\mu$  in diameter, and forms chains which may contain a large number of members, especially when it is growing in fluids (Fig. 52). The chains vary somewhat in length in different specimens, and on this ground varieties have been distinguished, e.g. the *streptococcus brevis* and *streptococcus longus* (*vide infra*). As division may take place in many of the cocci in a chain at the same time, the appearance of

a chain of diplococci is often met with. In young cultures the cocci are fairly uniform in size, but after a time they present considerable variations, many swelling up to twice their normal diameter. These are to be regarded as involution forms. In its



staining reactions the streptococcus resembles the staphylococci described, being readily coloured by Gram's method.

*Cultivation.*—In cultures outside the body the streptococcus pyogenes grows much more slowly than the staphylococci, and also dies out more readily, being in every respect a more delicate organism.

In *peptone gelatin* a stab culture shows, about the second day, a thin line, which in its subsequent growth is seen to be formed of a row of minute rounded colonies of whitish colour, which may be separate at the lower part of the puncture. They do not usually exceed the size of a small pin's head, this size being reached about the fifth or sixth day. The growth does not spread on the surface, and no liquefaction of the medium occurs. The colonies in gelatin plates have a corresponding appearance, being minute spherical points of whitish colour. A somewhat warm temperature is necessary for growth; even at 20° C. some varieties do not grow. On the *agar* media, growth takes place along the stroke as a collection of small circular discs of semi-translucent appearance, which show a great tendency to remain separate (Fig. 53). The separate colonies remain small, rarely exceeding 1 mm. in diameter. Cultures on agar kept at the body temperature may often be found to be dead after ten days. On *potato*, as a rule, no visible growth takes place. In *milk* it produces a strongly acid reaction but no clotting of the medium. It ferments lactose, saccharose, and salicin (Andrewes and Horder); it produces no fermentation of inulin in Hiss's serum-water-medium, in this respect differing from the pneumococcus. It has a strong hæmolytic action, as can be demonstrated by growing it in blood-agar plates (p. 43). In *bouillon*, growth forms numerous minute granules which afterwards fall to the bottom, the deposit, which is usually not very abundant, having a sandy appearance. The appearance in broth, however, presents variations which have been used as an aid to distinguish different species of streptococci. It has been



FIG. 53.—Culture of the streptococcus pyogenes on an agar plate, showing numerous colonies—three successive strokes. Twenty-four hours' growth. Natural size.



found that those which form the longest chains grow most distinctly in the form of spherical granules, those forming short chains giving rise to a finer deposit. To a variety which forms distinct spherules of minute size the term *streptococcus conglomeratus* has been given.

*Varieties of Streptococci.*—Formerly the *streptococcus pyogenes* and the *streptococcus erysipelatis* were regarded as two distinct species, and various points of difference between them were given. Further study, and especially the results obtained by modifying the virulence (p. 210), have shown that these distinctions cannot be maintained, and now practically all authorities are agreed that the two organisms are one and the same, erysipelas being produced when the *streptococcus pyogenes* of a certain standard of virulence gains entrance to the lymphatics of the skin. Petruschky, moreover, showed conclusively by inoculation that a *streptococcus* cultivated from pus could cause erysipelas in the human subject.

*Streptococci* have also been classified according to the length of the chains. Thus there have been distinguished (a) *streptococcus longus*, which occurs in long chains and is pathogenic to rabbits and mice; (b) *streptococcus brevis*, which is common in the mouth in normal conditions, and is usually non-pathogenic; and (c) *streptococcus conglomeratus*, so called from its forming in bouillon minute granules composed of very long chains. It may be stated that pathogenic *streptococci* obtained from the human subject usually form fairly long chains on agar, whilst the short *streptococci* obtained from the mouth and intestine are usually devoid of virulence. But to these statements exceptions occur, as short *streptococci* may be associated with grave lesions; it has also been found that the length of the chains is not a constant feature.

As in the case of other organisms attempts have also been made to differentiate *streptococci* by means of their fermentative properties. Mervyn Gordon introduced for this purpose nine tests, namely: (1) The clotting of milk, (2) the reduction of neutral red, (3–9) the fermentation with acid production of saccharose, lactose, raffinose, inulin, salicin, coniferin, and mannite. Andrewes and Horder by means of these have differentiated six varieties, of which five occur in the human subject. These are: (a) A short-chained form called *streptococcus mitis*, which occurs chiefly in the saliva and fæces as a saprophyte. It ferments saccharose and lactose, and sometimes the glucosides; it produces an acid reaction in milk but no clotting, and often reduces neutral-red. (b) The *streptococcus pyogenes*, which is the most important pathogenic variety, and has the characters described above. (c) The *streptococcus salivarius*, which corresponds to the *streptococcus brevis* of the mouth, and which, as regards fermentative action, seems to bear the same relation to the next



variety as the streptococcus mitis does to the streptococcus pyogenes. It ferments saccharose, lactose, and raffinose, sometimes the glucosides and rarely inulin; it clots milk and reduces neutral-red. (d) The *streptococcus anginosus*, which corresponds with the so-called streptococcus scarlatinae and the streptococcus conglomeratus. It ferments saccharose and lactose, and sometimes raffinose, reduces neutral-red, and is actively hæmolytic. It usually clots milk and does not grow on gelatin at 20° C. (e) The *streptococcus faecalis*, a short-chained form, which abounds in the intestine and which has great fermentative activity, and reacts positively to all Gordon's tests with the exception of raffinose and inulin. It forms sulphuretted hydrogen, and is devoid of hæmolytic action. (f) The sixth variety is the *streptococcus equinus*, which is common in the air and dust of towns, and appears to be derived from horse dung.<sup>1</sup> It ferments saccharose and the two glucosides, and forms little or no acid in milk. It is, however, to be noted that to all these varieties variants are met with.

Schottmüller has employed the appearance of the colonies of streptococci on blood agar as a means of separating varieties, the medium used consisting of two parts human blood and five parts melted agar. He distinguishes the *streptococcus longus* or *erysipelatis*, which forms grey colonies and has a marked hæmolytic action; a *streptococcus mitior* or *viridans*, a short-chained organism, which produces small green colonies and very little hæmolysis; and a *streptococcus mucosus encapsulatus*, which, as its name indicates, shows well-marked capsules and produces colonies which have a slimy consistence. Mandelbaum adds to these the *streptococcus saprophyticus*, which is without hæmolytic action. It should be noted that on blood agar the pneumococcus forms green colonies and produces little or no hæmolysis. Levy finds that a 2.5 per cent. solution of taurocholate of sodium in bouillon produces complete bacteriolysis of the pneumococcus and the streptococcus mucosus, while it has no effect on other varieties of streptococcus. He considers the streptococcus mucosus to be a variety of pneumococcus. The general statement may be made that most of the streptococci from lesions in the human subject have hæmolytic action, but that occasionally streptococci without this property are found even in severe infections.

It will be thus seen from this account that the streptococcus pyogenes as described above is the organism most frequently associated with the pathogenic processes, and that short-chained forms are common saprophytes in the human body, although they may be associated with conditions of disease; these may be subdivided according to their fermentative activity as detailed. And lastly, there is the streptococcus conglomeratus (anginosus), which is specially abundant in the throat in scarlet fever, though it also occurs in other acute catarrhal states. No definite statement can yet be made as to the etiological relation of streptococci to scarlet fever; we can only say that streptococci are almost invariably present in the fauces, and that to them many of the complications of the disease are due.

<sup>1</sup> For further details, reference must be made to the original papers, *Lancet*, September 1906, ii. 708, etc.



**Bacillus coli communis.**—The microscopic and cultural characters are described in the chapter on typhoid fever. The *bacillus lactis aerogenes* and the *bacillus pyogenes fetidus* closely resemble it; they are either varieties or closely related species. The former is distinguished by producing more abundant gas formation, and by its growth on gelatin, etc., being thicker and whiter than that of the *bacillus coli*.

**Bacillus aerogenes encapsulatus** sometimes invades the tissues before death, and is characterised by the formation of bubbles of gas in the infected parts. Its characters are described in Chapter XVII.

**Bacillus pyocyaneus.**—This organism occurs in the form of minute rods 1.5 to 3  $\mu$  in length and less than .5  $\mu$  in thickness (Fig. 54).

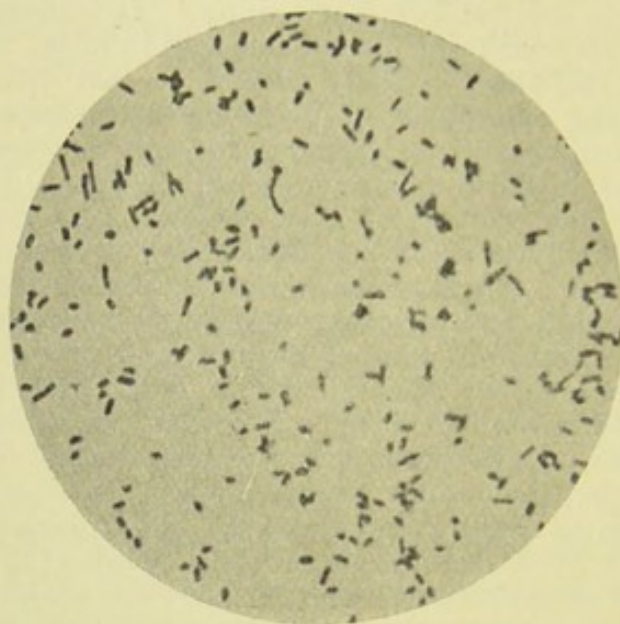


FIG. 54.—*Bacillus pyocyaneus*; young culture on agar. Stained with weak carbol-fuchsin.  $\times 1000$ .

Occasionally two or three are found attached end to end. They are actively motile, and do not form spores. They stain readily with the ordinary basic stains, but are decolorised by Gram's method.

**Cultivation.**—It grows readily on all the ordinary media at the room temperature, the cultures being distinguished by the formation of a greenish pigment. In puncture cultures in peptone-gelatin a greyish line appears in twenty-four hours, and at its upper part a small cup of liquefaction forms within forty-eight hours. At this time a slightly greenish tint is seen in the superficial part of the gelatin. The liquefaction

extends pretty rapidly, the fluid portion being turbid and showing masses of growth at its lower part. The green colour becomes more and more marked, and diffuses through the gelatin. Ultimately liquefaction reaches the wall of the tube. In plate cultures the colonies appear as minute whitish points, those on the surface being the larger. Under a low power of the microscope they have a brownish-yellow colour and show a nodulated surface, the superficial colonies being thinner and larger. Liquefaction soon occurs, the colonies on the surface forming shallow cups with small irregular masses of growth at the bottom, the deep colonies small spheres of liquefaction. Around the colonies a greenish tint appears. On agar the growth forms an abundant slimy greyish layer which afterwards becomes greenish, and a bright green colour diffuses through the whole substance of the medium. On potatoes the growth is an abundant reddish-brown layer resembling that of the glanders bacillus, and the potato sometimes shows a greenish discoloration.

From the cultures there can be extracted by chloroform a coloured body, pyocyanin, which belongs to the aromatic series, and crystallises in the form of long, delicate bluish-green needles. On the addition of a weak acid its colour changes to a red.



This organism has distinct pathogenic action in certain animals. Subcutaneous injection of small doses in rabbits may produce a local suppuration, but if the dose be large, spreading hæmorrhagic œdema results, which may be attended by septicæmia. Intravenous injection may produce, according to the dose, rapid septicæmia with nephritis, or sometimes a more chronic condition of wasting attended by albuminuria.

**Micrococcus tetragenus.**—This organism, first described by Gaffky, is characterised by the fact that it divides in two planes at right angles to one another (Fig. 55), and is thus generally found in the tissues in groups of four, or tetrads, which are often seen to be surrounded by a capsule. The cocci measure  $1\mu$  in diameter. They stain readily with all the ordinary stains, and also retain the stain in Gram's method.

It grows readily on all the media at the room temperature. In a puncture culture on peptone-gelatin a pretty thick whitish line forms along the track of the needle, whilst on the surface there is a thick rounded disc of whitish colour. The gelatin is not liquefied. On the surface of agar and of potato the growth is an abundant moist layer of the same colour. The growth on all the media has a peculiar viscid or tenacious character, owing to the gelatinous character of the sheaths of the cocci.

White mice are exceedingly susceptible to this organism. Subcutaneous injection is followed by a general septicæmia, the organism being found in large numbers in the blood throughout the body. Guinea-pigs are less susceptible; sometimes only a local abscess with a good deal of necrotic change results; sometimes there is also septicæmia.

**Experimental Inoculation.**—We shall consider chiefly the staphylococcus pyogenes aureus and the streptococcus pyogenes, as these have been most fully studied.

It may be stated at the outset that the occurrence of suppuration depends upon the number of organisms introduced into the tissues, the number necessary varying not only in different animals, but also in different parts of the same animal,—a smaller number producing suppuration in the anterior chamber of the eye, for example, than in the peritoneum. The virulence of the organism also may vary, and corresponding results may be produced. Especially is this so in the case of the streptococcus pyogenes.

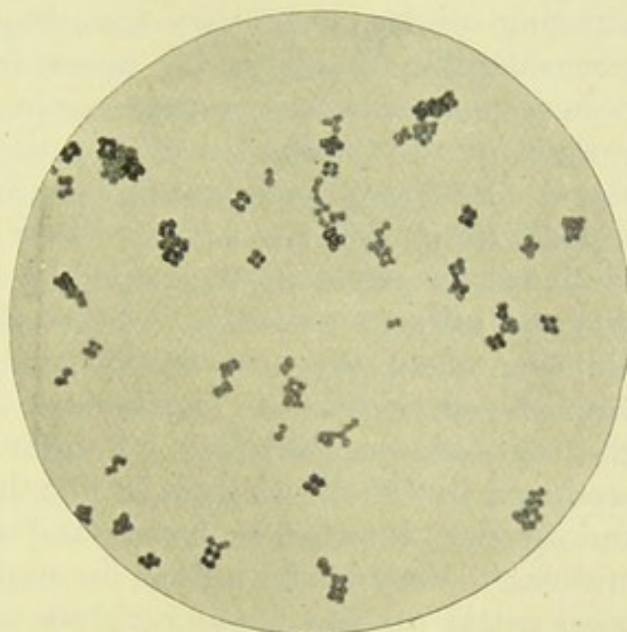


FIG. 55.—*Micrococcus tetragenus*; young culture on agar, showing tetrads. Stained with weak carbol-fuchsin.  $\times 1000$ .



The *staphylococcus aureus*, when injected *subcutaneously* in suitable numbers, produces an acute local inflammation, which is followed by suppuration, in the manner described above. If a large dose is injected, the cocci may enter the blood stream in sufficient numbers to cause secondary suppurative foci in internal organs (cf. intravenous injection).

*Intravenous injection* in rabbits, for example, produces interesting results, which vary according to the quantity used. If a considerable quantity be injected, the animal may die in twenty-four hours of a general septicaemia, numerous cocci being present in the capillaries of the various organs, often forming plugs. If a smaller quantity be used, the cocci gradually disappear from the circulating blood; some become destroyed, while others settle in the capillary walls in various parts and produce minute abscesses. These are most common in the kidneys, where they occur both in the cortex and medulla as minute yellowish areas surrounded by a zone of intense congestion and hæmorrhage. Similar small abscesses may be produced in the heart wall, in the liver, under the periosteum, and in the interior of bones, and occasionally in the striped muscles. Very rarely indeed, in experimental injection, do the cocci settle on the healthy valves of the heart. If, however, when the organisms are injected into the blood, there be any traumatism of a valve, or of any other part of the body, they show a special tendency to settle at these weakened points.

Experiments on the *human subject* have also proved the pyogenic properties of those organisms. Garré inoculated scratches near the root of his finger-nail with a pure culture, a small cutaneous pustule resulting; and by rubbing a culture over the skin of the forearm he caused a carbuncular condition which healed only after some weeks. Confirmatory experiments of this nature were made by Bockhart, Bumm, and others.

When tested experimentally, the *staphylococcus pyogenes albus* has practically the same pathogenic effects as the *staphylococcus aureus*.

The *streptococcus pyogenes* is an organism the virulence of which varies much according to the diseased condition from which it has been obtained, and also one which loses its virulence rapidly in cultures. Even highly virulent cultures, if grown under ordinary conditions, in the course of time lose practically all pathogenic power. By passage from animal to animal, however, the virulence may be much increased, and *pari passu* the effects of inoculation are correspondingly varied. Marmorek, for example, found that the virulence of a streptococcus can be



enormously increased by growing it alternately (*a*) in a mixture of human blood serum and bouillon (*vide* p. 42), and (*b*) in the body of a rabbit; ultimately, after several passages it possesses a super-virulent character, so that even an extremely minute dose introduced into the tissues of a rabbit produces rapid septicaemia, with death in a few hours. It has been proved by Marmorek's experiments, and those of others, that the same species of streptococcus may produce at one time merely a passing local redness, at another a local suppuration, at another a spreading erysipelatous condition, or again a general septicæmic infection, according as its virulence is artificially increased. Such experiments are of extreme importance as explaining to some extent the great diversity of lesions in the human subject with which streptococci are associated.

**Bacillus Coli Communis.**—The virulence of this organism also varies much, and can be increased by passage from animal to animal. Injection into the serous cavities of rabbits produces a fibrinous inflammation which becomes purulent if the animal lives sufficiently long. If, however, the virulence of the organism be of a high order, death takes place before suppuration is established, and there is a septicæmic condition, the organisms occurring in large numbers in the blood. Intravenous injection of a few drops of a virulent bouillon culture usually produces a rapid septicaemia with scattered hæmorrhages in various organs.

**Lesions in the Human Subject.**—The following statement may be made with regard to the occurrence of the chief organisms mentioned, in the various suppurative and inflammatory conditions in the human subject. The account is, however, by no means exhaustive, as clinical bacteriology has shown that practically every part of the body may be the site of a lesion produced by the pyogenic bacteria. It may also be noted that acute catarrhal conditions of cavities or tubes are in many cases also to be ascribed to their presence.

The *staphylococci* are the most common causal agents in localised abscesses, in pustules on the skin, in carbuncles, boils, etc., in acute suppurative periostitis, in catarrhs of mucous surfaces, in ulcerative endocarditis, and in various pyæmic conditions. They may also be present in septicaemia.

*Streptococci* are especially found in spreading inflammation with or without suppuration; in diffuse phlegmonous and erysipelatous conditions, suppurations in serous membranes and in joints (Fig. 56). They also occur in acute suppurative periostitis and ulcerative endocarditis. Secondary abscesses in



lymphatic glands and lymphangitis are also, we believe, more frequently caused by streptococci than staphylococci. They also produce fibrinous exudation on the mucous surfaces, leading

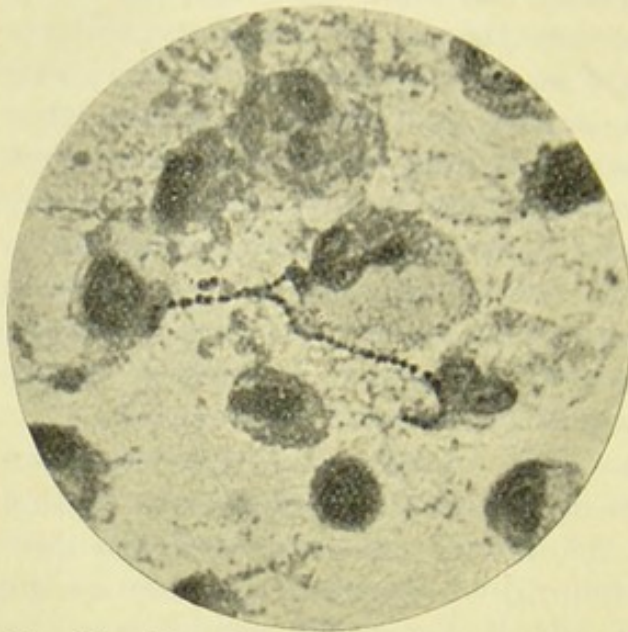


FIG. 56.—Streptococci in acute suppuration. Corrosive film; stained by Gram's method and safranin.  $\times 1000$ .

to the formation of false membrane in many of the cases of non-diphtheritic inflammation of the throat, which are met with in scarlatina<sup>1</sup> and other conditions, and they are also the organisms most frequently present in acute catarrhal inflammations in this situation. In puerperal peritonitis they are frequently found in a condition of purity, and they also appear to be the most frequent cause of puerperal septicaemia, in which condition they may be found after

death in the capillaries of various organs. In pyaemia they are frequently present, though in most cases associated with other pyogenic organisms. Some cases of enteritis in infants—streptococcus enteritis—are also apparently due to a streptococcus, which, however, presents in cultures certain points of difference from the streptococcus pyogenes.

The *bacillus coli communis* is found in a great many inflammatory and suppurative conditions in connection with the alimentary tract—for example, in suppuration in the peritoneum, or in the extraperitoneal tissue with or without perforation of the bowel, in the peritonitis following strangulation of the bowel, in appendicitis and the lesions following it, in suppuration in and around the bile ducts, etc. It may also occur in lesions in other parts of the body,—endocarditis, pleurisy, etc., which in some cases are associated with lesions of the intestine, though in others such cannot be found. It is also frequently present in inflammation of the urinary passages, cystitis, pyelitis, abscesses in the kidneys, etc., these lesions being in fact most frequently caused by this or closely allied organisms.

In certain cases of enteritis it is probably the causal agent,

<sup>1</sup> True diphtheria may also occasionally be associated with this diseases usually as a sequel.



though this is difficult of proof, as it is much increased in numbers in practically all abnormal conditions of the intestine. We may remark that it has been repeatedly proved that the bacillus coli cultivated from various lesions is more virulent than that in the intestine, its virulence having been heightened by growth in the tissues.

The *micrococcus tetragenus* is often found in suppurations in the region of the mouth or in the neck, and also occurs in various lesions of the respiratory tract, in phthisical cavities, abscesses in the lungs, etc. Sometimes it is present alone, and probably has a pyogenic action in the human subject under certain conditions. In other cases it is associated with other organisms. Cases of pyæmia have been described in which this organism was found in a state of purity in the pus in various situations. In this latter condition the pus has been described as possessing an oily, viscous character, and as being often blood-stained.

The *bacillus pyocyaneus* is rarely found alone in pus, though it is not infrequent along with other organisms. We have met with it several times in cases of multiple abscesses, in association with the staphylococcus pyogenes aureus. Lately some diseases in children have been described in which the bacillus pyocyaneus has been found throughout the body; in these cases the chief symptoms have been fever, gastro-intestinal irritation, pustular or petechial eruptions on the skin, and general marasmus. It has also been said to be constantly present in pemphigus, and it certainly occurs in some cases of this disease.

Inflammatory and suppurative conditions, associated with the organisms of special diseases, will be described in the respective chapters.

**Mode of Entrance and Spread.**—Many of the organisms of suppuration have a wide distribution in nature, and many also are present on the skin and mucous membranes of healthy individuals. Staphylococci are commonly present on the skin, and also occur in the throat and other parts, and streptococci can often be cultivated from the secretions of the mouth in normal conditions. The pneumococcus of Fraenkel and the pneumobacillus of Friedländer have also been found in the mouth and in the nasal cavity, whilst the bacillus coli communis is a normal inhabitant of the intestinal tract. The entrance of these organisms into the deeper tissues when a surface lesion occurs can be readily understood. Their action will, of course, be favoured by any condition of depressed vitality. Though in normal conditions the blood is bacterium-free, we must suppose



that from time to time a certain number of such organisms gain entrance to it from trifling lesions of the skin or mucous surfaces, the possibilities of entrance from the latter being especially numerous. In most cases they are killed by the action of the healthy serum or cells of the body, and no harm results. If, however, there be a local weakness, they may settle in that part and produce suppuration, and from this other parts of the body may be infected. Such a supposition as this is necessary to

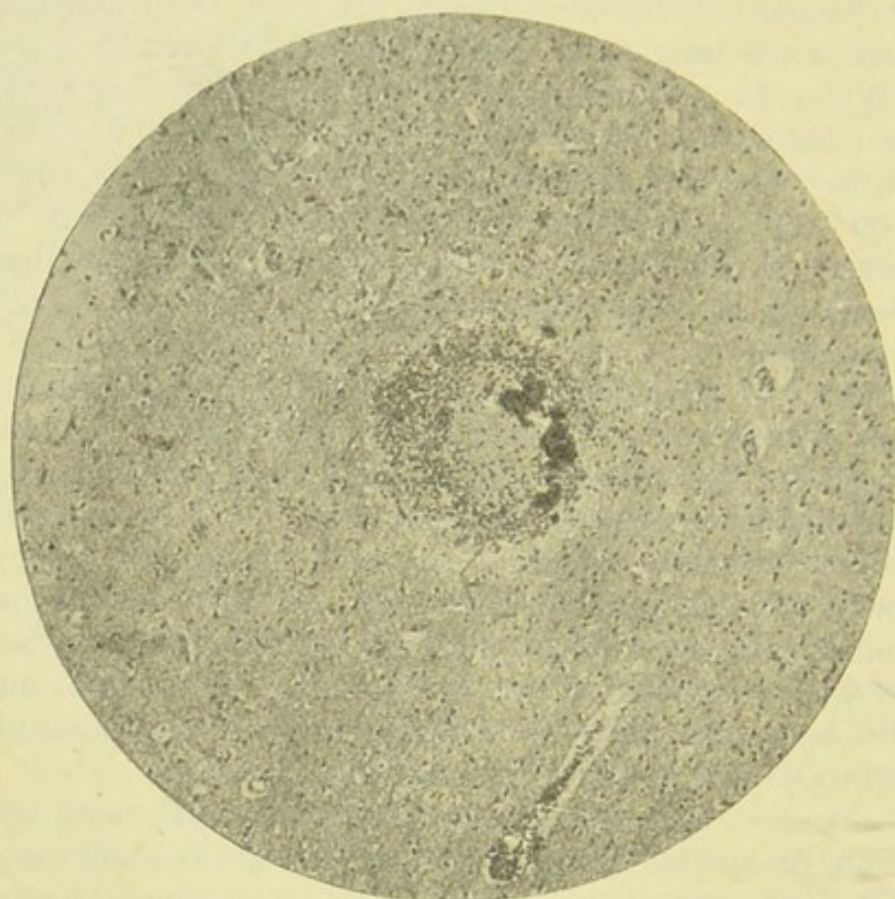


FIG. 57.—Minute focus of commencing suppuration in brain—case of acute ulcerative endocarditis. In the centre a small hæmorrhage; to right side dark masses of staphylococci; zone of leucocytes at periphery.

Alum carmine and Gram's method.  $\times 50$ .

explain many inflammatory and suppurative conditions met with clinically. In some cases of multiple suppurations due to staphylococcus infection, only an apparently unimportant surface lesion is present; whilst in others no lesion can be found to explain the origin of the infection. The term *cryptogenetic* has been applied by some writers to such cases in which the original point of infection cannot be found, but its use is scarcely necessary.

The paths of secondary infection may be conveniently summarised thus: First, by lymphatics; in this way the lymphatic



glands may be infected, and also serous sacs in relation to the organs where the primary lesion exists. Second, by natural channels, such as the ureters and the bile ducts, the spread being generally associated with an inflammatory condition of the lining epithelium. In this way the kidneys and liver respectively may be infected. Third, by the blood vessels: (*a*) by a few organisms gaining entrance to the blood from a local lesion, and



FIG. 58.—Secondary infection of a glomerulus of kidney by the staphylococcus aureus, in a case of ulcerative endocarditis. The cocci (stained darkly) are seen plugging the capillaries and also lying free. The glomerulus is much swollen, infiltrated by leucocytes, and partly necrosed.

Paraffin section; stained by Gram's method and Bismarck-brown.  $\times 300$ .

settling in a favourable nidus or a damaged tissue, the original path of infection often being obscure; (*b*) by a septic phlebitis with suppurative softening of the thrombus and resulting embolism; and we may add (*c*), by a direct extension along a vein, producing a spreading thrombosis and suppuration within the vein. In this way suppuration may spread along the portal vein to the liver from a lesion in the alimentary canal, the condition being known as pylephlebitis suppurativa.



Although many of the lesions produced by the bacteria under consideration have already been mentioned, certain conditions may be selected for further consideration on account of their clinical importance or bacteriological interest.

**Endocarditis.**—There is now strong presumptive evidence that all cases of endocarditis are due to bacterial infection. In the simple or vegetative form, so often the result of acute rheumatism, the micrococcus rheumaticus (p. 221) has been cultivated from the valves in a certain number of cases, and is probably the causal agent in most instances.

Endocarditis of the ulcerative type may be produced by various organisms, chiefly pyogenic. Of these the staphylococci and streptococci are most frequently found. In some cases of ulcerative endocarditis following pneumonia the pneumococcus (Fraenkel's) is present; in these the vegetations often reach a large size and have not so much tendency to break down as in the case of staphylococcus infections. Other organisms have been cultivated from different cases of the disease, and some of these have received special names; for example, the diplococcus endocarditis encapsulatus, bacillus endocarditidis griseus (Weichselbaum), and others. In some cases the bacillus coli communis has been found, and occasionally in endocarditis following typhoid the typhoid bacillus has been described as the organism present, but further observations on this point are desirable. The gonococcus also has been shown to affect the heart valves (p. 256), though this is a very rare occurrence. Tubercle nodules on the heart valves have been found in a few cases of acute tuberculosis, though no vegetative or ulcerative condition is produced.

In some cases, though we believe not often, the organisms may attack healthy valves, producing a *primary* ulcerative endocarditis, but more frequently the valves have been the seat of previous endocarditis, *secondary* ulcerative endocarditis being thus produced. In some cases, especially when the valves have been previously diseased, the source of the infection is quite obscure. It is evident that as the vegetations are composed for the most part of unorganised material, they do not offer the same resistance to the growth of bacteria, when a few reach them, as a healthy cellular tissue does. On microscopic examination of the diseased valves the organisms are usually to be found in enormous numbers (Fig. 59). By their action breaking down of the vegetations occurs, and the emboli thus produced act as the carriers of infection to other organs, and give rise to secondary suppurations.



*Experimental.*—Occasionally ulcerative endocarditis is produced by the simple intravenous injection of staphylococci and streptococci into the circulation, but this is a very rare occurrence. It often follows, however, when the valves have been previously injured. Orth and Wyssokowitsch at a comparatively early date produced the condition by damaging the aortic cusps by a glass rod introduced through the carotid, and afterwards injecting staphylococci into the circulation. Similar experiments have since been repeated with streptococci, pneumococci, and other organisms, with like result. Ribbert found that if a potato culture of

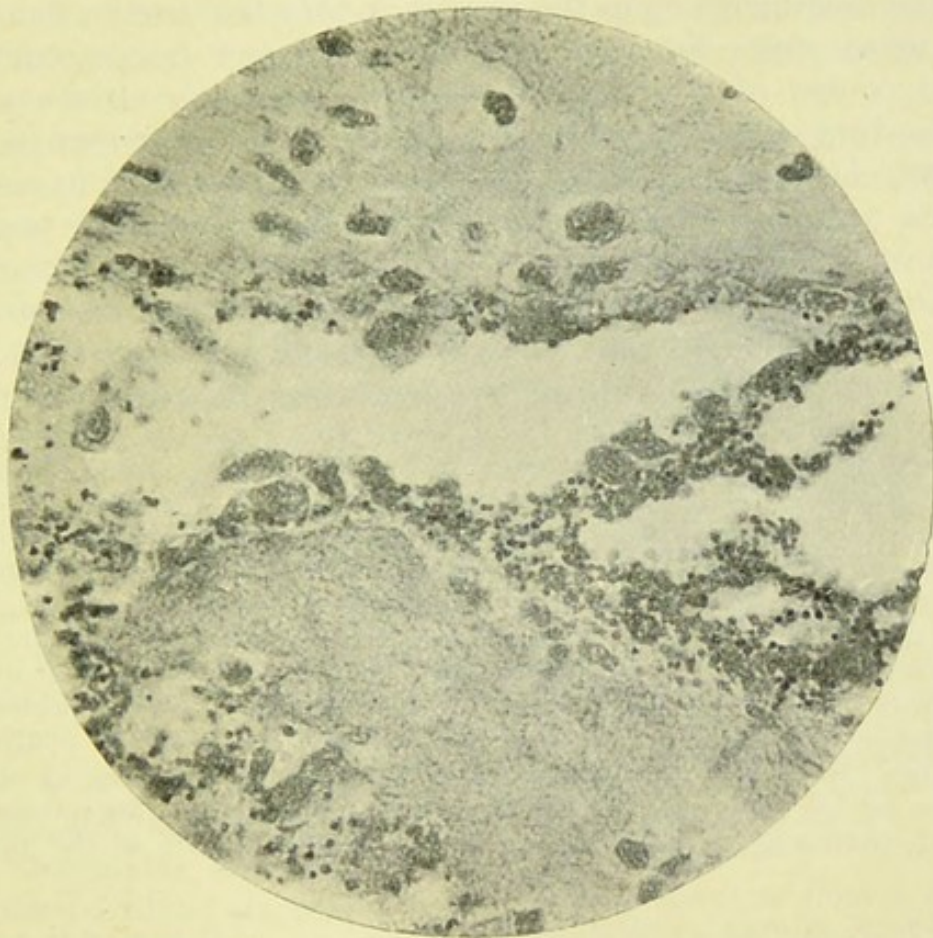


FIG. 59.—Section of a vegetation in ulcerative endocarditis showing numerous staphylococci lying in the spaces. The lower portion is a fragment in process of separation.

Stained by Gram's method and Bismarck-brown.  $\times 600$ .

the staphylococcus aureus were rubbed down in salt solution so as to form an emulsion, and then injected into the circulation, some minute fragments became arrested at the attachment of the chordæ tendineæ and produced an ulcerative endocarditis.

**Acute Suppurative Periostitis and Osteomyelitis.**—Special mention is made of this condition on account of its comparative frequency and gravity. The great majority of cases are caused by the pyogenic cocci, of which one or two varieties may be



present, the staphylococcus aureus, however, occurring most frequently. Pneumococci have been found alone in some cases, and in a considerable number of cases following typhoid fever the bacillus typhosus has been found alone. In others, again, the bacillus coli communis is present.

The affection of the periosteum or interior of the bones by these organisms, which is especially common in young subjects, may take place in the course of other affections produced by the same organisms or in the course of infective fevers, but in a great many cases the path of entrance cannot be determined. In the course of this disease serious secondary infections are always very liable to follow, such as small abscesses in the kidneys, heart-wall, lungs, liver, etc., suppurations in serous cavities, and ulcerative endocarditis; in fact, some cases present the most typical examples of extreme general staphylococcus infection. The entrance of the organisms into the blood stream from the lesion of the bone is especially favoured by the arrangement of the veins in the bone and marrow.

*Experimental.*—Multiple abscesses in the bones and under the periosteum may occur in simple intravenous injection of the pyogenic cocci into the blood, and are especially liable to be formed when young animals are used. These abscesses are of small size, and do not spread in the same way as in the natural disease in the human subject.

In experiments on healthy animals, however, the conditions are not analogous to those of the natural disease. We must presume that in the latter there is some local weakness or susceptibility which enables the few organisms which have reached the part by the blood to settle and multiply. Moreover, if a bone be experimentally injured, *e.g.* by actual fracture or by stripping off the periosteum, before the organisms are injected, then a much more extensive suppuration occurs at the injured part.

**Erysipelas.**—A spreading inflammatory condition of the skin may be produced by a variety of organisms, but the disease in the human subject in its characteristic form is almost invariably due to a streptococcus, as was shown by Fehleisen in 1884. He obtained pure cultures of the organism, and gave it the name of streptococcus erysipelatis; and, further, by inoculations on the human subject as a therapeutic measure in malignant disease, he was able to reproduce erysipelas. As stated above, however, one after another of the supposed points of difference between the streptococcus of erysipelas and that of suppuration has broken down, and it is now generally held that erysipelas is produced by the streptococcus pyogenes of a certain degree of virulence. It must be noted, however, that erysipelas passes from patient to patient as erysipelas, and purulent con-



ditions due to streptococci do not appear liable to be followed by erysipelas. On the other hand, the connection between erysipelas and puerperal septicaemia is well established clinically.

In a case of erysipelas the streptococci are found in large numbers in the lymphatics of the cutis and underlying tissues, just beyond the swollen margin of the inflammatory area. As the inflammation advances they gradually die out, and after a time their extension at the periphery comes to an end. The streptococci may extend to serous and synovial cavities and set up inflammatory or suppurative change,—peritonitis, meningitis, and synovitis may thus be produced.

**Conjunctivitis.**—A considerable number of organisms are concerned in the production of conjunctivitis and its associated lesions. Of these a number appear to be specially associated with this region. Thus a small organism, generally known as the Koch-Weeks bacillus, is the most common cause of acute contagious conjunctivitis, especially prevalent in Egypt, but also common in this country. This organism is very minute, being little more than  $1\ \mu$  in length, and morphologically resembles the influenza bacillus; its conditions of growth are even more restricted, as it rarely grows on blood agar, the best medium being serum agar. On this medium it produces minute transparent colonies like drops of dew. The obtaining of pure cultures is a matter of considerable difficulty, and it is nearly always accompanied by the xerosis bacillus. It can readily be found in the muco-purulent secretion by staining films with weak (1 : 10) carbol-fuchsin, and is often to be seen in the interior of leucocytes (Fig. 60). Another organism exceedingly like the previous, apparently differing from it only in the rather wider conditions of growth, is Müller's bacillus. It was cultivated by him in a considerable proportion of cases of trachoma, but its



FIG. 60.—Film preparation from a case of acute conjunctivitis, showing Koch-Weeks bacilli, chiefly contained within a leucocyte. (From a preparation by Dr. Inglis Pollock.)  
× 1000.

being serum agar. On this medium it produces minute transparent colonies like drops of dew. The obtaining of pure cultures is a matter of considerable difficulty, and it is nearly always accompanied by the xerosis bacillus. It can readily be found in the muco-purulent secretion by staining films with weak (1 : 10) carbol-fuchsin, and is often to be seen in the interior of leucocytes (Fig. 60). Another organism exceedingly like the previous, apparently differing from it only in the rather wider conditions of growth, is Müller's bacillus. It was cultivated by him in a considerable proportion of cases of trachoma, but its



relation to this condition is still matter of dispute. Another bacillus which is now well recognised is the diplo-bacillus of conjunctivitis first described by Morax. It is especially common in the more subacute cases of conjunctivitis. Eyre found it in 2.5 per cent. of all cases of conjunctivitis. Its cultural characters are given below. The xerosis bacillus, which is a small diphtheroid organism (Fig. 119), has been found in xerosis of the conjunctiva, in follicular conjunctivitis, and in other conditions; it appears to occur sometimes also in the normal conjunctiva. It is doubtful whether it has any pathogenic action of importance. Acute conjunctivitis is also produced by the pneumococcus, epidemics of the disease being sometimes due to this organism, and also by streptococci and staphylococci. True diphtheria of

the conjunctiva caused by the Klebs-Löffler bacillus also occurs, whilst in gonorrhœal conjunctivitis, often of an acute purulent type, the gonococcus is present (p. 255).



FIG. 61.—Film preparation of conjunctival secretion, showing the Morax diplo-bacillus of conjunctivitis.  $\times 1000$ .

**Diplo-bacillus of Conjunctivitis.**—This organism, discovered by Morax, is a small plump bacillus, measuring  $1 \times 2 \mu$ , and usually occurring in pairs, or in short chains of pairs (Fig. 61). It is non-motile, does not form spores, and is decolorised by Gram's method. It does not grow on the ordinary gelatin and agar media, the addition of blood or serum being

necessary. On serum it forms small rounded colonies which produce small pits of liquefaction; hence it has been called the *bacillus lacunatus*. In cultures it is distinctly pleomorphic, and involution forms also occur. It is non-pathogenic to the lower animals.

**Acute Rheumatism.**—There are many facts which point to the infective nature of this disease, and investigations from this point of view have yielded important results. Of the organisms isolated, the one which appears to have strongest claims is a small coccus observed by Triboulet, and by Westphal and Wassermann, the characters and action of which were first investigated in this country by Poynton and Paine. It is now



usually spoken of as the *micrococcus rheumaticus*. The organism is sometimes spoken of as a diplococcus, but it is best described as a streptococcus growing in short chains; in the tissues, however, it usually occurs in pairs. It is rather smaller than the streptococcus pyogenes, and although it can be stained by Gram's method, it loses the colour more readily than the streptococcus. In the various media it produces a large amount of acid, and usually clots milk after incubation for two days; on blood agar it alters the hæmoglobin to a brownish colour. Its growth on media generally is more luxuriant than that of the streptococcus, and it grows well on gelatin at 20° C. Injection of pure cultures in rabbits often produces polyarthritis and synovitis, valvulitis and pericarditis, without any suppurative change—lesions which it is stated are not produced by the ordinary streptococci (Beattie). In one or two instances choreiform movements have been observed after injection. The organism is most easily obtained from the substance of inflamed synovial membrane where it is invading the tissues; a part where there is special congestion should be selected as being most likely to give positive results. It is only occasionally to be obtained from the fluid in joints. It has also been cultivated from the blood in rheumatic fever, from the vegetations on the heart valves, and from other acute lesions; in many cases, however, cultures from the blood give negative results. Beattie in a recent paper has shown that in rabbits the arthritis produced reproduces the main features of acute or subacute rheumatism in man, namely, the rapidity with which the affection flies from joint to joint, the tendency to relapses, the contributory effects of exposure to cold, and the absence of gross anatomical changes in the joints. Poynton and Paine cultivated the streptococcus from the cerebro-spinal fluid in three cases where chorea was present, and also detected it in the membranes of the brain. They consider that this disease is probably of the nature of a slight meningo-myelitis produced by the organism. The facts already accumulated speak strongly in favour of this organism being causally related to rheumatic fever, though this cannot be considered completely proved. Andrewes finds that the organism has the same cultural characters and fermentative effects as the streptococcus faecalis, a common inhabitant of the intestine. Even, however, if the two organisms were the same, it might well be possible that rheumatic fever is due to an infection of the tissues by this variety of streptococcus. The clinical data, in fact, rather point to rheumatic fever being due to an infection by some organism frequently present in the



body, brought about by some state of predisposition or acquired susceptibility.

**Vaccination Treatment of Infections by the Pyogenic Cocci.**

—From his study of the part played by phagocytosis in the successful combat of the pyogenic bacteria by the body, Wright was led to advocate the treatment of such infections during their course by active immunisation by means of dead cultures of the infecting agent (for methods of preparation see p. 133). The treatment is applicable when the infection is practically local, as in acne pustules, in boils, etc. (For the theoretical questions raised, see Immunity.) For an isolated faruncle, Wright recommends a dose of 100 million staphylococci to be followed three or four days later by the injection of 250 to 300 millions, and for an incipient streptococcic lymphangitis a dose of 2,000,000 streptococci. In chronic infections the number of bacteria used for an injection is from 250,000,000 to 500,000,000, but a smaller number may give a good result, and the general principle to be adopted is to use the smallest dose necessary for a therapeutic effect. If repeated injections are necessary, Wright recommends that the opsonic index should be observed every few days and the injections only practised during a positive phase. If it is not practicable to use the strain derived from the lesion for the preparation of the vaccine, then laboratory cultures or the stock vaccines which are now in the market may be used; in such cases it is well to use a vaccine made from a mixture of strains; in skin infections a mixture of staphylococcus aureus and albus may be employed. Such means have been extensively used in the treatment of acne, boils, sycosis, infections of the genito-urinary tract by the *b. coli*, infections of joints by the gonococcus, and in many cases considerable success has followed the treatment. Sometimes acute affections, *e.g.* puerperal septicæmia, have been treated by vaccines. It is difficult here to judge of the results which have been attained, but in any case only very small doses of the vaccine should at first, at any rate, be administered.

**Methods of Examination in Inflammatory and Suppurative Conditions.**—These are usually of a comparatively simple nature, and include (1) microscopic examination, (2) the making of cultures.

(1) The pus or other fluids should be examined microscopically, first of all by means of film preparations in order to determine the characters of the organisms present. The films should be stained (*a*) by one of the ordinary solutions, such as carbol-thionin-blue (p. 105), or a saturated watery solution of



methylene-blue; and (b) by Gram's method. The use of the latter is of course of high importance as an aid in the recognition.

(2) As most of the pyogenic organisms grow readily on the gelatin media at ordinary temperatures, pure cultures can be readily obtained by the ordinary plate methods. But in many cases the separation can be affected much more rapidly by the method of successive streaks on agar tubes, which are then incubated at 37° C. When the presence of pneumococci is suspected, this method ought always to be used, and it is also to be preferred in the case of streptococci. Inoculation experiments may be carried out as occasion arises.

In cases of suspected blood infection the examination of the blood is to be carried out by the methods already described (p. 72).



## CHAPTER VIII.

### INFLAMMATORY AND SUPPURATIVE CONDITIONS, *CONTINUED*: THE ACUTE PNEUMONIAS, EPI- DEMIC CEREBRO-SPINAL MENINGITIS.

**Introductory.**—The term Pneumonia is applied to several conditions which present differences in pathological anatomy and in origin. All of these, however, must be looked on as varieties of inflammation in which the process is modified in different ways, depending on the special structure of the lung or of the parts which compose it. There is, first of all, and, in adults, the commonest type, the acute croupous or lobar pneumonia, in which an inflammatory process attended by abundant fibrinous exudation affects, by continuity, the entire tissue of a lobe or of a large portion of the lung. It departs from the course of an ordinary inflammation in that the reaction of the connective tissue of the lung is relatively slight, and there is usually no tendency for organisation of the inflammatory exudation to take place. Secondly, there is the acute catarrhal or lobular pneumonia, where a catarrhal inflammatory process spreads from the capillary bronchi to the air vesicles, and in these a change, consisting largely of proliferation of the endothelium of the alveoli, takes place which leads to consolidation of patches of the lung tissue. Up till 1889 acute catarrhal pneumonia was comparatively rare except in children. In adults it was chiefly found as a secondary complication to some condition such as diphtheria, typhoid fever, etc. Since, however, influenza in an epidemic form has become frequent, catarrhal pneumonia has been of much more frequent occurrence in adults, has assumed a very fatal tendency, and has presented the formerly quite unusual feature of being sometimes the precursor of gangrene of the lung. Besides these two definite types other forms also occur. Thus instead of a fibrinous material the exudation may be of a serous, hemorrhagic, or purulent character. Cases of mixed fibrinous and catarrhal pneumonia also occur, and



in the catarrhal there may be great leucocytic emigration. Hæmorrhages also may occur here.

Besides the two chief types of pneumonia there is another group of cases which are somewhat loosely denominated septic pneumonias, and which may arise in two ways: (1) by the entrance into the trachea and bronchi of discharges, blood, etc., which form a nidus for the growth of septic organisms; these often set up a purulent capillary bronchitis and lead to infection of the air cells and also of the interstitial tissue of the lung; (2) from secondary pyogenic infection by means of the blood stream from suppurative foci in other parts of the body. (See chapter on Suppuration, etc.) In these septic pneumonias various changes, resembling those found in the other types, are often seen round the septic foci.

In pneumonias, therefore, there may be present a great variety of types of inflammatory reaction. We shall see that with all of them bacteria have been found associated. Special importance is attached to acute croupous pneumonia on account of its course and characters, but reference will also be made to the other forms.

**Historical.**—Acute lobar pneumonia for long was supposed to be an effect of exposure to cold; but many observers were dissatisfied with this view of its etiology. Not only did cases occur where no such exposure could be traced, but it had been observed that the disease sometimes occurred epidemically, and was occasionally contracted by hospital patients lying in beds adjacent to those occupied by pneumonia cases. Further, the sudden onset and definite course of the disease conformed to the type of an acute infective fever; it was thus suspected by some to be due to a specific infection. This view of its etiology was promulgated in 1882–83 by Friedländer, whose results were briefly as follows. In pneumonic lungs there were cocci, adherent usually in pairs, and possessed of a definitely contoured capsule. These cocci could be isolated and grown on gelatin, and on inoculation in mice they produced a kind of septicæmia with inflammation of the serous membranes. The blood and the exudation in serous cavities contained numerous capsulated diplococci. There is little doubt that many of the organisms seen by Friedländer were really Fraenkel's pneumococcus, to be presently described.

By many observers it had been found that the sputum of healthy men, when injected into animals, sometimes caused death, with the same symptoms as in the case of the injection of Friedländer's coccus; and in the blood and serous exudations of such animals capsulated diplococci were found. A. Fraenkel found that the sputum of pneumonic patients was much more fatal and more constant in its effects than that of healthy individuals. The cocci which were found in animals dead of this "sputum septicæmia," as it was called, differed from Friedländer's cocci in several respects, to be presently studied. Fraenkel further investigated a few cases of pneumonia, and isolated from them cocci identical in microscopic appearances, cultures, and pathogenic effects,



with those isolated in sputum septicæmia. The most extensive investigations on the whole question were those of Weichselbaum, published in 1886. This author examined 129 cases of the disease, including cases not only of acute croupous pneumonia, but of lobular and septic pneumonia. From them he isolated four groups of organisms. (1) *Diplococcus pneumoniae*. This he described as an oval or lancet-formed coccus, corresponding in appearance and growth characters to Fraenkel's coccus. (2) *Streptococcus pneumoniae*. This on the whole presented similar characters to the last, but it was more vigorous in its growth, and could grow below 20° C., though it preferred a temperature of 37° C. (3) *Staphylococcus pyogenes aureus*. (4) *Bacillus pneumoniae*. This was a rod-shaped organism, and was identical with Friedländer's pneumococcus. Of these organisms the diplococcus pneumoniae was by far the most frequent. It also occurred in all forms of pneumonia. Next in frequency was the streptococcus pneumoniae, and lastly the bacillus pneumoniae. Inoculation experiments were also performed by Weichselbaum with each of the three characteristic cocci he isolated. The diplococcus pneumoniae and the streptococcus pneumoniae both gave pathogenic effects of a similar kind in certain animals.

The general result of these earlier observations was to establish the occurrence in connection with pneumonia of two species of organisms, each having its distinctive characters, viz. :—

1. *Fraenkel's pneumococcus*, which is recognised to be identical with the coccus of "sputum septicæmia," with Weichselbaum's diplococcus pneumoniae, and with his streptococcus pneumoniae.

2. *Friedländer's pneumococcus* (now known as Friedländer's pneumobacillus), which is almost certainly the bacillus pneumoniae of Weichselbaum.

We shall use the terms "Fraenkel's pneumococcus" and "Friedländer's pneumobacillus," as these are now usually applied to the two organisms.

#### **Microscopic Characters of the Bacteria of Pneumonia.—**

*Methods.*—The organisms present in acute pneumonia can best be examined in film preparations made from pneumonic lung (preferably from a part in a stage of acute congestion or early hepatisation), or from the gelatinous parts of pneumonic sputum (here again preferably when such sputum is either rusty or occurs early in the disease), or in sections of pneumonic lung. Such preparations may be stained by any of the ordinary weak stains, such as a watery solution of methylene-blue, but Gram's method is to be preferred, with Bismarck-brown or Ziehl-Neelsen carbol-fuchsin (one part to ten of water) as a contrast stain; with the latter it is best either to stain for only a few seconds, or to overstain and then decolorise with alcohol till the ground of the preparation is just tinted. The capsules can also be stained by the methods already described (p. 109). In such preparations as the above, and even in specimens taken from



the lungs immediately after death (as may be quite well done by means of a hypodermic syringe), putrefactive and other bacteria may be present, but those to be looked for are capsulated organisms, which may be of either or both of the varieties mentioned.

(1) *Fraenkel's Pneumococcus*.—This organism occurs in the form of a small oval coccus, about  $1\ \mu$  in longest diameter, arranged generally in pairs (diplococci), but also in chains of four to ten (Fig. 62). The free ends are often pointed like a lancet, hence the term *diplococcus lanceolatus* has also been applied to it. These cocci, in their typical form, have round them a capsule, which, in films stained by ordinary methods, usually appears as an unstained halo, but is sometimes stained more deeply than the ground of the preparation. This difference in staining depends, in part at least, on the amount of decolorisation to which the preparation has been subjected. The capsule is rather broader than the body of the coccus, and has a sharply defined external margin. Often in sputum and even in the lung no capsule can be demonstrated. The organism takes up the basic aniline stains with great readiness, and also *retains the stain in Gram's method*. It is the organism of by far the most frequent occurrence in true croupous pneumonia, and in fact may be said to be rarely absent.



FIG. 62.—Film preparations of pneumonic sputum, showing numerous pneumococci (Fraenkel's) with unstained capsules; some are arranged in short chains. See also Plate I., Fig. 2.

Stained with carbol fuchsin.  $\times 1000$ .

(2) *Friedländer's Pneumobacillus*.—As seen in the sputum and tissues, this organism, both in its appearance and arrangement, as also in the presence of a capsule, somewhat resembles Fraenkel's pneumococcus, and it was at first described as the "pneumococcus." The form, however, is more of a short rod-shape, and it has blunt rounded ends; it is also rather broader than Fraenkel's pneumococcus. It is now classed amongst the bacilli, especially in view of the fact that elongated rod forms



may occur (Fig. 63). The capsule has the same general characters as that of Fraenkel's organism. Friedländer's pneumobacillus stains readily with the basic aniline stains, but *loses the stain in Gram's method*, and is accordingly coloured with the contrast stain,—fuchsin or Bismarck-brown, as above recommended. A valuable means is thus afforded of distinguishing it from Fraenkel's pneumococcus in microscopic preparations.

FIG. 63.—Friedländer's pneumobacillus, showing the variations in length, also capsules. Film preparation from exudate in a case of pneumonia.  $\times 1000$ .

pneumonia than Fraenkel's; sometimes it is associated with the latter; very rarely it occurs alone.

In sputum preparations the capsule of both pneumobacteria may not be recognisable, and the same is sometimes true of lung preparations. This is probably due to changes which occur in the capsule as the result of changes in the vitality of the organisms. Sometimes in preparations stained by ordinary methods the difficulty of recognising the capsule when it is present, is due to the refractive index of the fluid in which the specimen is mounted being almost identical with

Friedländer's organism is much less frequently present in

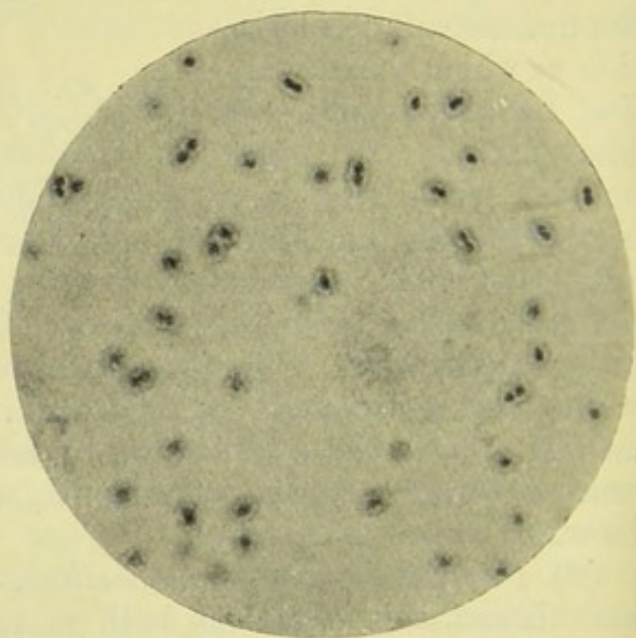


FIG. 64.—Fraenkel's pneumococcus in serous exudation at site of inoculation in a rabbit, showing capsules stained. Stained by Rd. Muir's method.  $\times 1000$ .

which the specimen is mounted being almost identical with



that of the capsule. This difficulty can always be overcome by having the groundwork of the preparation tinted.

**The Cultivation of Fraenkel's Pneumococcus.**—It is usually difficult, and sometimes impossible, to isolate this coccus directly from pneumonic sputum. On culture media it has not a vigorous growth, and when mixed with other bacteria it is apt to be overgrown by the latter. To get a pure culture it is best to insert a small piece of the sputum beneath the skin of a rabbit or a mouse. In about twenty-four to forty-eight hours the animal will die, with numerous capsulated pneumococci throughout its blood. From the heart-blood cultures can be easily obtained. Cultures can also be got *post mortem* from the lungs of pneumonic patients by streaking a number of agar or blood-agar tubes with a scraping taken from the area of acute congestion or commencing red hepatisation, and incubating them at 37° C. The colonies of the pneumococcus appear as almost transparent small discs which have been compared to drops of dew (Fig. 65). This method is also sometimes successful in the case of sputum.



FIG. 65.—Stroke culture of Fraenkel's pneumococcus on blood agar. The colonies are large and unusually distinct. Twenty-four hours' growth at 37° C. Natural size.

The appearances presented in cultures by different varieties of the pneumococcus vary somewhat. It always grows best on blood serum or on Pfeiffer's blood agar. It usually grows well on ordinary agar or in bouillon, but not so well on glycerin agar. In a stroke culture on *blood serum* growth appears as an almost transparent pellicle along the track, with isolated colonies at the margin. On *agar* media it is more manifest, but otherwise has similar characters. On agar plates colonies are very transparent, but under a low power of the microscope appear to have a compact finely granular centre and a pale transparent periphery. The appearances are similar to those of a culture of streptococcus pyogenes, but the growth is less vigorous, and is more delicate in appearance. A similar statement also applies to cultures in *gelatin* at 22° C., growth in a stab culture appearing as a row of minute points which remain of small size; there is, of course, no liquefaction of the medium. In *bouillon*, growth forms a slight turbidity, which



settles to the bottom of the vessel as a slight dust-like deposit. On *potatoes*, as a rule, no growth appears. Cultures may be maintained for one or two months, if fresh sub-cultures are made every four or five days, but they tend ultimately to die out. They also rapidly lose their virulence, so that four or five days after isolation from an animal's body their pathogenic action is already diminished. Eyre and Washbourn, however, have succeeded in maintaining cultures in a condition of constant virulence for at least three months by growing the organisms



FIG. 66.—Fraenkel's pneumococcus from a pure culture on blood agar of twenty-four hours' growth, some in pairs, some in short chains. Stained with weak carbol-fuchsin.  $\times 1000$ .

on agar smeared with rabbit blood. The agar must be prepared with Witte's peptone, must not be heated over  $100^{\circ}$  C., and after neutralisation (rosolic acid being used as the indicator) must have .5 per cent. of normal sodium hydrate added. The tubes when inoculated are to be kept at  $37^{\circ}\cdot 5$  C. and sealed to prevent evaporation. In ordinary artificial media pneumococci usually appear as diplococci without a capsule, but in preparations made from the surface of agar

or from bouillon, shorter or longer chains may be observed (Fig. 66). After a few days' growth they lose their regular shape and size, and involution forms appear. Usually the pneumococcus does not grow below  $22^{\circ}$  C., but forms in which the virulence has disappeared often grow well at  $20^{\circ}$  C. Its optimum temperature is  $37^{\circ}$  C., its maximum  $42^{\circ}$  C. It is preferably an aerobe, but can exist without oxygen. It prefers a slightly alkaline medium to a neutral, and does not grow on an acid medium. In ordinary media the pneumococcus does not usually appear to develop a capsule, but according to Hiss, the absence of a capsule is often only apparent, and if in making cover-glass preparations off such media some ox or rabbit serum be used as the diluent, and the films stained by his copper-sulphate method (p. 109), a capsule can be demonstrated. Capsulation frequently appears in fluid serum media, *e.g.*, can be readily recognised if the



organism be grown in rabbit or human serum which has been obtained under aseptic precautions and heated for half an hour at 55° C.

The pneumococcus ferments saccharose, raffinose, and lactose, and a similar fermentative action on inulin is important, as ordinary streptococci do not so readily ferment this sugar. Apparently some samples of inulin are more readily acted on than others. Usually the test is carried out with Hiss's inulin serum water medium, in which coagulation of the serum results (p. 47), but some investigators have had more success with inulin bouillon, acid production being estimated by titration against soda with a phenolphthalein indicator.

There has been described by Eyre and Washbourn a non-pathogenic type of the pneumococcus which may be found in the healthy mouth, and which may also be produced during the saprophytic growth of the virulent form. From the latter it differs generally in its more vigorous growth, in producing a uniform cloud in bouillon, in slowly liquefying gelatin, and in growing on potato. The facts that in cultures the pneumococcus often grows in chains, and that occasionally streptococci are found to develop capsules, have raised the question of the relationship of the pneumococcus to other streptococci. When, however, biological characters are taken along with morphological, relatively little difficulty arises in the recognition of a true pneumococcus. Here the reaction in inulin is important. It may be said that the capacity of a capsulated organism to produce acid from this sugar makes its being a true pneumococcus extremely probable. That the pneumococcus may be related to other streptococci is, however, shown by the fact that both sets of organisms tend to originate common group agglutinins.

Considerable attention has been devoted to a bacterium originally described by Schottmüller, and called by him the *Streptococcus mucosus*. This organism has been isolated from a variety of suppurative conditions and also from certain cases of pneumonia. In culture, it differs from the pneumococcus in the colonies being more clear, transparent, and dewdrop-like, showing great tendency to confluence, and being more slimy than those of the pneumococcus. It coagulates the serum in Hiss's inulin serum water medium. It is pathogenic to white mice, but its pathogenicity in the rabbit seems to be less than that of the true pneumococcus. Its agglutinative reactions are somewhat peculiar. Unlike the pneumococcus, it produces in animals only a weak agglutinating serum, but such a serum often can agglutinate pneumococci. Further, antipneumococcal sera frequently



agglutinate the streptococcus mucosus more readily than other streptococci. All the facts seem to point to this organism being closely allied to the true pneumococcus.

**The Cultivation of Friedländer's Pneumobacillus.**—This organism, when present in sputum or in a pneumonic lung, can

be readily separated by making ordinary gelatin plate cultures, or a series of successive strokes on agar tubes. The surface colonies always appear as white discs which become raised from the surface so as to resemble little knobs of ivory. From these, pure cultures can be readily obtained. The appearance of a stab culture in gelatin is very characteristic. At the site of the puncture, there is on the surface a white growth heaped up, it may be fully one-eighth of an inch, above the level of the gelatin; along the needle track there is a white granular appearance, so that the whole resembles a white round-headed nail driven into the gelatin (Fig. 67). Hence the name "nail-like" which has been applied. Occasionally bubbles of gas develop along the line of growth. There is no liquefaction of the medium. On sloped *agar* it forms a very white growth with a shiny lustre, which, when touched with a platinum needle, is found to be of a viscous consistence. In cultures much longer rods are formed than in the tissues of the body (Fig. 68). On the surface of *potatoes* it forms an abundant moist white layer. It is non-motile. Friedländer's bacillus has active fermenting powers on sugars, though varieties isolated by different observers vary in the degree in which such powers are



FIG. 67.—Stab culture of Friedländer's pneumobacillus in peptone gelatin, showing the nail-like appearance; ten days' growth. Natural size.

possessed. It always seems capable of acting on dextrose, lactose, maltose, dextrin, and mannite, and sometimes also on glycerin. The substances produced by the fermentation vary with the sugar fermented, but include ethylic alcohol, acetic acid, lævolactic acid, succinic acid, along with hydrogen and carbonic acid gas. The amount of acid produced from lactose



seems only exceptionally sufficient to cause coagulation of milk. With regard to indol formation the results of different observers vary. Here, as with other reactions, it is to be noted that only strains isolated from cases of pneumonia are to be taken into account. It is said by some that the bacillus is identical with an organism common in sour milk, and also a normal inhabitant of the human intestine, namely, the bacterium *lactis aerogenes* of Escherich.

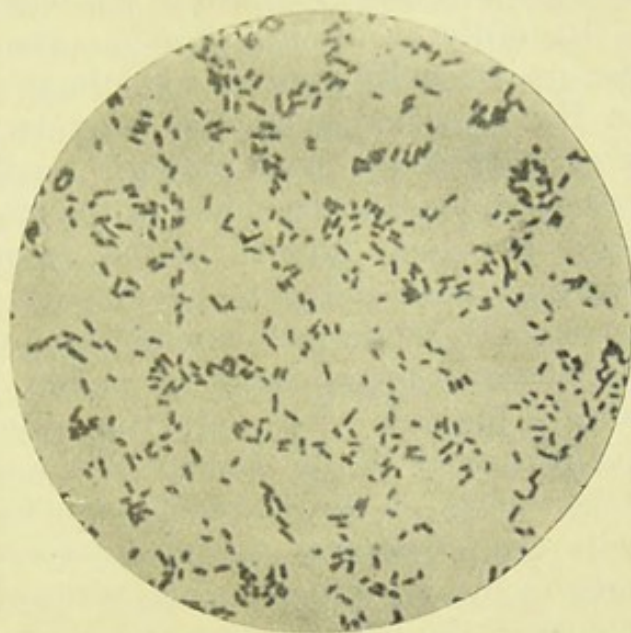


FIG. 68.—Friedländer's pneumobacillus,<sup>1</sup> from a young culture on agar, showing some rod-shaped forms. Stained with thionin-blue.  $\times 1000$ .

**The Occurrence of the Pneumobacteria in Pneumonia and other Conditions.**—Capsulated organisms have been found in every variety of the disease—in acute croupous pneumonia, in broncho-pneumonia, in septic pneumonia. In the great majority of these it is Fraenkel's pneumococcus which both microscopically and culturally has been found to be present. Friedländer's pneumobacillus occurs in only about 5 per cent. of the cases. It may be present alone or associated with Fraenkel's organism. In a case of croupous pneumonia the pneumococci are found all through the affected area in the lung, especially in the exudation in the air-cells. They also occur in the pleural exudation and effusion, and in the lymphatics of the lung. The greatest number are found in the parts where the inflammatory process is most recent, *e.g.* in an area of acute congestion in a case of croupous pneumonia, and therefore such parts are preferably to be selected for microscopic examination, and as the source of cultures. Sometimes there occur in pneumonic consolidation areas of suppurative softening, which may spread diffusely. In such areas the pneumococci occur with or without ordinary pyogenic organisms, streptococci being the commonest concomitants. In

<sup>1</sup> The apparent size of this organism, on account of the nature of its sheath, varies much according to the stain used. If stained with a strong stain, *e.g.* carbol-fuchsin, its thickness appears nearly twice as great as is shown in the figure.



other cases, especially when the condition is secondary to influenza, gangrene may supervene and lead to destruction of large portions of the lung. In these a great variety of bacteria, both aerobes and anaerobes are to be found.

In ordinary broncho-pneumonias also Fraenkel's pneumococcus is usually present, sometimes along with pyogenic cocci; in the broncho-pneumonias secondary to diphtheria it may be accompanied by the diphtheria bacillus, and also by pyogenic cocci; in typhoid pneumonias the typhoid bacillus or the *b. coli* may be alone present or be accompanied by the pneumococcus, and in influenza pneumonias the influenza bacillus may occur. In septic pneumonias the pyogenic cocci in many cases are the only organisms discoverable, but the pneumococcus may also be present. Especially important, as we shall see, from the point of view of the etiology of the disease, is the occurrence in other parts of the body of pathological conditions associated with the presence of the pneumococcus. By direct extension to neighbouring parts empyema, pericarditis, and lymphatic enlargements in the mediastinum and neck may take place; in the first the pneumococcus may occur either alone or with pyogenic cocci. But distant parts may be affected, and the pneumococcus may be found in suppurations and inflammations in various parts of the body (subcutaneous tissue, peritoneum (especially in children), joints, kidneys, liver, etc.), in otitis media, ulcerative endocarditis (p. 216), and meningitis. In fact, there is practically no inflammatory or suppurative condition in the body in which the pneumococcus in pure culture may not be found. These conditions may take place either as complications of pneumonia, or they may constitute the primary disease. The occurrence of meningitis is of special importance, for next to the lungs the meninges appear to be the parts most liable to attack by the pneumococcus. A large number of cases have been investigated by Netter, who gives the following tables of the relative frequency of the primary infections by the pneumococcus in man:—

(1) In adults—

Pneumonia	.	.	.	.	.	.	65·95 per cent.
Broncho-pneumonia	}	.	.	.	.	.	15·85 „
Capillary bronchitis		.	.	.	.	.	
Meningitis	.	.	.	.	.	.	13·00 „
Empyema	.	.	.	.	.	.	8·53 „
Otitis	.	.	.	.	.	.	2·44 „
Endocarditis	.	.	.	.	.	.	1·22 „
Liver abscess	.	.	.	.	.	.	1·22 „

(2) In children 46 cases were investigated. In 29 the primary affection was otitis media, in 12 broncho-pneumonia, in 2 meningitis, in 1 pneumonia, in 1 pleurisy in 1 pericarditis.



Thus in children the primary source of infection is in a great many cases an otitis media, and Netter concludes that infection takes place in such conditions from the nasal cavities.

As bearing on the occurrence of pneumococcal infections secondary to such a local lesion as pneumonia, it is important to note that in a large proportion of cases of the latter disease the pneumococcus can be isolated from the blood.

**Experimental Inoculation.**—The *pneumococcus* of Fraenkel is pathogenic to various animals, though the effects vary somewhat with the virulence of the race used. The susceptibility of different species, as Gamaleia has shown, varies to a considerable extent. The rabbit, and especially the mouse, are very susceptible; the guinea-pig, the rat, the dog, and the sheep occupy an intermediate position; the pigeon is immune. In the more susceptible animals the general type of the disease produced is not pneumonia, but a general *septicæmia*. Thus, if a rabbit or a mouse be injected subcutaneously with pneumonic sputum, or with a scraping from a pneumonic lung, death occurs in from twenty-four to forty-eight hours. There is some fibrinous infiltration at the point of inoculation, the spleen is often enlarged and firm, and the blood contains capsulated pneumococci in large numbers (Fig. 69). If the seat of inoculation be in the lung, there generally results pleuritic effusion on both sides, and in the lung there may be a process somewhat resembling the early stage of acute croupous pneumonia in man. There are often also pericarditis and enlargement of spleen. We have already stated that cultures of the pneumococcus on artificial media in a few days begin to lose their virulence. Now, if such a partly attenuated culture be injected subcutaneously into a rabbit, there is greater local reaction; pneumonia, with exudation of lymph on the surface of the pleura, and a similar condition in the peritoneum, may occur. In sheep greater immunity is marked by the occurrence, after subcutaneous inoculation, of an enormous local sero-fibrinous exudation, and by the fact that few pneumococci are found in the blood stream. Intra-pulmonary injection in sheep is followed by a typical pneumonia, which is generally fatal. The dog is still more immune; in it also intra-pulmonary injection is followed by a fibrinous pneumonia, which is only sometimes fatal. Inoculation by inhalation appears only to have been performed in the susceptible mouse and rabbit; here also septicæmia resulted.

The general conclusion to be drawn from these experiments thus is that in highly susceptible animals virulent pneumococci



produce a general septicaemia ; whereas in more immune species there is an acute local reaction at the point of inoculation, and if the latter be in the lung, then there may result pneumonia, which, of course, is merely a local acute inflammation occurring in a special tissue, but identical in essential pathology with an inflammatory reaction in any other part of the body. When a dose of pneumococci sufficient to kill a rabbit is injected sub-

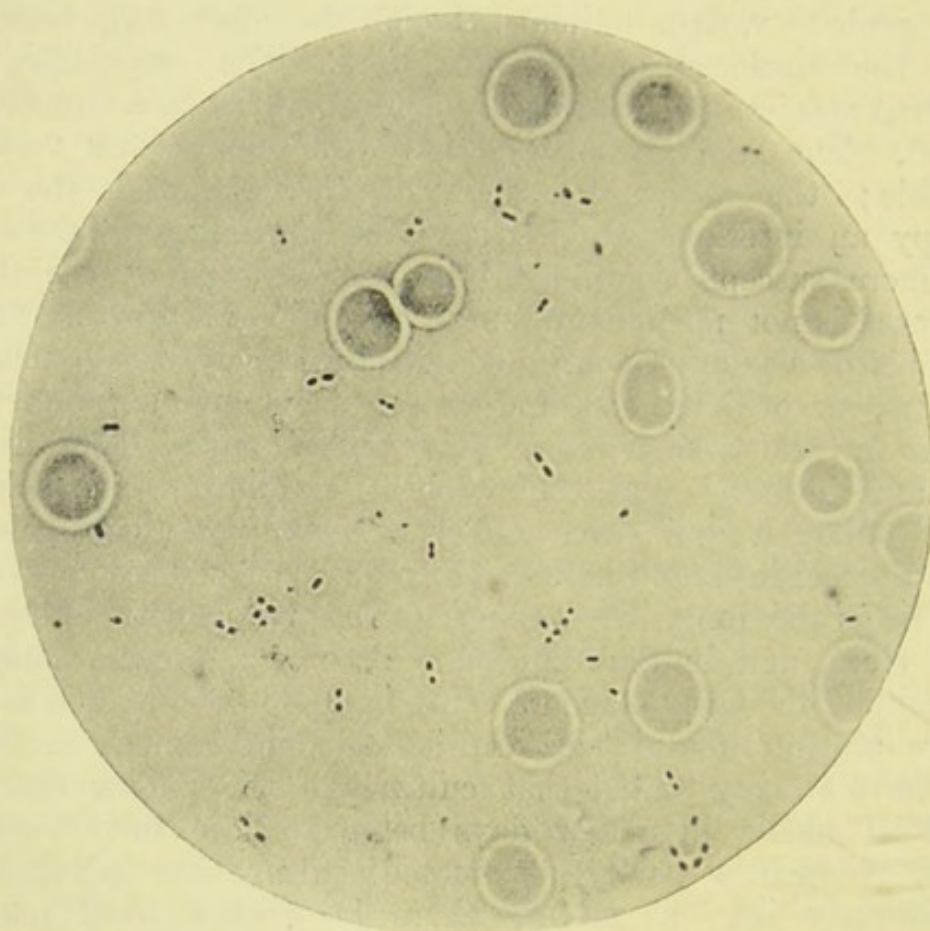


FIG. 69.—Capsulated pneumococcus in blood taken from the heart of a rabbit, dead after inoculation with pneumonic sputum.

Dried film, fixed with corrosive sublimate. Stained with carbol-fuchsin and partly decolorised.  $\times 1000$ .

cutaneously in the human subject, it gives rise to a local inflammatory swelling with redness and slight rise of temperature, all of which pass off in a few days. It is therefore justifiable to suppose that man occupies an intermediate place in the scale of susceptibility, probably between the dog and the sheep, and that when the pneumococcus gains an entrance to his lungs the local reaction in the form of pneumonia occurs. In this connection the occurrence of manifestations of general infection associated with pneumonia in man is of the highest import-



ance. We have seen that meningitis and other inflammations are not very rare complications of the disease, and such cases form a link connecting the local disease in the human subject with the general septicæmic processes which may be produced artificially in the more susceptible representatives of the lower animals.

A fact which at first appeared rather to militate against the pneumococcus being the cause of pneumonia was the discovery of this organism in the saliva of healthy men. This fact was early pointed out by Pasteur, and also by Fraenkel, and the observation has been confirmed by many other observers. It can certainly be isolated from the mouths of a considerable proportion of normal men, from their nasal cavities, etc., being probably in any particular individual more numerous at some times (especially, it is stated, during the winter months, *i.e.* a little before the period of the greatest prevalence of pneumonia) than at others, and sometimes being entirely absent. This can be proved, of course, by inoculation of susceptible animals. Such a fact, however, only indicates the importance of predisposing causes in the etiology of the disease, and it is further to be observed that we have corresponding facts in the case of the diseases caused by pyogenic staphylococci, streptococci, the bacillus coli, etc. It is probable that by various causes the vitality and power of resistance of the lung are diminished, and that then the pneumococcus gains an entrance. In relation to this possibility we have the very striking fact that in the irregular forms of pneumonia, secondary to such conditions as typhoid and diphtheria, the pneumococcus is very frequently present, alone or with other organisms. Apparently the effects produced by such bacteria as the *b. typhosus* and the *b. diphtheriæ* can devitalise the lung to such an extent that secondary infection by the pneumococcus is more likely to occur and set up pneumonia. We can therefore understand how much less definite devitalising agents such as cold, alcoholic excess, etc., can play an important part in the causation of pneumonia. In this way also other abnormal conditions of the respiratory tract, a slight bronchitis, etc., may play a similar part.

It is more difficult to explain why sometimes the pneumococcus is associated with a spreading inflammation, as in croupous pneumonia, whilst at other times it is localised to the catarrhal patches in broncho-pneumonia. It is quite likely that in the former condition the organism is possessed of a different order of virulence, though of this we have no direct proof. We have,



however, a closely analogous fact in the case of erysipelas; this disease, we have stated reasons for believing, is produced by a streptococcus which, when less virulent, causes only local inflammatory and suppurative conditions.

*Summary.*—We may accordingly summarise the facts regarding the relation of Fraenkel's pneumococcus to the disease by saying that it can be isolated from nearly all cases of acute croupous pneumonia, and also from a considerable proportion of other forms of pneumonia. When injected into the lungs of moderately insusceptible animals it gives rise to pneumonia. If, in default of the crucial experiment of intra-pulmonary injection in the human subject, we take into account the facts we have discussed, we are justified in holding that it is the chief factor in causing croupous pneumonia, and also plays an important part in other forms. Pneumonia, in the widest sense of the term, is, however, not a specific affection, and various inflammatory conditions in the lungs can be set up by the different pyogenic organisms, by the bacilli of diphtheria, of influenza, etc.

The possibility of Friedländer's *pneumobacillus* having an etiological relationship to pneumonia has been much disputed. Its discoverer found that it was pathogenic towards mice and guinea-pigs, and to a less extent towards dogs. Rabbits appeared to be immune. The type of the disease was of the nature of a septicæmia. No extended experiments, such as those performed by Gamaleia with Fraenkel's coccus, have been done, and therefore we cannot say whether any similar pneumonic effects are produced by it in partly susceptible animals. The organism appears to be present alone in a small number of cases of pneumonia, and the fact that it also appears to have been the only organism present in certain septicæmic complications of pneumonia, such as empyema and meningitis, render it possible that it may be the causal agent in a few cases of the disease. It is also stated to have been observed in certain cases of appendicitis and occasionally in pyæmic cases.

In the septic pneumonias the different pyogenic organisms already described are found, and sometimes in ordinary pneumonias, especially the catarrhal forms, other organisms, such as the *b. coli* or its allies, may be the causal agents.

**The Pathology of Pneumococcus Infection.**—The effects of the action of the pneumococcus, at any rate in a relatively insusceptible animal such as man, seem to indicate that toxins may play an important part. Pneumonia is a disease which presents in many respects the character of an acute poisoning. In very few cases does death take place from the functions of



the lungs being interfered with to such an extent as to cause asphyxia. It is from cardiac failure, from grave interference with the heat-regulating mechanism, and from general nervous depression that death usually results. These considerations, taken in connection with the fact that in man the organisms are found in the greatest numbers in the lung, suggest that a toxic action is at work. Various attempts have been made to isolate toxins from cultures of the pneumococcus, *e.g.*, by precipitating bouillon cultures with alcohol or ammonium sulphate, and poisonous effects have been produced by certain substances thus derived; but the effects produced are, as in so many other similar cases, of a non-specific character, and to be classed as interferences with general metabolism. The general conclusion has been that the toxins at work in pneumonia are intracellular; but no special light has been thrown on the common effects of the members of this group of bacterial poisons.

*Immunisation against the Pneumococcus.*—Animals can be immunised against the pneumococcus by inoculation with cultures which have become attenuated by growth on artificial media, or with the naturally attenuated cocci which occur in the sputum after the crisis of the disease. Netter effected immunisation by injecting an emulsion of the dried spleen of an animal dead of pneumococcus septicaemia. Virulent cultures killed by heating at 62° C., rusty sputum kept at 60° C. for one to two hours and then filtered, and filtered or unfiltered bouillon cultures similarly treated have also been used. In all cases one or two injections, at intervals of several days, are sufficient for immunisation, but the immunity has often been observed to be of a fleeting character and may not last more than a few weeks. The serum of such immunised animals protects rabbits against subsequent inoculation with pneumococci, and if injected within twenty-four hours after inoculation, may prevent death. A protective serum was obtained by Washbourn, who employed pneumococcus cultures of constant virulence. This observer immunised a pony by using successively (1) broth cultures killed by one hour's exposure to 60° C.; (2) living agar cultures; (3) living broth cultures. From this animal there was obtained a serum which protected susceptible animals against many times an otherwise fatal dose, and which also had a limited curative action. It is stated that the serum of patients who have recovered from pneumonia has in a certain proportion of cases a protective effect against the pneumococcus in rabbits, similar to that exhibited by the serum of immune animals.

The Klemperers treated a certain number of cases of human



pneumonia by serum derived from immune animals, and apparently with a certain measure of success, and sera prepared by Washbourn and by others have also been used. The results obtained by different observers have, however, been rather contradictory. The use of these sera apparently causes the temperature in some cases to fall, and even may hasten a crisis, but further experience is necessary before their value in therapeutics can be properly estimated.

There has been considerable difference of opinion as to the explanations to be given of the facts observed regarding immunisation against the pneumococcus and especially regarding the properties of immune sera. At first these sera were supposed to possess antitoxic qualities—largely on the ground that no bactericidal effect was produced by them on the bacterium *in vitro*. As no specific toxin has been proved to be concerned in the action of the organism, the development of an antitoxin during immunisation must, in the present state of knowledge, be looked on as not yet proved. To explain the action of a serum in preventing and curing pneumococcal infections, it has been thought to have the complex character seen in anti-typhoid sera in which two substances—immune body and complement (see Immunity)—are concerned, and the variability in the therapeutic results obtained has been accounted for on the view that there might be a deficiency of complement, such as occurs in other similar cases. The absence of bactericidal effect, however, raises several difficult points. It is stated that no such effect is observable either in immune sera, or in the serum of patients who have successfully come through an attack of the natural disease. Some effect of the kind would be expected to be present if the anti-pneumonic serum were quite comparable to the anti-typhoid serum. Within recent times many have turned to the opsonic property of sera to account for the facts observed. In this connection Mennes observed that normal leucocytes only become phagocytic towards pneumococci when they are lying in the serum of an animal immunised against this bacterium. Wright had in his early papers looked to the phagocytosis of sensitised bacteria to explain their destruction in the absence of bactericidal qualities in the serum alone, and Neufeld and Rimpau have described the occurrence of an opsonic effect in the action of an anti-pneumococcic serum. Further work may show that along these lines lies the explanation of the facts observed.

¶ In studying further the relationship of the opsonic effect to pneumococcal infection, inquiry has been directed to the opsonic



qualities of the blood of pneumonic patients, especially with a view to throwing light on the nature of the febrile crisis. According to some results, the opsonic index as compared with that of a healthy person is not above normal, but if the possible phagocytic capacities of the whole blood of the sick person be taken into account, these will probably be much above normal in consequence of the leucocytosis which usually accompanies a successful resistance to this infection. It has been observed, however, that as the crisis approaches in a case which is to recover the opsonic index rises, and after defervescence gradually falls to normal. As bearing on the factors involved in the successful resistance of the organism to the pneumococcus, it has been noted that avirulent pneumococci are more readily opsonised than more virulent strains. It is further stated that avirulent cultures of the pneumococcus can be made to resist phagocytosis if they are treated with the products of the autolysis of virulent strains or with washings from such strains, and that virulent cocci if washed with saline become capable of being readily phagocyted. Further observations along such lines are to be looked for with interest, and it may be said that Wright's vaccination methods have been applied to the treatment of pneumonic cases, and in certain instances are said to have been followed by favourable result. It may be noted here, in conclusion, that in man it is probable that immunity against pneumonia may be short-lived, as in a good many cases of pneumonia a history of a previous attack is elicited.

*Agglutination of the Pneumococcus.*—If a small amount of a culture of Fraenkel's pneumococcus be placed in an anti-pneumococcic serum, an aggregation of the bacteria into clumps occurs. Such an agglutination, as it is called, is frequently observed under similar circumstances with other bacteria. The phenomenon is not invariably associated with the presence of protective bodies in a serum, but it has been used for diagnostic purposes in the differentiation of sore throats due to pneumococcus infection from those due to other bacteria. Whether the method is reliable has still to be proved. It has been shown that a serum which agglutinates the pneumococcus may also agglutinate streptococci isolated from various sources. Such organisms are, however, not so uniformly agglutinated by a pneumococcus serum as are pneumococci isolated from pneumonic cases.

**Methods of Examination.**—These have been already described, but may be summarised thus: (1) Microscopic. Stain films from the densest part of the sputum or from the area of spreading inflammation in the lung by Gram's method



and by carbol-fuchsin, etc. (pp. 105, 108), in the latter case it is usually best not to decolorise the groundwork of the preparation.

(2) By cultures. (a) *Fraenkel's pneumococcus*. With similar material make successive strokes on agar, blood agar, or blood serum. The most certain method, however, is to inject some of the material containing the suspected cocci into a rabbit. If the pneumococcus be present the animal will die, usually within forty-eight hours, with numerous capsulated pneumococci in its heart blood. With the latter inoculate tubes of the above media and observe the growth. In some cases of severe pneumococcic infection the organism may be cultivated from the blood obtained by venesection (p. 72). (b) *Friedländer's pneumobacillus* can be readily isolated either by ordinary gelatin plates or by successive strokes on agar media.

#### EPIDEMIC CEREBRO-SPINAL MENINGITIS.

As the result of observations on this disease in different parts of the world, it has been now established that the causal agent



FIG. 70.—Film preparation of exudation from a case of meningitis, showing the diplococci within leucocytes. See also Plate I., Fig. 3. Stained with carbol-thionin-blue.  $\times 1000$ .

is the *diplococcus intracellularis meningitidis*, first described by Weichselbaum, and now often known as the meningococcus. This organism is a small coccus measuring about  $1\ \mu$  in diameter and usually occurs in pairs, the adjacent sides being somewhat flattened against each other. In most cases the cocci are chiefly contained within polymorphonuclear leucocytes in the exudation (Fig. 70); in some cases, however, the majority may be lying free. It stains readily with basic

aniline dyes, but loses the stain in Gram's method. Both in appearance and in its staining reactions it is similar to the gonococcus (*vide infra*). The organism can readily be cultivated outside the body, but the conditions of growth



are somewhat restricted—agar with an admixture of serum or blood (preferably human) is most suitable (p. 43). Strains separated in different epidemics appear to present slight individual variations, but the following description may be taken as summing up the common characters:—Growth takes place best at the temperature of the body, and practically ceases at 25° C. On serum agar the colonies are circular discs of somewhat transparent appearance, and possessing a smooth, shining surface; they have little tendency to become confluent. When examined under a low magnification the colour is seen to be somewhat yellowish, and the margins usually are smooth and regular, though on some media slight crenation may appear. The colonies may be of considerable size, reaching sometimes a diameter of 2 to 3 mm. on the third day. On plain agar the colonies are very much smaller, and sometimes no growth occurs; subcultures especially often fail to give any growth on this medium. In serum bouillon the organism produces a general turbidity with formation of some deposit after a day or two. It ferments

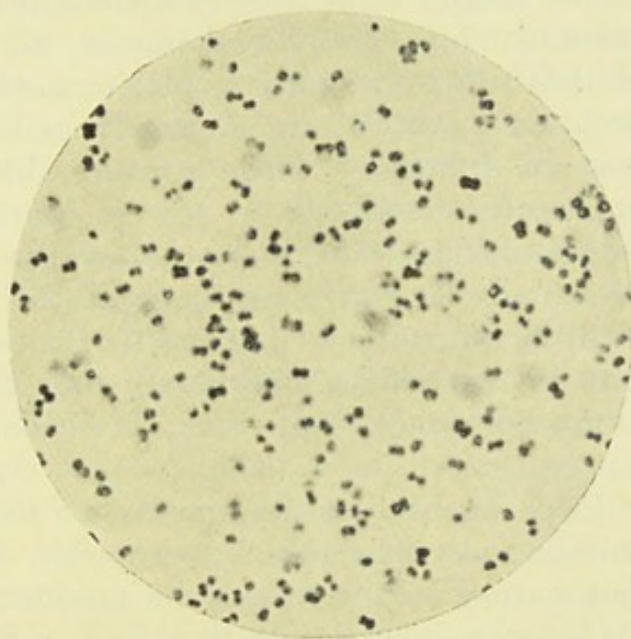


FIG. 71.—Pure culture of diplococcus intracellularis, showing involution forms.

maltose and dextrose with acid production, a property which distinguishes it from the micrococcus catarrhalis (*vide infra*). Fermentation tests are most satisfactorily carried out by means of solid serum media containing 1 per cent. of the sugar to be tested (p. 80). In all cases growth occurs best when the medium has a neutral or very slightly alkaline reaction. In cultures the organism presents the same appearance as in the body, and often shows tetrad formation. There is also a great tendency to the production of involution forms (Fig. 71), many of the cocci becoming much swollen, staining badly, and afterwards undergoing disintegration. This change, according to Flexner's observations, would appear to be due to the production of an autolytic enzyme, and he has also found that this substance has the property of producing dissolution of the bodies of other



bacteria. The life of the organism in cultures is a comparatively short one; after a few days cultures will often be found to be dead, but, by making sub-cultures every three or four days, strains can be maintained alive for considerable periods. The organism is readily killed by heat at  $60^{\circ}$  C., and it is also very sensitive to weak antiseptics; drying for a period of a day has been found to be fatal to it. The facts established accordingly show it to be a somewhat delicate parasite.

As stated above, the organism occurs in the exudate in the meninges and in the cerebro-spinal fluid, and it can usually be obtained by lumbar puncture. In acute cases, especially in the earlier stages, it is usually abundant; but in the later stages of cases of more sub-acute character, its detection may be a matter of difficulty, and only a few examples may be found after a prolonged search; in extremely acute cases also the organism may be difficult to demonstrate. In most cases the disease is practically restricted to the nervous system, but occasionally complications occur, and in these the organism may sometimes be found. It has been observed, for example, in arthritis, pericarditis, pneumonic patches in the lung, and in other inflammatory conditions associated with the disease. In a certain number of cases it has been obtained from the blood during life, but cultures in most instances give negative results.

Experimental inoculation shows that the ordinary laboratory animals are relatively insusceptible to this organism. An inflammatory condition may be produced in mice and guinea-pigs by intra-peritoneal injection, and a fatal result may follow; in such cases the organism does not seem to undergo very active multiplication, though it may sometimes be cultivated from the blood, and none of the lesions in the nervous system are reproduced. Flexner and Stuart M'Donald have shown that cerebro-spinal meningitis may be produced in monkeys by injections of the organism into the spinal canal, the latter observer finding that exudate containing meningococci was more effective than cultures. In such experiments the organism extends upwards to the brain, and produces meningitis within a very short time. The resulting lesions, both as regards their distribution and general characters, and also as regards the histological changes, resemble the disease in the human subject. Even these animals, however, are manifestly less susceptible than the human subject.

Many questions of great importance with regard to the spread of the disease still require further investigation. The organism has been obtained by culture from the throat and nasal cavities



of those suffering from the disease in a considerable number of instances. It has also been obtained from the same positions in healthy individuals, during an epidemic of the disease. In some epidemics also a pharyngitis has been found to occur, and the organism has been obtained from the affected fauces. The majority of workers at this subject are inclined to believe that the organism spreads by means of the lymphatics from the pharynx or nose to the base of the brain, but direct evidence that this occurs has not been supplied. On the other hand, the facts established with regard to other infections make it quite probable that the organism gains entrance to the blood stream from the upper respiratory passages, and then settles in the meninges. Infection by the alimentary canal, the organisms thereafter reaching the spinal meninges by the lymphatics, has been suggested as a possibility, but such a view does not appear to have much support. Whatever may be found to be the path by which the organism reaches the brain, the evidence at present tends to show that the entrance of the organism into the body is by the naso-pharynx, and that this usually results by inhalation of the organism distributed in fine particles of expectoration, etc. In fact, as regards the mode and conditions of infection, an analogy would appear to hold between this disease and influenza.

Apart from the epidemic form of the disease, cases of sporadic nature also occur, in which the lesions are of the same nature, and in which the meningococcus is present. The facts stated would indicate that the origin and spread of the disease in the epidemic form depend on certain conditions which produce an increased virulence of the organism. We are, however, as yet entirely ignorant as to what these conditions may be. In simple posterior basal meningitis in children a diplococcus is present, as described by Still, which has the same microscopic and cultural characters as the diplococcus intracellularis; it has been regarded as probably an attenuated variety of the latter. Recently, however, Houston and Rankin have found that the serum of a patient suffering from epidemic meningitis does not exert the same opsonic and agglutinative effects on the diplococcus of basal meningitis as on the diplococcus intracellularis; and this result points to the two organisms being distinct, though closely allied, species.

**Serum Reactions.**—An agglutination reaction towards the diplococcus intracellularis is given by the serum of patients suffering from the disease, where life is prolonged for a sufficient length of time. It usually appears about the fourth day, when



the serum may give a positive reaction in a dilution of 1 : 50 ; at a later stage it has been observed in so great a dilution as 1 : 1000. Specific opsonins may appear in the blood about the same time, and though they are not always proportional in amount to the agglutinins, the two classes of substances have pretty much the same significance, and may occasionally be of use in diagnosis when lumbar puncture fails to give positive results. Although their presence in large amounts may be said to indicate a marked reaction, they do not supply information of much value in relation to prognosis. Immune-bodies, as shown by bactericidal and deviation of complement tests (p. 126, 130), may also be developed in considerable amount in the course of the disease.

*Anti-sera* for therapeutical purposes have been introduced by various workers, and of these the one which has been most extensively used is that of Flexner and Jobling. This serum is prepared from the horse by repeated injections in increasing doses of dead cultures, followed by injections of culture autolysate and of living cultures, these two latter being best administered by the subcutaneous method. Several strains of meningococci are mixed together for purposes of injection, and the immunisation is continued over a period of several months. For treatment of the disease the serum is injected under the spinal dura, 30 c.c. being generally used for an injection, this being continued for several days. This serum has been used on a large scale in various parts of the world, and there is general agreement as to its favourable effects—the mortality of the disease, which is generally 70 to 80 per cent., having been reduced to about 30 per cent. or even less. By means of its use the tendency to the occurrence of chronic lesions has also been markedly diminished. The action of such anti-sera cannot as yet be fully explained. They certainly contain opsonins, agglutinins, immune-bodies which bind complement, and possibly also anti-endotoxins. After the injection the number of meningococci becomes markedly reduced, probably as a result of increased phagocytosis ; there can scarcely be any direct bactericidal action owing to the absence of complement. The standardisation of such anti-sera is a matter of some difficulty ; at first the deviation of complement method was used (p. 130), but now the opsonic index is regarded with more favour as an index of the potency of the serum. Mackenzie and Martin have treated cases by the intra-spinal injection of the fresh serum of patients suffering from the disease or who have recovered from it, such serum being in



many cases rich in immune-bodies for the meningococcus, and possessing a greatly increased bactericidal action as compared with normal serum. Though the number of cases treated by this method is not yet large, a distinctly favourable result has been obtained.

**Allied Diplococci.**—In the naso-pharynx there occur other Gram-negative diplococci which have a close resemblance to the diplococcus intracellularis. These occur in the healthy state, but are especially abundant in catarrhal conditions. Of these the *diplococcus catarrhalis* has the closest resemblance to the diplococcus intracellularis. In addition to occurring in health this organism has also been found in large numbers in epidemic catarrh. Its microscopic appearances are practically similar to those described above, and it also occurs within leucocytes. Its colonies on serum agar are more opaque than those of the diplococcus intracellularis, and often have a somewhat firm though friable consistence, so that they are sometimes removed *en masse* by the platinum needle. The organism grows on gelatin at 20° C. without liquefying the medium, and it has none of the fermentative properties described above as belonging to the diplococcus intracellularis. The *diplococcus pharyngis siccus* (v. Lingelsheim) also grows at room temperature, and its colonies are very tough and adhere to the surface of the medium; it can thus readily be distinguished from the meningococcus. It has marked fermentative properties, acting on glucose, maltose, saccharose, and lævulose. The *diplococcus mucosus* has colonies of slimy consistence; it grows at room temperature, and it forms capsules, which can be demonstrated by the method of Hiss. There are other Gram-negative diplococci which are chromogenic, and thus can readily be distinguished. The points of difference between the meningococcus and the gonococcus are given on p. 252. A Gram-positive diplococcus called the *diplococcus crassus* is also of common occurrence; it is rather larger than the diplococcus intracellularis, and especially in sub-cultures may tend to assume staphylococcal forms.

Apart from the epidemic form of the disease, meningitis may be produced by almost any of the organisms described in the previous chapter, as associated with inflammatory conditions. A considerable number of cases, especially in children, are due to the pneumococcus. In many instances where no other lesions are present the extension is by the Eustachian tube to the middle ear. In other cases the path of infection is from some other lesion by means of the blood stream. This organism also infects the meninges not infrequently in lobar pneumonia, and in some



cases with head symptoms we have found it present where there was merely a condition of congestion. The pneumobacillus also has been found in a few cases. Meningitis is not infrequently produced by streptococci, especially when middle-ear disease is present, less frequently by one of the staphylococci; occasionally more than one organism may be concerned. In meningitis following influenza the influenza bacillus has been found in a few instances, but sometimes the pneumococcus is the causal agent. Cases of meningitis occur associated with organisms which resemble the influenza bacillus morphologically and also in presenting haemophilic culture reactions, but which possess pathogenic properties for rabbits and guinea-pigs. These bacilli frequently both in the cerebro-spinal fluid and in cultures show a tendency to produce long filamentous forms and also under both circumstances may show a beading of the protoplasm which gives them a diphtheroid appearance. Gram-negative anaerobic bacilli have also been found in cases of meningitis. An invasion of the meninges by the anthrax bacillus occurs, but is a rare condition; it is attended by diffuse hæmorrhage in the subarachnoid space. In tubercular meningitis the tubercle bacillus, of course, is present, especially in the nodules along the sheaths of the vessels.

In conclusion, here it may be stated that mixed infections may occur in meningitis. Thus the pneumococcus has been found associated with the tubercle bacillus and also with the diplococcus intracellularis.

**Methods of Examination.**—During life these involve the microscopic investigation of the centrifuged lumbar puncture fluid and making cultures therefrom. For the former, Gram-stained smears make the recognition of the meningococcus relatively easy, and the presence of Gram-negative cocci, especially within cells, is practically diagnostic of a case of cerebro-spinal fever. Tubes of serum-agar, nasgar (pp. 42, 43), or agar containing 25 per cent. of ascitic or ovarian fluid, may then be inoculated. The difficult cases are those where no bacteria can be found microscopically in the lumbar fluid. Here the character of the exudate may give help. A predominance of polymorphonuclear cells is usually manifest in meningococcic, pneumococcic, and influenzal cases, whereas in tubercular meningitis the exudate is chiefly lymphocytic. In such circumstances, besides other media, a tube of blood-smeared agar should be inoculated in case the pneumococcus or the influenza bacillus is the causal organism. To speak generally, if with a polymorphonuclear exudate no growth occurs in the media mentioned the case is most likely to be due to the meningococcus. In tubercular cases it is sometimes impossible to demonstrate the bacilli microscopically in the exudate, though on careful search they may often be found.



## CHAPTER IX.

### GONORRHOEA AND SOFT SORE.

#### GONORRHOEA.

**Introductory.**—The micrococcus now known to be the cause of gonorrhœa, and called the *gonococcus*, was first described by Neisser, who in 1879 gave an account of its microscopical characters as seen in the pus of gonorrhœal affections, both of the urethra and of the conjunctiva. He considered that this organism was peculiar to the disease, and that its characters were distinctive. Later it was successfully isolated and cultivated on solidified human serum by Bumm and others. Its characters have since been minutely studied, and by inoculations of cultures on the human subject its causal relationship to the disease has been conclusively established.

**The Gonococcus.—Microscopical Characters.**—The organism of gonorrhœa is a small micrococcus which usually is seen in the diplococcus form, the adjacent margins of the two cocci being flattened, or even slightly concave, so that between them there is a small oval interval which does not stain. An appearance is thus presented which has been compared to that of two beans placed side by side (*vide* Fig. 72). When division takes place in the two members of a diplococcus, a tetrad is formed, which, however, soon separates into two sets of diplococci—that is to say, arrangement as diplococci is much commoner than as tetrads. Cocci in process of degeneration are seen as spherical elements of varying size, some being considerably swollen.

These organisms are found in large numbers in the pus of acute gonorrhœa, both in the male and female, and for the most part are contained within the leucocytes. In the earliest stage, when the secretion is glairy, a considerable number are lying free, or are adhering to the surface of desquamated epithelial cells, but when it becomes purulent the large proportion within leucocytes is a very striking feature. In the leucocytes they lie



within the protoplasm, especially superficially, and are often so numerous that the leucocytes appear to be filled with them, and their nuclei are obscured. As the disease becomes more chronic,



FIG. 72.—Portion of film of gonorrhœal pus, showing the characteristic arrangement of the gonococci within leucocytes. See also Plate I., Fig. 5.

Stained with fuchsin.  $\times 1000$ .

the gonococci gradually become diminished in number, though even in long-standing cases they may still be found in considerable numbers. They are also present in the purulent secretion of gonorrhœal conjunctivitis, also in various parts of the female genital organs when these parts are the seat of true gonorrhœal infection, and they have been found in some cases in the secondary infections of the joints in the disease, as will be described below.

*Staining.*—The gonococcus stains readily and

deeply with a watery solution of any of the basic aniline dyes—methylene-blue, fuchsin, etc. It is, however, easily decolorised, and it completely loses the stain by Gram's method—an important point in the microscopical examination.

**Cultivation of the Gonococcus.**—This is attended with some difficulty, as the conditions of growth are somewhat restricted. The most suitable media are "blood-agar" and the serum media already described for the purpose (pp. 42, 43). It is advisable to inoculate the media within half an hour after obtaining the material from the body, and place the tubes at once in the incubator. Growth takes place best at the temperature of the body, and ceases altogether at  $25^{\circ}\text{C}$ . Cultures are obtained by taking some pus on the loop of the platinum needle and inoculating one of the media mentioned by leaving minute quantities here and there on the surface. The medium may be used either as ordinary "sloped tubes" or as a thin layer in a Petri's capsule. The young colonies are usually visible within forty-eight hours, and often within twenty-four hours; it is important, however, to note that sometimes growth may not appear till the fourth day. They appear around the



points of inoculation as small semi-transparent discs of rounded shape. The colonies vary somewhat in size, and tend to

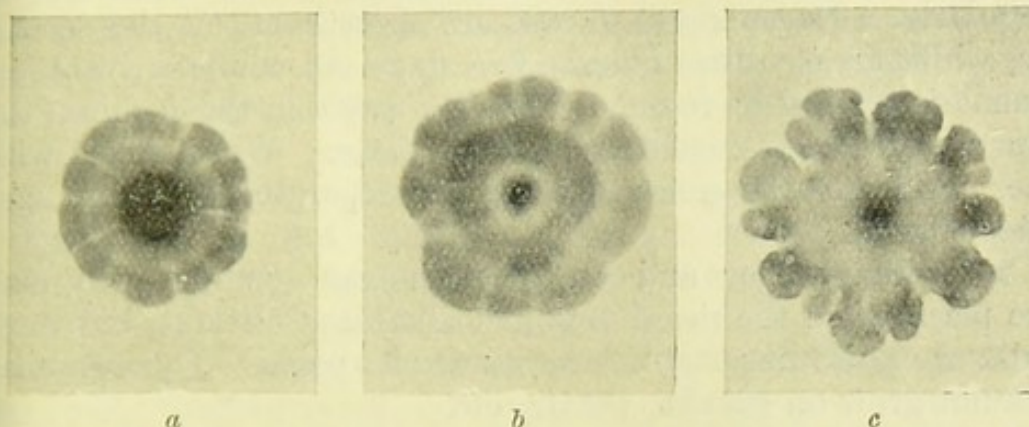


FIG. 73.—Colonies of gonococcus on serum agar; (a) three days' growth; (b) and (c) five days' growth.  $\times 9$ .  
From photographs by Dr. W. B. M. Martin.

remain more or less separate. Later, the margin tends to be undulated and the centre more opaque; a radial marking may be present (Fig. 73). The first cultures die out somewhat quickly, but in sub-cultures, kept at  $37^{\circ}\text{C}$ ., the organism remains alive for a considerable time, sometimes three weeks. After a week more active foci of growth may appear in some of the colonies in the form of heaped-up opaque points, thus giving an appearance suggestive of contamination. In the early stage of the disease the organism is present in the male urethra in practically pure condition, and if the meatus of the urethra be sterilised by washing with weak solution of corrosive sublimate and then with absolute alcohol, and the material for inoculation be expressed from the deeper part of the urethra, cultures may often be obtained which are pure from the first. In culture the

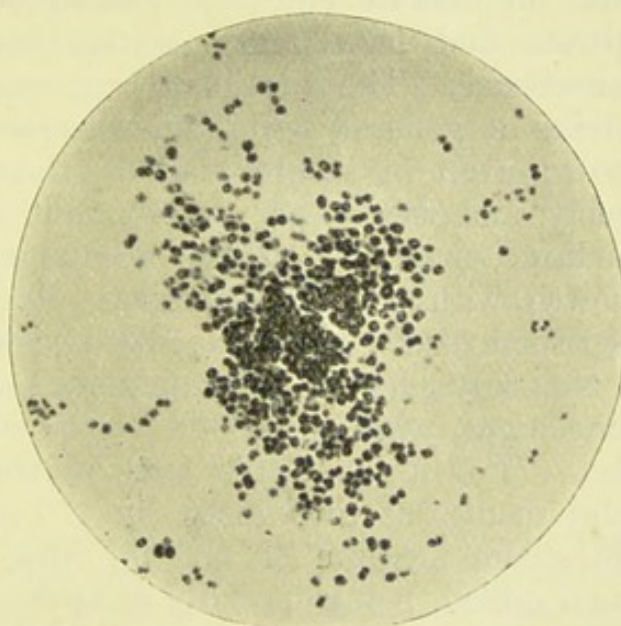


FIG. 74.—Gonococci, from a pure culture on blood agar of twenty-four hours' growth. Some already are beginning to show the swollen appearance common in older cultures.  
Stained with carbol-thionin blue.  $\times 1000$ .



organisms have similar microscopic characters to those described (Fig. 74), but show a remarkable tendency to undergo degeneration, becoming swollen and of various sizes, and staining very irregularly. Degenerated forms are seen even on the second day, whilst in a culture four or five days old comparatively few normal cocci may be found. The less suitable the medium the more rapidly does degeneration take place. When mixed with other organisms the gonococcus may be separated by serum agar plates (p. 43).

On ordinary agar and on glycerin-agar some growth may take place when the reaction is just alkaline to litmus, but these media are quite unsuitable for ordinary purposes. The organism does not grow on gelatin, potato, etc.

**Comparison with Meningococcus.**—The morphological and cultural characters of the gonococcus and meningococcus are in many respects closely similar; the following points are of importance in distinguishing them. The conditions of growth of the gonococcus are more restricted than those of the meningococcus. The gonococcus usually does not grow on the ordinary agar media, whereas the meningococcus grows well, at least after the first sub-culture. The colonies of the latter are more opaque and have more regular margins than those of the gonococcus. The meningococcus grows well in bouillon, producing a general turbidity, whereas the gonococcus does not grow; even in serum bouillon the latter organism flourishes feebly, and the scanty growth falls to the bottom leaving the medium clear, whilst the meningococcus produces abundant growth with general turbidity. The fermentative effects have also been studied, and the chief results obtained are that glucose is the only sugar usually employed which is fermented by the gonococcus, whereas the meningococcus always ferments maltose also. (For fermentative tests in the case of the gonococcus, solid media, as introduced by v. Lingelsheim, should be used, the serum medium of Martin, with litmus and the particular sugar added, being specially suitable.)

Specific serum reactions — agglutination, opsonic action, bactericidal action, and fixation of complement—have been studied by Torrey, Elser and Huntoon, and Martin, in the case of the two organisms. The general results obtained are that each organism represents a somewhat heterogeneous group showing considerable variations as regards the tests mentioned. An anti-gonococcus serum produced by injecting one strain of gonococcus has the maximum effect on that strain, whilst its action on other strains may be much feebler; so also with an



anti-meningococcus serum in relation to different strains of meningococci. An anti-gonococcus serum may have some effect, though usually very slight, on a meningococcus and *vice versa*; this indicates that there are some receptors common to the two organisms. These results are in many ways comparable with the facts established with regard to members of the typhoid-coli group, and are of course quite compatible with the gonococcus and the meningococcus being distinct species.

**Relations to the Disease.**—The gonococcus is invariably present in the urethral discharge in gonorrhœa, and also in other parts of the genital tract when these are the seat of true gonorrhœal infection. Its presence in these different positions has been demonstrated not only by microscopic examination but also by culture. From the description of the conditions of growth in culture it will be seen that a life outside the body in natural conditions is practically impossible—a statement which corresponds with the clinical fact that the disease is always transmitted directly by contagion. Inoculations of pure cultures on the urethra of lower animals, and even of apes, is followed by no effect, but a similar statement can be made with regard to inoculations of gonorrhœal pus itself. In fact, hitherto it has been found impossible to reproduce the disease by any means in the lower animals. On a considerable number of occasions inoculations of pure cultures have been made on the human urethra, both in the male and female, and the disease, with all its characteristic symptoms, has resulted. (Such experiments have been performed independently by Bumm, Steinschneider, Wertheim, and others.) The causal relationship of the organism to the disease has therefore been completely established, and it is interesting to note how the conditions of growth and the pathogenic effects of the organism agree with the characters of the natural disease.

Intraperitoneal injections of pure cultures of the gonococcus in white mice produce a localised peritonitis with a small amount of suppuration, the organisms being found in large numbers in the leucocytes (Wertheim). They also penetrate the peritoneal lining and are found in the sub-endothelial connective tissue, but they appear to have little power of proliferation, they soon disappear, and the inflammatory condition does not spread. Injection of pure cultures into the joints of rabbits, dogs, and guinea-pigs causes an acute inflammation, which, however, soon subsides, whilst the gonococci rapidly die out; a practically similar result is obtained when dead cultures are used. These experiments show that while the organism, when present in large numbers, can produce a certain amount of inflammatory change in these animals, it has little or no power of multiplying and spreading in their tissues.

**Toxin of the Gonococcus.**—De Christmas has cultivated the gonococcus



in a mixture of one part of ascitic fluid and three parts of bouillon, and has found that the fluid after twelve days' growth has toxic properties. At this period all the organisms are dead; such a fluid constitutes the "toxin." The toxic substances are precipitated along with the proteids by alcohol, and the precipitate after being desiccated possesses the toxic action. In young rabbits injection of the toxin produces suppuration; this is well seen in the anterior chamber of the eye, where hypopyon results. The most interesting point, however, is with regard to its action on mucous surfaces; for, while in the case of animals it produces no effect, its introduction into the human urethra causes acute catarrh, attended with purulent discharge. He found that no tolerance to the toxin resulted after five successive injections at intervals. In a more recent publication he points out that the toxin on intracerebral injection has marked effects; he also claims to have produced an antitoxin. He states that the toxin diffuses out in the culture medium, and does not merely result from disintegration of the organisms. This has, however, been called in question by other investigators.

**Distribution in the Tissues.**—The gonococcus having been thus shown to be the direct cause of the disease, some additional facts may be given regarding its presence both in the primary and secondary lesions. In the human urethra the gonococci penetrate the mucous membrane, passing chiefly between the epithelial cells, causing a loosening and desquamation of many of the latter and inflammatory reaction in the tissues below, attended with great increase of secretion. There occurs also a gradually increasing emigration of leucocytes, which take up a large number of the organisms. The organisms also penetrate the subjacent connective tissue and are especially found, along with extensive leucocytic emigration, around the lacunæ. Here also many are contained within leucocytes. Even, however, when the gonococci have disappeared from the urethral discharge, they may still be present in the deeper part of the mucous membrane of the urethra, possibly also in the prostate, and may thus be capable of producing infection. The prostatic secretion may sometimes be examined by making pressure on the prostate from the rectum when the patient has almost emptied his bladder, the secretion being afterwards discharged along with the remaining urine. (Foulerton.) In acute gonorrhœa there is often a considerable degree of inflammatory affection of the prostate and vesiculæ seminales, but whether these conditions are always due to the presence of gonococci in the affected parts we have not at present the data for determining. A similar statement also applies to the occurrence of orchitis and also of cystitis in the early stage of gonorrhœa. Gonococci have, however, been obtained in pure culture from peri-urethral abscess and from epididymitis; it is likely that



the latter condition, when occurring in gonorrhœa, is usually due to the actual presence of gonococci. During the more chronic stages other organisms may appear in the urethra, aid in maintaining the irritation, and may produce some of the secondary results. The bacillus coli, the pyogenic cocci, etc., are often present, and may extend along the urethra to the bladder and set up cystitis, though in this they may be aided by the passage of a catheter. It may be mentioned here that Wertheim cultivated the gonococcus from a case of chronic gonorrhœa of two years' standing, and by inoculation on the human subject proved it to be still virulent.

In the disease in the female, gonococci are almost invariably present in the urethra, the situation affected next in frequency being the cervix uteri. They do not appear to infect the lining epithelium of the vagina of the adult unless some other abnormal condition be present, but they do so in the gonorrhœal vulvovaginitis of young subjects. They have also been found in suppurations in connection with Bartholini's glands, and sometimes produce an inflammatory condition of the mucous membrane of the body of the uterus. They may also pass along the Fallopian tubes and produce inflammation of the mucous membrane there. From the pus in cases of pyosalpinx they have been cultivated in a considerable number of cases. According to the results of various observers they are present in one out of four or five cases of this condition, usually unassociated with other organisms. Further, in a large proportion of the cases in which the gonococcus has not been found, no organisms of any kind have been obtained from the pus, and in these cases the gonococci may have been once present and have subsequently died out. Lastly, they may pass to the peritoneum and produce peritonitis, which is usually of a local character. It is chiefly to the methods of culture supplied by Wertheim that we owe our extended knowledge of such conditions.

In *gonorrhœal conjunctivitis* the mode in which the gonococci spread through the epithelium to the subjacent connective tissue is closely analogous to what obtains in the case of the urethra. Their relation to the leucocytes in the purulent secretion is also the same. Microscopic examination of the secretion alone in acute cases often gives positive evidence, and pure cultures may be readily obtained on blood-agar. As the condition becomes more chronic the gonococci are less numerous and a greater proportion of other organisms may be present.

*Relations to Joint-Affections, etc.*—The relations of the gono-



coccus to the sequelæ of gonorrhœa form a subject of great interest and importance, and the application of recent methods of examination shows that the organism is much more frequently present in such conditions than the earlier results indicated. The following statements may be made with regard to them: First, in a large number of cases of arthritis following gonorrhœa pure cultures of the gonococcus may be obtained. A similar statement applies to inflammation of the sheaths of tendons following gonorrhœa. Secondly, in a considerable proportion of cases no organisms have been found. It is, however, possible that in many of these the gonococci may have been present in the synovial membrane, as it has been observed that they may be much more numerous in that situation than in the fluid. Thirdly, in some cases, especially in those associated with extensive suppuration, occasionally of a pyæmic nature, various pyogenic cocci have been found to be present. In the instances in which the gonococcus has been found in the joints, the fluid present has usually been described as being of a whitish yellow tint, somewhat turbid, and containing shreds of fibrin-like material, sometimes purulent in appearance. In one case Bordoni-Uffreduzzi cultivated the gonococcus from a joint-affection, and afterwards produced gonorrhœa in the human subject by inoculating with the cultures obtained. In another case, in which pleurisy was present along with arthritis, the gonococcus was cultivated from the fluid in the pleural cavity. The existence of a *gonorrhœal endocarditis* has been established by recent observations. Cases apparently of this nature occurring in the course of gonorrhœa had been previously described, but the complete bacteriological test has now been satisfied in several instances. In one case Lenhartz produced gonorrhœa in the human subject by inoculation with the organisms obtained from the vegetations. That a true *gonorrhœal septicæmia* may occur has also been established, cultures of the gonococcus having been obtained from the blood during life on more than one occasion (Thayer and Blumer, Thayer and Lazear, Ahmann).

**Methods of Diagnosis.**—For microscopical examination, dried films of the suspected pus, etc., may be stained by any of the simple solutions of the basic aniline stains. We prefer methylene- or thionin-blue, as they do not overstain, and the films do not need to be decolorised. Staining for one minute is sufficient. It is also advisable to stain by Gram's method, and it is a good plan to put at one margin of the cover-glass a small quantity of culture of *staphylococcus aureus* if available, in order to have



a standard by which to be certain that the supposed gonococci are really decolorised. Regarding the value of microscopic examination alone, we may say that the presence of a large number of micrococci in a urethral discharge having the characters, position, and staining reactions described above, is practically conclusive that the case is one of gonorrhœa. There is no other condition in which the sum-total of the microscopical characters is present. We consider that it is sufficient for purposes of clinical diagnosis, and therefore of great value; in the acute stage a diagnosis can thus be made earlier than by any other method. The mistake of confusing gonorrhœa with such conditions as a urethral chancre with urethritis, will also be avoided. Even in chronic cases the typical picture is often well maintained, and microscopic examination alone may give a definite positive result. When other organisms are present, and especially when the gonococci are few in number, it is difficult, and in some cases impossible, to give a definite opinion, as a few gonococci mixed with other organisms cannot be recognised with certainty. This is often the condition in chronic gonorrhœa in the female. Microscopic examination, therefore, though often giving positive results, will sometimes be inconclusive. As regards lesions in other parts of the body, microscopical examination alone is quite insufficient; it is practically impossible, for example, to distinguish by this means the gonococcus from the diplococcus intracellularis of meningitis. Cultures alone supply the test, and the points above detailed are to be attended to.

#### SOFT SORE.

The bacillus of soft sore was first described by Ducrey in 1889, who found it in the purulent discharge from the ulcerated surface; and later, in 1892, Unna described its appearance and distribution as seen in sections through the sores. The statements of these observers regarding the presence and characters of this organism have been fully confirmed by other observers.

*Microscopical Characters.*—The organism occurs in the form of minute oval rods measuring about  $1.5 \mu$  in length, and  $.5 \mu$  in thickness (Fig. 75). It is found mixed with other organisms in the purulent discharge from the surface, and is chiefly arranged in small groups or in short chains. When studied in sections through the ulcer, it is found in the superficial part of the floor, but more deeply situated than other organisms, and may be present in a state of purity amongst the leucocytic infiltration.



In this position it is usually arranged in chains, which may be of considerable length, and which are often seen lying in parallel rows between the cells. The bacilli chiefly occur in the free condition, but occasionally a few may be contained within leucocytes.

There is no doubt that in many cases the organism is present in the buboes in a state of purity; it has been found there by microscopic examination, and cultures have also been obtained



FIG. 75.—Film preparation of pus from soft chancre, showing Ducrey's bacillus, chiefly arranged in pairs. Stained with carbolfuchsin and slightly decolorised.  $\times 1500$ .

from this source. The negative results of some observers are probably due to the organism having died off. On the whole the evidence goes to show that the ordinary bubo associated with soft sore is to be regarded as another lesion produced by Ducrey's bacillus. Sometimes the ordinary pyogenic organisms become superadded.

This bacillus takes up the basic aniline stains fairly readily, but loses the colour very rapidly when a decolorising agent is applied. Accordingly,

in film preparations when dehydration is not required, it can be readily stained by most of the ordinary combinations, though Löffler's or Kühne's methylene-blue solutions are preferable, as they do not overstain. In sections, however, great care must be taken in the process of dehydration, and the aniline-oil method (*vide* p. 100) should be used for this purpose, as alcohol decolorises the organism very readily. A little of the methylene-blue or other stain may be with advantage added to the aniline-oil used for dehydrating.

**Cultivation.**—Although for a long period of time attempts to obtain cultures were unsuccessful, success has been attained within recent years. Bezançon, Griffon, and Le Sourd obtained pure cultures in four cases, the medium used being a mixture of rabbit's blood and agar, in the proportion of one part of the former to two of the latter. The blood is added to the agar in the melted condition at  $45^{\circ}$  C., and the tubes are then sloped.



Davis confirms these results, and finds that another good medium is freshly-drawn human blood distributed in small tubes; this method is specially suitable, as the blood inhibits the growth of various extraneous organisms. On the solid medium (blood-agar) the growth appears in the form of small round globules, which attain their complete development in forty-eight hours, having then a diameter of 1 to 2 mm.; the colonies do not become confluent. Microscopic examination of these colonies, which are dissociated with some difficulty, shows appearances similar to those observed when the organism is in the tissues (Fig. 76), but occasionally long undivided filaments are observed which Davis regards as degenerative forms. Within a comparatively short period cultures undergo marked degenerative changes, and great irregularities of form and shape are to be found. It would appear that a comparatively large amount of blood is

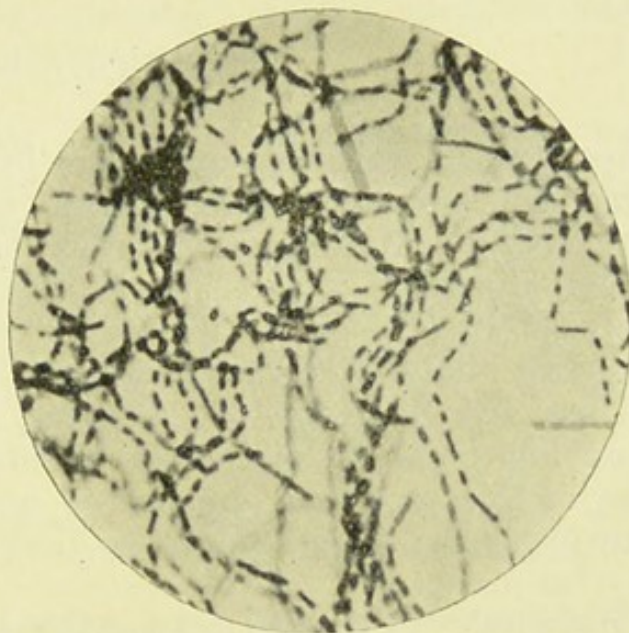


FIG. 76.—Ducrey's bacillus from a 24-hour culture in blood-bouillon.  $\times 1500$ .<sup>1</sup>

necessary for the growth of this organism, and even sub-cultures on the ordinary media, including blood-serum media, give negative results. Inoculation of the ordinary laboratory animals is not attended by any result, but it has been found that some monkeys are susceptible, small ulcerations being produced by superficial inoculation, and in these the organism can be demonstrated. Tomaszewski cultivated the organism for several generations, and reproduced the disease by inoculation of the human subject. The causal relationship of this bacillus must therefore be considered as completely established, and the conditions under which it grows show it to be a strict parasite—a fact which is in conformity with the known facts as to the transmission of the disease.

<sup>1</sup> We are indebted to Dr. Davis for the use of Figs. 75 and 76.



## CHAPTER X.

### TUBERCULOSIS.

THE cause of tubercle was proved by Koch in 1882 to be the organism now universally known as the tubercle bacillus. Probably no other single discovery has had a more important effect on medical science and pathology than this. It has not only shown what is the real cause of the disease, but has also supplied infallible methods for determining what are tubercular lesions and what are not, and has also given the means of studying the modes and paths of infection. A definite answer has in this way been supplied to many questions which were previously the subject of endless discussion.

**Historical.**—By the work of Armanni and of Cohnheim and Salomonsen (1870–80) it had been demonstrated that tubercle was an infective disease. The latter observers found on inoculation of the anterior chamber of the eye of rabbits with tubercular material, that in many cases the results of irritation soon disappeared, but that after a period of incubation, usually about twenty-five days, small tubercular nodules appeared in the iris; afterwards the disease gradually spread, leading to a tubercular disorganisation of the globe of the eye. Later still, the lymphatic glands became involved, and finally the animal died of acute tuberculosis. The question remained as to the nature of the virus, the specific character of which was thus established, and this question was answered by the work of Koch.

The announcement of the discovery of the tubercle bacillus was made by Koch in March 1882, and a full account of his researches appeared in 1884 (*Mitth. a. d. K. Gsndhtsamte.*, Berlin). Koch's work on this subject will remain as a classical masterpiece of bacteriological research, both on account of the great difficulties which he successfully overcame and the completeness with which he demonstrated the relations of the organism to the disease. The two chief difficulties were, first, the demonstration of the bacilli in the tissues, and, secondly, the cultivation of the organism outside the body. For, with regard to the first, the tubercle bacillus cannot be demonstrated by a simple watery solution of a basic aniline dye, and it was only after prolonged staining for twenty-four hours, with a solution of methylene-blue with caustic potash added, that he was able to reveal the presence of the organism. Then, in the second place, all attempts to cultivate it on the ordinary media failed, and he only



succeeded in obtaining growth on solidified blood serum, the method of preparing which he himself devised, inoculations being made on this medium from the organs of animals artificially rendered tubercular. The fact that growth did not appear till the tenth day at the earliest, might easily have led to the hasty conclusion that no growth took place. All difficulties were, however, successfully overcome. He cultivated the organism by the above method from a great variety of sources, and by a large series of inoculation experiments on various animals, performed by different methods, he conclusively proved that bacilli from these different sources produced the same tubercular lesions and were really of the same species. His work was the means of showing conclusively that such conditions as lupus, "white swelling" of joints, scrofulous disease of glands, etc., are really tubercular in nature.

**Tuberculosis in Animals.**—Tuberculosis is not only the most widely spread of all diseases affecting the human subject, and produces a mortality greater than any other, but there is probably no other disease which affects the domestic animals so widely. We need not here describe in detail the various tubercular lesions in the human subject, but some facts regarding the disease in the lower animals may be given, as this subject is of great importance in relation to the infection of the human subject.

Amongst the domestic animals the disease is commonest in cattle (bovine tuberculosis), in which animals the lesions are very various, both in character and distribution. In most cases the lungs are affected, and contain numerous rounded nodules, many being of considerable size; these may be softened in the centre, but are usually of pretty firm consistence and may be calcified. There may be in addition caseous pneumonia, and also small tubercular granulations. Along with these changes in the lungs, the pleuræ are also often affected, and show numerous nodules, some of which may be of large size, firm and pedunculated, the condition being known in Germany as *Perlsucht*, in France as *pommelière*. Lesions similar to the last may be chiefly confined to the peritoneum and pleuræ. In other cases, again, the abdominal organs are principally involved. The udder becomes affected in a certain proportion of cases of tuberculosis in cows—in 3 per cent. according to Bang—but primary affection of this gland is very rare. Tuberculosis is also a comparatively common disease in pigs, in which animals it in many cases affects the abdominal organs, in other cases produces a sort of caseous pneumonia, and sometimes is met with as a chronic disease of the lymphatic glands, the so-called "scrofula" of pigs. Tubercular lesions in the muscles are less rare in pigs than in most other animals. In the horse the abdominal organs are usually the primary seat of the disease, the spleen being often enormously enlarged and crowded with nodules of various shapes and sizes; sometimes, however, the primary lesions are pulmonary. In sheep and goats tuberculosis is of rare occurrence, especially in the former animals. It may occur spontaneously in dogs, cats, and in the large carnivora. It is also sometimes met with in monkeys in confinement, and leads to a very rapid and widespread affection in these animals, the nodules having a special tendency to soften and break down into a pus-like fluid.



Tuberculosis in fowls (avian tuberculosis) is a common and very infectious disease, nearly all the birds in a poultry-yard being sometimes affected.

From these statements it will be seen that the disease in animals presents great variations in character, and may differ in many respects from that met with in the human subject. The relation of the different forms of tuberculosis is discussed below.

**Tubercle Bacillus—Microscopical Characters.**—Tubercle bacilli are minute rods which usually measure  $2.5$  to  $3.5 \mu$  in length, and  $.3 \mu$  in thickness, *i.e.* in proportion to their length they are comparatively thin organisms (Figs. 77 and 78). Some-

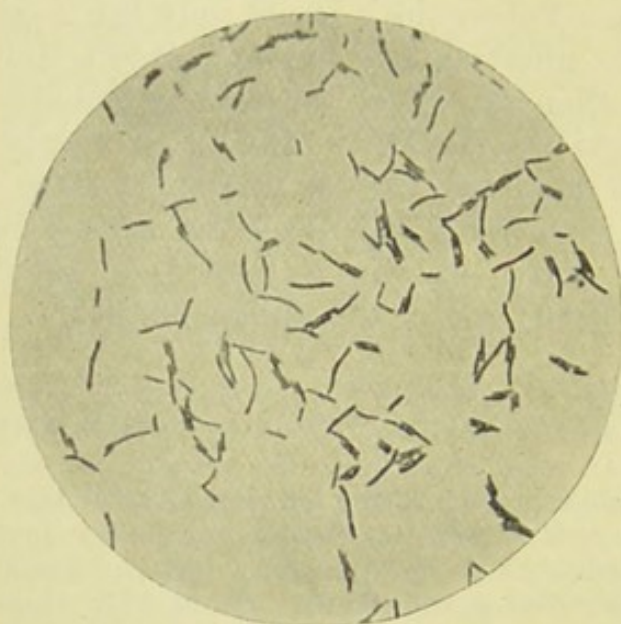


FIG. 77.—Tubercle bacilli, from a pure culture on glycerin agar. Stained with carbol-fuchsin.  $\times 1000$ .

times, however, longer forms, up to  $5 \mu$  or more in length, are met with, both in cultures and in the tissues. They are straight or slightly curved, and are of uniform thickness, or may show slight swelling at their extremities. When stained they appear uniformly coloured, or may present small uncoloured spots along their course, with darkly stained parts between. In such a minute organism it is extremely difficult to determine the exact nature of the unstained

points. Accordingly, we find that some observers consider these to be spores, while others find that it is impossible to stain them by any means whatever, and consider that they are really of the nature of vacuoles. Against their being spores is also the fact that many occur in one bacillus. Others again hold that some of the condensed and highly stained particles are spores. It is impossible to speak definitely on the question at present. We can only say that the younger bacilli stain uniformly, and that in the older forms inequality in staining is met with; this latter condition is, however, not found to be associated with greater powers of resistance.

The bacilli in the tissues occur scattered irregularly or in little masses. They are usually single, or two are attached end to end and often form in such a case an obtuse angle. True



chains are not formed, but occasionally short filaments are met with. In cultures the bacilli form masses in which the rods are closely applied to one another and arranged in a more or less parallel manner. Tubercle bacilli are quite devoid of motility.

*Aberrant Forms.*—Though such are the characters of the organism as usually met with, other appearances are sometimes found. In old cultures, for example, very much larger elements



FIG. 78.—Tubercle bacilli in phthysical sputum ; they are longer than is often the case. See also Plate II., Fig. 7.

Film preparation, stained with carbol-fuchsin and methylene-blue.  
× 1000.

may occur. These may be in the form of long filaments, sometimes swollen or clubbed at their extremities, may be irregularly beaded, and may even show the appearance of branching. Such forms have been studied by Metchnikoff, Maffucci, Klein, and others. Their significance has been variously interpreted, for while some look upon them as degenerated or involution forms, others regard them as indicating a special phase in the life history of the organism, allying it with the higher bacteria. Recent observations, however, go to establish the latter view, and this is now generally accepted by authorities. It has also



been found that under certain circumstances tubercle bacilli in the tissues produce a radiating structure closely similar to that of the actinomyces. This was found by Babes and also by Lubarsch to be the case when the bacilli were injected under the dura mater and directly into certain solid organs, such as the kidneys in the rabbit. Club-like structures may be present at the periphery; these are usually not acid-fast, but they retain the stain in the Weigert-Gram method. Similar results obtained with other acid-fast bacilli will be mentioned below, and these organisms would appear to form a group closely allied to the streptothriceæ, the bacillary parasitic form being one stage of the life history of the organism. This group is often spoken of as the *mycobacteria*.

*Staining Reactions.* — The tubercle bacillus takes up the ordinary stains very slowly and faintly, and for successful staining one of the most powerful solutions ought to be employed, *e.g.* gentian-violet or fuchsin, along with aniline-oil water or solution of carbolic acid. Further, such staining solutions require to be applied for a long time, or the staining must be accelerated by heat, the solution being warmed till steam arises and the specimen allowed to remain in the hot stain for two or three minutes. One of the best and most convenient methods is the Ziehl-Neelsen method (see p. 108). The bacilli present this further peculiarity, however, that after staining has taken place they resist decolorising by solutions which readily remove the colour from the tissues and from other organisms which may be present. Such decolorising agents are sulphuric or nitric acid in 20 per cent. solution. Preparations can thus be obtained in which the tubercle bacilli alone are coloured by the stain first used, and the tissues can then be coloured by a contrast stain. Within recent years certain other bacilli have been discovered which present the same staining reactions as tubercle bacilli; they are therefore called "acid-fast" (*vide infra*). The spores of many bacilli become decolorised more readily than tubercle bacilli, though some retain the colour with equal tenacity.

Bulloch and Macleod, by treating tubercle bacilli with hot alcohol and ether, extracted a wax which gave the characteristic staining reactions of the bacilli themselves. The remains of the bacilli, further, when extracted with caustic potash, yielded a body which was probably a chitin, and which was acid-fast when stained for twenty-four hours with carbol-fuchsin.

It had long been recognised that it might not be possible to detect by microscopic methods tubercle bacilli in old tubercular



lesions, and yet the material from such was virulent on inoculation. This was supposed to be due either to the paucity of the bacilli or possibly to the presence of spores. Recently observations have been brought forward by Much which may throw important light on this subject. Briefly put, his conclusions are that the tubercle virus exists in three forms—(a) the ordinary bacillary form stainable by the Ziehl method; (b) a fine bacillary form which is not acid-fast, often showing granules in its interior; and (c) free granules which also fail to stain with the Ziehl method. The two last forms can be stained by Gram's method when the stain is applied for a long time. Much gives three modifications of Gram's method, the following being one which has been found by others to be specially suitable:—

Methyl violet B.N. 10 c.c. of a saturated alcoholic solution in 100 c.c. of a 2 per cent. watery solution of carbolic acid; stain by boiling over the flame for a few minutes or at 37° C. for 24–48 hours, then treat with Lugoe's iodine for 1–5 minutes, 5 per cent. hydrochloric acid for one minute, 3 per cent. hydrochloric acid 10 seconds, and complete the decolorisation with a mixture of acetone and alcohol in equal parts.

Much claims that by such a method bacilli and granules can be found in tubercular lesions when the Ziehl method gives a negative result. He also found that, when bacilli from a culture were added to sterilised milk and incubated, the acid-fast forms disappeared whilst those stainable with Gram's method remained; and that when this had occurred the milk when injected into an animal produced tuberculosis in which acid-fast bacilli were demonstrable. His statements have received confirmation by other observers, *e.g.* Wirths and Treuholtz, but as yet it is not possible to give a definite pronouncement on the whole subject. If the bacillus can pass into a form not demonstrable by the method usually employed but still virulent, we have manifestly to deal with a fact of the highest importance. There seems to be no doubt that in certain conditions more tubercle bacilli can be demonstrated in the tissues by Much's method than by the ordinary carbol-fuchsin method.

**Cultivation.**—The medium first used by Koch was *inspissated blood serum* (*vide* p. 40). If inoculations are made on this medium with tubercular material free from other organisms, there appear in from ten to fourteen days minute points of growth of dull whitish colour, rather irregular, and slightly raised above the surface (it is advisable to plant on the medium an actual piece of the tubercular tissue and to fix it in a wound of the



surface of the serum). Koch compared the appearance of these to that of small dry scales. In such cultures the growths usually reach only a comparatively small size and remain separate, becoming confluent only when many occur close together. In sub-cultures, however, growth is more luxuriant and may come to form a dull wrinkled film of whitish colour, which may cover

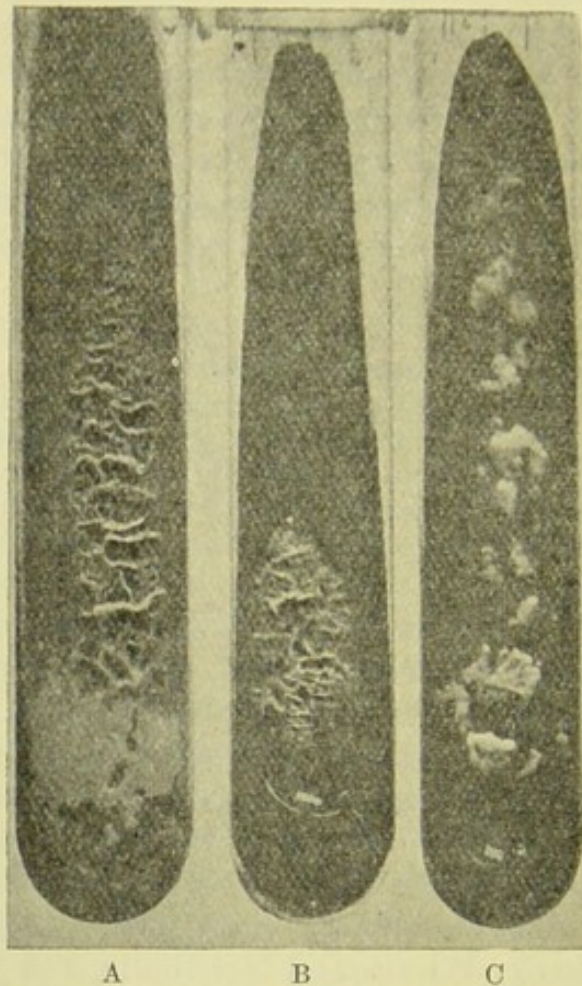


FIG. 79.—Cultures of tubercle bacilli on glycerin agar.

A and B. Mammalian tubercle bacilli; A is an old culture, B one of a few weeks' growth.  
C. Avian tubercle bacilli. The growth is whiter and smoother on the surface than the others.

the greater part of the surface of the serum and at the bottom of the tube may grow over the surface of the condensation water on to the glass (Fig. 79, A). The growth is always of a dull appearance, and has a considerable degree of consistence, so that it is difficult to dissociate a portion thoroughly in a drop of water. In older cultures the growth may acquire a slightly brownish or buff colour. When the small colonies are examined under a low power of the microscope, they are seen to be extending at the periphery in the form of wavy or sinuous streaks which radiate outward, and which have been compared to the flourishes of a pen. The central part shows similar markings closely interwoven. These

streaks are composed of masses of the bacilli arranged in a more or less parallel manner.

On *glycerin agar*, which was first introduced by Nocard and Roux as a medium for the culture of the tubercle bacillus, growth takes place in sub-cultures at an earlier date and progresses more rapidly than on serum, but this medium is not suitable for obtaining cultures from the tissues, inoculations with tubercular material usually yielding a negative result.



The growth has practically the same characters as on serum, but is more luxuriant. In *glycerin broth*, especially when the layer is not deep, tubercle bacilli grow readily in the form of little white masses, which fall to the bottom and form a powdery layer. If, however, the growth be started on the surface, it spreads superficially as a dull whitish wrinkled pellicle which may reach the walls of the flask; this mode of growth is specially suitable for the production of tuberculin (*vide infra*). The culture has a peculiar fruity and not unpleasant odour. On ordinary agar and on gelatin media no growth takes place. The use of animal tissues in glycerine bouillon as a medium for the growth of the tubercle bacillus has been recently introduced by Frugoni, and is one which gives excellent results. He recommends that small wedges of rabbit's lung should be sterilised in the autoclave, and placed in tubes of glycerine bouillon in such a way that their surface is kept moist by the medium, without the fragments being submerged. The growth is probably more rapid and luxuriant than in any other method.

*Use of Egg Media.*—Within recent years media containing either the yolk or both the yolk and the white of egg have been used for the culture of the tubercle bacillus by Dorset and others. The following is Dorset's method: The contents of four eggs are well beat, 25 c.c. of water are added and thoroughly mixed, the mixture being passed through muslin to remove air bells. The fluid is then filled into tubes, and these are heated for four hours in the sloped position at 70° C. Before the inoculation of a tube, two drops of sterilised water are placed on the surface. The inoculation material is well rubbed over the surface of the medium, the tubes are sealed with a few drops of paraffin on the top of the plug and are incubated in the sloped position. Vigorous growth takes place on such media, having, generally speaking, the naked eye characters seen in blood serum cultures.

It was at one time believed that the tubercle bacillus would only grow on media containing animal fluids, but of late years it has been found that growth takes place also on a purely vegetable medium, as was first shown by Pawlowsky in the case of potatoes. Sander found that the bacillus grew readily on potato, carrot, macaroni, and on infusion of these substances, especially when glycerin was added. He also found that cultures from tubercular lesions could be obtained on glycerin potato (p. 46).

The optimum temperature for growth is 37° to 38° C. Growth ceases about 42° and usually below 28°, but on long-continued cultivation outside the body and in special circumstances, growth may take place at a lower temperature, *e.g.*



Sander found that growth took place in glycerin-potato broth even at 22° to 23° C.

**Powers of Resistance.**—Tubercle bacilli have considerable powers of resistance to external influences, and can retain their vitality for a long time outside the body in various conditions; in fact, in this respect they may be said to occupy an intermediate position between spores and spore-free bacilli. Dried phthisical sputum has been found to contain still virulent bacilli (or their spores) after two months, and similar results are obtained when the bacilli are kept in distilled water for several weeks. So also they resist for a long time the action of putrefaction, which is rapidly fatal to many pathogenic organisms. Sputum has been found to contain living tubercle bacilli even after being allowed to putrefy for several weeks (Fraenkel, Baumgarten), and the bacilli have been found to be alive in tubercular organs which have been buried in the ground for a similar period. They are not killed by being exposed to the action of the gastric juice for six hours, or to a temperature of  $-3^{\circ}$  C. for three hours, even when this is repeated several times. It has been found that when completely dried they can resist a temperature of  $100^{\circ}$  C. for an hour, but, on the other hand, exposure in the moist condition to  $70^{\circ}$  C. for the same time is usually fatal. It may be stated that raising the temperature to  $100^{\circ}$  C. kills the bacilli in fluids and in tissues, but in the case of large masses of tissue care must be taken that this temperature is reached throughout. They are killed in less than a minute by exposure to 5 per cent. carbolic acid, and both Koch and Straus found that they are rapidly killed by being exposed to the action of direct sunlight.

**Action on the Tissues.**—The *local lesion* produced by the tubercle bacillus is the well-known tubercle nodule, the structure of which varies in different situations and according to the intensity of the action of the bacilli. After the bacilli gain entrance to a connective tissue such as that of the iris, their first action appears to be on the connective-tissue cells, which become somewhat swollen and undergo mitotic division, the resulting cells being distinguishable by their large size and pale nuclei—the so-called epithelioid cells. These proliferative changes may be well seen on the fifth day after inoculation or even earlier. A small focus of proliferated cells is thus formed in the neighbourhood of the bacilli, and about the same time numbers of leucocytes—chiefly lymphocytes—begin to appear at the periphery and gradually become more numerous. Soon, however, the action of the bacilli as cell-poisons comes into prominence. The epithelioid cells become swollen and somewhat hyaline, their



outlines become indistinct, whilst their nucleus stains faintly, and ultimately loses the power of staining. The cells in the centre, thus altered, gradually become fused into a homogeneous substance, and this afterwards becomes somewhat granular in appearance. If the central necrosis does not take place quickly, then giant-cell formation may occur in the centre of the follicle, this constituting one of the characteristic features of the tubercular lesion; or after the occurrence of caseation giant-cells may be formed in the cellular tissue around. The centre of a giant-cell often shows signs of degeneration, such as hyaline change and vacuolation, or it may be more granular than the rest of the cell. The exact mode of formation of a tubercle follicle varies, however, in different tissues.

Though there has been a considerable amount of discussion as to the mode of origin of the giant-cells, we think there can be little doubt that in most cases they result from enlargement of single epithelioid cells, the nucleus of which undergoes proliferation without the protoplasm dividing. These epithelioid cells may sometimes be the lining cells of capillaries. Some consider that the giant-cells result from a fusion of the epithelioid cells; but, though there are occasionally appearances which indicate such a mode of formation, it cannot be regarded as of common occurrence. In some cases of acute tuberculosis, when the bacilli become lodged in a capillary, the endothelial cells of its wall may proliferate, and thus a ring of nuclei may be seen round a small central thrombus. Such an occurrence gives rise to an appearance closely resembling a typical giant-cell. There can be no doubt that the cell necrosis and subsequent caseation depend upon the products of the bacilli, and are not due to the fact that the tubercle nodule is non-vascular. This non-vascularity itself is to be explained by the circumstance that young capillaries cannot grow into a part where tubercle bacilli are active, and that the already existing capillaries become thrombosed, owing to the action of the bacillary products on their walls, and ultimately disappear. At the periphery of tubercular lesions there may be considerable vascularity and new formation of capillaries.

The *general symptoms of tuberculosis*—pyrexia, perspiration, wasting, etc., are to be ascribed to the absorption and distribution throughout the system of the toxic products of the bacilli; in the case of phthisical cavities and like conditions where other bacteria are present, the toxins of the latter also play an important part. The occurrence of waxy change in the organs is believed by some to be chiefly due to the products of other,



especially pyogenic, organisms, secondarily present in the tubercular lesions. This matter, however, requires further elucidation.

*Presence and Distribution of the Bacilli.*—A few facts may be stated regarding the presence of bacilli, and the numbers in which they are likely to be found in tubercular lesions. They are usually very few in number in chronic lesions, whether these are tubercle nodules with much connective tissue

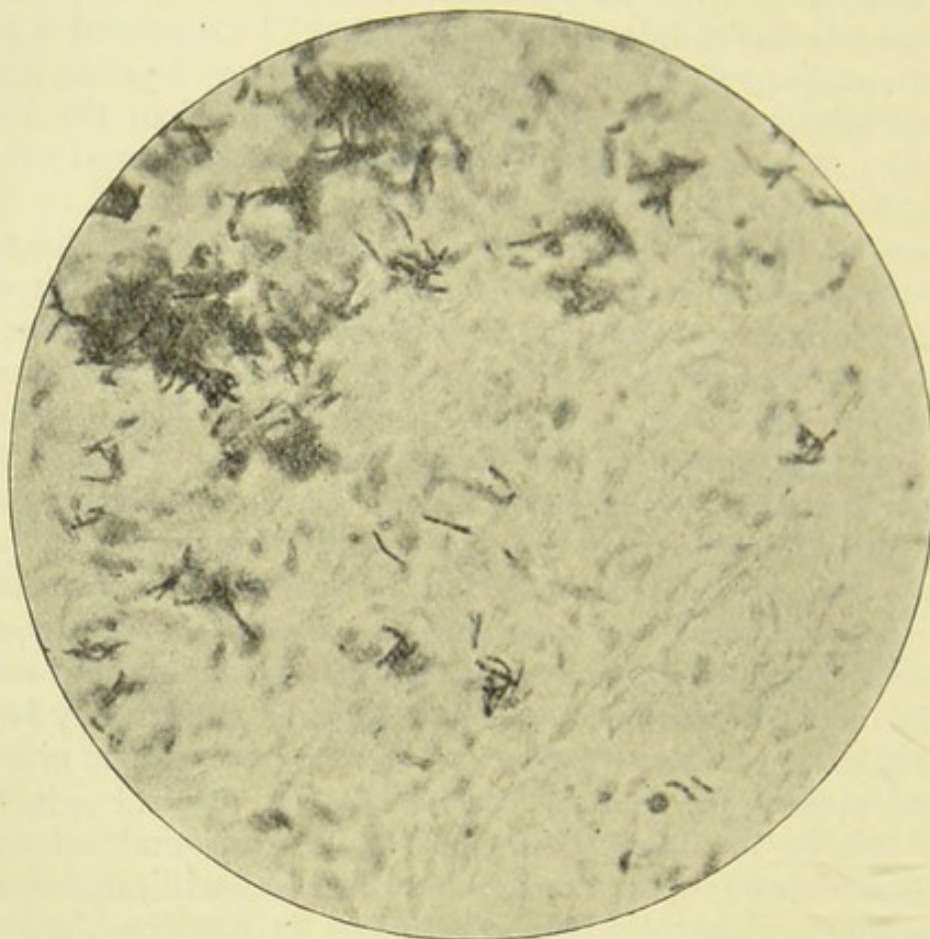


FIG. 80.—Tubercle bacilli in section of human lung in acute phthisis. The bacilli are seen lying singly, and also in large masses to left of field. The pale background is formed by caseous material. Stained with carbol-fuchsin and Bismarck-brown.  $\times 1000$ .

formation or old caseous collections. In caseous material one can sometimes see a few bacilli faintly stained, along with very minute unequally stained granular points, some of which may possibly be spores of the bacilli. Whether they are spores or not, the important fact has been established, that tubercular material in which no bacilli can be found microscopically, may be proved, on experimental inoculation into animals, to be still virulent. In such cases the bacilli may be present in numbers so small as to escape observation, or it may be that their spores only



are present. In subacute lesions, with well-formed tubercle follicles and little caseation, the bacilli are generally scanty. They are most numerous in acute lesions, especially where caseation is rapidly spreading, for example, in such conditions as caseous catarrhal pneumonia (Fig. 80), acute tuberculosis of the spleen in children, which is often attended with a good deal of rapid caseous change, etc.; in such conditions they often form

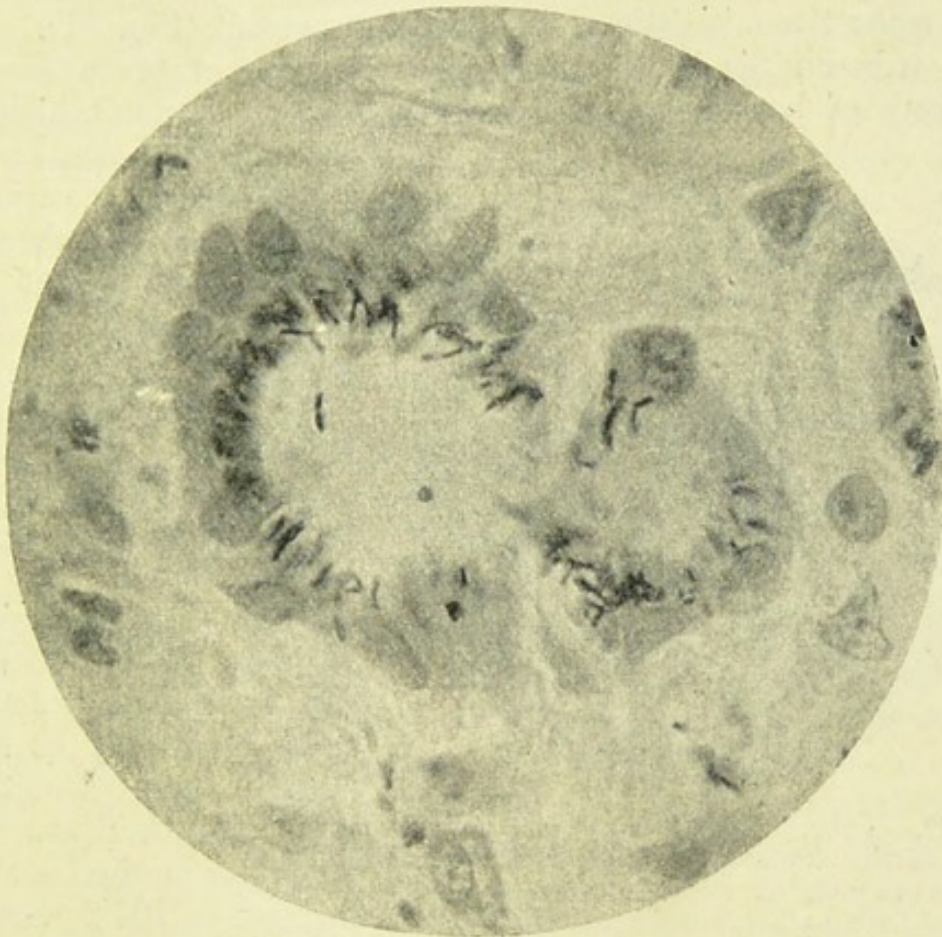


FIG. 81.—Tubercle bacilli in giant-cells, showing the radiate arrangement at the periphery of the cells. Section of tubercular udder of cow.

Stained with carbol-fuchsin and Bismarck-brown.  $\times 1000$ .

large masses which are easily seen under a low power of the microscope. In acute miliary tuberculosis a few bacilli can generally be found in the centre of the follicles; but here they are often much more scanty than one would expect. The tubercle bacillus is one which not only has comparatively slow growth, but retains its form and staining power for a much longer period than most organisms. As a rule the bacilli are extra-cellular in position. Occasionally they occur within the giant-cells, in which they may be arranged in a somewhat radiate



manner at the periphery, occasionally also in epithelioid cells and in leucocytes.

The above statements, however, apply only to tuberculosis in the human subject, and even in this case there are exceptions. In the ox, on the other hand, the presence of tubercle bacilli within giant-cells is a very common occurrence; and it is also common to find them in considerable numbers scattered irregularly throughout the cellular connective tissue of the lesions, even when there is little or no caseation present (Fig. 81).

In tuberculosis in the horse and in avian tuberculosis the numbers of bacilli may be enormous, even in lesions which are not specially acute; and considerable variation both in their number and in their site is met with in tuberculosis of other animals.

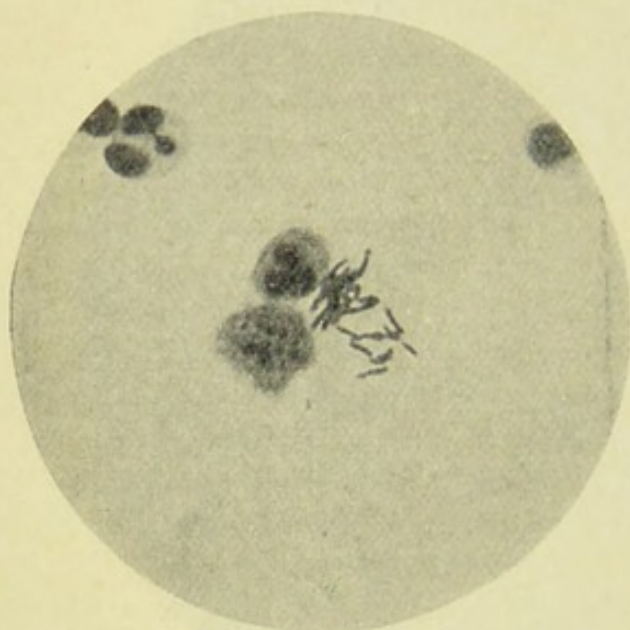


FIG. 82.—Tubercle bacilli in urine; showing one of the characteristic clumps, in which they often occur. Stained with carbol-fuchsin and methylene-blue.  $\times 1000$ .

In discharges from tubercular lesions which are breaking down, tubercle bacilli are usually to be found. In the sputum of phthisical patients their presence can be demonstrated almost invariably at some period, and sometimes their numbers are very large (for method of staining, see p. 107). Several examinations may, however, require to be made;

this should always be done before any conclusion as to the non-tubercular nature of a case is come to. In cases of genito-urinary tuberculosis they are usually present in the urine; but as they are much diluted it is difficult to find them unless a deposit is obtained by means of the centrifuge. This deposit is examined in the same way as the sputum. The bacilli often occur in little clumps, as shown in Fig. 82. In tubercular ulceration of the intestine their presence in the fæces may be demonstrated, as was first shown by Koch; but in this case their discovery is usually of little importance, as the intestinal lesions, as a rule, occur only in advanced stages when diagnosis is no longer a matter of doubt.



**Experimental Inoculation.**—Tuberculosis can be artificially produced in animals by infection in a great many different ways—by injection of the bacilli into the subcutaneous tissue, into the peritoneum, into the anterior chamber of the eye, into the veins; by feeding the animals with the bacilli; and, lastly, by making them inhale the bacilli suspended in the air.

The exact result, of course, varies in different animals and according to the method of inoculation, but we may state generally that when introduced into the tissues of a susceptible animal, the bacilli produce locally the lesions above described, terminating in caseation; that there occurs a tubercular affection of the neighbouring lymphatic glands, and that lastly there may be a rapid extension of the bacilli to other organs by the blood stream and the production of general tuberculosis. Of the animals generally used for the purpose, the guinea-pig is most susceptible.

When a guinea-pig is inoculated subcutaneously with tubercle bacilli from a culture, or with material containing them, such as phthisical sputum, a local swelling gradually forms which is usually well marked about the tenth day. This swelling becomes softened and caseous, and may break down, leading to the formation of an irregularly ulcerated area with caseous lining. The lymphatic glands in relation to the parts can generally be found to be enlarged and of somewhat firm consistence, about the end of the second or third week. Later, in them also caseous change occurs, and a similar condition may spread to other groups of glands in turn, passing also to those on the other side of the body. During the occurrence of these changes, the animal loses weight, gradually becomes cachectic, and ultimately dies, sometimes within six weeks, sometimes not for two or three months. *Post mortem*, in addition to the local and glandular changes, an acute tuberculosis is usually present, the spleen being specially affected. This organ is swollen, and is studded throughout by numerous tubercle nodules, which may be minute and grey, or larger and of a yellowish tint. If death has been long delayed, calcification may have occurred in some of the nodules. Tubercle nodules, though rather less numerous, are also present in the liver and in the lungs, the nodules in the latter organs being usually of smaller size though occasionally in large numbers. The extent of the general infection varies; sometimes the chronic glandular changes constitute the outstanding feature. Statements as to differences in the pathogenic effects of bacilli from human and bovine sources will be found below (p. 274).



**Varieties of Tuberculosis.** 1. *Human and Bovine Tuberculosis.*—Up till recent years it was generally accepted that all mammalian tuberculosis was due to the same organism, and, in particular, that tuberculosis could be transmitted from the ox to the human subject. The matter became one of special interest owing to Koch's address at the Tuberculosis Congress in 1901, in which he stated his conclusion that human and bovine tuberculosis are practically distinct, and that if a susceptibility of the human subject to the latter really exists, infection is of very rare occurrence,—so rare that it is not necessary to take any measures against it. Previously to this, Theobald Smith had pointed out differences between mammalian and bovine tubercle bacilli, the most striking being that the latter possess a much higher virulence to the guinea-pig, rabbit, and other animals, and in particular that human tubercle bacilli, on inoculation into oxen, produce either no disease or only local lesions without any dissemination. Koch's conclusions were based chiefly on the result of his inoculations of the bovine species with human tubercle bacilli, the result being confirmatory of Smith's, and also on the supposition that infection of the human subject through the intestine is of very rare occurrence.

Since the time of Koch's communication an enormous amount of work has been done on this subject, and Commissions of inquiry have been appointed in various countries. We may summarise the chief facts which have been established. Practically all observers are agreed that there are two chief types of tubercle bacilli, which differ both in their cultural characters and in their virulence—a bovine type and a human type. The bacilli of the bovine type, when cultivated, are shorter and thicker and more regular in size; whilst their growth on various culture media is scantier than that of the human type. From the latter character the British Royal Commission have applied the term *dysgonic* to the bovine and *eugonic* to the human type. As already stated, there is also a great difference in virulence towards the lower animals, the bacillus from the ox having a much higher virulence. This organism when injected in suitable quantities into the ox produces a local tubercular lesion, which is usually followed by a generalised and fatal tuberculosis; whereas injection of human tubercle bacilli produces no more than a local lesion, which undergoes retrogression. (In certain experiments, *e.g.* those of Delépine, Hamilton, and Young, general tuberculosis has been produced in the bovine species by tubercle bacilli from the human subject, but these results are exceptional.) Corresponding differences come out



in the case of the rabbit; in fact, intravenous injection of suitable quantities in this animal is the readiest method of distinguishing the two types—an acute tuberculosis resulting with the bovine, but not with the human type. In guinea-pigs and monkeys a generalised tuberculosis may result from subcutaneous injection of bacilli of the human type, but in this case also the difference in favour of the greater virulence of the bovine type is made out. With regard to the distribution of the two types of organisms, it may be stated that, so far as we know, the bacillus obtained from bovine tuberculosis is always of the bovine type, and the same may be said to be true of tuberculosis in pigs; in fact this seems to be the prevalent organism in animal tuberculosis. In human tuberculosis the bacilli in a large majority of the cases are of the human type; but, on the other hand, in a certain proportion bacilli of the bovine type are present, the bacilli when cultivated being indistinguishable by any means at our disposal from those obtained from bovine tuberculosis. The Royal Commission found the bovine type in 14 out of 60 cases of human tuberculosis—a somewhat higher proportion than has been obtained by most other investigators—and in all of these, with one exception, the bacilli were obtained either from caseous cervical glands, or from the lesions of primary abdominal tuberculosis, that is from cases where there was evidence of infection by alimentation. It is also to be noted that almost all the tubercular lesions from which the bovine type has been obtained have been in children. The general result accordingly is that bovine tubercle bacilli are present in a certain proportion of cases of tuberculosis in young subjects, and that these are especially cases where infection by the alimentary canal has occurred. It must thus be held as established that tuberculosis is transmissible from the ox to man, and that the milk of tubercular cows is a common vehicle of transmission.

Although most of the bacilli which have been cultivated correspond to one of the two types, as above described, it is also to be noted that intermediate varieties are met with. It has also been found that the type characters of the bacillus are not constant. Various observers have found it possible to modify bacilli of the human type by passing them through the bodies of certain animals, *e.g.* guinea-pigs, sheep, and goats, so that they acquire the characters of bovine bacilli. In view of these facts, it is probable that bovine bacilli will undergo corresponding modifications in the tissues of the human subject—what period of time is necessary for such a change we



cannot say. It is thus possible that the cases of human tuberculosis from which the bovine type has been obtained do not represent the full number where infection from the ox has occurred. It is quite likely that although the bovine bacilli are more virulent to the lower animals than the human bacilli are, this does not also hold in the case of the human subject. In fact, the comparative chronicity of the primary abdominal lesions in children, in the first instance, would point rather to a low order of virulence towards the human subject. We may also add that there are cases, notably those of Ravenel, in which accidental inoculation of the human subject with bovine tubercle has resulted in the production of tuberculosis.

2. *Avian Tuberculosis*.—In the tubercular lesions in birds there are found bacilli which correspond in their staining reactions and in their morphological characters with those in mammals, but differences are observed in cultures, and also on experimental inoculation. These differences were first described by Maffucci and by Rivolta, but special attention was drawn to the subject by a paper read by Koch at the International Medical Congress in 1890. Koch stated that he had failed to change the one variety of tubercle bacillus into the other, though he did not conclude therefrom that they were quite distinct species. The following points of difference may be noted:—

On glycerin agar and on serum, the growth of tubercle bacilli from birds is more luxuriant, has a moister appearance (Fig. 79, C), and, moreover, takes place at a higher temperature,  $43.5^{\circ}\text{C}$ ., than is the case with mammalian tubercle bacilli. Experimental inoculation brings out even more distinct differences. Tubercle bacilli derived from the human subject, for example, when injected into birds, usually fail to produce tuberculosis, whilst those of avian origin very readily do so. Birds are also very susceptible to the disease when fed with portions of the organs of birds containing tubercle bacilli, but they can consume enormous quantities of phthisical sputum without becoming tubercular (Strauss, Wurtz, Nocard). No doubt, on the other hand, there are cases on record in which the source of infection of a poultry-yard has apparently been the sputum of phthisical patients. Again, tubercle bacilli cultivated from birds have not the same effect on inoculation of mammals as ordinary tubercle bacilli have. When guinea-pigs are inoculated subcutaneously they usually resist infection, though occasionally a fatal result follows. In the latter case, usually no tubercles visible to the naked eye are found, but numerous bacilli may be present in internal organs, especially in the spleen, which is much swollen. Further, intravenous injection even of large quantities of avian tubercle bacilli, in the case of dogs, leads to no effect, whereas ordinary tubercle bacilli produce acute tuberculosis. [The rabbit, on the other hand, is comparatively susceptible to avian tuberculosis (Nocard).]

There is, therefore, abundant evidence that the bacilli derived



from the two classes of animals show important differences, and, reasoning from analogy, we might infer that probably the human subject also would be little susceptible to infection from avian tuberculosis. The question remains, are these differences of a permanent character? The matter seems conclusively settled by the experiments of Nocard, in which mammalian tubercle bacilli have been made to acquire all the characters of those of avian origin. The method adopted was to place bacilli from human tuberculosis in small collodion sacs (*v. p.* 144) containing bouillon, and then to insert each sac in the peritoneal cavity of a fowl. The sacs were left *in situ* for periods of from four to eight months. They were then removed, cultures were made from their contents, fresh sacs were inoculated from these cultures and introduced into other fowls. In such conditions the bacilli are subjected only to the tissue juices, the wall of the sac being impervious both to bacilli and to leucocytes, etc. After one sojourn of this kind, and still more so after two, the bacilli are found to have acquired some of the characters of avian tubercle bacilli, but are still non-virulent to fowls. After the third sojourn, however, they have acquired this property, and produce in fowls the same lesions as bacilli derived from avian tuberculosis. It therefore appears that the bacilli of avian tuberculosis are not a distinct and permanent species, but a variety which has been modified by growth in the tissues of the bird. It is also interesting to note that Rabinowitch has cultivated tubercle bacilli of the mammalian type from some cases of tuberculosis in parrots kept in confinement.

3. *Tuberculosis in the Fish*.—Bataillon, Dubard, and Terre cultivated from a tubercle-like disease in a carp, a bacillus which, in staining reaction and microscopic characters, closely agrees with the tubercle bacillus. The lesion with which it was associated was an abundant growth of granulation tissue in which numerous giant-cells were present. It forms, however, luxuriant growth at the room temperature, the growth, being thick and moist like that of avian tubercle bacilli (Fig. 84, *c*). Growth does not occur at the body temperature, though by gradual acclimatisation a small amount of growth has been obtained up to 36° C. Furthermore, the organism appears to undergo no multiplication when injected into the tissues of mammals, and attempts to modify this characteristic have so far been unsuccessful. Weber and Taute have cultivated this organism from mud, and also from organs of healthy frogs. It is thus probably to be regarded as a saprophyte which is only occasionally associated with disease in the fish. According to



the results of different experimenters, it is possible to modify human tubercle bacilli by allowing them to sojourn in the tissues of cold-blooded animals, *e.g.* the frog, blind-worm, etc., so that they flourish at lower temperatures. These results have, however, been recently called in question, as it has been stated the organisms obtained were not modified tubercle bacilli, but other acid-fast bacilli which may be found in the tissues of normal cold-blooded animals. This question must accordingly be considered still an open one.

All the above facts taken together indicate that tubercle bacilli may become modified in relative virulence and in conditions of growth by sojourn in the tissues of various animals. This modification appears slight, though of definite character in the case of bovine tuberculosis, more distinct in the case of avian tuberculosis, and much more marked, if not permanent, in the case of fish tuberculosis, that is, of course, in their relations to the bacilli from the human subject.

**Other Acid-fast Bacilli.**—Within recent years a number of bacilli presenting the same staining reaction as the tubercle bacilli have been discovered. Such bacilli have a comparatively wide distribution in nature, as they have been obtained from various species of grass, from butter and milk, from manure, and from the surfaces of animal bodies. Microscopically, they agree more or less closely with tubercle bacilli, though most of them are shorter and plumper; many of them show filamentous and branching forms under certain conditions of culture. Moreover, on injection, they produce granulation-tissue nodules which may closely resemble tubercles, although on the whole there is a greater tendency to softening and suppuration, and usually the lesions are localised to the site of inoculation. The most important point of distinction is the fact that their multiplication on artificial media is much more rapid, growth usually being visible within forty-eight hours and often within twenty-four hours at 37° C. Furthermore, in most instances growth occurs at the room temperature. The general character of the cultures in this group is a somewhat irregular layer, often with wrinkled surface, dry or moist in appearance, and varying in tint from white to yellow or reddish brown. The number of such organisms is constantly being added to, but the following may be mentioned as examples:—

*Moeller's Grass Bacilli I. and II.*—The former was found in infusions of Timothy-grass (*Phleum pratense*). It is extremely acid-fast, morphologically resembles the tubercle bacillus, and in cultures may show club formation and branching. The lesions produced closely resemble



tubercles. The colonies, visible in thirty-six hours, are scale-like and of greyish-white colour (Fig. 84, *a*). Moeller's bacillus II. was obtained from the dust of a hay-loft. The colonies at first are moist and somewhat tenacious, but afterwards run together, and are of a dull yellowish colour. The general results of inoculation resemble those of grass bacillus I., but are less marked. Moeller also obtained a similar organism from milk. He also discovered a third acid-fast bacillus, which he obtained from manure and therefore called the "Mistbacillus" (dung bacillus). This organism has analogous characters, though presenting minor differences. It also has pathogenic effects.

Petri and Rabinowitch independently cultivated an acid-fast bacillus from butter ("butter bacillus"), in which it occurs with comparative



FIG. 83.—Moeller's Timothy-grass bacillus.  
From a culture on agar.  
Stained with carbol-fuchsin, and treated  
with 20 per cent. sulphuric acid.  
× 1000.

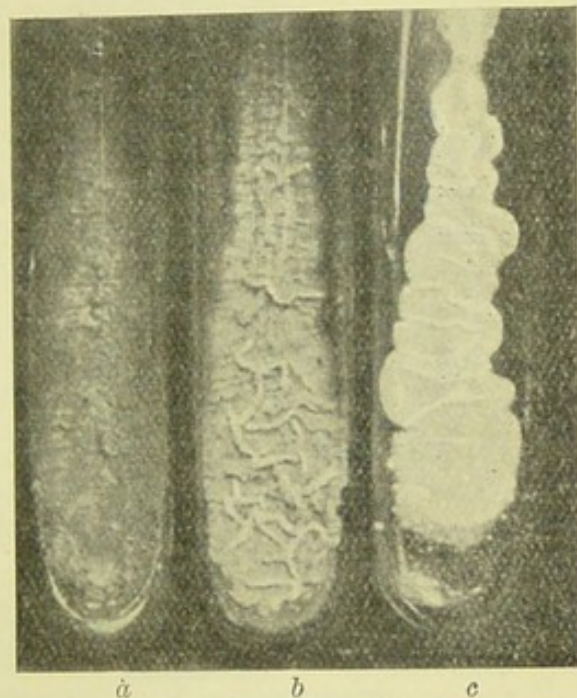


FIG. 84.—Cultures of acid-fast bacilli  
grown at room temperature.

- (*a*) Moeller's Timothy-grass bacillus I.  
(*b*) The Petri-Rabinowitch butter bacillus.  
(*c*) Bacillus of fish tuberculosis.

frequency. The organism resembles the tubercle bacillus, although it is on the whole shorter and thicker. Its lesions closely resemble tuberculosis, especially when injection of the organism is made into the peritoneal cavity of guinea-pigs, along with butter,—the method usually adopted in searching for tubercle bacilli in butter. This organism produces pretty rapidly a wrinkled growth (Fig. 84, *b*) not unlike that of Moeller's grass bacillus II. Korn has also obtained other two bacilli from butter which he holds to be distinct from one another and from Rabinowitch's bacillus. The points of distinction are of a minor character. Other more or less similar bacilli have been cultivated by Tobler, Coggi, and others.<sup>1</sup>

Another bacillus of considerable interest is Johne's bacillus or the

<sup>1</sup> For further details on this subject, *vide* Potet, *Études sur les bacilles dites acidophiles*. Paris, 1902.



bacillus of "chronic bovine pseudo-tuberculous enteritis," the lesions produced by it being corrugated thickenings of the mucous membrane, especially of the small intestine. The disease has now been observed in various countries, and several cases in Britain have been recorded by M'Fadyean. The bacilli occur in large numbers in the lesions, and can readily be found in scrapings from the surface. They resemble the tubercle bacillus in appearance, but on the whole are rather shorter; they are equally acid-fast. The organism has not yet been cultivated outside the body.

**Smegma Bacillus.**—This organism is of importance, as in form and staining reaction it somewhat resembles the tubercle bacillus and may be



FIG. 85.—Smegma bacilli. Film preparation of smegma. Ziehl-Neelsen stain.  $\times 1000$ .

mistaken for it. It occurs often in large numbers in the smegma præputiale and in the region of the external genitals, especially where there is an accumulation of fatty matter from the secretions. Morphologically it is a slender, slightly curved organism, like the tubercle bacillus, but usually distinctly shorter (Fig. 85). Like the tubercle bacillus, it stains with some difficulty and resists decolorisation with strong mineral acids. Most observers ascribe the latter fact to the fatty matter with which it is surrounded, and find that if the specimen is treated with alcohol the organism is easily decolorised.

Czaplewski, however, who claims to have cultivated it on various media, finds that in culture it shows resistance to decolorisation both with alcohol and with acids, and considers, therefore, that the reaction is not due to the surrounding fatty medium. We have found that in smegma it can be readily decolorised by a minute's exposure to alcohol after the usual treatment with sulphuric acid, and thus can be readily distinguished from the tubercle bacillus. We, moreover, believe that minor points of difference in the microscopic appearances of the two organisms are quite sufficient to make the experienced observer suspicious if he should meet with the smegma bacillus in urine, and lead him to apply the decolorising test. Difficulty will only occur when a few scattered bacilli retaining the fuchsin are found.

Its cultivation, which is attended with some difficulty, was first effected by Czaplewski. On serum it grows in the form of yellowish-grey irregularly rounded colonies about 1 mm. in diameter, sometimes becoming confluent to form a comparatively thick layer. He found that it also grew on glycerin agar and in bouillon. It is non-pathogenic to various animals which have been tested.

Cowie has recently found that acid-fast bacilli are of common occur-



rence in the secretions of the external genitals, mammæ, etc., in certain of the lower animals, and that these organisms vary in appearance. He considers that the term "smegma bacillus" probably represents a number of allied species.

The question may be asked—do these results modify the validity of the staining reaction of tubercle bacilli as a means of diagnosis? The source of any acid-fast bacilli in question is manifestly of importance, and it may be stated that when these have been obtained from some source outside the body, or where contamination from without has been possible, their recognition as tubercle bacilli cannot be established by microscopic examination alone. In the case of material coming from the interior of the body, however,—sputum, etc.,—the condition must be looked on as different, and although an acid-fast bacillus (not tubercle) has been found by Rabinowitch in a case of pulmonary gangrene, we have no sufficient data for saying that acid-fast bacilli other than the tubercle bacillus flourish within the tissues of the *human* body, except in such rare instances as to be practically negligible. (To this statement the case of the leprosy bacillus is of course an exception.) Accordingly, up till now, the microscopic examination of sputum, etc., cannot be said to have its validity shaken, and we have the results of enormous clinical experience that such examination is of practically unvarying value. Nevertheless the facts established with regard to other acid-fast bacilli must be kept carefully in view, and great care must be exercised when only one or two bacilli are found, especially if they deviate in their morphological characters from the tubercle bacillus.

**Action of dead Tubercle Bacilli.**—The remarkable fact has been established by independent investigators, that tubercle bacilli in the dead condition, when introduced into the tissues in sufficient numbers, can produce tubercle-like nodules. Prudden and Hodenpyl, by intravenous injection in rabbits of cultures sterilised by heat, produced in the lungs small nodules in which giant-cells, but no caseation, were occasionally present, and which were characterised by more growth of fibrous tissue than in ordinary tubercle. The subject was very fully investigated with confirmatory results by Straus and Gamaleia, who found that, if the number of bacilli introduced into the circulation were large, there resulted very numerous tubercle nodules with well-formed giant-cells, and occasionally traces of caseation. The bacilli can be well recognised in the nodules by the ordinary staining method. In these experiments the bacilli were killed by exposure to a temperature of 115° C. for ten minutes before being injected. Similar nodules can be produced by intra-



peritoneal injection. Subcutaneous injection, on the other hand, produces a local abscess, but in this case no secondary tubercles are found in the internal organs. Further, in many of the animals inoculated by the various methods, a condition of marasmus sets in and gradually leads to a fatal result, there being great emaciation before death. These experiments, which have been confirmed by other observers, show that even after the bacilli are dead they preserve their staining reactions in the tissues for a long time, and also that there are apparently contained in the bodies of the dead bacilli certain substances which act locally, producing proliferative and, to a less extent, degenerate changes, and which also markedly affect the general nutrition. S. Stockman has found that an animal inoculated with large numbers of dead tubercle bacilli afterwards gives the tuberculin reaction.

**Practical Conclusions.**—From the facts above stated with regard to the conditions of growth of the tubercle bacilli, their powers of resistance, and the paths by which they can enter the body and produce disease (as shown by experiment), the manner by which tuberculosis is naturally transmitted can be readily understood. Though the experiments of Sander show that tubercle bacilli can multiply on vegetable media to a certain extent at warm summer temperature, it is doubtful whether all the conditions necessary for growth are provided to any extent in nature. At any rate, the great multiplying ground of tubercle bacilli is the animal body, and tubercular tissues and secretions containing the bacilli are the chief, if not the only, means by which the disease is spread. The tubercle bacilli leave the body in large numbers in the sputum of phthisical patients, and when the sputum becomes dried and pulverised they are set free in the air. Their powers of resistance in this condition have already been stated. As examples of the extent to which this takes place, it may be said that their presence in the air of rooms containing phthisical patients has been repeatedly demonstrated. Williams placed glass plates covered with glycerine in the ventilating shaft of the Brompton Hospital, and after five days found, by microscopic examination, tubercle bacilli on the surface, whilst Klein found that guinea-pigs kept in the ventilating shaft became tubercular. Cornet produced tuberculosis in rabbits by inoculating them with dust collected from the walls of a consumptive ward. Tubercle bacilli are also discharged in considerable quantities in the urine in tubercular disease of the urinary tract, and also by the bowel when there is tubercular ulceration; but, so far as the human subject is concerned, the great means



of disseminating the bacilli in the outer world is dried phthisical sputum, and the source of danger from this means can scarcely be overestimated. Every phthisical patient ought to be looked upon as a fruitful source of infection to those around, and should only expectorate on pieces of rag which are afterwards to be burnt, or into special receptacles which are to be then sterilised either by boiling or by the addition of a 5 per cent. solution of carbolic acid.

Another great source of infection is in all probability the milk of cows affected with tuberculosis of the udder. In such cases the presence of tubercle bacilli in the milk can usually be readily detected by centrifugalising it, and then examining the deposit microscopically, or by inoculating an animal with it. As pointed out by Woodhead and others, the milk from cows thus affected is probably the great source of *tabes mesenterica*, which is so common in young subjects. In these cases there may be tubercular ulceration of the intestine, or it may be absent. Woodhead found that out of 127 cases of tuberculosis in children, the mesenteric glands showed tubercular affection in 100, and that there was ulceration of the intestine in 43. It is especially in children that this mode of infection occurs, as in the adult ulceration of the intestine is rare as a primary infection, though it is common in phthisical patients as the result of infection by the bacilli in the sputum which has been swallowed. There is less risk of infection by means of the flesh of tubercular animals, for, in the first place, tuberculosis of the muscles of oxen being very rare, there is little chance of the bacilli being present in the flesh unless the surface has been smeared with the juice of the tubercular organs, as in the process of cutting up the parts; and, in the second place, even when present they will be destroyed if the meat is thoroughly cooked.

We may state, therefore, that the two great modes of infection are by inhalation, and by ingestion, of tubercle bacilli. By the former method the tubercle bacilli will in most cases be derived from the human subject; in the latter, probably from tubercular cows, though inhaled tubercle bacilli may also be swallowed and contamination of food by tubercular material from the human subject may occur. Alike when inhaled and when ingested, tubercle bacilli may lodge about the pharynx and thus come to infect the pharyngeal lymphoid tissue, tonsils, etc., tubercular lesions of these parts being much more frequent than was formerly supposed. Thence the cervical lymphatic glands may become infected, and afterwards other groups of glands, bones, or joints, and internal organs.



**The Specific Reactions of Tubercle Bacillus.**—The tubercle bacillus belongs to the group of organisms which do not secrete soluble toxins into the media in which they are growing. It shares with other members of the group the capacity to induce serious changes in the metabolism of an animal. As, in other similar cases, we are in the dark as to how these changes come about, and thus can only summarise the chief effects which, by present methods, can be detected as occurring in the bodies of infected animals. These effects which, it may be remarked, are of value in the diagnosis of tubercular affections, consist on the one hand (*a*) of certain phenomena of supersensitiveness, and on the other (*b*) of certain changes in the blood serum of tubercular patients resulting from reactions of immunity. The former are seen when the bacilli or substances artificially derived from their bodies are introduced into the tissues of those suffering from tuberculosis, and were first demonstrated by Koch in his work on tuberculin. In recent times, examples of similar effects are the ophthalmic reaction of Calmette and the cutaneous reaction of von Pirquet. The changes in the blood serum of infected persons depend on the presence of anti-substances in the blood. These may be of the nature of (*a*) immune bodies which lead to fixation of complement, and (*b*) precipitins, (*c*) agglutinins, (*d*) opsonins. These may now be severally discussed in detail.

(1) **Phenomena of Supersensitiveness.** (*a*) *Koch's Old Tuberculin.*—Koch (1890-1) stated that if in a guinea-pig suffering from the effects of a subcutaneous inoculation with tubercle bacilli, a second subcutaneous inoculation of tubercle bacilli was practised in another part of the body, superficial ulceration occurred in the primary tubercular nodule, the wound healed, and the animal did not succumb to tuberculosis. This reaction was further studied by means of tuberculin, which consisted of a concentrated glycerin bouillon culture of tubercle in which the bacilli had been killed by heat. Its essential components probably were the dead and often macerated bacilli and the substances indestructible by boiling which existed in these bacilli, or which were formed during their growth. The injection of .25 c.c. of tuberculin into a healthy man causes, in from three to four hours, malaise, tendency to cough, laboured breathing, and moderate pyrexia; all of which pass off in twenty-four hours. The subcutaneous injection, however, of .01 c.c. into a tubercular person gives rise to similar symptoms (now known as the *tuberculin reaction*), but in a much more aggravated form, and in addition there occurs around any tubercular focus great inflammatory reaction, resulting in necrosis



and a casting off of the tubercular mass, when this is possible, as for instance in the case of lupus. The bacilli are, it was shown, not killed in the process.

The hopes which the introduction of tuberculin raised, that a curative agent against tuberculosis had been discovered, were soon found not to be justified. It was very difficult to see how the necrosed material which was produced and which contained the still living bacilli, could be got rid of either naturally, as would be necessary in the case of a small tubercular deposit in a lung or a lymphatic gland, or artificially, as in a complicated joint-cavity where surgical interference could be undertaken. Not only so, but the ulceration which might be the sequel of the necrosis appeared to open a path for fresh infection. Soon facts were reported which justified these criticisms. Cases where rapid acute tubercular conditions ensued on the use of tuberculin were reported, and in a few months the treatment was practically abandoned.

(b) *The Cutaneous Tuberculin reaction of von Pirquet and the Ophthalmo-reaction of Calmette.*—In recent times the diagnosis of tuberculosis has been considerably aided by the introduction of these two tests. Both are essentially of the same nature, and depend like the original tuberculin reaction on the sensitiveness of the tissues of tubercular patients to tuberculin.

The *cutaneous* test is carried out as follows: The skin, usually that of the flexor aspect of the forearm, is well cleansed with ether and then allowed to dry. Two drops of tuberculin are placed on the prepared surface about four inches apart, and then midway between the two drops a small spot is scarified with a small metal bore constructed for the purpose. This serves as a control, any reaction which follows in it being merely a traumatic one. Similar scarification is effected through the drops of tuberculin, so that the scarified spots are exposed to its action. Small portions of cotton wool are placed over the drops to prevent the tuberculin from running off, and the latter is allowed to act for ten minutes. After that time the cotton wool is removed; no dressing is required. In the process of scarification only the epidermis should be injured and blood should not be drawn. The "old" tuberculin of Koch is that used. In the case of a positive reaction an inflammatory redness and swelling make their appearance round the sites of tuberculin inoculation, generally within a few hours, and at the end of twenty-four hours there is a distinct inflammatory papule about half an inch in diameter, with a somewhat paler centre like a spot of urticaria; sometimes in the centre there are minute vesicles. The maximum



effect usually occurs within forty-eight hours, and after that time the reaction gradually recedes. Such is the typical reaction, but of course slighter, and also more intense reactions are met with. In a negative reaction all three points of scarification show merely a slight traumatic redness which soon passes off.

For the *ophthalmo-reaction* Calmette uses a purified tuberculin. The tuberculin is prepared as in Koch's original method, and is precipitated with 95 per cent. alcohol; the precipitate is then dissolved in water. This process is repeated other two times, and the final precipitate is made up as a 1 per cent. solution in distilled water. For use, in the case of an adult, a drop of this solution is placed in the conjunctival sac and the fluid allowed to spread over the surface; for children about half this quantity is sufficient. In the case of a positive reaction the ocular conjunctiva is congested, the lids become somewhat swollen and their inner surface presents a bright red colour, there is increased secretion of tears and a varying amount of fibrinous exudation. The reaction usually reaches its maximum in from six to ten hours after the instillation, and commences to pass off in from twenty-four to thirty-six hours,—in children a little sooner.

The general results obtained by these two reactions appear to correspond closely. A distinct positive result obtained by either is practically conclusive as to the presence of a tubercular lesion. In cases of latent tuberculosis the reaction is sometimes obtained, sometimes not. Again, in very advanced cases of tuberculosis, especially a short time before death, a negative result may be got; in some of these cases v. Pirquet has met with a colourless papule or a livid spot without exudation, conditions which he describes as indicating a "cachectic reaction." The *ophthalmo-reaction* is the more easily applied, at least in adults, but its use is contra-indicated when there is any abnormal condition of the conjunctiva. Even apart from this, however, inflammatory symptoms of disagreeable severity sometimes supervene. It should also be noted that a second test ought not to be applied to the same eye, as the first may produce a condition of super-sensibility (p. 284). V. Pirquet claims for his method that in the case of children it can be satisfactorily carried out with greater ease than the *ophthalmic* test.

It will be recognised that the processes underlying the original tuberculin reaction on the one hand, and the cutaneous and *ophthalmic* reactions on the other, are analogous. In the former there is the occurrence of local inflammation with metabolic changes and fever; in the latter, of mild inflammatory effects,—in both cases the phenomena being found only in tubercular



subjects. The original explanation given by Koch of the tuberculin reaction was that the essential constituent of tuberculin being toxic products of the tubercle bacillus, the action of these was superadded to the toxins produced at the focus of infection. The combined action of the toxins from these two sources caused a rapid necrosis of the newly formed cells, and opened the way for the dead tissue being rapidly cast off. This explanation was, however, not generally accepted, for it was found that other substances, such as albumoses, when injected into animals suffering from local tuberculosis, gave rise to modified effects of the same kind as those produced by tuberculin. This dissatisfaction with regard to the original explanation is accentuated by a consideration of the effects seen in the Calmette and v. Pirquet tests, as these clearly indicate that the sensitiveness in a tubercular subject is not confined to tissues actually affected with the tubercular process, but is also manifested in parts of the body distant from the site of actual infection.

Further, it has been found, first, that the injection of tuberculin directly into a tubercular focus does not produce the tuberculin reaction (a fact which militates against the idea of concentrations of toxins), and, secondly, that the injection of living or dead tubercle bacilli into healthy animals produces anaphylactic phenomena similar to those originated by foreign albumins generally. At the present time, therefore, although no full explanation can be given of the tuberculin reaction and of similar reactions, it is likely that in tuberculosis a general hypersensitiveness is developed, and may be the underlying phenomenon. These reactions must therefore be considered in the light of what will be set forth on the subject of hypersensitiveness in the chapter on Immunity.

*The Use of Old Tuberculin in the Diagnosis of Tuberculosis in Cattle.*—In cattle, tuberculosis may be present without giving rise to apparent symptoms. It is thus important from the point of view of human infection that an early diagnosis should be made. The method is applied as follows:—The animals are kept twenty-four hours in their stalls, and the temperature is taken every three hours, from four hours before the injection till twenty-four after. The average temperature in cattle is  $102.2^{\circ}$  F. ; 30 to 40 centigrammes of tuberculin are injected, and if the animal be tubercular the temperature rises  $2^{\circ}$  or  $3^{\circ}$  F. in eight to twelve hours, and continues elevated for ten to twelve hours. Bang, who has worked most at the subject, lays down the principle that the more nearly the temperature approaches  $104^{\circ}$  F. the more reason for suspicion is there. He gives a record of 280 cases where the value of the method was tested by subsequent *post-mortem* examination. He found that with proper precautions the error was only 3.3 per cent. The method has been largely practised in all parts of the world, and is of great value.



(2) **Immunity Phenomena in Tuberculosis.**—*Koch's Tuberculin-R.* The study of immunity phenomena in tuberculosis dates from the introduction by Koch in 1897 of the substance denominated by him "Tuberculin-R." Koch's new researches consisted (1) of an attempt to immunise animals against the tubercle bacillus by employing its intracellular toxins; (2) of trying to utilise such an immunisation to aid the tissues of an animal already attacked with tubercle the better to combat the effects of the bacilli. The method of obtaining the intracellular toxins was as follows: Bacilli from young virulent cultures were dried *in vacuo*, and disintegrated in an agate mill, treated with distilled water and centrifugalised. The clear fluid was decanted, and is called by Koch "Tuberculin-O." The remaining deposit was again dried, ground, treated with water and centrifugalised; the clear fluid being again decanted, and this process was repeated with successive residues till no residue remained. These fluids put together constitute the "Tuberculin-R." From the fact that tuberculin-O gave no cloudiness when glycerin was added, Koch concluded that it contained the substances present in the glycerin-bouillon extracts originally used by him, and he held this was borne out by the readiness with which a tuberculin reaction could be caused by it. Similarly, as tuberculin-R gave a cloudiness with glycerin and did not readily originate a reaction, he considered that it contained different products of the bacillus. When injected into animals in repeated and increasing doses,  $\frac{1}{500}$  mgrm. being the initial dose, tuberculin-R was said to produce immunity against the original extract, against tuberculin-O, and against living and virulent tubercle bacilli. Further research has not confirmed this last result.

*Koch's New Tuberculin.*—Another preparation has also been introduced, known as "Koch's new tuberculin" (*Bazillenemulsion*). This is an emulsion of ground tubercle bacilli in water containing 50 per cent. of glycerin; it thus really contains both tuberculin-O and tuberculin-R.

Scientific enquiry into the action of these new tuberculin preparations has resulted in attempts being made to recognise in their effects phenomena similar to those produced by organisms such as the typhoid and cholera bacteria, the investigation of which has brought out the complex processes at work in the reaction of an organism against invading bacteria. The phenomena manifested in such cases consist in the formation of immune-bodies, precipitins, agglutinins, and opsonins.

(1) *Immune-bodies and Precipitins.*—Evidence for the existence of these in tuberculosis has been sought by applying



the method of complement fixation (see p. 130), *e.g.* the serum of a tubercular animal being mixed with tuberculin, the mixture is tested for its capacity of absorbing complement. Following this line, Wasserman and others have found evidence of the presence of an *antituberculin* in tubercular foci, and this is taken as an indication of the occurrence of a vital reaction against the poisons of an invading organism. Generally speaking, such an antituberculin is absent from the blood serum of most tubercular patients. It is present, however, in the serum of such individuals after they have been subjected to repeated tuberculin injections. Here it is chiefly seen when a patient is losing the capacity for reacting to the injections. Another immunity phenomenon which may be observed is the formation of a *precipitate* when some of the serum of a tuberculous patient is added to a solution of tuberculin, the mixture being allowed to stand for twenty-four hours (precipitin reaction). The exact relationship of such precipitins to immune-bodies is still doubtful; that it is a close one is shown by the fact that such precipitates have the property of absorbing complement. At present it is enough to say that there is evidence in tubercular infection of a vital reaction resulting in the formation of antagonistic bodies, which may include both immune-bodies and precipitins. In support of the view that immune-bodies exist against the tubercle bacillus, it may be said that the sera of certain animals, *e.g.* rabbit and ox, when mixed with tuberculin, become capable of deviating complement from a hæmolytic combination.

(2) *Agglutinins*.—The serum of tubercular patients has been found to exert an agglutinating action on the tubercle bacillus. A convenient method is to add different amounts of serum, commencing with, say, 1 c.c., to quantities of a dilution of the new tuberculin (*Bazillenemulsion*) equivalent to 1 part of the bacterial bodies to 10,000 of diluent, and leave the mixture for twenty-four hours before observing. As with other agglutinative observations, it is difficult to correlate the degree of agglutinating power of the serum with the degree of immunisation possessed by the individual from which it was derived. The method has been used by some as a means of diagnosis, but its value is doubtful and is certainly inferior to the methods depending on supersensitiveness.

(3) *Opsonins*.—The serum of most normal men and of several species of animals normally contains opsonins to the tubercle bacillus. The opsonic effect is also manifested in varying degree by the serum during the course of natural infection; such variations are considered below.



In considering the relationships of the specific immune reactions against the tubercle bacillus, it is to be noted that while the existence of such reactions has been established, the development of these to an extent likely to benefit an infected animal is limited, and the production of such a lasting immunity as would enable it to resist an infection or to throw off an infection already established is extremely difficult or impossible. There are probably factors in the pathology of the tubercular process which militate against such an occurrence. This process seems to differ from what occurs in more acute infections, in that a local lesion may be in existence for a very considerable period without other parts of the body being much or at all concerned. This is especially marked in certain tubercular manifestations, the outstanding example of which is lupus, in which for years, while the bacilli are present and active in the skin, even the adjacent lymphatic glands may show no signs of disease. What underlies this apparent independence of the body generally in relation to a serious condition affecting one locality is unknown. Other examples of a similar process are found in leprosy and also in certain chronic suppurations of the skin.

**Therapeutic Applications of the Tuberculins.**—We have already stated that the use of the old tuberculin to mechanically remove local foci of tuberculosis through the use of large doses of the reagent was soon found to be impracticable, but both this preparation and its modifications have been largely used in what is now denominated "vaccine-therapy." It has been already pointed out that the tubercular process is peculiar in that the disease may exist locally without much affecting the general health of the infected individual. The principle of vaccine-therapy may roughly be said to be to bring into play the potential but latent defensive mechanisms of the body with the object of so reinforcing the cells locally attacked as to enable them to destroy the invading bacteria. This is effected by introducing into the body small doses of the infecting agent, and is in reality an immunisation carried through after infection has already taken place. For this purpose all the tuberculin preparations, but especially tuberculin-R and the "new tuberculin," have been used. In the case of both the latter, doses commencing with from  $\frac{1}{400}$  to  $\frac{1}{500}$  mgrm., gradually increased, were given every second day, and the rule originally laid down for the regulation of the dosage was that no amount should be given which raised the temperature more than  $5^{\circ}$  F. Opinion varied as to the efficacy of such treatment. There was little



doubt that in certain cases of local conditions, such as lupus, tubercular joints, glands, and genito-urinary tuberculosis, improvement followed its application; but where febrile conditions indicated that general disturbances were in existence, there was little justification for its being applied, and even in many local conditions the absence of benefit was so marked that by many physicians the method had been abandoned.

*Active Immunisation associated with Opsonic Observations.*—The credit of rehabilitating the vaccine-therapy of tuberculosis and of defining its scope belongs to Wright, who directed attention to the possibility of controlling the use of the tuberculin by observations of its effect on the opsonic qualities of the serum. Early in his work he showed that tubercle bacilli when sensitised by an appropriate serum, were readily phagocyted by the polymorpho-nucleate leucocytes, and the relative sensitising capacities of serum from tubercular and non-tubercular cases has been widely studied. According to Wright, in strictly localised tuberculosis, the opsonic index is persistently low, varying from  $\cdot 1$  to  $\cdot 9$ , while in tuberculosis with general disturbances it fluctuates greatly from day to day, being sometimes below, sometimes above unity. To take the former and simpler case, he holds that if the treatment with injections of tuberculin be controlled by noting the effect produced on the opsonic index, great improvement in the patient's condition may result. Wright's interpretation of what occurs is briefly as follows: For reasons unknown the opsonic qualities of the body fluids may become abnormally low, and the tubercle bacilli, if they gain admission to the body, can multiply locally. This multiplication is associated with a still further local diminution of the opsonins. By the introduction of such a substance as tuberculin, the bodily mechanism, whatever it is, which produces the opsonins is stimulated, and a rise in the general opsonic index occurs. Naturally this is accompanied by a passing to the site of infection of fluids more rich in opsonins than previously, the activity of the phagocytes comes into play, and the tubercle bacilli are destroyed. But any such vaccination process must be controlled by constant observations of the opsonic index, and it is only by this means, not only that good results can be obtained, but that the production of harmful effects can be prevented. The reason of this is that in a great many cases the injection of a bacterial vaccine is followed by a decrease in the opsonic qualities of the serum,—the occurrence of a negative phase. During such a period of depression there is probably an increased susceptibility to the action of the



bacilli. Now, in order to get permanent benefit from the vaccination process, repeated injections of the tuberculin must be practised, and if an injection be given during a negative phase, actual harm may be done. The course of a successful vaccination is that, after the passing off of the negative phase, the opsonic index should rise to above its original level,—the occurrence of a positive phase. It is when this positive phase is fully developed that a fresh inoculation can be practised with success. The new negative phase which will now occur may not cause a drop to below the level of the original state of the serum, and the hope is that its succeeding positive phase will carry the opsonic index still higher and ensure a still greater resistance to the bacterium. There are very great variations in the capacities shown by tubercular patients to react to a vaccination process. In certain cases good positive phases are readily and quickly produced, while in others, after an inoculation the negative phase is long continued and may even show no tendency to pass into a positive phase. The irregularities in the opsonic index in cases where there is a general disturbance of metabolism, Wright explains by supposing that they result from very irregular auto-infections of the patient's body by tubercular products from the local lesions,—positive and negative phases being produced without the purposive quality which ought to characterise a successful therapeutic vaccination. Such auto-infections may come about in various ways, and Wright is of opinion that exercise, for instance, may disseminate both tubercular products and tubercle bacilli,—he having noticed in certain patients a fall in the opsonic index after muscular exertion. For ordinary cases with low opsonic index and no evidence of constitutional disturbance, an amount of tuberculin corresponding to from one-thousandth to a six-hundredth of a milligramme of tubercle powder is a sufficient dose, and if any dose seems to produce a pronounced negative phase, then a smaller dose ought to be tried at the next inoculation. For cases clinically tubercular, where the index is about normal, then smaller doses, say, the equivalent of a two-thousandth of a milligramme or less, ought to be used,—the effect on the index being carefully watched. In any case, the dose which is found to give the highest positive phase is the optimum dose, and one which need not necessarily be increased. Cases where there is constitutional disturbance should be as a rule left untreated.

With regard to the results obtained, many cases have been brought forward by Wright and others where benefit has followed the putting into practice of the principles enunciated,



and there is little doubt that the work done has given a fresh start to the active immunisation method in the treatment of tuberculosis. An outstanding event of Wright's work in this field has been his insistence on the good effects produced by extremely small doses of tuberculin (down to the four-thousandth of a milligramme) given at fairly long intervals (say ten days or more). With regard to the efficacy of the opsonic method as affording an index to the progress of a case, it must be recognised that the method is still on its trial, and it is doubtful if even in the work of the most careful observers the limits of the experimental error of the opsonic method have been sufficiently defined.

Great controversy has taken place as to whether it is justifiable, in the treatment of tubercular cases with tuberculin, merely to rely on the observation of the clinical effects, without having recourse to the constant estimation of the opsonic index, which Wright considers advisable. There is no doubt that in all complicated cases of tuberculosis, such as lung affections and cases of multiple foci in the body, the treatment ought to be in the hands of an expert. In cases of strictly localised tubercle, however, such as adenitis, arthritis, cystitis, or lupus, Wright admits that in many cases, without much risk, an uncontrolled treatment may be undertaken. The injections ought to begin with doses of one-twenty-thousandth of a milligramme, with ten-day intervals intervening between each dose. If clinical improvement occurs, the dose may be gradually increased until it reaches one four-thousandth of a milligramme after six months. If the treatment of any other form of tuberculosis be undertaken along similar lines, the preliminary injection should not consist of more than one-fifty-thousandth of a milligramme.

The whole question of the immunisation treatment of tuberculosis presents many difficulties, and it is the merit of Wright's work that it has shed fresh light on some of these. One great difficulty arises from the great chronicity of the results of the infection in the majority of human cases. It is probably true not only of man but of many species of animals used in experimental inquiries, that many individuals are on the border-line between immunity and susceptibility. From the widespread distribution of the bacilli in centres of human population, it is certain that the opportunity for infection arises in a very large proportion of the race; in many cases no results follow infection, and in many others small lesions occur which do not develop further; this has actually been shown by morbid anatomists to be the case. The disease being thus so often characterised by transient



local effects without constitutional disturbance, the course of an immunisation may be expected to be rather different from that obtaining in an ordinary acute affection, though the underlying processes may be of the same nature. It is difficult, for instance, on account of the slowness of tubercular processes, to define recovery from an attack of the disease, or to speak of an animal recovering from the effect of an inoculation during an immunisation. It follows that little is known regarding an attenuation of the tubercle bacillus analogous to what is an important feature in immunisations against other organisms.

**Antitubercular Sera.**—Several attempts have been made to treat tuberculosis with the serum of animals immunised by the tubercle bacillus or its products. The most successful is perhaps that of Maragliano. This author distinguishes between the toxic materials contained in the bodies of the bacilli (which withstand, unchanged, a temperature of 100° C.) and those secreted into the culture fluid (which are destroyed by heat). The substance used by him for immunising his animals consists of three parts of the former and one of the latter. The animals employed are the dog, the ass, the horse. The serum obtained from these is capable of protecting healthy animals against an otherwise fatal dose of tuberculin, but very little importance can be attached to this result. Maragliano does not appear to have studied the effects of this serum on tubercular animals, but it has been tried in a great number of cases of human tuberculosis, 2 c.c. being injected subcutaneously every two days. Improvement is said to have taken place in a certain proportion, especially of mild non-febrile cases.

An antitubercular serum has also been introduced by Marmorek. This observer considers that the tubercle bacillus cannot produce in ordinary media the toxins which it originates when exposed to the antagonism of the bodily cells. He tries to make good this defect by first growing it in a serum antagonistic to some of the phagocytic cells of the body; for this a leucotoxic serum is used. When the bacillus has grown in this presumably favourable soil, it is transferred to a medium containing a substance which may be unfavourable; and for this there is employed a medium containing liver extract, the liver being an organ in which in man tubercular lesions are comparatively rare. The bacilli being thus accustomed to an unfavourable surrounding are used for immunising animals, the serum of which is now suitable for the treatment of human tuberculosis. There is considerable diversity of opinion as to the efficacy of Marmorek's serum as a therapeutic agent.



**Methods of Examination.**—(1) *Microscopic Examination.* Tuberculosis is one of the comparatively few diseases in which a diagnosis can usually be definitely made by microscopic examination alone. In the case of sputum, one of the yellowish fragments which are often present ought to be selected; dried films are then prepared in the usual way, and stained by the Ziehl-Neelsen stain (p. 108). In the case of urine or other fluids, a deposit should first be obtained by centrifugalising a quantity in a test-tube, or by allowing the fluids to stand in a tall glass vessel (an ordinary burette is very convenient). Film preparations are then made with the deposit and treated as before. If a negative result is obtained in a suspected case, repeated examination should be undertaken. To avoid risk of contamination with the smegma bacillus, the meatus of the urethra should be cleansed and the urine first passed should be rejected, or the urine may be drawn off with a sterile catheter. As stated above, it is only exceptionally that difficulty will arise to the experienced observer from this cause. (For points to be attended to, *vide* p. 280). The detection of tubercle bacilli by microscopical methods in sputum, pus, fæces, and even tissues, has been greatly facilitated by the recent introduction of a preparation called "antiformin." This is a mixture of equal parts of liquor sodæ chlorinatæ (B.P.) and of a 15 per cent. solution of caustic soda. It has a remarkable disintegrative and dissolving action on the tissues, etc., so that after it has been allowed to act on sputum, for example, and the mixture is centrifugalised, the resulting deposit is scanty and the tubercle bacilli, if present, are accordingly greatly concentrated. The time necessary may be judged of by the appearance of the mixture, but it will generally be found that the desired result will be obtained if one part of antiformin be added to five or six parts of sputum and allowed to act for two or three hours.

(2) *Inoculation.*—The guinea-pig is the most suitable animal. If the material to be tested is a fluid, it is injected subcutaneously or into the peritoneum; if solid or semi-solid, it is placed in a small pocket in the skin, or it may be thoroughly broken up in sterile water or other fluid and the emulsion injected. By this method, material in which no tubercle bacilli can be found microscopically may sometimes be shown to be tubercular.

(3) *Cultivation.*—Owing to the difficulties this is usually quite impracticable as a means of diagnosis, and it is also unnecessary. The best method to obtain pure cultures is to produce tuberculosis in a guinea-pig by inoculation with tubercular material, and then, killing the animal after four or



five weeks, to inoculate tubes of solidified blood serum, under strict aseptic precautions, with portions of a tubercular organ, *e.g.* the spleen. The portions of tissue should be fairly large, and should be well rubbed into the broken surface of the medium. Cultures may, however, be obtained from sputum by means of antiformin, as this substance readily kills most of the ordinary bacteria and has comparatively slight effect on the tubercle bacillus. Antiformin should be allowed to act on sputum in the proportion and for the time mentioned in paragraph (1), the mixture should then be centrifugalised, the supernatant fluid removed, and the deposit washed with sterile water and again centrifugalised, these processes being repeated several times. If, then, inoculations be made from the deposit on blood serum or on Dorset's egg medium, pure cultures of the tubercle bacillus may, in some instances, be obtained. The method is one which gives good results. Another somewhat similar method is that introduced by Twort; in this, portions of sputum are exposed to the action of a 2 per cent. solution of ericolin (a glucoside) for an hour at 38° C., and thereafter cultures are made on Dorset's medium.

(4) *Reactive phenomena*.—The presence of immune-substances in the blood and the tuberculin reaction, along with the methods of applying the respective tests, have been described above (p. 284).



## CHAPTER XI.

### LEPROSY.

LEPROSY is a disease of great interest, alike in its clinical and pathological aspects; whilst from the bacteriological point of view, also, it presents some striking peculiarities. The invariable association of large numbers of characteristic bacilli with all leprosy lesions is a well-established fact, and yet, so far, attempts to cultivate the bacilli outside the body, or to produce the disease experimentally in animals, have been attended with failure. Leprosy, so far as is known, is a disease which is confined to the human subject, but it has a very wide geographical distribution. It occurs in certain parts of Europe—Norway, Russia, Greece, etc., but is commonest in Asia, occurring in Syria, Persia, etc. It is prevalent in Africa, being especially found along the coast, in the Pacific Islands, in the warmer parts of North and South America, and also to a small extent in the northern part of North America. In all these various regions the disease presents the same general features, and the study of its pathological and bacteriological characters, wherever such has been carried on, has yielded similar results.

**Pathological Changes.**—Leprosy is characteristically a chronic disease, in which there is a great amount of tissue change, with comparatively little necessary impairment of the general health. In other words, the local effects of the bacilli are well marked, often extreme, whilst the toxic phenomena are proportionately at a minimum.

There are two chief forms of leprosy. The one, usually called the tubercular form,—*lepra tuberosa* or *tuberculosa*,—is characterised by the growth of granulation tissue in a nodular form or as a diffuse infiltration in the skin, in mucous membranes, etc., great disfigurement often resulting. In the other form, the anæsthetic,—maculo-anæsthetic of Hansen and Looft,—the outstanding changes are in the nerves, with consequent anæsthesia, paralysis of muscles, and trophic disturbances.



In the *tubercular* form, the disease usually starts with the appearance of erythematous patches attended by a small amount of fever, and these are followed by the development of small nodular thickenings in the skin, especially of the face, of the backs of hands and feet, and of the extensor aspects of arms and legs. These nodules enlarge and produce great distortion of the surface, so that, in the case of the face, an appearance is produced

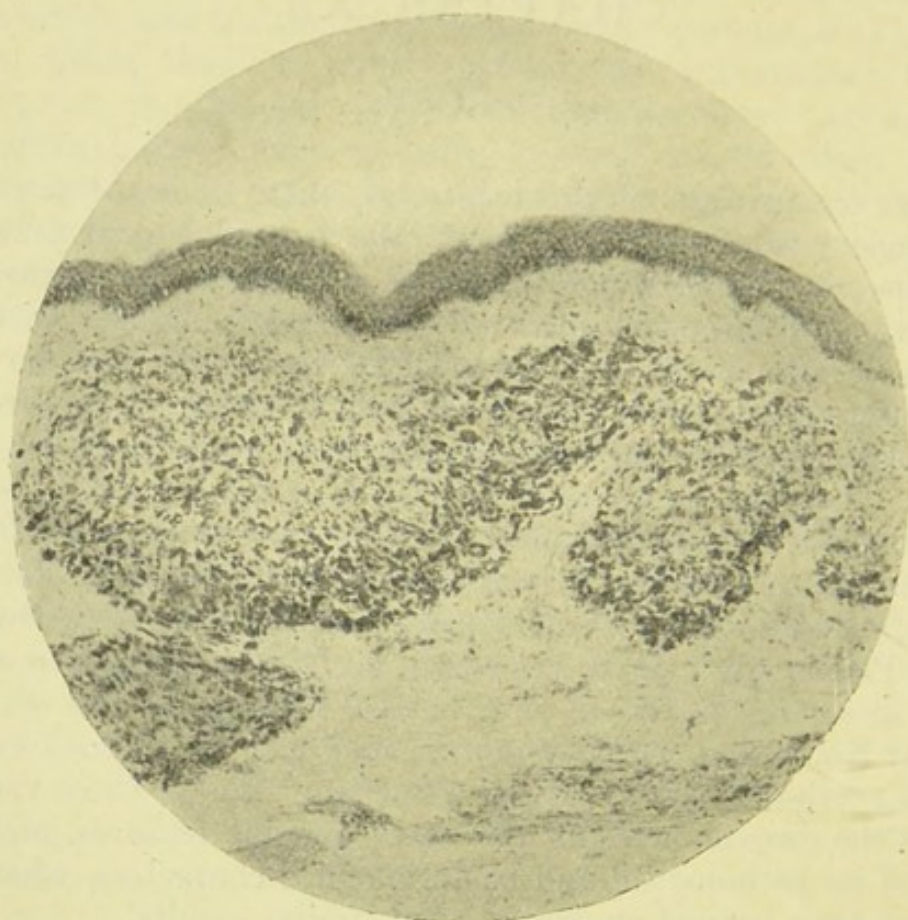


FIG. 86.—Sections through leprosy skin, showing the masses of cellular granulation tissue in the cutis; the dark points are cells containing bacilli deeply stained.

Paraffin section; Ziehl-Neelsen stain.  $\times 80$ .

which has been described as “leonine.” The thickenings occur chiefly in the cutis (Fig. 86), to a less extent in the subcutaneous tissue. The epithelium often becomes stretched over them, and an oozing surface becomes developed, or actual ulceration may occur. The cornea and other parts of the eye, the mucous membrane of the mouth, larynx, and pharynx, may be the seat of similar nodular growths. Internal organs, especially the spleen, liver, and testicles, may become secondarily affected. In all situations the change is of the same nature, consisting in an



abundant formation of granulation tissue, nodular or diffuse in its arrangement. In this tissue a large proportion of the cells are of rounded or oval shape, like hyaline leucocytes; a number of these may be of comparatively large size, and may show vacuolation of their protoplasm and a vesicular type of nucleus. These are often known as "lepra-cells." Amongst the cellular elements there is a varying amount of stroma, which in the earlier lesions is scanty and delicate, but in the older lesions may be very dense. Periarteritis is a common change, and very frequently the superficial nerves become involved in the nodules, and undergo atrophy. The tissue in the leprosy lesions is comparatively vascular, at least when young, and, unlike tubercular lesions, never shows caseation. Some of the lepra cells may contain several nuclei, but we do not meet with cells resembling in their appearance tubercle giant-cells, nor does an arrangement like that in tubercle follicles occur.

In the *anæsthetic* form, the lesion of the nerves is the outstanding feature. These are the seat of diffuse infiltrations, which lead to the destruction of the nerve fibres. In the earlier stages, in which the chief symptoms are pains along the nerves, there occur patches on the skin, often of considerable size, the margins of which show a somewhat livid congestion. Later, these patches become pale in the central parts, and the periphery becomes pigmented. There then follow remarkable series of trophic disturbances, in which the skin, muscles, and bones are especially involved. The skin often becomes atrophied, parchment-like, and anæsthetic; frequently pemphigoid bullæ or other skin eruptions occur. Partly owing to injury to which the feet and arms are liable from their anæsthetic condition, and partly owing to trophic disturbances, necrosis and separation of parts are liable to occur. In this way great distortion results. The lesions in the nerves are of the same nature as those described above, but the granulation tissue is scantier, and has a greater tendency to undergo cicatricial contraction. This is to be associated with the fact that the bacilli are present in fewer numbers.

**Bacillus of Leprosy.**—This bacillus was first observed in leprosy tissues by Hansen in 1871, and was the subject of several communications by him in 1874 and later. Further researches, first by Neisser in 1879, and afterwards by observers in various parts of the world, agreed in their main results, and confirmed the accuracy of Hansen's observations. The bacilli, as seen in scrapings of ulcerated leprosy nodules, or in sections, have the following characters:—They are thin rods of practically



the same size as tubercle bacilli, which they also resemble both in appearance and in staining reaction. They are straight or slightly curved, and usually occur singly, or two may be attached end to end; but they do not form chains. When stained they may have a uniform appearance, or the protoplasm may be fragmented, so that they appear like short rows of cocci. They often appear tapered at one or both extremities; occasionally

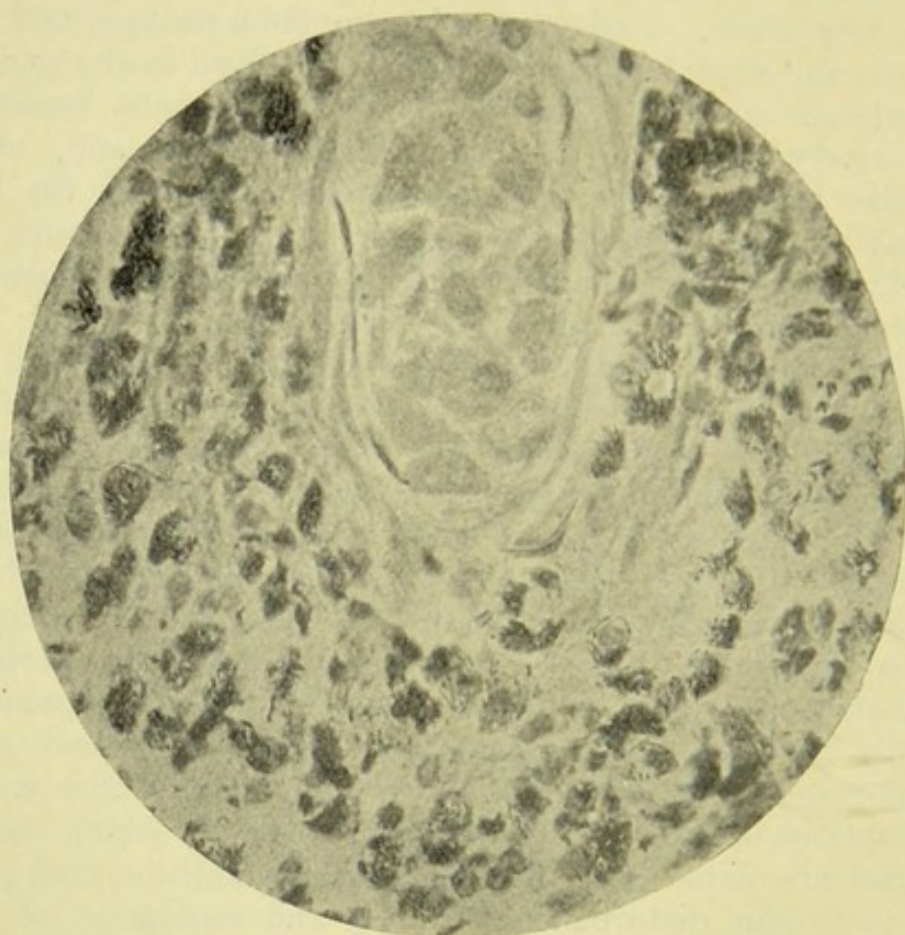


FIG. 87.—Superficial part of leprosy skin; the cells of the granulation tissue appear as dark patches, owing to the deeply stained bacilli in their interior. In the upper part a process of epithelium is seen. Paraffin section; stained with carbol-fuchsin and Bismarck-brown.  $\times 500$ .

there is slight club-like swelling. Degenerated and partially broken-down forms are also seen. They take up the basic aniline stains more readily than tubercle bacilli, but in order to stain them deeply, a powerful stain, such as carbol-fuchsin, is necessary. When stained, they strongly resist decolorising, though they are more easily decolorised than tubercle bacilli (p. 108). The bacilli are also readily stained by Gram's method. Regarding the presence of spores, practically nothing is known, though some of the unstained or stained points may be of this



nature. We have, however, no means of testing their powers of resistance. Leprosy bacilli are non-motile.

**Position of the Bacilli.**—They occur in enormous numbers in the leprous lesions, especially in the tubercular form—in fact, so numerous are they that the granulation tissue in sections, properly stained as above, presents quite a red colour under a low power of the microscope (Plate II., Fig. 8). The bacilli



FIG. 88.—High-power view of portion of leprous nodule, showing the arrangement of the bacilli within the cells of the granulation tissue. Paraffin section ; stained with carbol-fuchsin and methylene-blue  $\times 1100$ .

occur for the most part within the protoplasm of the round cells of the granulation tissue, and are often so numerous that the structure of the cells is quite obscured (Fig. 87). They are often arranged in bundles which contain several bacilli lying parallel to one another, though the bundles lie in various directions (Fig. 88 and Plate II., Fig. 9). The appearance thus presented by the cells filled with bacilli is very characteristic. Bacilli are also found free in the lymphatic spaces, but the greater number are undoubtedly contained within the cells. They are



also found in spindle-shaped connective-tissue cells, in endothelial cells, and in the walls of blood vessels. They are for the most part confined to the connective tissue, but a few may be seen in the hair follicles and glands of the skin. Occasionally one or two may be found in the surface epithelium, where they probably have been carried by leucocytes, but this position is, on the whole, exceptional. They also occur in large numbers in the lymphatic glands associated with the affected parts. In the internal organs,—liver, spleen, etc.,—when leprosy lesions are present, the bacilli are also found, though in relatively smaller numbers. In the nerves in the anaesthetic form they are comparatively few, and in the sclerosed parts it may be impossible to find any. There are few also in the skin patches referred to above as occurring in this form of the disease.

Their spread is chiefly by the lymphatics, though distribution by the blood stream also occurs. They are said to have been found in the blood during the presence of fever and the eruption of fresh nodules, and they have also been observed in the blood vessels *post mortem*, chiefly contained within leucocytes. Recent observations (*e.g.* those of Doutrelepon and Wolters) show that the bacilli may be more widely spread throughout the body than was formerly supposed. A few may be detected in some cases in various organs which show no structural change, especially in the capillaries. The brain and spinal cord are almost exempt, but in some cases bacilli have been found even within nerve cells.

**Relations to the Disease.**—Attempts to obtain pure cultures of the leprosy bacillus have so far been unsuccessful. Clegg has recently attempted to grow the organism in association with amoebæ and other bacteria on agar plates, and has obtained a short acid-fast bacillus which does not grow on ordinary media, and which has been carried through several generations in the conditions mentioned. The identity of this organism with the leprosy bacillus has, however, not been established. Attempts to transfer the disease to animals, including monkeys, have been unsuccessful. When a small portion of leprosy material is transplanted, a nodule may result in which leprosy bacilli may be demonstrated for some time, but this probably represents merely the reaction to a foreign body; there is no sufficient evidence that the bacilli undergo multiplication, and it is impossible to continue such lesions in fresh animals. The only exception to this statement is afforded by the experiments of Melcher and Orthmann, who inoculated the anterior chamber of the eye of rabbits with leprosy material, the inoculation being followed by



an extensive growth of nodules in the lungs and internal organs, which they affirmed contained leprosy bacilli. It has been questioned, however, by several authorities whether the organisms in the nodules were really leprosy bacilli, and up to the present we cannot say that there is any satisfactory proof that the disease can be transmitted to any of the lower animals. Diphtheroid bacilli of more than one variety have been cultivated from the blood and tissues of leprosy patients by Babes and others. Their presence would appear to be by no means infrequent, but it is not possible to say at present what their significance is.

It is interesting to note that a disease occurs under natural conditions in rats which presents many points of close similarity to leprosy. It is very widespread, having been observed in Europe, Asia, America, and Australia; an excellent description has been given by Dean. In this affection there are lesions in the skin which resemble those in leprosy, and the cells contain enormous numbers of an acid-fast bacillus. The disease can be transmitted to rats by inoculation with the tissue juices containing the bacilli, but not to animals of other species. All attempts to cultivate the characteristic organism outside the body have failed, but Dean has obtained a diphtheroid bacillus—a result of interest in relation to what has been found in leprosy. Whether this disease has any relation to leprosy in the human subject is very doubtful, but the facts which have been ascertained may prove of high importance in connection with the pathology of the latter disease.

It would also appear that the disease is not readily inoculable in the human subject. In a well-known case described by Arning, a criminal in the Sandwich Islands was inoculated in several parts of the body with leprosy tissue. Two or three years later, well-marked tubercular leprosy appeared, and led to a fatal result. This experiment, however, is open to the objection that the individual before inoculation had been exposed to infection in a natural way, having been frequently in contact with lepers. In other cases, inoculation experiments on healthy subjects and inoculations in other parts of leprosy individuals have given negative results. It has been supposed by some that the failure to obtain cultures and to reproduce the disease experimentally may be partly due to the bacilli in the tissues being dead. That many of the leprosy bacilli are in a dead condition is quite possible, in view of the long period during which dead tubercle bacilli introduced into the tissues of animals retain their form and staining reaction. There is also the fact that from time to



time in leprosy subjects there occur febrile attacks, which are followed by a fresh outbreak of nodules, and it would appear that especially at these times multiplication of the bacilli takes place more actively.

The facts stated with regard to cultivation and inoculation experiments go to distinguish the leprosy bacillus all the more strongly from other organisms. Some have supposed that leprosy is a form of tubercle, or tubercle modified in some way, but for this there appears to us to be no evidence. It should also be mentioned that tubercle is a not uncommon complication in leprosy subjects, in which case it presents the ordinary characters. It has been found that a considerable proportion of lepers react to tuberculin like tubercular patients. This result has been variously interpreted, some considering that tuberculosis is also present in such cases, whilst others maintain that the reaction may be given in the absence of tubercle. If, as is probable, the latter is the case, the result most likely depends on the close relationship of the organisms of the two diseases; it by no means proves their identity. Another curious fact is that the Wassermann reaction (p. 131) may be given by the serum of leprosy patients (in about 50 per cent., according to some observers); this would seem to be quite independent of the concurrent presence of syphilis, but it is not possible at present to give an explanation of the phenomenon.

The mode by which leprosy is transmitted has been the subject of great controversy, and is one on which authorities still hold opposite opinions. Some consider that it is a hereditary disease, or at least that it is transmitted from a parent to the offspring; others, again, that it is transmitted by direct contact. There appears to be no doubt, however, that on the one hand leprosy subjects may bear children free from leprosy, and that on the other hand healthy individuals entering a leprosy district may contract the disease, though this rarely occurs. Of the latter occurrence there is the well-known instance of Father Damien, who contracted leprosy after going to the Sandwich Islands. In view of all the facts, there can be little doubt that leprosy in certain conditions may be transmitted by direct contact, though its contagiousness is not of a high order.

**Methods of Diagnosis.**—Film preparations should be made with the discharge from any ulcerated nodule which may be present, or from the scraping of a portion of excised tissue, and should be stained as above described. The presence of large numbers of bacilli situated within the cells and giving the staining reaction of leprosy bacilli, is conclusive. It is more satisfactory,



however, to make microscopic sections through a portion of the excised tissue, when the structure of the nodule and the arrangement of the bacilli can be readily studied. The points of difference between leprosy and tubercle have already been stated, and in most cases there is really no difficulty in distinguishing the two conditions.



## CHAPTER XII.

### GLANDERS AND RHINOSCLEROMA.

#### GLANDERS.

THE bacillus of glanders (*bacillus mallei*; Fr., *bacille de la morve*; Ger., *Rotzbacillus*) was discovered by Löffler and Schutz, the announcement of this discovery being made towards the end of 1882. They not only obtained pure cultures of this organism from the tissues in the disease, but by experiments on horses and other animals conclusively established its causal relationship. These have been fully confirmed. The same organism has also been cultivated from the disease in the human subject, first by Weichselbaum in 1885, who obtained it from the pustules in a case of acute glanders in a woman, and by inoculation of animals obtained results similar to those of Löffler and Schutz.

Within more recent times a substance, *mallein*, has been obtained from the cultures of the glanders bacillus by a method similar to that by which tuberculin was prepared, and has been found to produce effects in animals suffering from glanders corresponding to those produced by tuberculin in tuberculous animals.

**The Natural Disease.**—Glanders chiefly affects the equine species—horses, mules, and asses. Horned cattle, on the other hand, are quite immune, whilst goats and sheep occupy an intermediate position, the former being rather more susceptible and occasionally suffering from the natural disease. It also occurs in some of the carnivora—cats, lions and tigers in menageries, which animals are infected from the carcasses of animals affected with the disease. Many of the small rodents are highly susceptible to inoculation (*vide infra*).

Glanders is also found in man as the result of direct inoculation on some wound of the skin or other part by means of the discharges or diseased tissues of an animal affected, and hence is commonest amongst grooms and others whose work brings them into contact with horses; even amongst them it is a comparatively rare disease.



In horses the lesions are of two types, to which the names "glanders" proper and "farcy" have been given, though both may exist together. In glanders proper, the septum nasi and adjacent parts are chiefly affected, there occurring in the mucous membrane nodules which are at first firm and of somewhat translucent grey appearance. The growth of these is attended usually by inflammatory swelling and profuse catarrhal discharge. Afterwards the nodules soften in the centre, break down, and give rise to irregular ulcerations. Similar lesions, though in less degree, may be found in the respiratory passages. Associated with these lesions there is usually implication of the lymphatic glands in the neck, mediastinum, etc.; and there may be in the lungs, spleen, liver, etc., nodules of the size of a pea or larger, of greyish or yellow tint, firm or somewhat softened in the centre, and often surrounded by a congested zone. The term "farcy" is applied to the affection of the superficial lymphatic vessels and glands, which is specially seen where infection takes place through an abrasion of the skin, such as is often produced by the rubbing of the harness. The lymphatic vessels become irregularly thickened, so as to appear like knotted cords, and the associated lymphatic glands become enlarged and firm, though suppurative softening usually follows, and there may be ulceration. These thickenings are often spoken of as "farcy buds" and "farcy pipes." In farcy, also, secondary nodules may occur in internal organs and the nasal mucous membrane. The disease is often present in a "latent form," and its presence can only be detected by the mallein test (*vide infra*). In the ass the disease runs a more acute course than in the horse.

In man the disease is met with in two forms, an acute and a chronic—though intermediate forms also occur, and chronic cases may take on the characters of the acute disease. The site of inoculation is usually on the hand or arm,—by means of some scratch or abrasion, or possibly by infection along a hair follicle,—sometimes on the face, and occasionally on the mucous membrane of the mouth, nose, or eye. In the *acute* form there appears at the site of inoculation inflammatory swelling, attended usually with spreading redness, and the lymphatics in relation to the part also become inflamed, the appearances being those of a "poisoned wound." These local changes are soon followed by marked constitutional disturbance, and by a local or widespread eruption on the surface of the body, at first papular and afterwards pustular, and later there may form in the subcutaneous tissue and muscles larger masses which soften and suppurate, the pus being often mixed with blood; suppuration may occur also in the joints. In some cases the nasal mucous membrane may be secondarily infected, and thence inflammatory swelling may spread to the tissues of the face. The patient usually dies in two or three weeks, sometimes sooner, with the symptoms of rapid pyæmia. In addition to the lesions mentioned, there may be foci, usually suppurative, in the lungs (attended often with pneumonic consolidation), in the spleen, liver, bone-marrow,



salivary glands, etc. In the *chronic* form a local *granulomatous* condition may occur, which usually breaks down and gives rise to the formation of an irregular ulcer with thickened margins, and sanious, often foul, discharge. The ulceration spreads deeply as well as superficially, and the thickened lymphatics also have a great tendency to ulcerate, though the lymphatic system is not so prominently affected as in the horse. Deposits may form in the subcutaneous tissue and muscles, and the mucous membrane may become affected. The disease may run a very chronic course, lasting for months or even years, and recovery may

occur; on the other hand, such a case may at any time take on the characters of the acute form of the disease and rapidly become fatal.

**The Glanders Bacillus.** — *Microscopical Characters.* — The glanders bacilli are minute rods, straight or slightly curved, with rounded ends, and about the same length as tubercle bacilli, but distinctly thicker (Fig. 89). They show, however, considerable variations in size and in appearance, and their protoplasm is often broken up into a num-



FIG. 89.—Glanders bacilli,—several contained within leucocytes,—from peritoneal exudate in a guinea-pig. Stained with weak carbol-fuchsin.  $\times 1000$ .

ber of deeply-stained portions with unstained intervals between. These characters are seen both in the tissues and in cultures, but, as in the case of many organisms, irregularities in form and size are more pronounced in cultures (Fig. 90); short filamentous forms 8 to 12  $\mu$  in length are sometimes met with, but these are on the whole rare. The organism is non-motile and does not form spores, though some have considered certain of the non-staining portions of the protoplasm to be of that nature.

In the tissues the bacilli usually occur irregularly scattered amongst the cellular elements; a few may be contained within leucocytes and connective-tissue corpuscles, but the position of most is extracellular. They are most abundant in the acute lesions, in which they may be found in considerable numbers;



but in the chronic nodules, especially when softening has taken place, they are few in number, and it may be impossible to find any in sections.

**Staining.**—The glanders bacillus differs widely from the tubercle bacillus in its staining reactions. It stains with simple watery solutions of the basic stains, but somewhat faintly (better when an alkali or a mordant, such as carbolic acid, is added), and even when deeply stained it readily loses the colour when a decolorising agent such as alcohol is applied. We have obtained the best results by carbol-thionin-blue (p. 105), and we prefer to dehydrate by the aniline-oil method. In film preparations of fresh glanders nodules the bacilli can be readily found by staining with any of the ordinary combinations, *e.g.*, carbol-thionin-blue or weak carbol-fuchsin. By using a stain of suitable strength no decolorising agent is necessary, the film being simply washed in water, dried, and mounted.



FIG. 90.—Glanders bacilli, from a pure culture on glycerin agar. Stained with carbol-fuchsin and partially decolorised to show segmentation of protoplasm.  $\times 1000$ .

McFadyean recommends that after sections have been stained in Löffler's methylene-blue and slightly decolorised in weak acetic acid, they should be treated for fifteen minutes with a saturated solution of tannic acid; thereafter they are washed thoroughly in water, and as a contrast stain a 1 per cent. solution of acid fuchsin may be applied for half a minute; they are then dehydrated, cleared, and mounted. Gram's method is quite inapplicable, the glanders bacilli rapidly losing the stain in the process.

**Cultivation.**—(For the methods of separation, *vide infra*.) The glanders bacillus grows readily on most of the ordinary media, but a somewhat high temperature is necessary, growth taking place most rapidly at  $35^{\circ}$  to  $37^{\circ}$  C. Though a certain amount of growth occurs down to  $21^{\circ}$  C., a temperature above  $25^{\circ}$  C. is always desirable.

On *agar* and *glycerin-agar* in stroke cultures growth appears



along the line as a uniform streak of greyish-white colour and somewhat transparent appearance, with moist-looking surface, and when touched with a needle is found to be of rather slimy consistence. Later it spreads laterally for some distance, and the layer becomes of slightly brownish tint.

On *serum* the growth is somewhat similar but more transparent, the separate colonies being in the form of round and almost clear drops. In sub-cultures on these media at the body temperature growth is visible within twenty-four hours, but when fresh cultures are made from the tissues it may not be visible till the second day. Serum or potato, however, is much more suitable for cultivating from the tissues than the agar media; on the latter it is sometimes difficult to obtain growth.

In *broth*, growth forms at first a uniform turbidity, but soon settles to the bottom, and after a few days forms a pretty thick flocculent deposit of slimy and somewhat tenacious consistence.

On *potato* at 30° to 37° C. the glanders bacillus flourishes well and produces a characteristic appearance, incubation at a high temperature, however, being necessary. Growth proceeds rapidly, and on the third day has usually formed a transparent layer of slightly yellowish tint, like clear honey in appearance. On subsequent days, the growth still extends and becomes darker in colour and more opaque, till about the eighth day it has a reddish-brown or chocolate tint, while the potato at the margin of the growth often shows a greenish-yellow staining. The characters of the growth on potato, along with the microscopical appearances, are quite sufficient to distinguish the glanders bacillus from every other known organism (sometimes the cholera organism and the *b. pyocyaneus* produce a somewhat similar appearance, but they can be readily distinguished by their other characters). Potato is also a suitable medium for starting cultures from the tissues; in this case minute transparent colonies become visible on the third day, and afterwards present the appearances just described.

**Powers of Resistance.**—The glanders bacillus is not killed at once by drying, but usually loses its vitality after fourteen days in the dry condition, though sometimes it lives longer. It is not quickly destroyed by putrefaction, having been found to be still active after remaining two or three weeks in putrefying fluids. In cultures the bacilli retain their vitality for three or four months, if, after growth has taken place, they are kept at the temperature of the room; on the other hand, they are often found to be dead at the end of two weeks when kept constantly at the body temperature. They have comparatively feeble



resistance to heat and antiseptics. Löffler found that they were killed in ten minutes in a fluid kept at 55° C., and in from two to three minutes by a 5 per cent. solution of carbolic acid. Boiling water and the ordinarily used antiseptics are very rapid and efficient disinfectants.

We may summarise the characters of the glanders bacillus by saying that in its morphological characters it resembles somewhat the tubercle bacillus, but is thicker, and differs widely from it in its staining reactions. For its cultivation the higher temperatures are necessary, and the growth on potato presents most characteristic features.

**Experimental Inoculation.**—In horses, subcutaneous injection of the glanders bacillus in pure culture reproduces all the important features of the disease. This fact was established at a comparatively early date by Löffler and Schutz, who, after one doubtful experiment, successfully inoculated two horses in this way, the cultures used having been grown for several generations outside the body. In a few days swellings formed at the sites of inoculation, and later broke down into unhealthy-looking ulcers. One of the animals died; after a few weeks, the other, showing symptoms of cachexia, was killed. In both animals, in addition to ulcerations on the surface with involvement of the lymphatics, there were found, post-mortem, nodules in the lungs, softened deposits in the muscles, and also affection of the nasal mucous membrane,—nodules, and irregular ulcerations. The ass is even more susceptible than the horse, the disease in the former running a more rapid course, but with similar lesions. The ass can be readily infected by simple scarification and inoculation with glanders secretion, etc. (Nocard).

Of small animals, field-mice and guinea-pigs are the most susceptible; on the other hand, house-mice and white mice enjoy an almost complete immunity. In field-mice, subcutaneous inoculation is followed by a very rapid disease, usually leading to death within eight days, the organisms becoming generalised and producing numerous minute nodules, especially in the spleen, lungs, and liver. In the guinea-pig the disease is less acute. At the site of inoculation an inflammatory swelling forms, which soon softens and breaks down, leading to the formation of an irregular crateriform ulcer with indurated margins. The lymphatic vessels become infiltrated, and the corresponding lymphatic glands become enlarged to the size of peas or small nuts, softened, and semi-purulent. The animal sometimes dies in two or three weeks, sometimes not for a longer period. Secondary nodules, in varying numbers in different cases, may be present in the



spleen, lungs, bones, nasal mucous membrane, testicles, ovaries, etc.; in some cases a few nodules are found in the spleen alone. Intraperitoneal injection in the male guinea-pig is followed, as pointed out by Straus, by a very rapid and semi-purulent affection of the tunica vaginalis, shown during life by great swelling and redness of the testicles, which changes may be noticeable in two or three days, or earlier if material from man has been used. This method of inoculation has been found of service for purposes of cultivation and diagnosis. Rabbits are less susceptible than guinea-pigs, and the effect of subcutaneous inoculation is somewhat uncertain. Accidental inoculation of the human subject with pure cultures of the bacillus has in more than one instance been followed by the acute form of the disease and a fatal result.

Mayer has found that when the glanders bacillus is injected along with melted butter into the peritoneum of a guinea-pig, it shows filamentous, branching, and club-shaped forms; in other words, it presents the characters of a streptothrix. Lubarsch, on the other hand, in a comparative study of the results of inoculation with acid-fast and other bacilli, found none of the above characters in the case of the glanders bacillus (cf. *Tubercle*, p. 263).

**Action on the Tissues.**—From the above facts it will be seen that in many respects glanders presents an analogy to tubercle as regards the general characters of the lesions and the mode of spread. When the tissue changes in the two diseases are compared, certain differences are found. The glanders bacillus causes a more rapid and more marked inflammatory reaction. There is more leucocytic infiltration and less proliferative change which might lead to the formation of epithelioid cells. Thus the centre of an early glanders nodule shows a dense aggregation of leucocytes, most of which are polymorpho-nuclear, whilst in the central parts many show fragmentation of nuclei with the formation of a deeply staining granular detritus. And further, the inflammatory change may be followed by suppurative softening of the tissue, especially in certain situations, such as the subcutaneous tissue and lymphatic glands. The nodules, therefore, in glanders, as Baumgarten puts it, occupy an intermediate position between miliary abscesses and tubercles. The diffuse coagulative necrosis and caseation which are so common in tubercle do not occur to the same degree in glanders, and typical giant cells are not formed. The nodules in the lungs show leucocytic infiltration and thickening of the alveolar walls, whilst the vesicles are filled with catarrhal cells; there may also be fibrinous exudation, whilst at the periphery of the nodules con-



nective-tissue growth is present in proportion to their age. The tendency to spread by the lymphatics is always a well-marked feature, and when the bacilli gain entrance to the blood stream, they soon settle in the various tissues and organs. Accordingly, even in acute cases it is usually quite impossible to detect the bacilli in the circulating blood, though sometimes they have been found. It is an interesting fact, shown by observations of the disease both in the human subject and in the horse, as well as by experiments on guinea-pigs, that the mucous membrane of the nose may become infected by means of the blood stream—another example of the tendency of organisms to settle in special sites.

**Mode of Spread.**—Glanders usually spreads from a diseased animal by direct contagion with the discharge from the nose or from the sores, etc. So far as infection of the human subject goes, no other mode is known. There is no evidence that the disease is produced in man by inhalation of the bacilli in the dried condition. Some authorities consider that pulmonary glanders may be produced in this way in the horse, whilst others maintain that in all cases there is first a lesion of the nasal mucous membrane or of the skin surface, and that the lung is affected secondarily. Babes, however, found that the disease could be readily produced in susceptible animals by exposing them to an atmosphere in which cultures of the bacillus had been pulverised. He also found that inunction of the skin with vaseline containing the bacilli might produce the disease, the bacilli in this case entering along the hair follicles.

**Serum Reactions.**—Shortly after the discovery of agglutination in typhoid fever, McFadyean showed that the serum of glandered horses possessed the power of agglutinating glanders bacilli. His later observations show that in the great majority of cases of glanders a 1 : 50 dilution of the serum produces marked agglutination in a few minutes, whilst in the great majority of non-glandered animals no effect is produced under these conditions. The test performed in the ordinary way is, however, not absolutely reliable, as exceptions occasionally occur in both directions, *i.e.* negative results by glandered animals and positive results by non-glandered animals. He found that a more delicate and reliable method is to grow the bacillus in bouillon containing a small proportion of the serum to be tested. In this way he obtained a distinct sedimenting reaction with a serum which did not agglutinate at all distinctly in the ordinary method. Within recent times the sedimentation test by the ordinary method (p. 120) has been most generally used. The general result seems to be that distinct sedimentation within thirty-six hours with a serum dilution of 1 : 1000 may be taken as a positive result, indicating the presence of glanders; whilst reactions with dilutions between this and 1 : 500 are highly suspicious but not conclusive. The deviation of complement test (p. 130) is also applicable in the case of glanders, and this has given valuable results in the hands of various observers; a precipitin reaction may also be obtained on the addition of



mallein to the serum of a glandered animal. These reactions, which of course depend on the presence of anti-substance in the blood in glanders, form important auxiliaries to the method of diagnosis by means of mallein.

**Mallein and its Preparation.**—Mallein is obtained from cultures of the glanders bacillus grown for a suitable length of time, and, like tuberculin, is really a mixture comprising (1) substances in the bodies of the bacilli, and (2) their soluble products, not destroyed by heat, along with substances derived from the medium of growth. It was at first obtained from cultures on solid media by extracting with glycerin or water, but is now usually prepared from cultures in glycerin bouillon. Such a culture, after being allowed to grow for three or four weeks, is sterilised by heat either in the autoclave at  $115^{\circ}$  C. or by steaming at  $100^{\circ}$  C. on successive days. It is then filtered through a Chamberland filter. The filtrate constitutes fluid mallein. Usually a little carbolic acid (.5 per cent.) is added to prevent it from decomposing. Of such mallein 1 c.c. is usually the dose for a horse (McFadyean). Foth has prepared a dry form of mallein by throwing the filtrate of a broth culture, evaporated to one-tenth of its bulk, into twenty-five or thirty times its volume of alcohol. A white precipitate is formed, which is dried over calcium chloride and then under an air-pump. A dose of this dry mallein is .05 to .07 gm.

**The Use of Mallein as a Means of Diagnosis.**—In using mallein for the diagnosis of glanders, the temperature of the animal ought to be observed for some hours beforehand, and, after subcutaneous injection of a suitable dose, it is taken at definite intervals,—according to McFadyean at the sixth, tenth, fourteenth, and eighteenth hours afterwards, and on the next day. Here both the local reaction and the temperature are of importance. In a glandered animal, at the site of inoculation there is a somewhat painful local swelling, which reaches a diameter of five inches at least, the maximum size not being attained until twenty-four hours afterwards. The temperature rises  $1.5^{\circ}$  to  $2^{\circ}$  C., or more, the maximum generally occurring in from eight to sixteen hours. If the temperature never rises as much as  $1.5^{\circ}$ , the reaction is considered doubtful. In the negative reaction given by an animal free from glanders, the rise of temperature does not usually exceed  $1^{\circ}$ , the local swelling reaches the diameter of three inches at most, and has much diminished at the end of twenty-four hours. In the case of dry mallein, local reaction is less marked. Veterinary authorities are practically unanimous as to the great value of the mallein test as a means of diagnosis. It has recently been shown that mallein instilled into the conjunctival sac, or inoculated by scarification into the skin of glandered animals, gives corresponding reactions to the ophthalmic and cutaneous tuberculin reactions in cases of tuberculosis (pp. 285, 286); in the case of glanders the conjunctival reaction would appear to be the more convenient and reliable.

**Methods of Examination.**—Microscopic examination in a case of suspected glanders will at most reveal the presence of bacilli corresponding in their characters to the glanders bacillus. An absolute diagnosis cannot be made by this method. Cultures may be obtained by making successive strokes on blood serum or on glycerin agar (preferably the former), and incubating at  $37^{\circ}$  C. The colonies of the glanders bacillus do not appear till two days after. This method often fails unless a considerable number of



the glanders bacilli are present. Another method is to dilute the secretion or pus with sterile water, to varying degrees, and then to smear the surface of potato with the mixture, the potatoes being incubated at the above temperature. The colonies on potatoes may not appear till the third day. The most certain method, however, is by inoculation of a guinea-pig, either by subcutaneous or intraperitoneal injection. By the latter method, as above described, lesions are much more rapidly produced, and are more characteristic. If, however, there have been other organisms present, the animal may die of a septic peritonitis, though even in such a case the glanders bacilli will be found to be more numerous in the tunica vaginalis, and may be cultivated from this situation. It is extremely doubtful whether the application of mallein to diagnosis of the disease in the human subject is justifiable. There is a certain risk that it may lead to the lesions assuming a more acute character; moreover, culture and inoculation tests are generally available. In the case of horses, etc., a diagnosis will, however, be much more easily and rapidly effected by means of mallein, or by one of the serum reactions described above. In some cases of acute glanders in the human subject the bacillus has been obtained in cultures from the blood during life.

#### RHINOSCLEROMA.

This disease is considered here as, from the anatomical changes, it also belongs to the group of infective granulomata. It is characterised by the occurrence of chronic nodular thickenings in the skin or mucous membrane of the nose, or in the mucous membrane of the pharynx, larynx, or upper part of the trachea. The nodules are of considerable size, sometimes as large as a pea; in the earlier stages they are comparatively smooth on the surface, but later they become shrunken and the centre is often retracted. The disease is scarcely ever met with in this country, but is of not very uncommon occurrence on the Continent, especially in Austria and Poland. In the granulation tissue of the nodules there are to be found numerous round and rather large cells, which have peculiar characters and are often known as the cells of Mikulicz. Their protoplasm contains a collection of somewhat gelatinous material which may fill the cell and push the nucleus to the side. Within these cells there is present a characteristic bacillus, occurring in little clumps or masses chiefly in the gelatinous material. A few bacilli also lie free in the lymphatic spaces around. This organism was first



observed by Frisch, and is now known as the bacillus of rhinoscleroma. The bacilli have the form of short oval rods, which, when lying separately, can be seen to possess a distinct capsule, and which in all their microscopical characters correspond closely with Friedländer's pneumobacillus. They are usually present in the lesions in a state of purity. It was at first stated that they could be stained by Gram's method, but more recent observations show that, like Friedländer's organism, they lose the stain.

From the affected tissues this bacillus can be easily cultivated by the ordinary methods. In the characters of its growth in the various culture media it presents a close similarity to that of the pneumobacillus, as it also does in its fermentative action in milk and sugar-containing fluids. The nail-like appearance of the growth on gelatin is said to be less distinct, and the growth on potatoes is more transparent and may show small bubbles of gas; but it is doubtful whether any distinct line of difference can be drawn between the two organisms so far as their microscopical and cultural characters are concerned.

The evidence that the organisms described are the cause of this disease consists in their constant presence and their special relation to the affected tissues, as already described. From these facts alone it would appear probable that they are the active agents in the production of the lesions. Experimental inoculation has thrown little light on the subject, though one observer has described the production of nodules on the conjunctivæ of guinea-pigs. The relation of the rhinoscleroma organism to that of Friedländer is, however, still a matter of doubt, and the matter has been further complicated by the fact that a bacillus possessing closely similar characters has been found to be very frequently present in ozæna, and is often known as the *bacillus ozæne*. The last-mentioned organism is said to have more active fermentative powers. From what has been stated it will be seen that a number of organisms, closely allied in their morphological characters, have been found in the nasal cavity in healthy or diseased conditions. There is no doubt that rhinoscleroma is a specific disease with well-marked characters, and it is quite possible that one member of this group of organisms may be the causal agent, though indistinguishable from others by culture tests. There is, however, a tendency on the part of recent investigators, *e.g.* Perkins, to consider the "bacillus of rhinoscleroma" to be identical with the pneumobacillus, and its presence in the affected tissues to represent merely a secondary invasion. The subject is one on which more light is still required,



## CHAPTER XIII.

### ACTINOMYCOSIS AND ALLIED DISEASES.

ACTINOMYCOSIS is the most important example of a group of diseases produced by streptothrix organisms. It is related, by the characters of the pathological changes, to the diseases which have been described. The disease affects man in common with certain of the domestic animals, though it is more frequent in the latter, especially in oxen, swine, and horses. The parasite was first discovered in the ox by Bollinger, and described by him in 1877, the name *actinomyces* or *ray fungus* being from its appearance applied to it by the botanist Harz. In 1878 Israel described the parasite in the human subject, and in the following year Ponfick identified it as being the same as that found in the ox. Since that time a large number of cases have been observed in the human subject, the result of investigation being to show that it affects man much more frequently than was formerly supposed.

It is, however, to be noted that the term "actinomyces," as originally used, does not represent one parasite but a number of closely allied species, as cultures obtained from various sources have presented considerable differences; and, further, it is noted that other distinct species of streptothrix have been cultivated from isolated cases of disease in the human subject where the lesions resembled more or less closely those of actinomycosis. In one or two instances the organism has been found to be "acid-fast," and there is no doubt that the actinomyces group is closely related through intermediate forms with the tubercle group (*vide* p. 264).

**Naked-Eye Characters of the Parasite.**—The actinomyces grows in the tissues in the form of little round masses or colonies, which, when fully developed, are easily visible to the naked eye, the largest being about the size of a small pin's head, whilst all sizes below this may be found. When suppuration is present, they lie free in the pus; when there is no suppuration, they are



embedded in the granulation tissue, but are usually surrounded by a zone of softer tissue. They may be transparent or jelly-like, or they may be opaque and of various colours—white, yellow, greenish, or almost black. The appearance depends upon their age and also upon their structure, the younger colonies being more or less transparent, the older ones being generally opaque. Their colour is modified by the presence of pigment and by degenerative change, which is usually accompanied by a yellowish coloration. They are generally of soft, sometimes tallow-like, consistence, though sometimes in the ox they are gritty, owing to the presence of calcareous deposit. They may be readily found in the pus by spreading it out in a thin layer on a glass slide and holding it up to the light. They are sometimes described as being always of a distinctly yellow colour, but this is only occasionally the case; in fact, in the human subject they occur much more frequently as small specks of semi-translucent appearance, and of greenish-grey tint.

**Microscopical Characters.**—The parasite, which is now generally regarded as belonging to the streptothrix group of the higher bacteria (p. 16), presents pleomorphous characters. In the colonies, as they grow in the tissues, three morphological elements may be described, namely, filaments, coccus-like bodies, and clubs.

1. The *filaments* are comparatively thin, measuring about  $\cdot 6 \mu$  in diameter, but they are often of great length. They are composed of a central protoplasm enclosed by a sheath. The latter, which is most easily made out in the older filaments with granular protoplasm, occasionally contains granules of dark pigment. In the centre of the colony the filaments interlace with one another, and form an irregular network which may be loose or dense; at the periphery they are often arranged in a somewhat radiating manner, and run outwards in a wavy or even spiral course. They also show true branching, a character which at once distinguishes them from the ordinary bacteria. Between the filaments there is a finely granular or homogeneous ground substance. Most of the colonies at an early stage are chiefly constituted by filaments loosely arranged; but later, part of the growth may become so dense that its structure cannot be made out. This dense part, starting excentrically, may grow round the colony to form a hollow sphere, from the outer surface of which filaments radiate for a short distance (Fig. 91). The filaments usually stain uniformly in the younger colonies, but some, especially in the older colonies, may be segmented so as to give the appearance of a chain of bacilli or of cocci, though



the sheath enclosing them may generally be distinguished. Rod-shaped and spherical forms may also be seen lying free.

2. *Spores or Gonidia*.—Like other species of streptothrix, the actinomyces when growing on a culture medium shows on its surface filaments growing upwards in the air, the protoplasm of which becomes segmented into rounded spores or gonidia. In natural conditions outside the body these gonidia become free, and act as new centres by growing out into filaments. They

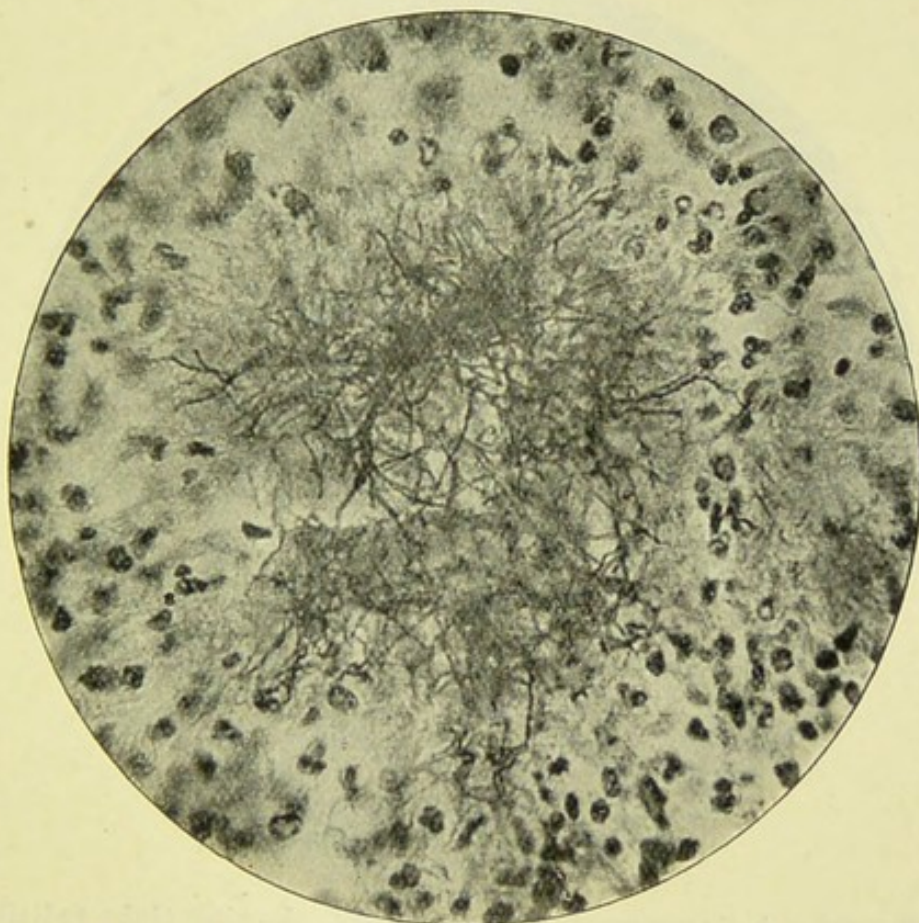


FIG. 91.—Actinomyces of human liver, showing a colony of the parasite composed of a felted mass of filaments surrounded by pus. Paraffin section; stained by Gram's method and with safranin.  $\times 500$ .

have somewhat higher powers of resistance than the filaments, though less than the spores of most of the lower bacteria. An exposure to  $75^{\circ}$  C. for half an hour is sufficient to kill most streptothrices or their spores; cultures containing spores can resist a temperature from five to ten degrees higher than spore-free cultures (Foulerton). It is probable that some of the spherical bodies formed within filaments when growing in the tissues have the same significance, *i.e.* are gonidia, whilst others may be merely the result of degenerative change. Both the



filaments and the spherical bodies are readily stained by Gram's method.

3. *Clubs*.—These are elongated pear-shaped bodies which are seen at the periphery of the colony, and are formed by a sort of hyaline swelling of the sheath around the free extremity of a filament (Figs. 92, 93). They are usually homogeneous and structureless in appearance. In the human subject the clubs are

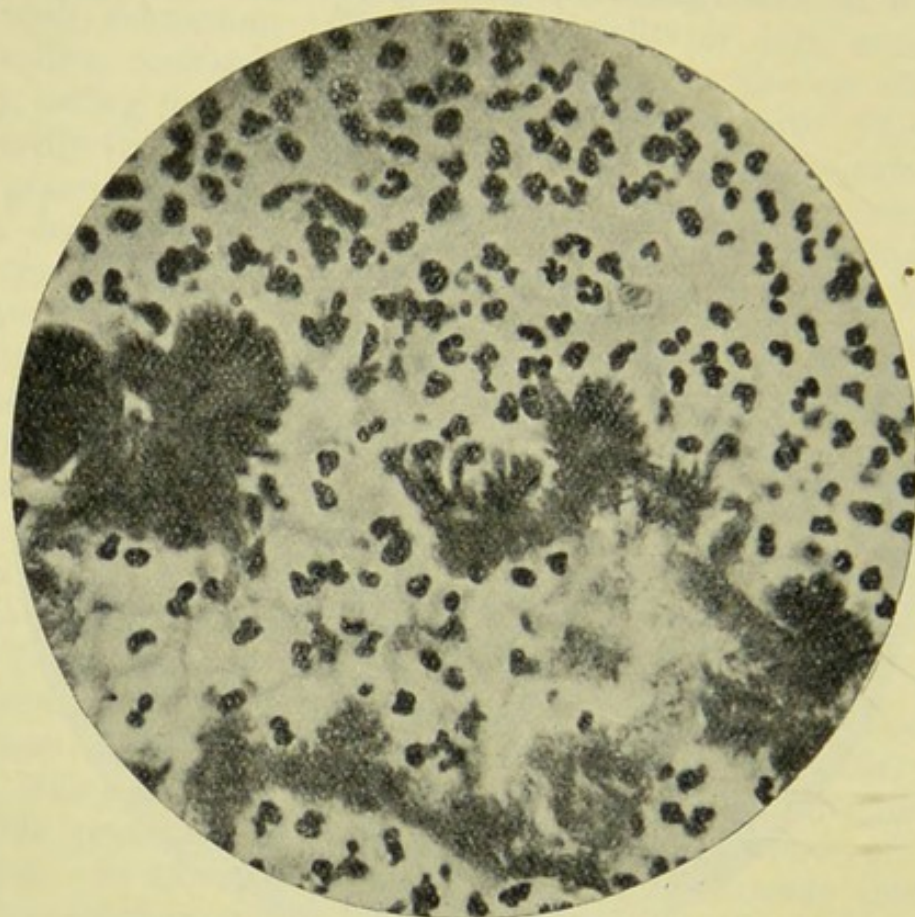


FIG. 92.—Actinomyces in human kidney, showing clubs radially arranged and surrounded by pus. The filaments had practically disappeared.

Paraffin section ; stained with hæmatoxylin and rubin.  $\times 500$ .

often comparatively fragile structures, which are easily broken down, and may sometimes be dissolved in water. Sometimes they are well seen when examined in the fresh condition, but in hardened specimens are no longer distinguishable. In specimens stained by Gram's method they are usually not coloured by the violet, but take readily a contrast stain, such as picric acid, rubin, etc. ; sometimes a darkly-stained filament can be seen running for a distance in the centre, and may have a knob-like extremity. In many of the colonies in the human subject the clubs are absent. In the ox, on the other hand, where there are



much older colonies, the clubs constitute the most prominent feature, whilst in most colonies the filaments are more or less degenerated, and it may sometimes be impossible to find any. They often form a dense fringe around the colony, and when stained by Gram's method retain the violet stain. They have, in fact, undergone some further chemical change which produces the altered staining reaction. Occasionally in very chronic

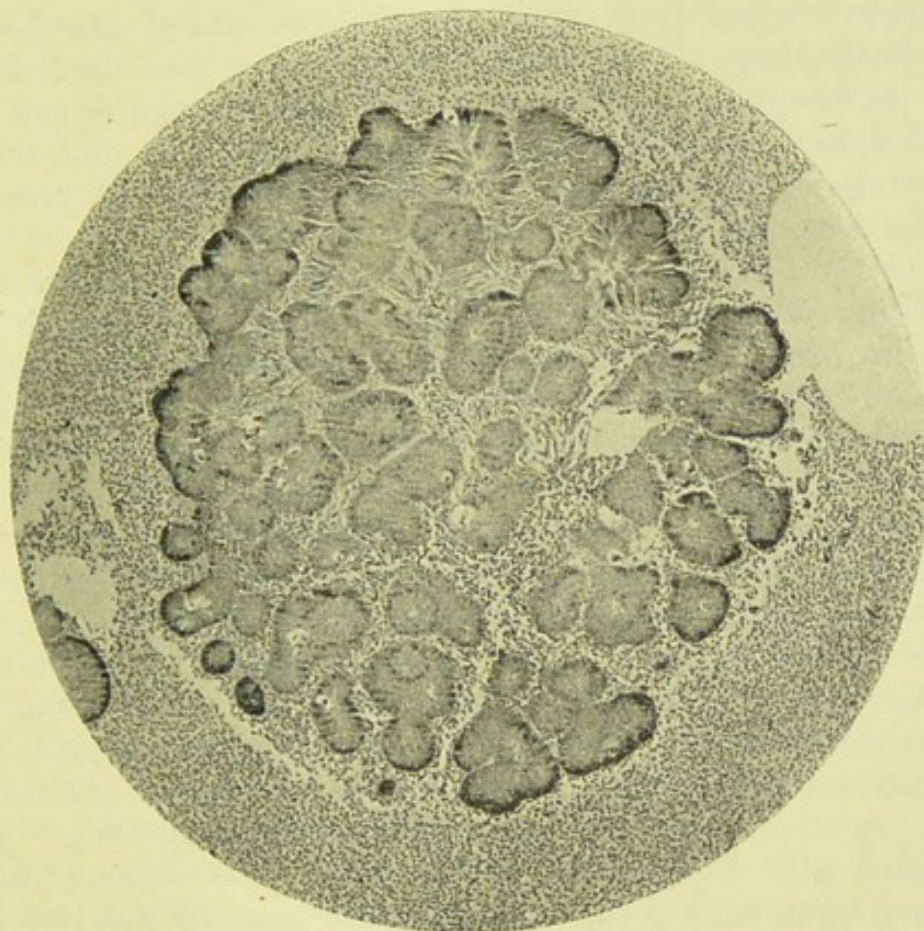


FIG. 93.—Colonies of actinomyces, showing general structural arrangement and clubs at periphery. From pus in human subject. Stained Gram and safranin.  $\times 60$ .

lesions in the human subject the clubs stain with Gram's method. Clubs showing intermediate staining reaction have been described in the ox by McFadyean. The club-formation probably represents a means of defence on the part of the parasite against the phagocytes of the tissue; the view, formerly held, that the clubs are organs of fructification has now been generally abandoned.

**Tissue Lesions.**—In the human subject the parasite produces by its growth a chronic inflammatory change, usually ending in a suppuration which slowly spreads. In some cases there



is a comparatively large production of granulation tissue, with only a little softening in the centre, so that the mass feels solid. This condition is sometimes found in the subcutaneous tissue, especially when the disease has not advanced far, and also in dense fibrous tissue. In most cases, however, and especially in internal organs, suppuration is the outstanding feature; this is associated with abundant growth of the parasite in the filamentous form. In an organ such as the liver, multiple foci of suppuration are seen at the spreading margin of the disease, often presenting a honeycomb appearance, whilst the colonies of the parasite may be seen in the pus with the naked eye. In the older parts the abscesses have become confluent, and formed large areas of suppuration. The pus is usually of greenish-yellow colour, and of somewhat slimy character.

In cattle the tissue reaction is more of a formative type, there being abundant growth of granulation tissue, which may result in large tumour-like masses, usually of more or less nodulated character, and often consisting of well-developed fibrous tissue containing areas of younger formation, in which, however, irregular abscess formation may be present. The cells immediately around the colonies are usually irregularly rounded, or may even be somewhat columnar in shape, whilst farther out they become spindle-shaped and concentrically arranged. It is not uncommon to find leucocytes or granulation tissue invading the substance of the colonies, and portions of the parasite, etc., may be contained within leucocytes or within small giant-cells, which are sometimes present. A similar invasion of old colonies by leucocytes is sometimes seen in human actinomycosis.

*Origin and Distribution of Lesions.*—The lesions in the human subject may occur in almost any part of the body, the paths of entrance being very various. In many cases the entrance takes place in the region of the mouth—probably around a decayed tooth—by the crypts of the tonsil, or by some abrasion. Swelling and suppuration may then follow in the vicinity and may spread in various directions. The periosteum of the jaw or the vertebræ may thus become affected, caries or necrosis resulting, or the pus may spread deeply in the tissues of the neck, and may even pass into the mediastinum. Occasionally the parasite may enter the tissues from the œsophagus, and in a considerable number of cases the primary lesion is in some part of the intestine, generally of the large intestine. The parasite penetrates the wall of the bowel, and may be found deeply between the coats, surrounded by purulent material. Thence it may spread to the peritoneum or to the



extraperitoneal tissue, the retrocaecal connective tissue and that around the rectum being not uncommonly seats of suppuration produced in this way. A peculiar affection of the intestine has been described, in which slightly raised plaques are found both in the large and small intestines, these plaques being composed almost exclusively of masses of the actinomyces along with epithelial cells. This, however, is a rare condition. The path of entrance may also be by the respiratory passages, the primary lesion being pulmonary or peribronchial; extensive suppuration in the lungs may result. Infection may also occur by the skin surface, and lastly, by the female genital tract, as in a case recorded by Grainger Stewart and Muir, in which both ovaries and both Fallopian tubes were affected.

When the parasite has invaded the tissues by any of these channels, secondary or "metastatic" abscesses may occur in internal organs. The liver is the organ most frequently affected, though abscesses may occur in the lungs, brain (where a primary meningitis may also occur), kidneys, etc. In such cases the spread takes place by the blood stream, and it is possible that leucocytes may be the carriers of the infection, as it is not uncommon to find leucocytes in the neighbourhood of a colony containing small portions of the filaments in their interior.

In the ox, on the other hand, the disease usually remains quite local, or spreads by continuity. It may produce tumour-like masses in the region of the jaw or neck, or it may specially affect the palate or tongue, in the latter producing enlargement and induration, with nodular thickening on the surface—the condition known as "woody tongue."

*Source of the Parasite.*—There is a considerable amount of evidence to show that outside the body the parasite grows on grain, especially on barley. Both in the ox and in the pig the parasite has been found growing around fragments of grain embedded in the tissues. There are besides, in the case of the human subject, a certain number of cases in which there was a history of penetration of a mucous surface by a portion of grain, and in a considerable proportion of cases the patient has been exposed to infection from this source. The position of the lesions in cattle is also in favour of such a view.

**Cultivation** (for methods of isolation see later).—The descriptions of the cultures obtained by various investigators differ in essential particulars, and there is no doubt that the organisms described are different. The following is the account of the organism as cultivated by Boström:—

On *agar* or *glycerin agar* at 37° C., growth is generally



visible on the third or fourth day in the form of little transparent drops which gradually enlarge and form rounded projections of a reddish-yellow tint and somewhat transparent appearance, like drops of amber. The growths tend to remain separate, and even when they become confluent, the nodular

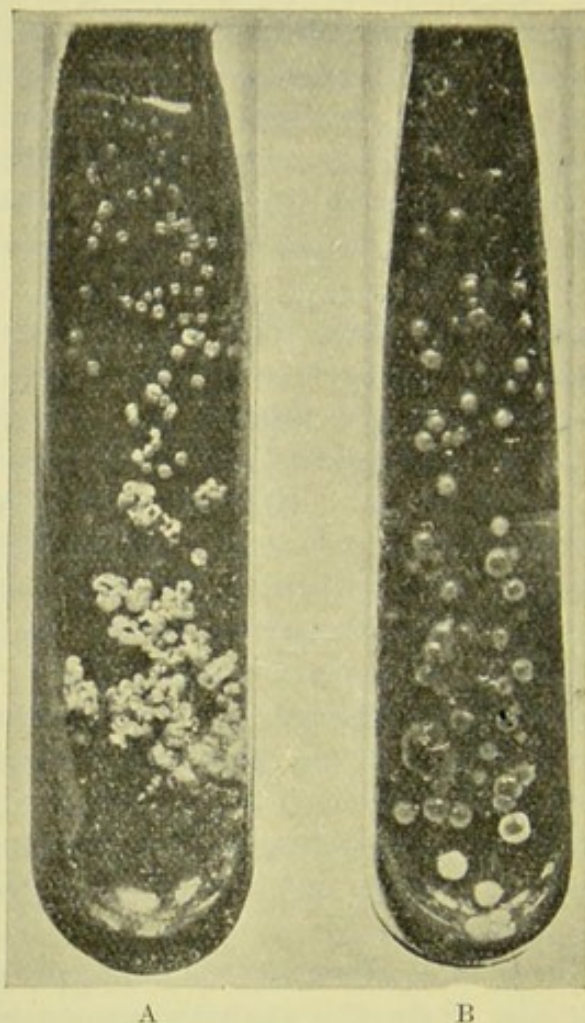


FIG. 94.—Cultures of the actinomyces on glycerin agar, of about three weeks' growth, showing the appearances which occur. The growth in A is at places somewhat corrugated on the surface. Natural size.

character is maintained. They have a tough consistence, being with difficulty broken up, and adhere firmly to the surface of the agar. Older growths often show on the surface a sort of corrugated aspect, and may sometimes present the appearance of having been dusted with a brownish-yellow powder (Fig. 94).

In the cultures at an early stage the growth is composed of branching filaments, which stain uniformly (Fig. 95), but later some of the superficial filaments may show segmentation into gonidia. Slight bulbous thickenings may be seen at the end of some of the filaments, but true clubs have not been observed.

On *gelatin* the same tendency to grow in little spherical masses is seen, and the medium becomes

very slowly liquefied. When this occurs the liquefied portion has a brownish colour and somewhat syrupy consistence, and the growths may be seen at the bottom, as little balls, from the surface of which filaments radiate.

The organism obtained in culture by Wolff and Israel (*vide infra*) is probably the same as the one which has been recently described in detail by J. H. Wright, who obtained it in pure condition from fifteen different cases of the disease. It differs markedly from Boström's organism in being almost a strict



anaerobe and in ceasing to grow at a temperature a little below that of the body. Under ordinary aerobic conditions either no growth occurs or it is of a very slight character. On the surface of agar under anaerobic conditions the organism produces dense rounded colonies of greyish-white colour, which sometimes assume a rosette form. A somewhat curious feature of growth is described by Wright, namely, that in a shake culture in glucose agar the colonies are most numerous and form a dense zone about half an inch from the surface of the medium, that is, at a level where there is presumably a mere trace of oxygen obtainable (Fig. 96). In bouillon, growth takes place at the bottom of the medium in rounded masses which afterwards undergo disintegration. Wright found when the organism was grown in the presence of serum or other animal fluids, that the formation of true clubs occurred at the extremity of some of the filaments (Fig. 97). From the conditions under which growth occurs, he is inclined to regard it as a true para-

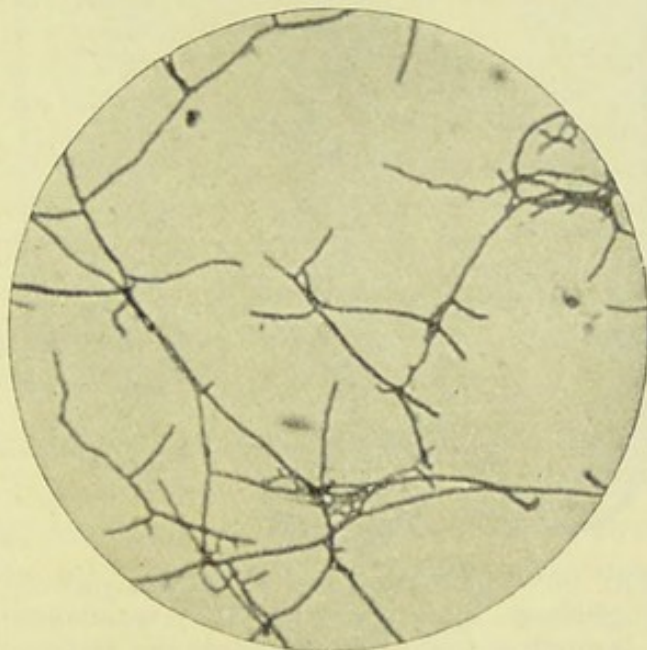


FIG. 95.—Actinomyces, from a culture on glycerin agar, showing the branching of the filaments. See also Plate III., Fig. 10. Stained with fuchsin.  $\times 1000$ .

site, and doubts whether it can have a saprophytic existence outside the body, *e.g.* on grain. He is also of opinion that all cases of true actinomycosis, *i.e.* cases where colonies visible to the naked eye are present, are probably produced by one species, and that the aerobic organisms obtained by Boström and others are probably accidental contaminations. It is quite evident that further investigations are required in the light of the results detailed. Certainly the parasite in many cases of actinomycosis in the human subject does not grow on ordinary media under aerobic conditions as Boström's organism does.

**Varieties of Actinomyces and Allied Forms.**—It is probable that in the cases of the disease described in the human subject there is more than one variety or species of parasite belonging to the same group. Gasperini has described several varieties of *actinomyces bovis* according to the colour



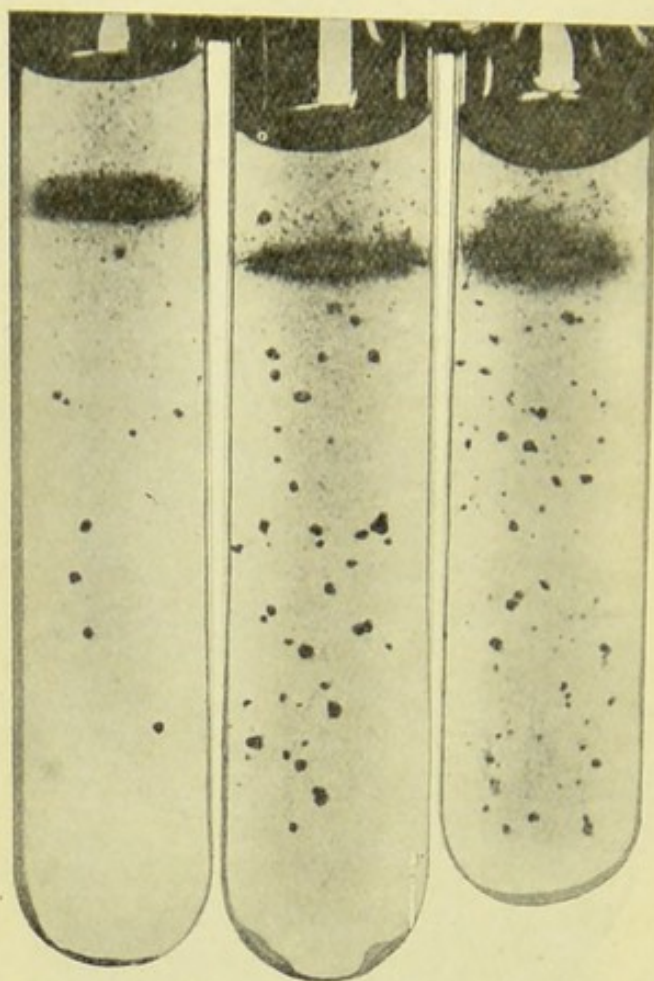


FIG. 96.<sup>1</sup>—Shake cultures of actinomyces in glucose agar, showing the maximum growth at some distance from the surface of the medium.

regarded as a distinct species. Another species was cultivated by Eppinger from a brain abscess, and called by him "*cladothrix asteroides*," from the appearance of its colonies on culture media. A case of general streptothrix infection in the human subject described by MacDonald was probably due to the same organism as Eppinger's. In the tissues it grows in a somewhat diffuse manner, and does not

of the growths, and a similar condition may obtain in the case of the human subject. Furthermore, a considerable number of *streptothrices* have been found in cases of disease in the human subject, the associated lesions varying in character from tubercle-like nodules on the one hand to suppurative processes on the other. The organisms cultivated from such sources differ according to their microscopic characters (for example, some form "clubs" whilst others do not) according to their conditions of growth, staining reactions, etc. Of these only a few examples may here be mentioned, but it may be noted that the importance of the streptothrices as causes of disease is constantly being extended. Wolff and Israel cultivated from two cases of "actinomycosis" in man a streptothrix which differs in so many important points from the actinomyces of Boström that it is now



FIG. 97.—Section of a colony of actinomyces from a culture in blood serum, showing the formation of clubs at the periphery.  $\times 1500$ .

<sup>1</sup> For Figs. 96 and 97 we are indebted to Dr. J. Homer Wright of Boston, U.S.A.



form clubs; in rabbits and guinea-pigs it produces tubercle-like lesions. Flexner observed a streptothrix in the lungs associated with lesions somewhat like a rapid phthisis, and applied the name "*pseudo-tuberculosis hominis streptothricea*"; an apparently similar condition has been described by Buchholz. Berestnew cultivated two species of streptothrix from suppurative lesions, one of which is acid-fast and grows only in anaerobic conditions. Birt and Leishman have described another acid-fast streptothrix obtained from cirrhotic nodules in the lungs of a man. This organism grows readily on ordinary media, forming a white powdery growth which afterwards assumes a pinkish colour; it is pathogenic for guinea-pigs, in which it causes caseous lesions. There is, further, the streptothrix *maduræ* described below.

In diseases of the lower animals several other forms have been found. For example, a streptothrix has been shown by Nocard to be the cause of a disease of the ox,—"farcin du bœuf,"—a disease in which also there occur tumour-like masses of granulation tissue. Dean has cultivated from a nodule in a horse another streptothrix, which produces tubercle-like nodules in the rabbit with club-formation; it has close resemblances to the organism of Israel and Wolff. The so-called diphtheria of calves and "bacillary necrosis" in the ox are probably both produced by another streptothrix or leptothrix, which grows diffusely in the tissues in the form of fine felted filaments. Further investigation may show that some of these or other species may occur in the human subject in conditions which are not yet differentiated.

**Experimental Inoculation.**—Inoculation of smaller animals, such as rabbits and guinea-pigs, has usually failed to give positive results. This was the case, for example, in the important series of experiments by Boström, and it may be assumed that these animals are little susceptible to the actinomyces. The disease has, however, been experimentally produced in the bovine species both by cultures from the ox and from the human subject. Inoculation with the organism of Israel and Wolff produces nodular lesions both in rabbits and in guinea-pigs, while Wright found that characteristic colonies and lesions resulted although the parasite did not grow to any great extent. Several of the other species of streptothrix have been found to possess active pathogenic properties.

**Methods of Examination and Diagnosis.**—As actinomycosis cannot be diagnosed with certainty apart from the discovery of the parasite, a careful examination of the pus in obscure cases of suppuration should always be undertaken. As already stated, the colonies can be recognised with the naked eye, especially when some of the pus is spread out on a piece of glass. If one of these is washed in salt solution and examined unstained, the clubs, if present, are at once seen on microscopic examination. Or the colony may be stained with a simple reagent such as picricarmin, and mounted in glycerin or Farrant's solution. To study the filaments, a colony should be broken down on a cover-



glass, dried, and stained with a simple solution of any of the basic aniline dyes, such as gentian-violet, though better results are obtained by carbol-thionin-blue, or by carbol-fuchsin diluted with five parts of water. If the specimen be over-stained, it may be decolorised by weak acetic acid. Cover-glass preparations of this kind, and also of cultures, are readily stained by these methods, but in the case of sections of the tissues, Gram's method, or a modification of it, should be used to show the filaments, etc., a watery solution of acid fuchsin being afterwards used to stain the clubs. By this method, very striking preparations may be obtained.

Cultures should be made both under aerobic and anaerobic conditions. Tubes of agar or glycerin agar should be inoculated and incubated at 37° C.; deep tubes of melted glucose agar should also be used, the inoculated material being diffused through the medium, separate colonies may thus be obtained. Owing to the slow growth of the actinomyces, however, the obtaining of pure cultures is somewhat difficult, unless the pus is free from contamination with other organisms.

#### MADURA DISEASE.

Madura disease or mycetoma resembles actinomycosis both as regards the general characters of the lesions and the occurrence of the parasite in the form of colonies or "granules." There is no doubt, however, that the two conditions are distinct, and it also appears established that the two varieties of Madura disease (*vide infra*) are produced by different organisms. This disease is comparatively common in India and in various other parts of the tropics: it has also been met with in Algiers and in America. Madura disease differs from actinomycosis not only in its geographical distribution but also in its clinical characters. Its course, for example, is of an extremely chronic nature, and though the local disease is incurable except by operation, the parasite never produces secondary lesions in internal organs. Vincent also found that iodide of potassium, which has a high value as a therapeutic agent in many cases of actinomycosis, had no effect in the case of Madura disease studied by him. It most frequently affects the foot; hence the disease is often spoken of as "Madura foot." The hand is rarely affected. In the parts affected there is a slow growth of granulation tissue which has an irregularly nodular character, and in the centre of the nodules there occurs purulent softening which is often followed by the formation of fistulous openings and ulcers. There are great



enlargement and distortion of the part and frequently caries and necrosis of the bones. Within the softened cavities and also in the spaces between the fibrous tissue, small rounded bodies or granules, bearing a certain resemblance to the actinomyces, are present. These may have a yellowish or pinkish colour, compared from their appearance to fish roe, or they may be black like grains of gunpowder, and may by their conglomeration form nodules of considerable size. Hence a *pale variety* and a *black variety* of the disease have been distinguished; in both varieties the granules mentioned reach a rather larger size than in actinomycosis. These two conditions will be considered separately.

**Pale Variety.**—When the roe-like granules are examined microscopically, they are found, like the actinomyces, to show in their interior an abundant mass of branching filaments with mycelial arrangement. There may also be present at the periphery club-like structures, as in actinomyces; sometimes they are absent. These structures often have an elongated wedge-shape, forming an outer zone to the colony, and in some cases the filaments can be found to be connected with them. Vincent obtained cultures of the parasite from a case in Algiers, and found it to be a distinct species: it is now known as the *streptothrix* or *discomyces Maduraæ*. Morphologically it closely resembles the actinomyces, but it presents certain differences in cultural characters. In gelatin it forms raised colonies of a yellowish colour, with umbilication of the centre, and there is no liquefaction of the medium. On agar the growth assumes a reddish colour; the organism flourishes well in various vegetable infusions in which the actinomyces does not grow. On all the media growth only takes place in aerobic conditions. Experimental inoculation of various animals has failed to reproduce the disease. There is therefore no doubt that the *streptothrix maduraæ* and the actinomyces are distinct species.

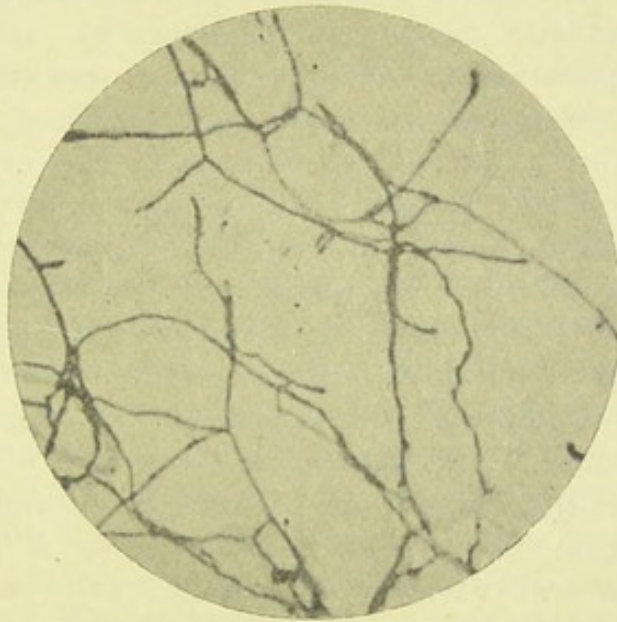


FIG. 98. — *Streptothrix Maduraë*, showing branching filaments. From a culture on agar. Stained with carbol-thionin-blue.  $\times 1000$ .



**Black Variety.**—The observations of J. H. Wright, who obtained pure cultures of a hyphomycete, show that this variety is a distinct affection from the pale variety. The pigment may be dissolved by soaking the granules for a few minutes in hypochlorite of sodium solution, and the granules may then be crushed out beneath a cover-glass and examined microscopically. The granules are composed of a somewhat homogeneous ground-substance impregnated with pigment, and in this there is a mycelium of thick filaments or hyphæ, many of the segments of which are swollen; at the periphery the hyphæ form a zone with radiate arrangement. In many of the older granules the parasite is largely degenerated and presents an amorphous appearance. Wright planted over sixty of the black granules in various culture media, and obtained cultures of a hyphomycete from about a third of these. The organism grows well on agar, bouillon, potato, etc.; on agar it forms a felted mass of greyish colour, and in old cultures black granules appear amongst the mycelium. Microscopically the parasite appears as a mycelium of thick branching filaments with delicate transverse septa; in the older threads the segments become swollen, so that strings of oval-shaped bodies result. No signs of spore-formation were noted. Inoculation of animals with cultures gave negative results, as did also direct inoculation with the black granules from the tissues. Brumpt, in a recent work, distinguishes several varieties of parasite concerned in Madura disease, and finds that a pale variety may be produced by a hyphomycete as well as by Vincent's streptothrix; in fact, with the exception of Vincent's organism, all the parasites are considered by him to be closely allied to *aspergillus*.



## CHAPTER XIV.

### ANTHRAX.<sup>1</sup>

*OTHER NAMES.*—SPLENIC FEVER, MALIGNANT PUSTULE, WOOL-SORTER'S DISEASE. *GERMAN*, MILZBRAND; *FRENCH*, CHARBON.<sup>2</sup>

**Introductory.**—Anthrax is a disease occurring epidemically among the herbivora, especially sheep and oxen, in which animals it has the characters of a rapidly fatal form of septicæmia with splenic enlargement, attended by an extensive multiplication of characteristic bacilli throughout the blood. The disease does not occur as a natural infection from man to man, but may be communicated to him directly or indirectly from animals, and it may then appear in one of three forms. In the first there is infection through the skin, in which a local lesion, the "malignant pustule," occurs. In the second form infection takes place through the respiratory tract. Here very aggravated symptoms centred in the thorax, with rapidly fatal termination, follow. Thirdly, an infection may occasionally take place through the intestinal tract, which is now the first part to give rise to symptoms. In all these forms of the affection in the human subject, the bacilli are in their distribution much more restricted to the local lesions than is the case in the ox, their growth and spread being attended by inflammatory œdema and often by hæmorrhages.

**Historical Summary.**—Historical researches leave little doubt that from the earliest times anthrax has occurred among cattle. For a long time its pathology was not understood, and it went by many names. Pollender in 1849 pointed out that the blood of anthrax animals contained numerous rod-shaped bodies which he conjectured had some

<sup>1</sup> In even recent works on surgery the term "anthrax" may be found applied to any form of carbuncle. Before its true pathology was known, the local variety of the disease which occurs in man, and which is now called "malignant pustule," was known as "malignant carbuncle."

<sup>2</sup> This must be distinguished from "charbon symptomatique," which is quite a different disease.



causal connection with the disease. In 1863 Davaine announced that they were bacteria, and originated the name *bacillus anthracis*. He stated that unless blood used in inoculation experiments on animals contained them, death did not ensue. Though this conclusion was disputed, still by the work of Davaine and others the causal relationship of the bacilli to the disease had been nearly established when the work of Koch appeared in 1876. This constituted that observer's first contribution to bacteriology, and did much to clear up the whole subject. Koch confirmed Davaine's view that the bodies were bacteria. He observed in the blood of anthrax animals the appearance of division, and from this deduced that multiplication took place in the tissues. He observed them under the microscope dividing outside the body, and noticed spore formation taking place. He also isolated the bacilli in pure culture outside the body, and, by inoculating animals with them, produced the disease artificially. In his earlier experiments he failed to produce death by feeding susceptible animals both with bacilli and spores, and as the intestinal tract was, in his view, the natural path of infection, he considered as incomplete the proof of this method of the spontaneous occurrence of anthrax in herds of animals. Koch's observations were, shortly afterwards, confirmed in the main by Pasteur, though controversy arose between them on certain minor points. Moreover, further research showed that the disease could be produced in animals by feeding them with spores, and thus the way in which the disease might spread naturally was explained.

**Bacillus Anthracis.**—Anthrax as a disease in man is of comparative rarity. Not only, however, is the bacillus anthracis easy of growth and recognition, but in its growth it illustrates many of the general morphological characters of the whole group of bacilli, and is therefore of the greatest use to the student. Further, its behaviour when inoculated in animals illustrates many of the points raised in connection with the general pathogenic effects of bacteria, immunity, etc. Hence an enormous amount of work has been done in investigating it in all its aspects.

If a drop of blood is taken immediately after death from an auricular vein of a cow, for example, which has died from anthrax, and examined microscopically, it will be found to contain a great number of large non-motile bacilli. On making a cover-glass preparation from the same source, and staining with watery methylene-blue, the characters of the bacilli can be better made out. They are about  $1.2\ \mu$  thick or a little thicker, and 6 to  $8\ \mu$  long, though both shorter and longer forms also occur. The ends are sharply cut across, or may be slightly dimpled so as to resemble somewhat the proximal end of a phalanx. Their protoplasm is very finely granular, and very frequently appears surrounded by a capsule whose external margin is not, however, so well defined as is the case with, *e.g.*, the pneumococcus. When several bacilli lie end to end in a thread, the capsule seems



common to the whole thread (Fig. 103). They stain well with all the basic aniline dyes and are not decolorised by Gram's method.

*Methylene Blue Reaction.*—This was introduced independently by McFadyean and by Heim with a view to the easy recognition of the bacilli in blood or other bodily fluids, and depends on a disintegration of the bacillary capsules which occurs when these are imperfectly fixed. Imperfect fixation is attained by drying a blood film on a *slide* and holding it three times for a second in a flame, film upwards (too great heating fixes the capsules and prevents the reaction from occurring). The preparation is stained for a few seconds with an old solution of methylene blue, 1 per cent. in water (*i.e.*, with a methylene blue possessing polychromatic qualities, see p. 113). It is washed in water and dried with filter paper,—preferably a cover glass is not applied. In such a preparation, between and near the bacteria there is a varying amount of an irregularly disposed amorphous or finely granular material of a violet or reddish-purple tint. Frequently the colour reaction in the preparation is so marked as to be recognisable naked-eye. McFadyean states that this reaction does not occur with putrefactive or other bacteria which might be present under circumstances where the recognition of the anthrax bacilli is the question under consideration.

*Plate Cultures.*—From a source such as that indicated, it is easy to isolate the bacilli by making gelatin or agar plates. If, after twelve hours' incubation at 37° C., the latter be examined under a low objective, colonies will be observed. They are to be recognised by beautiful wavy wreaths like locks of hair, radiating from the centre and apparently terminating in a point which, however, on examination with a higher power, is observed to be a filament which turns upon itself (Fig. 99). Graham Smith (*vide* p. 4) attributes the appearance to the toughness of the bacterial envelope, which prevents the separation of individuals from one another after division. The whole colony is, in fact, probably one long thread. Such colonies are very suitable for making impression preparations (*vide* p. 138) which preserve permanently the appearances



FIG. 99.—Surface colony of the anthrax bacillus on an agar plate, showing the characteristic appearance.  $\times 30$ .



described. On examining such with a high power, the wreaths are seen to be made up of bundles of long filaments lying parallel with one another, each filament consisting of a chain

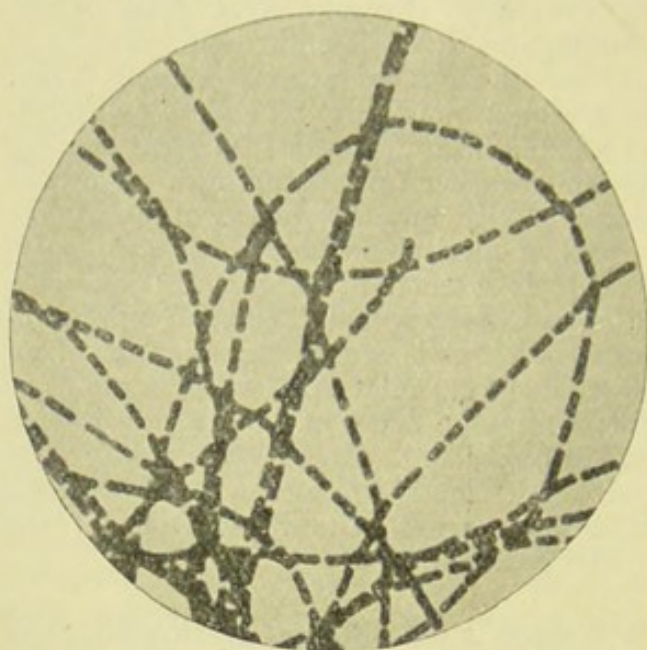


FIG. 100.—Anthrax bacilli, arranged in chains, from a twenty-four hours' culture on agar at 37° C.

Stained with fuchsin.  $\times 1000$ .

of bacilli lying end to end, and similar to those observed in the blood (Fig. 100).

On gelatin plates, after from twenty-four to thirty-six hours at 20° C., the same appearances manifest themselves, and later they are accompanied by liquefaction of the gelatin. In gelatin plates, however, instead of the characteristically wreathed appearance at the margin, the colonies sometimes give off radiating spikelets irregularly nodulated, which produce a star-like form. These spikelets are composed of spirally twisted threads.

From such plates the bacilli can be easily isolated, and the appearances of pure cultures on various media studied.

In *bouillon*, after twenty-four hours' incubation at 37° C., there is usually the appearance of irregularly spiral threads suspended in the liquid. These, on being examined, are seen

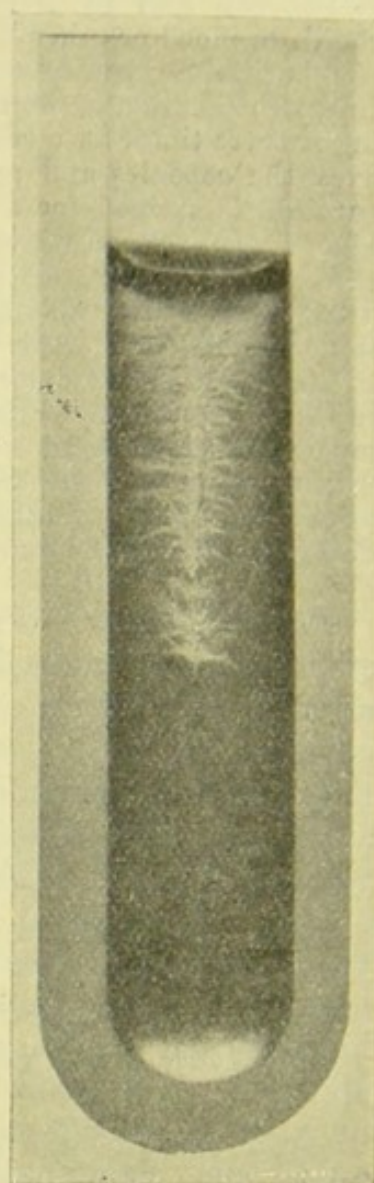


FIG. 101. Stab culture of the anthrax bacillus in peptone-gelatin; seven days' growth. It shows the "spiking," and also, at the surface, commencing liquefaction. Natural size.



to be made up of bundles of parallel chains of bacilli. Later, growth is more abundant, and forms a flocculent mass at the bottom of the fluid.

In *gelatin* stab cultures, the characteristic appearance can be best observed when a low proportion, say  $7\frac{1}{2}$  per cent., of gelatin is present, and when the tube is directly inoculated from anthrax blood. In about two days there radiate out into the medium from the needle track numberless very fine spikelets which enable the cultures to be easily recognised. These spikelets are longest at the upper part of the needle track (Fig. 101). Not much spread takes place on the surface of the gelatin, but here liquefaction commences, and gradually spreads down the stab and out into the medium, till the whole of the gelatin may be liquefied. Gelatin slope cultures exhibit a thick felted growth, the edges of which show the wreathed appearance seen in plate cultures. Liquefaction here soon ploughs a trough in the surface of the medium. Sometimes "spiking" does not take place in gelatin stab cultures, only little round particles of growth occurring down the needle tract, followed by liquefaction. As has been shown by Rd. Muir, this property of spiking can be restored by growing the bacillus for twenty-four hours on blood agar at  $37^{\circ}$  C. Agar sloped cultures have the appearance of similar cultures in gelatin, though, of course, no liquefaction takes place.

*Blood serum* sloped cultures present the same appearances as those on agar. The margin of the surface growth on any of the solid media shows the characteristic wreathing seen in plate colonies. The occurrence of capsulation of the bacilli in such cultures has been described.

On *potatoes* there occurs a thick felted white mass of bacilli showing no special characters. Such a growth, however, is useful for studying sporulation.

The anthrax bacillus will thus grow readily on any of the ordinary media. It can usually be sufficiently recognised by its microscopic appearance, by its growth on agar or gelatin plates, and by its growth in gelatin stab cultures. The growth on plates is specially characteristic, and is simulated by no other pathogenic organism.

**The Biology of the B. Anthracis.**—Koch found that the bacillus anthracis grows best at a temperature of  $35^{\circ}$  C. Growth, *i.e.* multiplication, does not take place below  $12^{\circ}$  C. nor above  $45^{\circ}$  C. In the spore-free condition the bacilli have comparatively low powers of resistance. They do not stand long exposure to  $60^{\circ}$  C., and if kept at ordinary temperature in the dry condition



they are usually found to be dead after a few days. The action of the gastric juice is rapidly fatal to them, and they are accordingly destroyed in the stomachs of healthy animals. They are also soon killed in the process of putrefaction. They can, however, be cooled below the freezing-point without dying. The bacillus can grow without oxygen, but some of its vital functions are best carried on in the presence of this gas. Thus in anthrax cultures the liquefaction of gelatin always commences at the surface and spreads downwards. Growth is more rapid in the presence of oxygen, and spore formation does not occur in its

absence. The organism may be classed as a facultative anaerobe.

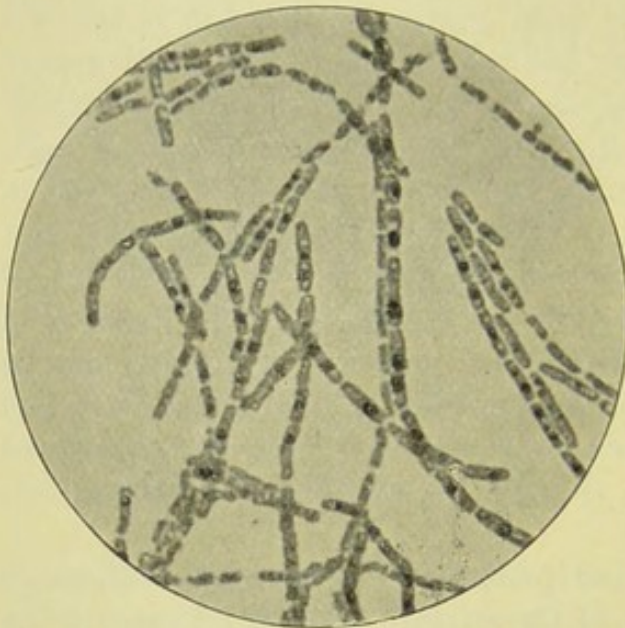


FIG. 102.—Anthrax bacilli containing spores (the darkly coloured bodies); from a three days' culture on agar at 37° C. See also Plate III., Fig. 2. Stained with carbol-fuchsin and methylene-blue.  $\times 1000$ .

*Sporulation.* — Under certain circumstances sporulation occurs in anthrax bacilli. The morphological appearances are of the ordinary kind. A little highly refractile speck appears in the protoplasm about the centre of the bacillus; this gradually increases in size until it forms an oval body about the same thickness as the bacillus lying in the bacillary protoplasm (Fig. 102). The latter gradually loses its staining capacities and finally disappears. The

spore thus lies free as an oval highly refractile body which does not stain by ordinary methods, but which can be easily stained by the special methods described for such a purpose (p. 109). When the spore is again about to assume the bacillary form the capsule is apparently absorbed, and the protoplasm within grows out, taking on the ordinary rod-shaped form.

According to most observers, sporulation never occurs within the body of an animal suffering from anthrax. Koch attributes this, probably rightly, to the absence of free oxygen. The latter gas he found necessary to the occurrence of spores in cultures outside the body. Many, however, are inclined to assign as the cause of sporulation the absence of the optimum pabulum. Be-



sides these conditions there is another factor necessary to sporulation, namely, a suitable temperature. The optimum temperature for spore production is  $30^{\circ}\text{C}$ . Koch found that spore formation did not occur below  $18^{\circ}\text{C}$ . Above  $42^{\circ}\text{C}$ . not only does sporulation cease, but Pasteur found that if bacilli were kept at this temperature for eight days they did not regain the capacity when again grown at a lower temperature. In order to make them again capable of sporing, it is necessary to adopt special measures, such as passage through the bodies of a series of susceptible animals.

Anthrax spores have extremely high powers of resistance. In a dry condition they will remain viable for a year or more. Koch found they resisted boiling for five minutes; and dry heat at  $140^{\circ}\text{C}$ . must be applied for several hours to kill them with certainty. Unlike the bacilli, they can resist the action of the gastric juice for a long period of time. They are often used as test objects by which the action of germicides is judged. For this purpose an emulsion is made by scraping off a surface culture and rubbing it up in a little sterile water. Into this sterile silk threads are dipped, which, after being dried over strong sulphuric acid in a desiccator, can be kept for long periods of time in an unchanged condition. For use they are placed in the germicidal solution for the desired time, then washed with water to remove the last traces of the reagent and laid on the surface of agar or placed in bouillon, in order that if death of the bacilli has not occurred growth may be observed (*see Chap. VI.*).

**Anthrax in Animals.**—Anthrax occurs from time to time epidemically in sheep, cattle, and, more rarely, in horses and deer. These epidemics are found in various parts of the world, although they are naturally most far-reaching where legal precautions to prevent the spread of infection are non-existent. All the countries of Europe are from time to time visited by the disease, but in some it is much more common than in others. In Britain the death-rate is small, but in France the annual mortality among sheep was probably 10 per cent. of the total number in the country, and among cattle 5 per cent. These figures, however, have been largely modified by the system of preventive treatment which will be presently described. In sheep and cattle the disease is specially virulent. An animal may suddenly drop down, with symptoms of collapse, quickening of pulse and respiration, and dyspnoea, and death may occur in a few minutes. In less acute cases the animal is apparently out of sorts, and does not feed; its pulse and respiration are



quickened; rigors occur, succeeded by high temperature; there is a sanguineous discharge from the bowels, and bloody mucus may be observed about the mouth and nose. There may be convulsive movements, and progressive weakness, with cyanosis, is followed by death in from twelve to forty-eight hours. In the more prolonged cases widespread œdema and extensive enlargement of lymphatic glands are marked features; and in the glands, especially about the neck, actual necrosis with ulceration may occur, constituting the so-called anthrax carbuncles. Such subacute conditions are especially found among horses, which are by nature not so susceptible to the disease as cattle and sheep.

On *post-mortem* examination of an ox dead of anthrax, the most noticeable feature—one which has given the name “splenic fever” to the disease—is the enlargement of the spleen, which may be two or three times its natural size. It is of dark-red colour, and on section the pulp is very soft and friable, sometimes almost diffuent. A cover-glass preparation may be made from the spleen and stained with watery methylene-blue. On examination it will be found to contain enormous numbers of bacilli mixed with red corpuscles and leucocytes, chiefly lymphocytes and the large mononucleated variety (Fig. 103). Pieces of the organ may be hardened in absolute alcohol, and sections cut in paraffin. These are best stained by Gram’s method. Microscopic examination of such shows that the structure of the pulp is considerably disintegrated, whilst the bacilli swarm throughout the organ, lying irregularly amongst the cellular elements. The liver is enlarged and congested, and may be in a state of acute cloudy swelling. The bacilli are present in the capillaries throughout the organ, but are not so numerous as in the spleen. The kidney is in a similar condition, and here the bacilli are chiefly found in the capillaries of the glomeruli, which often appear as if injected with them. The lungs are congested and may show catarrh, whilst bacilli are present in large numbers throughout the capillaries, and may also be found in the air cells, probably as the result of rupture of the capillaries. The blood throughout the body is usually fluid and of dark colour.

The lymphatic system generally is much affected. The glands, especially the mediastinal, mesenteric, and cervical glands, are enlarged and surrounded by œdematous tissue, the lymphatic vessels are swollen, and both glands and vessels may contain numberless bacilli. The heart-muscle may be in a state of cloudy swelling, and the blood in its cavities contains bacilli,



though in smaller numbers than that in the capillaries. The intestines are enormously congested, the epithelium more or less desquamated, and the lumen filled with a bloody fluid. From all the organs the bacilli can be easily isolated by stroke cultures on agar.

It is important to note the existence of great differences in susceptibility to anthrax in different species of animals. Thus



FIG. 103.—Scraping from spleen of guinea-pig dead of anthrax, showing the bacilli mixed with leucocytes, etc. (Same appearance as in the ox.)

“Corrosive film” stained with carbol-thionin-blue.  $\times 1000$ .

the ox, sheep (except those of Algeria, which only succumb to enormous doses of the bacilli), guinea-pig, and mouse are all very susceptible, the rabbit slightly less so. We have no data to determine whether the disease occurs among the last three in the wild state. Less susceptible than this group are the horse, deer, goat, in which the disease occurs from time to time in nature. Anthrax also occurs epidemically in the pig, often from the ingestion of the organs of other animals dead of the disease. It is, however, doubtful if all cases of disease in the pig described



on clinical grounds as anthrax are really such. A careful bacteriological examination is here always advisable, especially of any œdematous infiltration about the throat, or in the neighbouring lymphatic glands; often, in pigs dying of anthrax, bacilli may not occur in the blood. Any hæmorrhagic infarction in the spleen of a suspected animal should be carefully investigated. The human subject may be said to occupy a



FIG. 104.—Portion of kidney of a guinea-pig dead of anthrax, showing the bacilli in the capillaries, especially of the glomerulus. Paraffin section; stained by Gram's method and Bismarck-brown.  $\times 300$ .

medium position between the highly susceptible and the relatively immune animals. The white rat is highly immune to the disease, while the brown rat is susceptible. Adult carnivora are also very immune, and the birds and amphibia are in the same position.

With these differences in susceptibility there are also great variations in the pathological effects produced in the natural or artificial disease. This is especially the case when we consider the distribution of the bacilli in the bodies of the less susceptible



animals. Instead of the widespread occurrence described above, they may be confined to the point where they first gained access to the body and the lymphatic system in relation to it, or may be only very sparsely scattered in organs such as the spleen (which is often not enlarged), the lungs, or kidneys. Nevertheless the cellular structure of the organs even in such a case may show changes, a fact which is important when we consider the essential pathology of the disease.

*Experimental Inoculation.*—Of the animals commonly used in laboratory work, white mice and guinea-pigs are the most susceptible to anthrax, and are generally used for test inoculations. If a small quantity of anthrax bacilli be injected into the subcutaneous tissue of a guinea-pig, a fatal result follows, usually within two days. *Post-mortem*, around the site of inoculation the tissues, owing to intense inflammatory œdema, are swollen and gelatinous in appearance, small hæmorrhages are often present, and on microscopic examination numerous bacilli are seen. The internal organs show congestion and cloudy swelling, with sometimes small hæmorrhages, and their capillaries contain enormous numbers of bacilli, as has already been described in the case of the ox (Fig. 104); the spleen also shows a corresponding condition. Highly susceptible animals may be infected by being made to inhale the bacilli or their spores, and also by being fed with spores, a general infection rapidly occurring by both methods.

**Anthrax in the Human Subject.**—As we have noted, man occupies a middle position in the scale of susceptibility to anthrax. It is always communicated to him from animals, and usually is seen among those whose trade leads them to handle the carcasses or skins of animals which have died of the disease. It occurs in two principal forms, the main difference between which is due to the site of entrance of the organism into the body. In one, the path of entrance is through cuts or abrasions in the skin, or through the hair follicles. A local condition called a “malignant pustule” develops, which may lead to a general infection. This variety occurs chiefly among butchers and those who work among hides (foreign ones especially). In Britain the workers of the latter class chiefly liable are the hide-porters and hide-workers in South-Eastern London. In the other variety of the disease the site of infection is the trachea and bronchi, and here a fatal result almost always follows. The cause is the inhalation of dust or threads from wool, hair, or bristles, which have been taken from animals dead of the disease, and which have been contaminated with blood or secretions con-



taining the bacilli, these having afterwards formed spores. This variety is often referred to as "woolsorter's disease," from its occurring in the centres of the woolstapling trade (in England, chiefly in Yorkshire), but it also is found in places where there are hair and brush factories.

(1) *Malignant Pustule*.—This usually occurs on the exposed surfaces—the face, hands, fore-arms, and back, the last being a common site among hide-porters. One to three days after inoculation a small red painful pimple appears, soon becoming a vesicle, which may contain clear or blood-stained fluid, and is rapidly surrounded by an area of intense congestion. Central necrosis occurs and leads to the malignant pustule proper, which in its typical form appears as a black eschar often surrounded by an irregular ring of vesicles, these in turn being surrounded by a congested area. From this pustule as a centre subcutaneous œdema spreads, especially in the direction of the lymphatics; the neighbouring glands are enlarged. There is fever with general malaise. On microscopic section of the typical pustule, the central eschar is noticed to be composed of necrosed tissue and degenerating blood cells; the vesicles are formed by the raising of the stratum corneum from the rete Malpighi. Beneath them and in their neighbourhood the cells of the latter are swollen and œdematous, the papillæ being enlarged and flattened out and infiltrated with inflammatory exudation, which also extends beneath the centre of the pustule. In the tissue next the eschar necrosis is commencing. The subcutaneous tissue is also œdematous, and often infiltrated with leucocytes. The bacilli exist in the periphery of the eschar and in the neighbouring lymphatics, and, to a certain extent, in the vesicles. It is very important to note that widespread œdema of a limb, enlargement of neighbouring glands, and fever may occur while the bacilli are still confined to the immediate neighbourhood of the pustule. Sometimes the pathological process goes no further, the bacilli gradually die out, the eschar becomes a scab, the inflammation subsides, and recovery takes place. In the majority of cases, however, if the pustule be not excised, the œdema spreads, invasion of the blood stream may occur, and the patient dies with, in a modified degree, the pathological changes detailed with regard to the acute disease in cattle. In man the spleen is usually not much enlarged, and the organs generally contain few bacilli. The actual cause of death is therefore a toxic effect. The early excision of an anthrax pustule, especially when it is situated in the extremities, is followed, in a large proportion of cases, by recovery.



(2) *Woolsorter's Disease*.—The pathology of this affection was worked out in this country especially by Greenfield. The local lesion is usually situated in the lower part of the trachea or in the large bronchi, and is in the form of swollen patches in the mucous membrane, often with hæmorrhage into them,—small ulcers may also be seen. The tissues are oedematous, and the cellular elements are separated, but there is usually little or no necrosis. There is enormous enlargement and engorgement of the mediastinal and bronchial glands, and hæmorrhagic infiltration of the cellular tissue in the region. There are pleural and pericardial effusions, and hæmorrhagic spots occur beneath the serous membranes. The lungs show great congestion, collapse and œdema. There may be cutaneous œdema over the chest and neck, with enlargement of glands, and the patient rapidly dies with symptoms of pulmonary embarrassment, and with a varying degree of pyrexia. It is to be noted that in such cases, though numerous bacilli are present in the bronchial lesions, in the lymphatic glands, and affected tissues in the thorax, comparatively few may be present in the various organs, such as the kidney, spleen, etc., and sometimes it may be impossible to find any.

(3) Infection occasionally takes place through the intestine, probably by ingestion of spores as in the case of animals; but this condition is rare. In such cases there occur single or multiple local hæmorrhagic lesions in the intestinal mucous membrane, the central parts of the hæmorrhagic areas tending to be necrotic and yellowish, and there may be a corresponding affection of the mesenteric glands.

A considerable number of cases have been recorded in which hæmorrhagic meningitis, associated with the presence of the anthrax bacilli in large numbers, has occurred as a complication of various primary lesions.

**The Toxins of the Bacillus Anthracis.**—Various theories were formerly held as to the mode in which the anthrax bacillus produces its effects. One of the earliest was the mechanical, according to which it was supposed that the serious results were produced by extensive blocking of the capillaries in the various organs by the bacilli. According to another, it was supposed that the bacilli used up the oxygen of the blood, thus leading to starvation of the tissues. In modern times there has been a tendency to attribute the effects produced to toxic action. Sidney Martin investigated this subject, and isolated from cultures, proto-albumose, deuterio-albumose, traces of peptone, and alkaloidal bodies. By these, pathogenic effects were



produced in animals, closely similar to those produced by the bacilli themselves. Martin, to account for the symptoms of the disease, considered that the fever was mostly due to the albumoses, while the œdema and congestion were caused by the alkaloid acting as a local irritant. Hankin and Wesbrook arrived at the conclusion that the bacillus anthracis produces a ferment which, diffusing out into the culture fluid, elaborates albumoses from the proteids present in it. The bacilli also produce albumoses directly without the intervention of a ferment. Marmier isolated from cultures in peptone solution a body which gave no reactions of albuminoid matter, peptone, propeptone, or alkaloid, and which he considered to be the toxin. It was chiefly retained within the bacilli when these were growing in the most favourable conditions, and was not destroyed by heating to 110° C. The toxin produced by the *b. anthracis* growing in a fluid medium remains intimately associated with the bacterial protoplasm, as such cultures when filtered are relatively non-toxic.

It cannot be said that great light has been thrown on the pathology of the disease by these researches. The effects of infection by the *b. anthracis* are those shared by all other organisms producing inflammation, the tendency to œdema-production of an unwonted degree being the chief special feature. That toxic effects do occur in anthrax is probable, for frequently, while the bacilli are still locally confined, there may occur pyrexia and œdema spreading widely beyond the pustule, but we have no definite information as to how these effects are produced. In this disease we are probably dealing with another example of the action of intracellular toxins, regarding which, as in other cases, little is known.

**The Spread of the Disease in Nature.**—We have seen that the *b. anthracis* rarely, if ever, forms spores in the body, and if the bacilli could be confined to the blood and tissues of carcasses of animals dying of the disease, it is certain that anthrax in an epidemic form would rarely occur. For it has been shown by many observers that in the course of the putrefaction of such a carcase the anthrax bacilli rapidly die out, and that after ten days or a fortnight very few remain. But it must be remembered that while still alive an animal is shedding into the air by the bloody excretions from the mouth, nose, and bowel, myriads of bacilli which may rapidly spore, and thus arrive at a very resistant stage. These lie on the surface of the ground and are washed off by surface water. At certain seasons of the year the temperature is, however, sufficiently high to permit of their



germination, and also of their multiplication, as they can undoubtedly grow on the organic matter which occurs in nature. They can again form spores. It is in the condition of spores that they are dangerous to susceptible animals. In the bacillary stage, if swallowed, they will be killed by the acid gastric contents; but as spores they can pass uninjured through the stomach, and gaining an entrance into the intestine, infect its wall, and ultimately reach, and multiply in the blood. It is known that in the great majority of cases of the disease in sheep and oxen, infection takes place thus from the intestine. It was thought by Pasteur that worms were active agents in the natural spread of the disease by bringing to the surface anthrax spores. Koch made direct experiments on this point, and could get no evidence that such was the case. He thinks it much more probable that the recrudescence of epidemics in fields where anthrax carcasses have been buried is due to persistence of spores on the surface which has been infected by the cattle when alive. In Britain it is common to attribute the occurrence of sporadic outbreaks to infection by imported feeding stuffs. Scientific proof of such a method of infection being common is still wanting.

**The Disposal of the Carcasses of Animals dead of Anthrax.**—It is extremely important that anthrax carcasses should be disposed of in such a way as to prevent their becoming future sources of infection. If anthrax be suspected as the cause of death, no *post-mortem* examination should be made, but only a small quantity of blood removed from an auricular vein for bacteriological investigation. If such a carcass be now buried in a deep pit surrounded by quicklime, little danger of infection will be run. The bacilli being confined within the body will not spore, and will die during the process of putrefaction. The danger of sporulation taking place is, of course, much greater when an animal has died of an unknown disease, which, on *post-mortem* examination, has proved to be anthrax, but similar measures for burial must be here adopted. In some countries anthrax carcasses are burned, and this, if practicable, is of course the best means of treating them. The chief source of danger to cattle subsequently, however, proceeds from the infection of fields, yards, and byres with the offal and the discharge from the mouths of anthrax animals. All material that can be recognised as such should be burned along with the straw in which the animals have lain. The stalls or buildings in which the anthrax cases have been must be limewashed. Needless to say, the greatest care must be taken in the case of men who handle the animal or its carcass that they have no wounds on their persons, and that they thoroughly disinfect themselves by washing their hands, etc., in 1 to 1000 solution of corrosive sublimate or lysol, and that all clothes soiled with blood, etc., from anthrax animals be thoroughly boiled or steamed for half an hour before being washed.

**The Immunising of Animals against Anthrax.**—Having



ascertained that there was ground for believing that in cattle one attack of anthrax protected against a second, Pasteur (in the years 1880-82) elaborated a method by which a mild form of the disease could be given to animals, which rendered harmless a subsequent inoculation with virulent bacilli. He found that the continued growth of anthrax bacilli at 42° to 43° C. caused them to lose their capacity of producing spores, and also gradually to lose their virulence, so that after twenty-four days they could no longer kill either guinea-pigs, rabbits, or sheep. Such cultures constituted his *premier vaccin*, and protected against the subsequent inoculation with bacilli which had been grown for twelve days at the same temperature, and the attenuation of which had therefore not been carried so far. The latter constituted the *deuxième vaccin*. It was further found that sheep thus twice vaccinated now resisted inoculation with a culture which usually would be fatal. The method was to inoculate a sheep on the inner side of the thigh by the subcutaneous injection, from a hypodermic syringe, of about five drops of the *premier vaccin*; twelve days later to again inoculate with the *deuxième vaccin*; fourteen days later an ordinary virulent culture was injected without any ill result. This method was applicable also to cattle and horses, about double the dose of each vaccine being here necessary. Extended experiments in France generally confirmed earlier results, and the method was, before long, used to mitigate the disease, which in many *départements* was endemic and a very great scourge. Since that time the method has been regularly in use. It is difficult to arrive at a certain conclusion as to its merits. Undoubtedly a certain number of animals die of anthrax either after the first or second vaccination, or during the year following vaccination. At the end of a year the immunity is lost in about 40 per cent. of the animals vaccinated; and thus to be permanently efficacious the process would have to be repeated every year. Further, the immunity is much higher in degree if, after the first and second vaccinations, an inoculation with virulent anthrax is performed. Everything being taken into account, however, there is no doubt that the mortality from natural anthrax is much diminished by this system.

During the twelve years 1882-93, 3,296,815 sheep were vaccinated, with a mortality, either after the first or second vaccination, or during the subsequent twelve months, of 0·94 per cent., as contrasted with the ordinary mortality in all the flocks of the districts of 10 per cent. During the same time 438,824 cattle were vaccinated, with a mortality, of 0·34 per cent., as contrasted with a probable mortality of 5 per cent. if they had been unprotected.



The immunisation of animals against anthrax has always been found to be a difficult proceeding. The most usual technique has been to commence with Pasteur's vaccines, and to follow these by careful dosage with virulent cultures. Marchoux in this way produced immunity, and found that the serum of immune animals had a certain degree of protective and curative action. The most successful attempts in this direction have been those of Sclavo and of Sobernheim. The former observer, after trying various animals, came to the conclusion that the ass was the most suitable. He first employed a method similar to that of Marchoux; later, however, after noting the effects of the serum of an animal so immunised, he commenced the immunisation by injecting 5 to 15 c.c. of this serum along with a slightly attenuated culture of the bacilli. A few days later this was followed up with injections of virulent cultures which could now be periodically introduced for many months, and a high degree of immunity resulted. What was even more important, the serum of such an animal had strongly protective and curative properties. It has been extensively used in the treatment of anthrax in man. In a case of malignant pustule 30 to 40 c.c. are injected in quantities of 10 c.c. into the abdominal wall, and if necessary the injection is repeated on the following day. In cases treated by Sclavo himself the serum is alone employed, and its action is not aided by the excision of the pustule usually practised. The results obtained have been very good,—Sclavo, out of 164 cases, had only ten deaths or about a fourth of the ordinary mortality in Italy. Sobernheim independently elaborated an almost identical method of combining passive with active immunisation for the obtaining of a powerful anti-serum, and he has used this for the protective inoculation of cattle. The technique is to inject a mixed serum obtained from the ox, the horse, and the sheep, into one side of the neck or into one thigh and the culture (Pasteur's second vaccine) into the other side; the doses given are for cattle or horses 5 c.c. of serum and 0.5 c.c. culture, and for sheep 4 c.c. of serum and 0.25 c.c. culture. The method has been widely used in Germany and in Brazil, and its originator claims as its advantages simplification of application, in that one operation instead of two is sufficient, less risk of death following the immunisation procedure, and higher degree and more lasting character of the immunity resulting. During the development of active immunity it is likely in every case (see Immunity) that there is a period of increased susceptibility to the disease. Such a period would be more likely to occur with the Pasteur



method than with the Sobernheim procedure, where the presence in the animal's body of the protective serum might tide it over the stage when the action of the vaccine was lowering its resistance.

The effects of the *b. anthracis* have been much studied with a view to the shedding of light on the processes obtaining in resistance and the development of immunity. Many puzzling facts have long been known; for example, in the dog, which shows great natural resistance, the serum has little if any bactericidal action, while in the susceptible rabbit there is present a serum capable of killing the organism. Such observations have hitherto been without explanation. Again, the properties of the serum of immune animals have been much discussed. Sobernheim and others have been unable to detect in it any trace of special bactericidal action. Selavo found that the serum when heated to 55° C. did not lose its protective properties; as the serum might have been complemented (see Immunity) by the serum of the animal into which it was injected, he simultaneously introduced an anti-complementary serum and found that the heated serum was still effectual. From this he deduces that in the action of the serum substances of the nature of immune body and complement are not concerned. Many have thought that the serum had a stimulating effect on the leucocytes, but Cler has brought forward ground for supposing that its effect is a sensitising one on the bacteria, and that thus the effects are to be traced to opsonic action. With regard to the formation of the protective substances, it is stated that the spleen and bone-marrow are richer in these than the blood fluids. In this connection an interesting fact may be mentioned, namely, that Roger and Garnier found evidence of the liver and spleen having special capacities for killing anthrax bacilli; an otherwise fatal dose could be introduced into the portal vein or the splenic artery without causing death. It has been thought that the capsule frequently developed by the anthrax bacillus is a defensive mechanism against bactericidal and bacteriolytic capacities in an infected animal. It is stated that capsulation renders the bacillus less susceptible to phagocytosis. Opinion on the significance of capsule formation is at present divided.

**Methods of Examination.**—These include (a) microscopic examination; (b) the making of cultures; and (c) test inoculations.

(a) *Microscopic Examination.*—In a case of suspected malignant pustule, film preparations should be made from the fluid in the vesicles or from a scraping of the incised or excised



pustule, and stained with a watery solution of methylene-blue and also by Gram's method. By this method practically conclusive evidence may be obtained; but sometimes the result is doubtful, as the bacilli may be very few in number. McFadyean's methylene-blue method (p. 333) should also be applied. In all cases confirmatory evidence should be obtained by culture. Occasionally bacilli are so scanty that both film preparations made from different parts and even cultures may give negative results, and yet a few bacilli may be found when a section of the pustule is examined. It should be noted that the greatest care ought to be taken in manipulating a pustule before excision, as the diffusion of the bacilli into the surrounding tissues may be aided and the condition greatly aggravated. The examination of the blood in cases of anthrax in man usually gives negative results, with the exception of very severe cases, when a few bacilli may be found in the blood shortly before death, though even then they may be absent.

(b) *Cultivation*.—A small quantity of the material used for microscopic examination should be taken on a platinum needle, and successive strokes made on agar tubes, which are then incubated at 37° C. At the end of twenty-four hours anthrax colonies will appear, and can be readily recognised from their wavy margins by means of a hand lens. They should also be examined microscopically by means of film preparations.

(c) *Test Inoculation*.—A little of the suspected material should be mixed with some sterile bouillon or water, and injected subcutaneously into a guinea-pig or mouse. If anthrax bacilli are present, the animal usually dies within two days, with the changes in internal organs already described. The diagnosis of an organism as the anthrax bacillus cannot be said to be substantiated till its pathogenicity has been proved.



## CHAPTER XV.

### TYPHOID FEVER—BACILLI ALLIED TO THE TYPHOID BACILLUS.

**Introductory.**—The organism now known as the bacillus typhosus was first described in 1880-1 by Eberth, who observed its microscopic appearance in the intestinal ulcers and in the spleen in cases of typhoid fever (German, Abdominaltyphus). It was first isolated (from the spleen) in 1884 by Gaffky, and its cultural characters were then investigated. In 1885 Escherich observed a bacillus, now known as the bacillus coli communis, which occurs in the normal intestine, and which both microscopically and culturally closely resembles the typhoid bacillus. Ordinarily the b. coli is no doubt a harmless saprophyte, but under experimental conditions in animals and also naturally in man it may manifest pathogenic properties. Investigation has shown that these two bacilli belong to a widespread group of organisms isolated from various disease conditions, chiefly of the intestine, which all bear close resemblances to one another, and whose differentiation is often a matter of considerable difficulty. Other members of this group are the paratyphoid bacillus, the organism of bacillary dysentery, the b. enteritidis of Gaertner, the psittacosis bacillus, and the bacillus of hog cholera.

The general characters of the group are as follows: the organisms, which are microscopically indistinguishable, are thin non-sporing bacilli, which in cultures often show variation in length; they are mostly motile, but this quality varies in different members; they possess flagella springing from all round the bacillus; they stain with ordinary dyes, and are all Gram-negative; they are all facultative anaerobes, *i.e.* they grow best in the presence of oxygen, but can tolerate its absence; in growth characters on ordinary media they closely resemble one another, and, generally speaking, they do not liquefy gelatin; they show wide differences in fermentative capacities, and are chiefly to be distinguished by their immunity reactions.



## THE BACILLUS COLI COMMUNIS.

Although the discovery of the bacillus coli communis was subsequent to that of the bacillus typhosus, it is convenient to commence with a description of the former, as it presents more positive characters than any other member of the group to which it belongs.

**Bacillus Coli Communis.**—*Morphological Characters.*—These are best seen in cultures. The bacillus is ordinarily from 2 to 4  $\mu$  long and about .5  $\mu$  broad; longer forms up to 8 or 10  $\mu$  are not infrequent (Fig. 105). It is usually found to be motile, but the motility varies in different strains and under different growth conditions in the same strain. Here it is best to use bouillon cultures incubated at 37° C. for from six to twelve hours. The organism may stain somewhat faintly with watery dyes, but is readily demonstrated with carbol-fuchsin even in fairly weak solution (1 of the Ziehl-Neelsen stain in 20 of water); it is Gram-negative. By appropriate staining b. coli derived from cultures

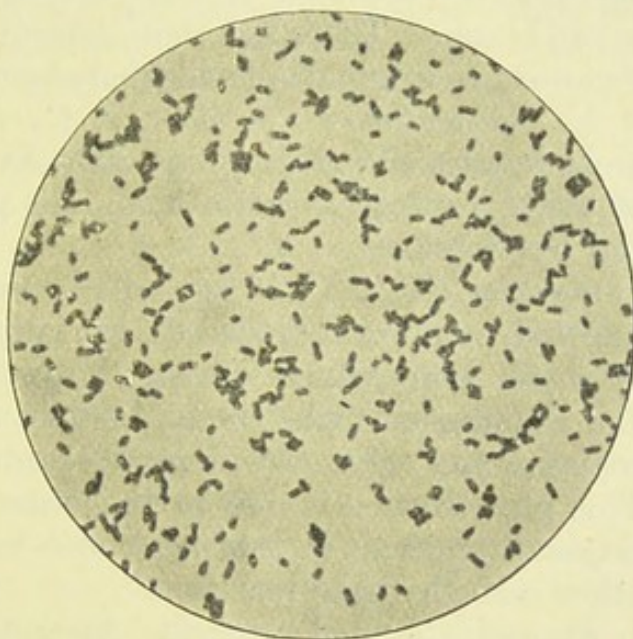


FIG. 105.—*Bacillus coli communis*. Film preparation from a young culture on agar. Stained with weak carbol-fuchsin.  $\times 1000$ .

can be shown to possess flagella springing from all round the organism, varying in number and occasionally rather short.

**Culture Reactions on Ordinary Media.**—The following are the appearances of the b. coli in the ordinary culture media:—

In *bouillon*, it produces a uniform turbidity. When grown in fluid gelatin, it is stated by Klein to tend to form flocculi floating on the surface rather than a uniform turbidity. In stab cultures on *peptone gelatin* an abundant film-like growth takes place on the surface, and there is a whitish or brownish-white line along the stab. No liquefaction of the gelatin occurs, but occasionally a few bubbles of gas develop (Fig. 109, C). In sloped *agar tubes* a somewhat dense, glistening, white or brownish-white growth occurs along the stroke. When *agar plates* are used



for the separation of the organism, the surface colonies are somewhat large, and it may be irregular in outline, but the deep colonies are smaller and lenticular in shape, and under a low power of the microscope appear rather dense to transmitted light. A similar growth occurs on *blood serum*. On *potatoes*, in forty-eight hours, there is a distinct film of growth of a brownish tint, sometimes with a moist surface, which rapidly spreads and becomes thicker. The appearance on potato, however, varies much with the different strains and also with the reaction of the potato.

**Culture Reactions on Special Media.**—A great variety of media have been used for the appreciation of special characters in the *b. coli*. These reactions depend upon the capacities of the organism to originate chemical changes in a variety of substances.

A. *Fermentative Reactions on Carbo-hydrates.*—*B. coli* shows great powers of splitting up carbo-hydrates with the formation of acids, especially lactic acid, and gas, chiefly carbon dioxide and hydrogen.

*Gelatin Shake Cultures.*—If a tube of gelatin be melted, infected with *b. coli*, shaken up, allowed to solidify, and kept at room temperature, distinct growth of the organism occurs, and round each little colony, bubbles of gas form, which in time coalesce and give the tube a readily recognised appearance. This phenomenon is due to the organism fermenting the sugars originally present in the meat, and is thus to be grouped with other carbo-hydrate reactions.

*Fermentation of Sugars.*—As stated on page 80, litmus or neutral-red peptone water, or dextrose-free bouillon in Durham's tubes is used, the sugar to be employed being added in the proportion of half to one per cent. The fermentative capacities of the *b. coli* are very wide. It produces acid and gas in glucose, lactose, lævulose, galactose, maltose, raffinose, mannite, dulcitate, sorbite, and very frequently in cane sugar (saccharose).<sup>1</sup> It produces a similar change in the glucosides, salicin, and arbutin.

The reactions of *b. coli* in some media other than simple sugar solutions likewise depend on sugar fermentation, and of these are the following:—

*Curdling of Milk.*—If the *b. coli* be grown in milk, preferably litmus milk, acid is produced from the lactose present which further curdles the milk. If litmus milk be used, the acid reaction should be permanent when growth is allowed to go on

<sup>1</sup> A strain of *b. coli* fermenting cane sugar was formerly referred to as *b. coli communior*, but this differentiating term has been discarded.



for some days. A similar reaction is observed if litmus whey is used (p. 51).

*Measuring of Gas Formation.*—As has been said, the gases produced by the *b. coli* in fermenting sugars are chiefly carbon dioxide and hydrogen. Many observers attach considerable importance, first, to the amount of gas formed from a given quantity of glucose in a given time, and, second, to the ratios of the two gases to one another, in such a fermentation. For the observation of this, MacConkey recommends the following method: fermentation tubes (p. 81, Fig. 36, c), with the closed limb graduated, containing 2 per cent. peptone (Witte) and 1 per cent. glucose in tap water, are inoculated and incubated for forty-eight hours at 37° C. The tube is allowed to cool and the total amount of gas noted. The bulb is then filled with 2 per cent. sodium hydrate solution, the opening closed with the thumb and thoroughly shaken. After the gas has been collected in the closed arm the thumb is removed and the ratio of the hydrogen left to the original gas volume is read off.

*Voges and Proskauer's Reaction.*—This is a reaction which is not given by the classical type of *b. coli*, but as it occurs with many members of the *coli* group it may be described here. It also depends on carbo-hydrate fermentation. A glucose peptone solution tube is inoculated and growth allowed to take place for three days. A solution of caustic potash is added and the tube allowed to stand for twenty-four hours at room temperature. A red fluorescent colour is produced, causing the medium to resemble a weak alcoholic solution of eosin.

*B. Action on Neutral-Red.*—When *b. coli* is grown on neutral-red lactose bouillon, a rosy red colour, the effect of the lactic acid upon the dye, is at first seen. Frequently this is succeeded by the appearance of a green fluorescence due to a direct action of the organism upon the dye. This is evidenced by the fact that the neutralisation of the lactic acid by an alkali does not lead to a reproduction of the original alkaline tint in the indicator. The reaction, however, varies with composition of the medium, the important factors being the percentage of sugar and the reaction.

*C. Production of Indol.*—The *b. coli* produces indol in peptone water. The methods have been given on page 82, and for the detection of the reaction the use of Ehrlich's rosindol test is preferable; (if the nitroso-indol reaction be used, a small quantity of a nitrite must be added). Two peptone tubes should always be inoculated, and if the reaction is not obtainable in one after two or three days' growth, the other should be incubated for from six to seven days and then tested. Where a faint reaction is obtained, it is well to corroborate the presence of indol by dissolving the rosindol out with amyl-alcohol as described.



D. *Reduction of Nitrates*.—The *b. coli* is frequently capable of reducing nitrates to nitrites. For this test, Savage recommends the use of a medium made by dissolving 10 gm. of peptone in 1 litre of ammonia-free distilled water, and adding 2 gm. of nitrite-free potassium nitrate. The medium is filtered, tubed, and sterilised for half an hour on three days. Tubes are infected and incubated for forty-eight hours, the formation of nitrites being now tested for by Ilosvay's method. The following solutions are required: (a) sulphanilic acid, .5 gm. dissolved in 150 c.c. dilute acetic acid (s.g. 1.04); (b) 1 gm.  $\alpha$ -naphthylamine is dissolved in 22 c.c. of water, the solution filtered, and 180 c.c. dilute acetic acid added. In using the test, 2 c.c. of each of these solutions is added to 10 c.c. of culture. If reduction of the nitrates has occurred, a rose pink colour should develop almost immediately. It is to be noted that the pink colour first produced sometimes disappears as it is formed or on shaking; in such a case further portions of the two reagents in equal quantities should be added.

**Agglutination Reactions of the *B. coli*.**—When the *b. coli* has produced a pathological condition in an animal, the serum of the infected animal frequently manifests specific agglutinative characters, especially towards the strain of the organism isolated from the lesions. Under certain circumstances, also, the serum of an animal infected by some other member of the *b. coli* group may also agglutinate strains of this organism. This subject will be treated of when we consider the differentiation of the members of the group one from another.

**Isolation of the *B. coli*.**—In the case of abscesses or coli infection of the kidney or bladder, etc. (p. 356), the isolation of the organism is usually easy, the use of agar plates being here sufficient. When, however, the organism is present along with other bacteria, as in the case of water, sewage, etc., special means must be adopted, the object of which usually is to inhibit the growth of all organisms except those belonging to the coli group. Formerly media containing small quantities of carbolic acid were used for this purpose, but now the inhibition is usually effected by the use of certain aniline dyes, by picric acid, or by bile salts. The media of Conradi-Drigalski, Conradi, Endo, Fawcett, and of MacConkey (pp. 47-51) are examples. All these media have their uses, and it is best to select that with which the worker has had most experience. In this country MacConkey's bile-salt lactose agar is perhaps most widely used. The methods of the application of these media and the appearances of *b. coli* have already been described (p. 47-51).



**The Recognition of typical B. coli.**—The work on *b. coli*, especially in relation to its occurrence in water, has revealed the existence of a very large number of varieties of the organism. These differ from one another in the absence of one or more of the characters which may be elucidated by the application of the biological methods given. Considerable difference of opinion exists as to what characters are to be looked upon as type characters, *i.e.* characters shared by the greatest number of varieties isolated. In this connection it is to be noted that as the *b. coli* was originally isolated from the human intestine, and as the detection of such intestinal bacteria outside the body constitutes a most important practical question, the inquiry for type characters is to a certain extent limited to an attempt to arrive at the type most frequently present in the human intestine.

Two standards may be alluded to. First, that of an English Committee which reported in 1904 on the standardisation of methods for the bacterioscopic examination of water. According to this, the *b. coli* is a small, motile, non-sporing bacillus, capable of growing at 37° C., decolorised by Gram, never liquefying gelatin, producing clot and permanent acidity of milk within seven days at 37°, fermenting glucose and lactose, with, in both, acid and gas formation,—subsidiary points being the formation of indol, the formation of a thick yellowish-brown growth on potato, production of fluorescence in neutral-red, reduction of nitrates, and fermentation of saccharose. A similar American Committee looked upon the typical organism as a non-sporing bacillus, motile, fermenting dextrose-broth, with the formation of about 50 per cent. of gas, of which about one-third is carbon dioxide, causing acid and clot in milk in forty-eight hours, not liquefying gelatin, producing indol and reducing nitrates. These two standards differ in the fact that the English Committee lay less weight on indol formation and the reduction of nitrates.

Generally speaking, the application in any case of the scheme of the English Committee is to be recommended, and organisms conforming to the tests laid down may be accepted in the majority of cases as probably having come from an intestinal source. The further differentiation of organisms conforming to this type will be dealt with later (p. 391). Meantime it may be said that, in addition to the type characters, lactose-fermenters from the human intestine usually ferment saccharose and dulcitate and have no effect on adonite, inulin and inosite, and it may be, no influence on mannite.

**Pathogenic Properties of the B. coli.**—In man, the *b. coli* has been found as the only organism present in various



suppurative conditions (see Chapter VII.), especially in connection with the intestine (*e.g.* appendicitis) and about the urinary tract. In the latter, it is also responsible for catarrhal conditions in the pelvis of the kidney and in the bladder, these being more common in the female, and frequently presenting chronic characters. As a practical point, it may be said that the treatment of the latter by vaccines, especially when made from the strain isolated from the lesion, has been attended with marked success. The *b. coli* is also apparently the cause of some cases of summer diarrhoea (*cholera nostras*), of infantile diarrhoea, and of some food poisonings.

**The Pathogenicity of the *B. coli* and its Relation to that of the Typhoid Bacillus.**—Intraperitoneal injection in guinea-pigs is often fatal. Subcutaneous injection may result in local abscesses, and sometimes in death from cachexia. Sanarelli found that the *b. coli* isolated from typhoid stools was much more virulent than when isolated from the stools of healthy persons. He holds that the increase in virulence is due to the effect of typhoid toxins. This increased virulence of the *b. coli* in the typhoid intestine makes it possible that some of the pathological changes in typhoid may be due, not to the typhoid bacillus, but to the *b. coli*. Some of the general symptoms may be intensified by the absorption of toxic products formed by it and by other organisms. Differences of behaviour of the two bacilli in connection with their pathological effects have been brought forward as confirmatory of the fact of their being distinct species. Thus Sanarelli accustomed the intestinal mucous membrane of guinea-pigs to toxins derived from an old culture of the *b. coli*, by introducing day by day small quantities of the latter into the stomach. When a relatively large dose could be tolerated, it was found that the introduction in the same way of a small quantity of typhoid toxin was still followed by fatal result. Pfeiffer also found that while the serum of convalescents from typhoid paralysed the typhoid bacilli, it had no more effect on similar numbers of *b. coli* than the serum of healthy men.

#### THE BACILLUS TYPHOSUS.

**Bacillus Typhosus.**—*Microscopic Appearances.*—It is sometimes difficult to find the typhoid bacilli in the organs of a typhoid patient. Numerous sections of different parts of a spleen, for example, may be examined before a characteristic group is found. The best tissues for examination are a Peyer's patch where ulceration has not yet commenced or where it is just commencing, the spleen, the liver, or a mesenteric gland. The spleen and liver are better than the other tissues named, as in the latter the presence of the *b. coli* is more frequent. From scrapings of such solid organs dried films may be prepared and stained for a few minutes in the cold by any of the strong



staining solutions, e.g. with carbol-thionin-blue, or with Ziehl-Neelsen carbol-fuchsin diluted with five parts of distilled water. As a rule, decolorising is not necessary. For the proper observation of the arrangement of the bacilli in the tissues, paraffin sections should be stained in carbol-thionin-blue for a few minutes, or in Löffler's methylene-blue for one or two hours. The bacilli take up the stain somewhat slowly, and as they are also easily decolorised, the aniline-oil method of dehydration may be used with advantage (*vide* p. 100).

In such preparations the characteristic appearance to be looked for is the occurrence of groups of bacilli lying between the cells of the tissue (Fig. 106). The individual bacilli are  $2\ \mu$  to  $4\ \mu$  long, with somewhat oval ends, and  $0.5\ \mu$  in thickness. Sometimes

filaments  $8\ \mu$  to  $10\ \mu$  long may be observed, though they are less common than in cultures. It is evident that one of the bacilli may frequently in a section be viewed endwise, in which case the appearance will be circular. This ap-

pearance accounts for some, at least, of the coccus-like forms which have been described. The bacilli are *decolorised* by *Gram's method*.

**Isolation and Appearances of Cultures.**—To grow the organism artificially it is best to isolate it from the spleen (for method, see p. 146) as it exists there in greater numbers than in the other solid organs, and may be the sole organism present even some time after death. Agar or gelatin plates or agar stroke cultures may be employed. On the agar media the growths are visible after twenty-four hours' incubation at  $37^{\circ}\text{C}$ . On agar plates the superficial colonies are thin and film-like, circular or slightly irregular at the margins, dull white by reflected light, bluish-grey by transmitted light. Colonies in the substance of

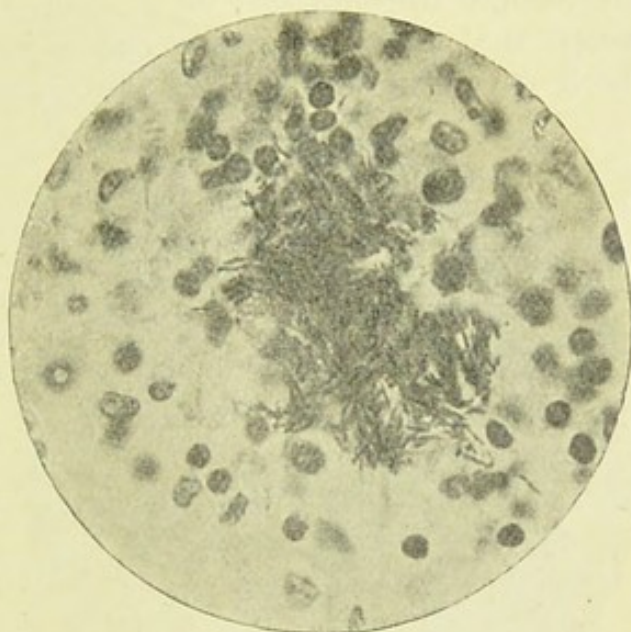


FIG. 106.—A large clump of typhoid bacilli in a spleen. The individual bacilli are only seen at the periphery of the mass. (In this spleen enormous numbers of typhoid bacilli were shown by cultures to be present in a practically pure condition.) Paraffin section; stained with carbol-thionin blue.  $\times 500$ .



the agar are small, and appear as minute round points. Under a low objective, the surface colonies are found to be very transparent (requiring a small diaphragm for their definition), finely granular in appearance, and with a very coarsely crenated and well-defined margin. The deep colonies are usually spherical, sometimes lenticular in shape, and are smooth or finely granular on the surface, and more opaque than the superficial colonies. In cover-glass preparations, the bacilli are found to present the same microscopic appearances as in preparations from solid organs, except that there may be a greater number of the longer forms which may almost be called filaments (Fig. 107). The same is



FIG. 107.—Typhoid bacilli, from a young culture on agar, showing some filamentous forms.

Stained with weak carbol-fuchsin.  $\times 1000$ .

true of films made from young gelatin cultures. Sometimes the diversity in the length of the bacilli is such as to throw doubt on the purity of the culture. As a general rule, in a young (twenty-four or forty-eight hours old) culture, grown at a uniform temperature, the bacilli are plump, and the protoplasm stains uniformly. In old cultures, or in cultures which have been exposed to changes of temperature, the protoplasm stains only in parts; there may be an appear-

ance of irregular vacuolation either at the centre or at the ends of the bacilli.

*Motility.*—In hanging-drop preparations the bacilli are found to be actively motile. The smaller forms have a darting or rolling motion, passing quickly across the field, whilst some show rapid rotatory motion. The filamentous forms have an undulating or serpentine motion, and move more slowly. Hanging-drop preparations ought to be made from agar or broth cultures not more than twenty-four hours old. In older cultures the movements are less active.

*Flagella.*—On being stained by the appropriate methods (*vide* p. 110), the bacilli are seen to possess many long wavy flagella which are attached all along the sides and to the ends



(Fig. 108). They are more numerous, longer, and more wavy than those of the *b. coli*.

*Characters of Culture.*—Generally speaking, on artificial media growths of the *b. typhosus* appear less dense than those of the *b. coli*. Stab cultures in *peptone gelatin* give a somewhat characteristic appearance. On the surface of the medium growth spreads outwards from the puncture as a thin leaf-like film or pellicle, with irregularly wavy margin (Fig.

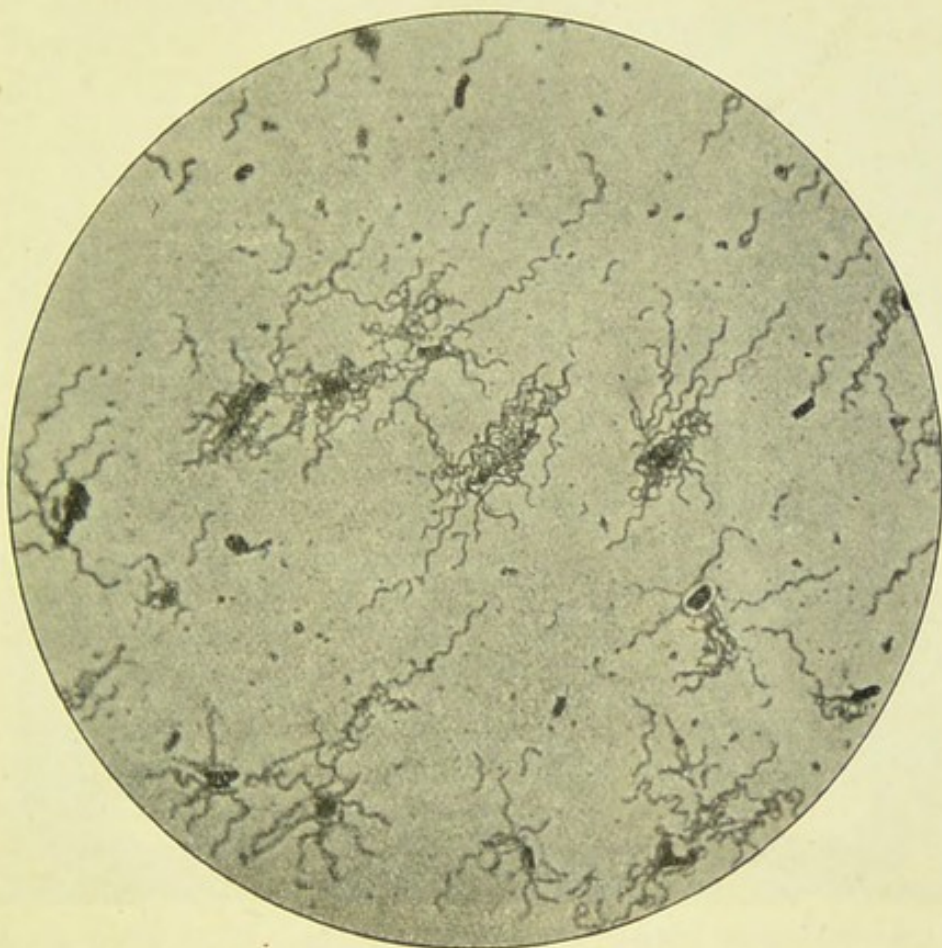


FIG. 108.—Typhoid bacilli, from a young culture on agar, showing flagella. See also Plate III., Fig. 20.  
Stained by Van Ermengem's method.  $\times 1000$ .

109, A). It is semi-transparent and of bluish-white colour. Ultimately this surface growth may reach the wall of the tube. Not infrequently, however, the surface growth is not well marked. Along the stab there is an opaque whitish line of growth, of finely nodose appearance. There is no liquefaction of the medium, and no formation of gas. In stroke cultures there is a thin bluish-white film, but it does not spread to such an extent as in the case of the surface growth of a stab culture (Fig. 109, B). In gelatin plates also the superficial and deep



colonies present corresponding differences. The former are delicate semi-transparent films, with wavy margin, and are much larger than the colonies in the substance, which appear as small round points (Fig. 110). These appearances, which are well seen on the third or fourth day, resemble those seen in agar plates, as already described in the method of isolation ; but on gelatin the surface colonies are rather more transparent than

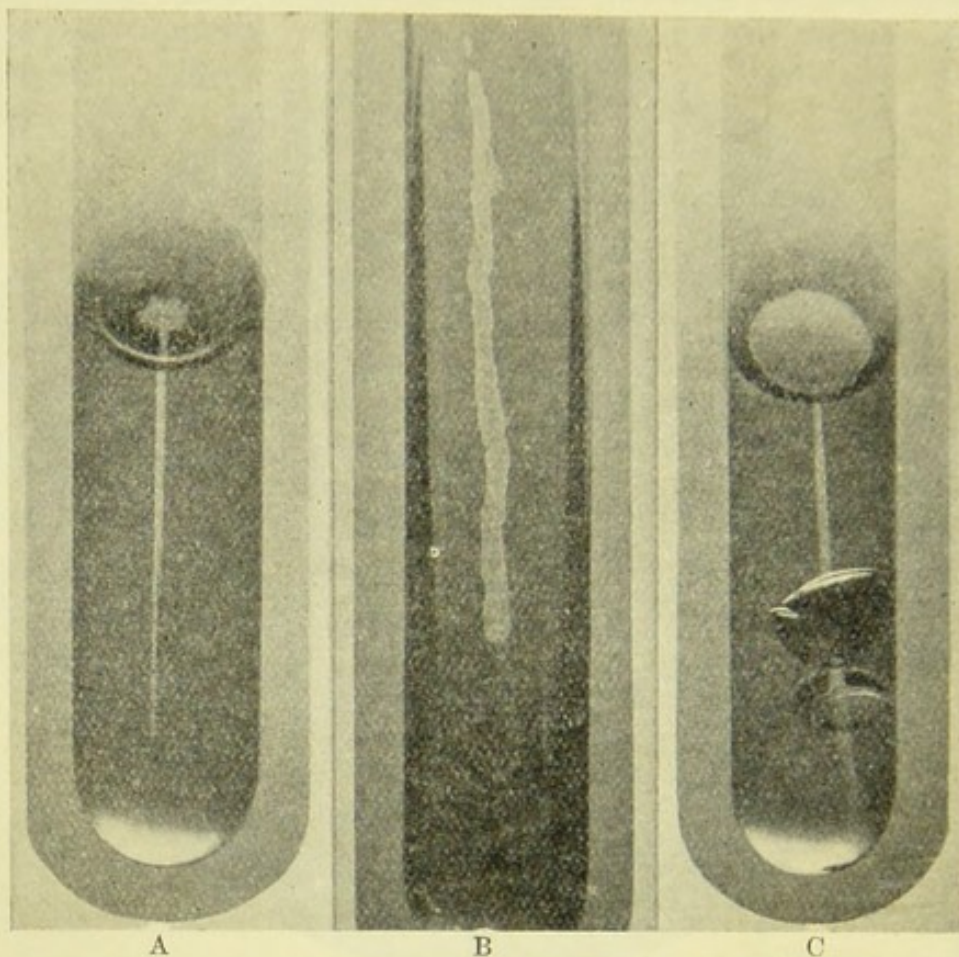


FIG. 109.

- A. Stab culture of the typhoid bacillus in gelatin, five days' growth.
- B. Stroke culture of the typhoid bacillus on gelatin, six days' growth.
- C. Stab culture of the bacillus coli in gelatin, nine days' growth ; the gelatin is split in its lower part owing to the formation of gas.

those on agar. Their characters, as seen under a low power of the microscope, also correspond. If a gelatin tube be inoculated and incubated at  $37^{\circ}$  C., a uniform turbidity is produced (cf. *b. coli*, p. 351).

In stroke cultures on *agar* there is a bluish-grey film of growth, with fairly regular margins, but without any characteristic features. This film is loosely attached to the surface, and can be easily scraped off.



The growth on *potatoes* is important. For several days (at incubation temperature) after inoculation there is apparently no growth. If looked at obliquely, the surface appears wet, and if it is scraped with the platinum loop, a glistening track is left; a cover-glass preparation shows numerous bacilli. Later, however, a slight pellicle with a dull, somewhat velvety surface may appear, and this may even assume a brown appearance. These characteristic appearances are only seen when a fresh potato with an acid reaction has been used.

In *bouillon* incubated at 37° C. for twenty-four hours there is simply a uniform turbidity. Cover-glass preparations made from such sometimes show filamentous forms of considerable length without apparent segmentation.

*Conditions of Growth, etc.*—The optimum temperature of the typhoid bacillus is about 37° C., though it also flourishes well at the room temperature. It will not grow below 9° C. or above 42° C. Its powers of resistance correspond with those of most non-sporing bacteria. It is killed by exposure for half an hour at 60° C., or for two or three minutes at 100° C. Typhoid bacilli kept in distilled or in ordinary tap water have usually been found to be dead after three weeks (Frankland).

**Biological Reactions.**—Very important means of identifying the typhoid bacillus are found in testing its capacities for growth on certain special media. This facilitates its being differentiated from the *b. coli* and the other members of the coli-typhoid group. The following results will be best appreciated if considered in relation to what is said regarding these other organisms, as the reactions of the typhoid bacilli in differentiating media are largely negative. (See Table, p. 394.)

The tests with sugars are important. The typhoid bacillus produces acid without gas in maltose, lævulose, glucose, and

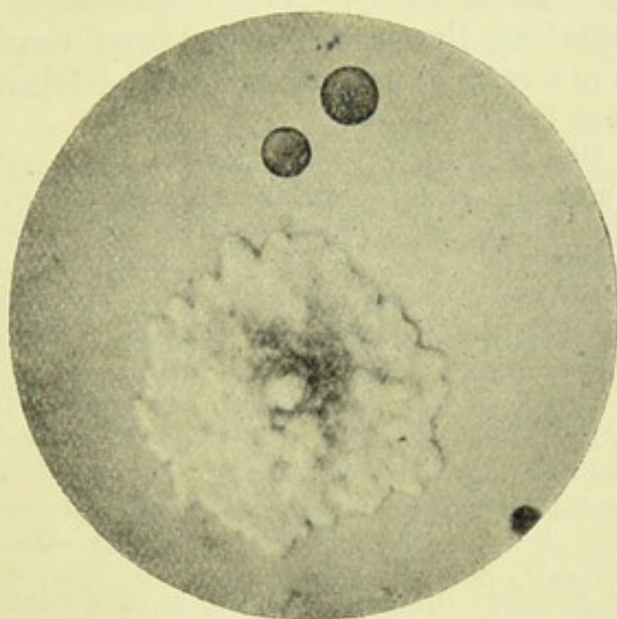


FIG. 110.—Colonies of the typhoid bacillus (one superficial and three deep) in a gelatin plate. Three days' growth at room temperature.  $\times 15$ .



mannite, but originates no change in lactose, cane-sugar, or dulcete. Further, no gas production is observed in gelatin shake cultures, and there is no curdling of milk, although in litmus milk slight acid production occurs; in a time varying from a few days to a month the acid change may be succeeded by alkali production. Under ordinary circumstances, the typhoid bacillus is incapable of producing indol in peptone-salt solution, and does not alter neutral-red in lactose bouillon.

A great many special tests were formerly in use for differentiating the *b. typhosus* from the *b. coli*. The use of these is not now so necessary, but the following may be described:—

*The Media of Capaldi and Proskauer.*—The first of these ("No. 1") is a medium free of albumin, in which *b. coli* grows well and freely produces acid, while the typhoid bacillus hardly grows at all, and certainly will produce no change in the reaction. Its composition is as follows: asparagin .2 parts, mannite .2, sodium chloride .02, magnesium sulphate .01, calcium chloride .02, potassium monophosphate .2, distilled water to 100 parts. The second medium ("No. 2") contains albumin, and in it the *b. coli* produces no acid, while the typhoid bacillus grows well and produces an acid reaction. It consists of Witte's peptone 2 parts, mannite .1, distilled water to 100 parts. After the constituents of each medium are mixed and dissolved, it is steamed for one and a half hours and then made neutral to litmus—the first medium, being usually naturally acid, by sodium hydrate, the second, being usually alkaline, by citric acid. The medium is then filtered, filled into tubes containing 5 c.c., and these are sterilised. After inoculation for twenty hours the reaction of the medium is tested by adding litmus.

The identification of the typhoid bacillus is best facilitated by means of agglutination reactions which will be treated of later (pp. 371, 393).

**Pathological Changes in Typhoid Fever.**—Here we confine our attention solely to the bacteriological aspects of the disease. The inflammation and ulceration in the *Peyer's patches and solitary glands of the intestine* are the central features. In the early stage there is produced an acute inflammatory condition, attended with extensive leucocytic emigration and sometimes with small hæmorrhages. At this period the typhoid bacilli are most numerous in the patches, groups being easily found between the cells. The subsequent necrosis is evidently in chief part the result of the action of the toxic products of the bacilli, which gradually disappear from their former positions, though they may still be found in the deeper tissues and at the spreading margin of the necrosed area. They also occur in the lymphatic spaces of the muscular coat. It is to be remarked that the number of the ulcers arising in the course of a case bears



no relation to its severity. Small ulcers may occur in the lymphoid follicles of the large intestine.

The *mesenteric glands* corresponding to the affected part of the intestine are usually enlarged, sometimes to a very great extent, the whole mesentery being filled with glandular masses. In such glands there may be acute inflammation, and occasionally necrosis in patches occurs. Sometimes on section the glands are of a pale-yellowish colour, the contents being diffuent and consisting largely of leucocytes. Typhoid bacilli may be isolated both from the glands and the lymphatics connected with them, but the *b. coli* is in addition often present.

The *spleen* is enlarged,—on section usually of a fairly firm consistence, of a reddish-pink colour, and in a state of congestion. Of all the solid organs it usually contains the bacilli in greatest numbers. They can be seen in sections, occurring in clumps between the cells, there being no evidence of local reaction round them (Fig. 106). Similar clumps may occur in the *liver* in any situation, and without any local reaction. In this organ, however, there are often small foci of leucocytic infiltration, in which, so far as our experience goes, bacilli cannot be demonstrated. The bacillus is found, often in large numbers, in the gall-bladder, where it may persist for years (*vide infra*). Clumps of bacilli may also occur in the *kidney*.

In addition to these local changes in the solid organs, there are also widespread *cellular degenerations* in the solid organs which suggest the action of toxic products.

In the *lungs* there may be bronchitis, patches of congestion and of acute broncho-pneumonia. In these, typhoid bacilli may sometimes be observed, but evidence of a toxic action depressing the powers of resistance of the lung tissue is found in the fact that the pneumococcus frequently occurs in such complications of typhoid fever.

The *nervous system* shows little change, though meningitis associated either with the typhoid bacillus, with the *b. coli*, or with the streptococcus pyogenes has been observed.

In typhoid fever the bacilli can in 90 per cent. of cases be isolated from the *blood* during the course of the illness. The local lesions are thus associated with a general septicæmic process. The bacilli have been found in the *roseolar spots* which occur in typhoid fever, but it cannot be yet stated that such spots are always due to the presence of the bacilli. The fact that the typhoid bacilli are usually confined to certain organs and tissues shows that they probably have a selective action on certain tissues.

To sum up the pathology of typhoid fever, we have in it a disease the centre of which lies in the lymphoid tissue in and connected with the intestine. In this situation we must have an irritant, against which the inflammatory reaction is set up,



and which in the intestine is sufficiently powerful to cause necrosis. The affections of the other organs of the body suggest the circulation in the blood of poisonous substances capable of depressing cellular vitality, and producing histological changes. The occurrence of bacilli in the blood and organs links typhoid fever with septicæmic processes.

**Suppuration occurring in connection with Typhoid Fever.**—

With regard to the relation of the typhoid bacillus to such conditions, statements as to its isolation from pus, etc., can be accepted only when all the points available for the diagnosis of the organism have been attended to. On this understanding the following summary may be given:—In a certain proportion of the cases examined the typhoid bacillus has been the only organism found. This has been the case in subcutaneous abscesses, in suppurative periostitis, suppuration in the parotid, abscesses in the kidneys, etc., and probably also in one or two cases of ulcerative endocarditis. But in the majority of cases other organisms, especially the *b. coli* and the pyogenic micrococci, have been obtained, the typhoid bacillus having been searched for in vain. It has, moreover, been experimentally shown, notably by Dmochowski and Janowski, that suppuration can be experimentally produced by injection in animals, especially in rabbits, of pure cultures of the typhoid bacillus, the occurrence of suppuration being favoured by conditions of depressed vitality, etc. These observers also found that when typhoid bacilli were injected along with pyogenic staphylococci, the former died out in the pus more quickly than the latter. Accordingly, in clinical cases where the typhoid bacillus is present alone, it is improbable that other organisms were present at an earlier date.

**Occurrence of Gallstones in those who have suffered from Typhoid Fever.**—As has been stated, foci of bacilli occur in the liver in typhoid fever, and these bacilli are excreted with the bile. In the gall-bladder they apparently not infrequently set up a catarrhal process in the biliary ducts and gall-bladder (*cholecystitis typhosa*), and are then in a better position for multiplication, in consequence of the presence of albuminous catarrhal secretions. There is evidence that the bacilli may persist in the gall-bladder for many years, and probably the catarrhal inflammation which they keep up is responsible for many of the cases of gallstones which occur—the albuminous matter produced causing a deposit of the bile in a solid form. Typhoid bacilli have actually been isolated from cases of gallstones operated on years after an attack of typhoid fever, and the bacilli have even been found within the calculi. They have also been demon-



strated in chronic suppurations occurring in the gall-bladder. It is to be noted that gallstones are more frequently found in women than in men, the proportion being about four to one, and probably a considerable proportion of the total number of cases of gallstones are traceable to the previous occurrence of typhoid fever.

**Pathogenic Effects produced in Animals by the Typhoid Bacillus.**—There is no disease of animals which can be said to be identical with typhoid, nor is there any evidence of the occurrence of the typhoid bacillus under ordinary pathological conditions in the bodies of animals. Attempts to communicate the disease to animals by feeding them on typhoid dejecta have been unsuccessful, and though pathogenic effects have been produced by introducing pure cultures in food, the disease has usually borne no resemblance to human typhoid. The most successful experiments have been those of Remlinger, who, by continuously feeding rabbits on vegetables soaked in water containing typhoid bacilli, produced in certain cases symptoms resembling those of typhoid fever (diarrhoea, remittent pyrexia, etc.). An agglutinating action was observed in the serum, and *post mortem* there was congestion of the Peyer's patches, and typhoid bacilli were isolated from the spleen.

Feeding experiments are thus unsatisfactory, and the same may be said of the results of subcutaneous or intraperitoneal infection. Here, again, pathogenic effects can easily be produced by the typhoid bacillus, but these effects are of the nature of a short acute illness characterised by pyrexia, rapid loss of weight, inability to take food, and frequently ending fatally in from twenty-four to forty-eight hours. The type of disease is thus very different from what occurs naturally in man. In such injection experiments the results vary considerably, sometimes scarcely any effect being produced by a large dose of a culture. This is no doubt due to the fact that different strains of the bacillus vary much in virulence. Ordinary laboratory cultures are often almost non-pathogenic. They can, however, be made virulent in various ways. Sanarelli used the method of injecting sterilised cultures of the *b. coli* intraperitoneally at the same time as the typhoid bacillus was introduced subcutaneously. After this procedure had been repeated through a series of animals, a typhoid culture of exalted virulence was obtained. Sidney Martin has obtained virulent cultures by passing bacilli, derived directly from the spleen of a person dead of typhoid fever, through the peritoneal cavities of a series of guinea-pigs.

Sanarelli, studying the effects of the intraperitoneal injection



of a few drops of a culture of highly exalted virulence, found that the Peyer's patches and solitary glands of the intestine were enormously infiltrated, sometimes almost purulent, and that they contained typhoid bacilli, as also did the mesenteric lymphatics and glands, and the spleen. These results are interesting, but have not been confirmed.

**The Toxic Products of the Typhoid Bacillus.**—Here very little light has been thrown on the pathology of the disease, but the general results may be outlined. We may state that there exist in the bodies of typhoid bacilli toxic substances, that in artificial cultures these do not pass to any great degree out into the surrounding medium, and that though they produce effects on the intestine, there is evidence that such effects are not characteristic and not peculiar to the toxins of the *b. typhosus*. Sidney Martin found that the bodies of bacteria killed by chloroform vapour were very toxic,—more so than filtered cultures. Diarrhoea was a constant symptom after injection, but no change in the Peyerian patches was observed. He also found that virulent cultures of the *b. coli* gave similar results when similarly treated. Allan Macfadyen, by grinding up typhoid bacilli frozen solid by liquid air, produced a fluid whose toxic effect he attributed to the presence of the intracellular poisons.

**The Immunisation of Animals against the Typhoid Bacillus.**—Earlier observers had been successful in accustoming mice to the typhoid bacillus by the successive injections of small and gradually increasing doses of living cultures of the bacillus. Later, Brieger, Kitasato, and Wassermann found that the bacillus when modified by being grown in a bouillon made from an extract of the thymus gland no longer killed mice and guinea-pigs. These animals after injection were moreover immune, and it was also found that the serum of a guinea-pig thus immunised could, if transferred to another guinea-pig, protect the latter from the subsequent injection of a dose of typhoid bacilli to which it would naturally succumb. Chantemesse and Widal, Sanarelli, and also Pfeiffer, succeeded in immunising guinea-pigs against the subsequent intraperitoneal injection of virulent living typhoid bacilli, by repeated and gradually increasing intraperitoneal or subcutaneous doses of dead typhoid cultures in bouillon. Experiments performed with serum derived from typhoid patients and convalescents indicate that similar effects occur in those who have successfully resisted the natural disease. The serum of such patients has *antibacterial* powers, but there is no evidence that it contains any *antitoxic* bodies (see chapter on Immunity). Pfeiffer, for example, found,



on adding serum from typhoid convalescents to typhoid bacilli killed by heat, and injecting the mixture into guinea-pigs, that death took place as in control animals which had received these toxic agents alone. Pfeiffer also found that by using the serum of immunised goats, he could, to a certain extent, protect other animals against the subsequent injection of virulent living typhoid bacilli. On trying to use the agent in a curative way, *i.e.* injecting it only after the bacilli had begun to produce their effects, he got little or no result.

**General View of the Relationship of the *B. typhosus* to Typhoid Fever.**—1. We see in typhoid fever a disease having its centre in and about the intestine, and acting secondarily on many other parts of the body. In the parts most affected there is always a bacillus present, microscopically resembling other bacilli, especially the *b. coli*, which is a normal inhabitant of the animal intestine. The bacillus can be isolated from the characteristic lesions of the disease and from other parts of the body as described, and further, it is found by culture and serum reactions to differ from other organisms. Here the important point is that a bacillus giving all the reactions of the typhoid bacillus has never been isolated except from cases of typhoid fever, or under circumstances that make it possible for the bacillus in question to have been derived from a case of typhoid fever.

2. A difficulty in the way of accepting the etiological relationship of the *b. typhosus* lies in the comparative failure of attempts to cause the disease in animals. We have noted, however, that in nature animals do not suffer from typhoid fever.

3. The observations of Pfeiffer and others on the protective power against typhoid bacilli shown, on testing in animals, to belong to the serum of typhoid patients and convalescents, and the peculiar action of such serum in immobilising and causing clumping of the bacilli (*vide infra*), are also of great importance as indicating an etiological relationship between the bacillus and the disease. Additional important evidence is found in the fact that vaccination by means of the dead bacilli (*vide infra*) has a marked effect in preventing the disease from arising in a population exposed to infection, and also in lowering the mortality when the fever attacks those who have been inoculated. These facts may thus be accepted as indirect but practically conclusive evidence of the pathogenic relationships of the typhoid bacillus to the disease.

According to our present results, we must thus hold that



the *b. typhosus* constitutes a distinct species of bacterium, and that it is the cause of typhoid fever. Evidence of an important nature confirmatory of this view is, we think, found in the fact that cases have occurred where bacteriologists have accidentally infected themselves by the mouth with pure cultures of the typhoid bacillus, and after the usual incubation period have developed typhoid fever. Several cases of this kind have been brought to our notice, and are not, we think, vitiated by the fact that other similar instances have occurred without the subsequent development of illness. These latter would be accounted for by a low degree of susceptibility on the part of the individual or to a want of pathogenicity in the cultures.

As there is thus strong evidence of the etiological relationship of the typhoid bacillus to typhoid fever, the view of the development of the disease usually taken has been that the bacilli, being ingested, multiply in the intestinal tract, cause inflammation and necrosis of the lymphoid tissue, and, gaining an entrance to the general circulation, produce the septicæmic phenomena which we have described.

Within recent years, considerable attention has been attracted to another view of the course of infection put forward by Forster and his co-workers in Strasburg. According to this, the process is primarily a septicæmia, and the intestinal manifestations are looked on as secondary. The bacilli are supposed to gain entrance to the circulation possibly through the tonsils, sore throat being a not uncommon initial symptom of typhoid fever. In the blood they multiply, and, passing through the liver, gain access to the gall-bladder, set up a catarrhal inflammation there on the products of which they flourish, and thence pass out to infect the intestine. The intestinal lesions are either due to an elective action of bacteria brought by the blood, or come from infection by the bacilli which pass out from the gall-bladder,—the former being apparently the alternative to which Forster leans. The evidence on which this view is based consists, firstly, in the results of animal experiments in which bacilli introduced intravenously have been subsequently found chiefly or solely in the gall-bladder,—it may be, persisting there for weeks. Further, it is stated that bacilli can be isolated from the blood during the later parts of the incubation stage of the disease, and before they can be demonstrated in the intestine, where they are said not to appear until sometime during the first week of active disease. And again it is stated that in the bodies of persons dying from typhoid fever, while bacilli are always present in the gall-bladder and in the upper parts of the small intestine, they are frequently absent from the lower part of the latter and from the colon. It cannot be said that this view of the disease has been satisfactorily established. Opinion differs as to the alleged late appearance of the bacilli in the intestine, and the infectivity noticed during the incubation stage must be explained. Further, there is strong reason for believing that multiplication of the bacilli in the intestine can take place. The evidence of this rests on the finding of bacilli, it may be in considerable numbers, in the fæces



and even in the blood of healthy individuals who have merely been in contact with typhoid cases or typhoid carriers, and who show no symptoms of the disease.

There is evidence that certain individuals are relatively insusceptible to typhoid fever. The cases of the occurrence of typhoid bacilli in the healthy intestine support this view, and it has been further shown that during an epidemic certain persons may suffer from slight intestinal symptoms with typhoid bacilli in the fæces without the disease going through its usual course. The so-called "ambulatory" cases of typhoid fever form a link between these mild infections and fully developed typhoid fever.

**The Epidemiology of Typhoid Fever.**—Generally speaking, the former prevalence of typhoid fever and the periodic outbreaks which still occur even in well-regulated communities, have depended on the capacity of the typhoid bacillus to live and it may be to multiply outside the human body. The investigation of the prevalence of the typhoid bacillus under such saprophytic conditions is a matter of great difficulty, as, for its proper study, the capacity of the organism to multiply when other intestinal and putrefactive organisms are present constitutes the essential problem. Enough is known, however, to show that the bacillus can remain viable under such circumstances for some days, and it may be weeks. This fact explains the occurrence of the epidemics due to water, and sometimes, it may be, to milk supplies becoming contaminated with the excreta from typhoid patients. Where surface wells are used, and where sewage, instead of being properly disposed of, finds its way into ash-heaps or cesspools, the way is opened up for communities becoming infected with typhoid fever.

**Typhoid Carriers.**—In the great majority of cases of typhoid fever, the bacilli disappear from the fæces within from two to ten weeks of convalescence, but in a certain proportion of cases, probably about 2 to 4 per cent., evidence is found of the persistence of the bacilli for many months, and in certain cases their existence has been demonstrated even thirty and, it may be, fifty years after the attack of illness. Persons in whom this phenomenon is present are a constant danger to those around them, as the infectivity of the bacilli frequently remains, and during recent years the importance of such "chronic" carriers has been recognised as explaining many outbreaks of the disease. The cases traceable to such an origin are of the type usually classed as sporadic. They arise amongst persons associated with carriers, especially when the latter are concerned in the prepara-



tion of food. From time to time, however, larger epidemics have arisen from a carrier having contaminated a milk supply in a dairy. The site of the multiplication of the bacteria in a great many of these carriers is probably the gall-bladder (see p. 364). As has been stated, the typhoid bacilli may persist there for many years, often giving rise to gallstones. The fact that women appear to be more liable to gallstones than men constitutes a serious factor in relation to the problem of the typhoid carrier, as women are more concerned in the preparation of food. An additional danger lies in the fact that carriers usually appear to be in perfect health or may only suffer from slight, and to them unimportant, pains in the region of the gall-bladder, it being well known that in only a proportion of patients suffering from gallstones do severe symptoms arise. An additional factor in the carrier problem lies in the fact stated above, that apparently certain persons ingest the typhoid bacilli, and the latter may multiply for some months in the intestinal tract without giving rise to typhoid fever. Such persons have been referred to as "paradoxical" carriers; they represent those who either are naturally insusceptible to typhoid fever or who have developed immunity in consequence of a previous attack; they may constitute a danger to susceptible persons with whom they may come in contact. The most serious danger to a community arises, however, from the "chronic" carrier. In certain carriers, the focus of multiplication of the typhoid bacillus may not be the bowel but the kidney or bladder, the bacilli in such cases passing out in the urine.

The tracking down of a typhoid carrier constitutes an important and difficult problem. Firstly, the serum of all suspicious persons ought to be subjected to the Widal test (*vide infra*). Usually speaking the carrier gives a positive reaction, but sometimes this is absent and sometimes is only obtained with a low dilution of the serum. Further, it has been shown in chronic carriers that the agglutinating capacity of the serum varies from time to time and sometimes may be absent. The proof of the presence of a carrier lies essentially in the isolation of the typhoid bacillus from the feces or the urine, and it is to be noted that, especially in the former, the organism is not constantly present,—in certain cases months of remission have been recorded. This of course may be due to the difficulties of the search, but whatever the explanation, it necessitates repeated examinations. Much work has been directed to the question of freeing the typhoid carrier from the organism, but although various methods, such as intestinal antisepsis, vaccination,



excision of the gall-bladder, have been tried, success has hitherto not been attained. From the public health standpoint, the prevention of carriers from occurring in a population has been considered, and it is a question whether in fever hospitals means ought not to be taken for retaining convalescents from typhoid until the bodily discharges are free from the typhoid bacillus. This has already been undertaken in the British army in India.

**The Serum Diagnosis of Typhoid Fever.**—This method of diagnosis is based on the fact that living and actively motile typhoid bacilli, if placed in the diluted serum of a patient suffering from typhoid fever, within a very short time lose their motility and become aggregated into clumps.

The methods by which the test can be applied have already been described (p. 118).

(1) It will be there seen that the loss of motility and clumping may be observed microscopically. If a preparation be made by the method detailed (typhoid serum in a dilution of, say, 1 : 30 having been employed), and examined at once under the microscope, the bacilli will usually be found actively motile, darting about in all directions. In a short time, however, these movements gradually become slower, the bacilli begin to adhere to one another, and ultimately become completely immobile and form clumps by their aggregation. When this occurs the reaction is said to be complete. If the clumps be watched still longer a swelling up of the bacilli will be observed, with a granulation of the protoplasm, so that their forms can with difficulty be recognised. In a preparation similarly made with non-typhoid serum the individual bacilli can be observed separate and actively motile for many hours.

(2) A corresponding reaction visible to the naked eye is obtained by the "sedimentation test," the method of applying which has also been described (p. 120). The test in this form has the disadvantage of taking longer time than the microscopic method, but it is useful as a control ; in nature it is similar.

Such is what occurs in the case of a typical reaction. The value of the method as a means of diagnosis largely depends on attention to several details. The *race of typhoid bacillus* employed is important. All races do not give uniformly the same results, though it is not known on what this difference of susceptibility depends. A race must therefore be selected which gives the best result in the greatest number of undoubted cases of typhoid fever, and which gives as little reaction as possible with normal sera or sera derived from other diseases.



This latter point is important, as some races react very readily to non-typhoid sera. Again, care must be taken as to the *state of the culture* used. The suitability of a culture may be impaired by varying the conditions of its growth. Continued growth of a race at 37° C. makes it less suitable for use in the test, as the bacilli tend naturally to adhere in clumps, which may be mistaken for those produced by the reaction. Wyatt Johnson recommended that the stock culture should be kept growing on agar at room temperature and maintained by agar sub-cultures made once a month. For use in applying the test, bouillon sub-cultures are made and incubated for twenty-four hours at 37° C. The relation of the *dilution of the serum* to the occurrence of clumping is most important. It has been found that if the degree of dilution be too small a non-typhoid serum may cause clumping. If possible, observations should always be made with dilutions of 1 : 10, 1 : 30, 1 : 60, 1 : 100. To speak generally, the more dilute the serum the longer time is necessary for a complete reaction. Some typhoid sera have, however, very powerful agglutinating properties, and may in a comparatively short time produce a reaction when diluted many hundreds of times. With a too dilute serum not only may the reaction be delayed, but it may be incomplete,—the clumps formed being small and many bacilli being left free. These latter may either have been rendered motionless or they may still be motile. No diagnosis is conclusive which is founded on the occurrence of such an incomplete clumping alone. Seeing that low dilutions sometimes give a reaction with non-typhoid sera, it is important to know what is the highest dilution at which complete clumping indicates a positive reaction. The general consensus of opinion, with which our own experience agrees, is that when a serum in a dilution of 1 : 30 causes complete clumping in half an hour, it may safely be said that it has been derived from a case of typhoid fever. Suspicion should be entertained as to the diagnosis if a lower dilution is required, or if a longer time is required.

The reaction given by the serum in typhoid fever usually begins to be observed about the seventh day of the disease, though occasionally it has been found as early as the fifth day, and sometimes it does not appear till the third week or later. Usually it becomes gradually more marked as the disease advances, and it is still given by the blood of convalescents from typhoid, but cases occur in which it may permanently disappear before convalescence sets in. How long it lasts after the end of the disease has not yet been fully determined, but in many cases



it has been found after several months or longer. As a rule, up to a certain point, the reaction is more marked where the fever is of a pronounced character, whilst in the milder cases it is less pronounced. In certain grave cases, however, the reaction has been found to be feeble or almost absent. In some cases, which from the clinical symptoms were almost certainly typhoid, the reaction has apparently been found to be absent. Such cases should always be investigated, from the point of view of their possibly being paratyphoid fever.

It has been found that the reaction is not only obtained with living bacilli, but in certain circumstances also with bacilli that have been killed by heating at  $60^{\circ}\text{C}$ . for an hour,—if a higher temperature be used, sensitiveness to agglutination is impaired. Dreyer has introduced a simple technique which enables an ordinary practitioner provided with dead cultures to carry out the test for himself. The capacity is also still retained if a germicide be employed. Here Widal recommends the addition of one drop of formalin to 150 drops of culture. The reaction, however, tends to be less complete.

Besides the blood serum, it has been found that the reaction is given in cases of typhoid fever by pericardial and pleural effusions, by the bile and by the milk, and also to a slight degree by the urine. The blood of a foetus may have little agglutinating effect, though that of its mother may have given a well-marked reaction; sometimes, however, the foetal blood gives a well-marked reaction. It may here also be mentioned that a serum will stand exposure for an hour at  $58^{\circ}\text{C}$ . without having its agglutinating power much diminished. Higher temperatures, however, cause the property to be lost.

*The Agglutination of Organisms other than the B. Typhosus by Typhoid Serum.*—It was at first thought that the reaction in typhoid fever would afford a reliable method of distinguishing the typhoid bacillus from the *b. coli*. Though many races of the latter give no reaction with a typhoid serum, there are others which react positively. Usually, however, a lower dilution and a longer time are required for a result to be obtained, and the reaction is often incomplete. It has also been found that other organisms belonging to the typhoid group (*v. p.* 382) react in a similar way. The reaction as a method of distinguishing between these forms is thus not absolutely reliable, but in certain cases it is of great value in giving confirmation to other tests. The important point here is the determination of the highest dilution with which clumping is obtained (for methods, see *p.* 120). There is a point in this connection regarding which further



light is required. Many races of *b. coli* in use have been isolated from typhoid cases, and we as yet do not know what effect this circumstance may have on its subsequent sensitiveness to agglutination by typhoid serum. Again, Christophers has pointed out that a large proportion of serum from normal persons or from those suffering from diseases other than typhoid will clump the *b. coli* in dilutions of from 1:20 to 1:200, and no doubt many of the reactions shown by typhoid sera towards *b. coli* are due to the pre-existence in the individuals of an agglutinative property towards the latter bacillus.

With regard to the value of the serum reaction there is little doubt. In nearly 95 per cent. of cases of typhoid it can be obtained in such a form that no difficulty is experienced if the precautions detailed above are observed. The causes of possible error may be summarised as follows: The serum of the person may naturally have the capacity of clumping typhoid bacilli; there may have been an attack of typhoid fever previously with persistence of agglutinative capacity; the case may be one of disease caused by an allied bacillus; the disease may have a quite different cause, and yet the serum may react with typhoid bacilli; the disease may be typhoid fever and yet no reaction may occur. The most important of these sources of error is that with which diseases caused by allied organisms are concerned, as it is probable that all the forms which these take in men have not been recognised. The very wide application of the reaction has elicited the fact that it is given in many cases of slight, transient, and ill-defined febriculæ, which occur especially when typhoid fever is prevalent. Some of these may be aborted typhoid, some may be paratyphoid. There is no doubt that, if all the facts are taken into account, the cases where the reaction gives undoubtedly correct information so far outnumber those in which an error may be made that it must be looked on as a most valuable aid to diagnosis. In conclusion, here we may say that the fact of a typhoid serum clumping allied bacilli in no way, so far as our present knowledge goes, justifies doubt being cast on the specific relation of the typhoid bacillus to typhoid fever.

In connection with the phenomenon that a serum either from a normal person or a typhoid patient may clump several varieties of bacteria, some points arise. The theoretical consideration of agglutination is reserved for the chapter on Immunity, but here it may be said that agglutinating properties may be present normally in a serum or they may be originated by an animal being infected with a particular bacterium. As the result of



injecting a bacterium, not only may agglutinins capable of acting on that bacterium appear in the serum, but the serum may become capable of agglutinating other, and especially kindred, bacteria; further, any normal agglutinins for the infecting bacterium present in the serum may be increased in amount. The agglutinin acting on the infecting organism has been called the primary or homologous agglutinin, while the others have been called the secondary or heterologous agglutinins. But besides what we know to be a fact, that infection by a single bacillary species can originate agglutinins acting both on itself and on allied species, we must consider the possibility of infections by more than one species occurring in an animal, *e.g.* *b. typhosus* with *b. coli* or with *b. paratyphosus* (*vide infra*). In such a case each organism may originate its primary agglutinin, so that the presence of multiple agglutinins in a serum may really be an indication of a mixed infection. Some attention has been directed to the diagnosis and differentiation of these conditions. Castellani introduced the absorption method for their investigation (for method, see p. 121), and by this means studied the primary and secondary agglutinins produced in infections in rabbits; he found that when an animal had been infected with *b. typhosus* this organism would absorb from its serum not only the primary typhoid agglutinins but also such secondary agglutinins as those acting on the *b. coli*. If, however, an animal had undergone infection with, say, both the *b. typhosus* and the *b. coli*, then the *b. typhosus* could not absorb from its serum the *b. coli* (primary) agglutinin. Castellani thus put forward the view that by this means primary could be differentiated from secondary agglutinins, and therefore pure could be differentiated from mixed infections. There is little doubt that this view possesses considerable validity, though it is probably not of universal applicability. Safe deductions can only be drawn when any serum is tested with several species of fairly closely related organisms, such as those of the coli group. Especially is it necessary that the highest dilutions in which agglutination occurs should be compared. If such precautions be adopted, the absorption method can be utilised for the differentiation of the typhoid and paratyphoid organisms and their infections, and for similar investigations.

**Vaccination against Typhoid.**—The principles of the immunisation of animals against typhoid bacilli have been applied by Wright and Semple to man in the following way:—Typhoid bacilli are obtained of such virulence that a quarter of a twenty-four hours' old sloped agar culture when administered hypo-



dermically will kill a guinea-pig of from 350 to 400 grammes. Vaccination can be accomplished by such a culture emulsified in bouillon, and killed by heating for five minutes at 60° C. For use, from one-twentieth to one-fourth of the dead culture is injected hypodermically, usually in the flank. The vaccine now used, however, actually consists of a portion of a bouillon culture similarly treated (see p. 133). The effects of the injection are some tenderness locally and in the adjacent lymphatic glands, and it may be local swelling, all of which come on in a few hours, and may be accompanied by a general feeling of restlessness and a rise of temperature, but the illness is over in twenty-four hours. During the next ten days the blood of the individual begins to manifest, when tested, an agglutination reaction, and further, Wright has found that usually after the injection there is a marked increase in the capacity of the blood serum to kill the typhoid bacillus *in vitro*. These observations, there is little doubt, indicate that the vaccinated person possesses a degree of immunity against the bacillus, a conclusion borne out by the results obtained in the use of the vaccine as a prophylactic against typhoid fever. Extensive observations have been made in the British army in India, and in the South African War the efficacy of the treatment was put to test. Though in isolated cases not much difference was observed among those treated as compared with those untreated, yet the broad general result may be said to leave little doubt that on the one hand protective inoculation diminishes the tendency for the individual to contract typhoid fever, and, on the other, if the disease be contracted, the likelihood of its having a fatal result is diminished. Thus, in India, of 4502 soldiers inoculated, .98 per cent. contracted typhoid, while of 25,851 soldiers in the same stations who were not inoculated, 2.54 per cent. took the disease. In Ladysmith during the siege there were 1705 soldiers inoculated, among whom 2 per cent. of cases occurred, and 10,529 uninoculated, among whom 14 per cent. suffered from typhoid. Wright has collected statistics dealing in all with 49,600 individuals, of whom 8600 were inoculated, and showed a case incidence of 2.25 per cent., with a case mortality of 12 per cent.; in the remaining 41,000 uninoculated the case incidence was 5.75 per cent. and the case mortality 21 per cent. The best results seemed to be obtained when ten days after the first inoculation a second similar inoculation was practised. Wright has found that in certain cases immediately after inoculation there is a fall in the bactericidal power of the blood (negative phase), and he is of opinion that this indicates a



temporary increased susceptibility to the disease. He therefore recommends that when possible the vaccination should be carried out some time previous to the exposure to infection. There can be little doubt that in this method an important prophylactic measure has been discovered.

**Vaccine Treatment of Typhoid Fever.**—As in the case of other acute infections, vaccines have been recently used in the treatment of typhoid fever during the acute stage (Leishman and Smallman). The method is to inject hypodermically 100 million dead typhoid bacilli, *i.e.* a fifth of the first dose used for the protective inoculation. If the temperature shows a tendency to fall, this may be repeated about every four days. The results obtained are hopeful, and justify the method being further applied.

**Antityphoid Serum.**—Chantemesse has immunised animals with dead cultures of the typhoid bacillus, and, having found that their sera had protective and curative effects in other animals, has used such sera in human cases of typhoid with apparent good result. In the hands of others, however, such a line of treatment has not been equally successful.

**Methods of Examination.**—The methods of microscopic examination, and of isolation of typhoid bacilli from the spleen *post mortem*, have already been described. They may be isolated from the Peyer's patches, lymphatic glands, etc., by a similar method.

During life, typhoid bacilli may be obtained in culture in the following ways:—

(a) *From the Blood.*—The typhoid bacillus can often be isolated from the blood, especially during the first week, by ordinary methods (see p. 72). A special method has also been used with success. In this 5 c.c. of blood are placed in 10 c.c. of sterilised ox bile. The mixture is incubated for from twenty-four hours to a week, and from time to time the presence of the bacillus is tested for by sub-culturing on such media as those of Conradi or MacConkey.

(b) *From the Spleen.*—This is the most certain method of obtaining the typhoid bacillus during the continuance of a case. The skin over the spleen is purified, and, a sterile hypodermic syringe being plunged into the organ, there is withdrawn from the splenic pulp a droplet of fluid, from which plates are made. In a large proportion of cases of typhoid the bacillus may be thus obtained, failure only occurring when the needle does not happen to touch a bacillus. Numerous observations have shown



that, provided the needle be not too large, the procedure is quite safe. Its use, however, is scarcely called for.

(c) *From the Urine.*—Typhoid bacilli are present in the urine in at least 25 per cent. of cases, especially late in the disease, probably chiefly when there are groups in the kidney substance. For methods of examining suspected urine, see p. 74.

(d) *From the Stools.*—During the first ten days of a case of typhoid fever, the bacilli can be isolated from the stools by the ordinary plate methods—preferably in McConkey's lactose bile-salt neutral-red agar, or in the other media described on pp. 47–53. After that period, though the continued infectiveness of the disease indicates that they are still present, their isolation is difficult. We have seen that after ulceration is fairly established by the sloughing of the necrosed tissue, the numbers present in the patches are much diminished, and therefore there are fewer cast off into the intestinal lumen, and that in addition there is a correspondingly great increase of the *b. coli*, which thus causes any typhoid bacilli in a plate to be quite outgrown. From the fact that the ulcers in a case of typhoid may be very few in number, it is evident that there may be at no time very many typhoid bacilli in the intestine. The microscopic examination of the stools is of course useless as a means of diagnosing the presence of the typhoid bacillus.

*Isolation from Water Supplies.*—A great deal of work has been done on this subject. It is evident that if it is difficult to isolate the bacilli from the stools, it must *a fortiori* be much more difficult to do so when the latter are enormously diluted by water. The *b. typhosus* has, however, been isolated from water during epidemics. The *b. coli* is, as might be expected, the organism most commonly present in such circumstances. In the case of both bacteria, the whole series of culture reactions must be gone through before any particular organism isolated is identified as the one or the other; probably there are saprophytes existing in nature which only differ from them in one or two reactions. In examining waters, the ordinary plate methods are generally used, but the McConkey or similar media may be employed with advantage. Klein filters a large quantity through a Berkefeld filter, and, brushing off the bacteria retained on the porcelain, makes cultures. A much greater concentration of the bacteria is thus obtained. From time to time various substances have been used with the object of inhibiting the growth of the *b. coli* without interfering with that of the *b. typhosus*. Most of these have not stood the test of experience. Lately caffeine has been used for this end. For use in examining waters the following is the method employed: To 900 c.c. of the suspected water there are added 10 grammes nutrose dissolved in 80 c.c. of sterile water, and 5 grammes of caffeine dissolved in sterile distilled water, heated to 80° C. and cooled to 55° C. before addition. After mixing the ingredients there is added 10 c.c. of .1 per cent. crystal violet. The flask is incubated at 37° C. for



twelve hours, and then plates of Conradi-Drigalski medium are inoculated from it. For investigation of faeces, a medium made up as above but with ordinary sterile water may be inoculated and a similar procedure followed. On the whole there is little to be gained from this attempt to isolate the typhoid bacillus from water in any particular case, and it is much more useful for the bacteriologist to bend his energies towards the obtaining of the indirect evidence of contamination of water by sewage, to the nature of which attention has been called in Chapter V.

#### THE PARATYPHOID AND FOOD-POISONING BACILLI.

In the *b. coli* we have an organism having a definite habitat in the animal intestine, and presenting certain cultural characters by which it may be recognised. We may look on the bacillus typhosus as an organism of the same class whose cultural reactions as compared with *b. coli* present somewhat negative characters, but which acquires definiteness from its association with a well-known clinical condition. We have now to deal with a group of organisms which occupy rather an intermediate position between the two organisms referred to, and whose cultural characters are such as to make their differentiation from either fairly practicable. The members of this group have been originally described in association with a variety of clinical conditions, but, notwithstanding, they resemble each other so closely that great difficulty arises, and the recognition of different types which in literature receive different names can only be effected by the application of the finest bacteriological tests. Although in cultures the different types present slight differences, these are not sufficient for the assignment of a name to an organism of the class isolated from some fresh source, and, as a matter of fact, in modern work relating to them, it is generally impossible in identifying an organism to rely on merely noting a correspondence with a described type. The method usually adopted is to obtain from other workers cultures of what may be called the historic strains isolated, and by comparing the organism under investigation with these, to attempt to place it in its proper position.

Organisms of the group are of great importance, not only from their producing ordinary infective disease in man, but because they are the agents at work in the great majority of the not infrequently occurring cases of illness usually described as "food poisoning."<sup>1</sup> Such poisoning is often referred to as "ptomaine poisoning," from the idea originally prevailing that

<sup>1</sup> A special type of food poisoning is associated with the *Bacillus botulinus*, *q.v.*



the symptoms were caused by alkaloidal substances produced during putrefactive processes occurring in meat. Certain cases of illness arising within an hour or two of the taking of tainted meat may be due to poisons of such a kind, but in the great majority of single or multiple cases of illness traceable to food, the symptoms do not appear so rapidly, and are associated with the multiplication in the intestine of organisms of the type now under consideration, and it may be also with an infection of the blood. In such cases, the meat at fault may not, to taste or smell, present any unusual features, but very often there can be isolated from it an organism identical with organisms derived from the sick individuals. Sometimes it has been proved that the animals from which the meat was derived have been suffering from illnesses probably due to the organisms subsequently found, but this has not always been the case, healthy meat being here contaminated by contact with infective matter. The foods giving rise to poisoning usually belong to the preserved food class, or consist of sausages or similar products. There is every reason to believe that the organisms in question may not be killed in the ordinary processes of cooking, in which the internal parts of the meat may not reach the temperature of blood coagulation.

The organisms included in the paratyphoid and food-poisoning group are as follows: The paratyphoid bacillus, varieties A and B, originally isolated from pathological conditions in man; bacillus enteriditis Gaertner, isolated from meat-poisoning cases; bacillus *Ærtryck*, also isolated from meat poisoning; bacillus *suipestifer* (Salmon's bacillus of hog cholera); psittacosis bacillus, occurring in a disease of parrots; bacillus *typhi murium*, isolated by Löffler from an epidemic of enteritis in mice; and Danysz's bacillus, isolated from an epidemic in field mice, and used by him for originating epidemics in rats. The pathological effects produced by these organisms include, on the one hand, general septicæmic manifestations, and, on the other, gastro-enteritis. The chief members of the group will be described below.

**The Characters of the Paratyphoid and Food-Poisoning Bacilli.**—These bacilli are all microscopically indistinguishable from the bacillus *typhosus*. They are Gram-negative, motile bacilli, the flagella being sometimes few in number, and they do not form spores. On ordinary media, growths have the general character of those of the *b. coli* and *b. typhosus*, some members in certain reactions resembling the one, and in others resembling the other. Opinion differs as to their capacity to form indol, but usually the reaction to this test is negative.



The methods for the isolation of the members of the group vary with the nature of the infected material to be examined. In the case of abscesses caused by the paratyphoid bacillus, the organism is usually accidentally discovered during the application of ordinary methods. When deliberate search for a member of the group is required, usually either the faeces or the blood constitutes the material to be examined. In the former case, advantage is taken of the fact that the food-poisoning bacilli do not ferment lactose. Thus, if McConkey bile-salt lactose-agar plates (p. 50) be used, the organisms sought for will appear as colourless colonies which can be picked off for systematic investigation. In the case of blood, ordinary methods will prove sufficient.

Capacity for fermenting sugars has been largely applied in work on this group. All the members produce practically the same reactions. They originate acid and gas in glucose, laevulose, sorbite, mannite, dextrin, maltose, dulcitol, galactose and arabinose, like *b. coli*, but produce no change in lactose, cane-sugar, salicin or inulin. Although differences in fermenting capacity have been noted in different strains, the existence of such cannot be relied upon for differentiating members of the group from one another. The sugar reactions are only of use in demarcating the lines between the food-poisoning group and *b. coli* on the one hand, and *b. typhosus* on the other. The differentiation of members of the group can only be effected by applying the agglutination tests to the serum of animals suffering from natural or artificial infection. The chief point here is that in such infections, the occurrence of group agglutinins in the serum is much in evidence. Herein lies the necessity for having at hand the historic strains of the organisms referred to above. In dealing with an organism, it is first of all advisable to take the serum of the infected individual, estimate the highest dilution with which it clumps the strain isolated, and compare the result obtained with the effect of the serum on the historic strains. The unknown strain is most likely to be allied to that strain which is agglutinated by a similar dilution of the serum used. Frequently, in the investigation of an organism, it is necessary to inject it into an animal and study the agglutinating properties of its serum on the infecting strain and upon allied organisms. Here considerable information may be obtained by the use of the absorption method. If from such a serum, for instance, an unknown organism has absorptive qualities similar to that of a historic Gaertner, its being named a Gaertner bacillus would be justified. It is customary in any



case to note the action of a typhoid serum on an organism under investigation, and also the action on the typhoid bacillus of an antiserum to the unknown organism.

**The Paratyphoid Bacillus.**—This organism, which was when first described often called the paracolon bacillus, was primarily isolated from abscesses occurring in apparently non-typhoid cases. Widal noted its resemblances to *b. typhosus* and *b. coli*, from the latter of which it differed in not producing indol and in not fermenting lactose. Gywnn first isolated it from the blood of a case presenting typhoid symptoms, and since then it has been recognised as being the probable cause of the disease effects in about 3 per cent. of cases which clinically are to be described as typhoid fever. The case mortality in paratyphoid fever is low, being only from 1 to 2 per cent. The organism has been isolated from the blood, the roseolar spots, and from the stools. Several strains showing slight differences in culture reactions have been obtained. Of these the two chief are "paratyphoid A" and "paratyphoid B," the latter being of commonest occurrence; these appear to present slight cultural differences. On gelatin, agar, and potato, A resembles *b. typhosus*, B resembles *b. coli*; in litmus milk A produces slight permanent acidity, while after the third day, in the case of B, acidity gives place to alkalinity; on sugars the fermentative activity of B is greater than that of A. Generally speaking, the characters of both are those of the group to which they belong. With regard to agglutinating reactions, the serum of a paratyphoid patient will agglutinate the bacillus in high dilutions. Observations on the behaviour of such sera towards the *b. typhosus* have in different cases yielded some discordant results, but usually a very much stronger concentration is necessary to give clumping, and often a paratyphoid serum will not clump the typhoid bacillus except in such concentrations as might give similar effects when normal sera are under observation. When any serum clumps both the paratyphoid and the typhoid bacilli, the more closely the maximal clumping dilutions correspond, the more likely is the case to be typhoid fever; on the other hand, if a high dilution will clump the paratyphoid bacillus, while a low dilution is necessary for the typhoid bacillus, then the case is likely to be paratyphoid fever. With regard to the effects of other sera on the paratyphoid bacillus, it may be said that usually a typhoid serum will require to be used in greater concentration to clump this bacillus than is necessary to obtain an effect with the typhoid bacillus itself. Similar effects are observed when the sera of animals immunised



against Gaertner's bacillus or the bacillus of psittacosis are used. In all serum tests the essential point is that deductions should only be based on comparative observations of the highest dilutions in which a clumping effect is produced with any series of organisms compared.

While the paratyphoid bacillus originates a disease resembling typhoid fever, it has also been found in the stools of typhoid patients, and mixed infections may thus occur. Both organisms have been observed together in the stools in typhoid carriers, and pure paratyphoid carriers are also stated to occur. A meat poisoning epidemic attributed to the paratyphoid bacillus has been reported. Besides the septic cases already alluded to, the organism has been isolated from cases of bone abscess, from orchitis, and in Widal's case from a thyroid abscess, and in such cases the history of a previous typhoid-like illness may not be elicited. It has also been found in ordinary fæces. In animal experiments it produces in rabbits and guinea-pigs a fatal illness of a septicæmic type with serous inflammations.

**Bacillus Enteritidis (Gaertner).**—In 1888, Gaertner, in investigating a number of cases of gastro-enteritis resulting from eating the flesh of a diseased cow, isolated, from the meat and from the spleen of a man who died, a bacillus closely resembling the typhoid bacillus. Since then, in a great number of similar outbreaks, similar bacilli have been found both in the stools and in the organs. The cultural characters are those of the group, except that in some strains the presence of an effect on lactose has been observed. Here again much information may be obtained from the agglutinating properties of the serum. It has also been found that the serum of persons suffering from meat poisoning sometimes clumps the typhoid bacillus, though a higher concentration is required than in the case of Gaertner's bacillus. The Gaertner group of organisms is very pathogenic for laboratory animals. Often, whatever the channel of infection, there is intense hæmorrhagic enteritis, and very usually there is a septicæmia with the occurrence of serous inflammations; the bacilli are recoverable from the solid organs and often from the blood. In man, as the name of the bacillus indicates, the symptoms are centred in the intestine, where there is usually marked inflammation of the mucous membrane, sometimes attended with hæmorrhage into it; evidence of a septicæmic condition may also exist. Infection may take place by the bacillus itself, and here the illness usually appears within twenty-four hours of the food being partaken of, but symptoms may appear almost at once, in which case they are no doubt due



to the action of toxins; here it is important to note that the poisons formed by this group of organisms are relatively heat-resisting, so that boiling for a time does not destroy the toxicity.

**The Psittacosis Bacillus.**—When parrots are imported from the tropics in large numbers, many die of a septicæmic condition in which an enteritis, it may be hæmorrhagic, is a marked feature. There is intense congestion of all the organs and peritoneal ecchymoses. From the spleen, bone marrow, and blood there has been isolated a bacillus having the group characters, except that here also an effect on lactose has been described. The parrot is most susceptible to its action, but it also causes a fatal hæmorrhagic septicæmia in guinea-pigs, rabbits, mice, pigeons, and fowls, the bacilli after death being chiefly in the solid organs. From affected parrots the disease appears to be readily communicable to man, chiefly, it is probable, from the feathers being soiled by infective excrement. Several small epidemics have been recognised and investigated in Paris. After about ten days' incubation, headache, fever, and anorexia occur, followed by great restlessness, delirium, vomiting, often diarrhœa, and albuminuria. Frequently broncho-pneumonia supervenes, and a fatal result has followed in about a third of the cases observed. The organism has been isolated from the blood of the heart. The psittacosis bacillus is evidently one of the typhoid group, a fact which is further borne out by the observation that it may be clumped by a typhoid serum. The clumping is, however, said often to be incomplete, as the bacilli between the clumps may retain their motility. It differs from the typhoid bacillus in its growth on potatoes and in its pathogenicity.

**Danysz's Bacillus and Rat Viruses.**—Danysz isolated from an epizootic in field mice an organism of this group, which he introduced for the purpose of killing rats by originating in them through feeding a similar epizootic, and several viruses of this kind are in commercial use for this purpose. These have been investigated by Bainbridge, who, however, finds that they owe any efficiency they possess to two organisms, the bacillus Aertryck and the bacillus enteriditis of Gaertner. The efficacy of such agents varies, and the mortality in artificially originated epizootics is from 20 to 50 per cent. Sometimes, apparently under natural conditions, rats develop an immunity to those viruses, and it is doubtful whether they are entirely innocuous to other animals which may partake of the food containing them.

### BACILLARY DYSENTERY.

Dysentery has for long been recognised as including a number of different pathological conditions, and within more recent times amœbic and non-amœbic forms have been distinguished. Of the latter, bacteria have been believed to be the causal agents, and an organism described by Shiga in 1898 has almost certainly been established as the cause of a large proportion of cases. Shiga's observations were made in Japan, and confirmatory results have been obtained by Kruse in Germany, by Flexner and by Strong and Harvie in the Philippine Islands, and more recently by Vedder



and Duval in the United States. It is now further recognised that the epidemics of dysentery which from time to time occur in lunatic asylums are usually due to bacilli of this type, and in America the organism has been demonstrated in summer diarrhoea in children. The evidence for the relationship of the organism to the disease consists chiefly in the apparently constant presence of the organism in the dejecta in this form of dysentery, and the agglutination of the organism by the serum of patients suffering from the disease, but confirmatory evidence has also come from animal experimentation. From different epidemics a great many different strains of the dysentery bacillus have been obtained, but these all possess common characters and are undoubtedly closely related to one another. The various strains resolve themselves into two chief groups, whose differences lie in their behaviour towards certain sugars, in their capacities of forming indol, and in their agglutinating reactions. The relation of amœbæ to dysentery will be discussed in the Appendix.

**Bacillus Dysenteriae.** — *Morphological Characters.* — This bacillus morphologically closely resembles the typhoid bacillus, but is on the whole somewhat plumper, and filamentous forms are comparatively rare. Involution forms sometimes occur, especially in glucose agar. Most observers have found no trace of motility, whilst others say that it is slightly motile. Vedder and Duval have, however, by a modification of Van Ermengen's process, demonstrated in the case of one strain the presence of numerous lateral flagella, which are of great fineness, but of considerable length. No spore formation occurs; the organism is stained readily by the ordinary dyes, but is decolorised by Gram's method.

*Cultural Characters.* — In these also considerable resemblance is presented to the typhoid bacillus. In *gelatin* a whitish line of growth occurs along the puncture, but the superficial film-like growth is usually absent, or at least poorly marked. In plate cultures also the superficial growths are smaller and have less of the film-like character, than those of the typhoid organism. On *agar*, growth occurs as a smooth film with regular margins, but after two or three days, especially if the surface be moist, Vedder and Duval describe an outgrowth of lateral offshoots on the surface of the medium. On agar plates the colonies resemble those of the typhoid organism, being of smaller size and less opaque than those of the bacillus coli. In *peptone bouillon* a uniform haziness is produced. As has been indicated, different strains of the bacillus behave differently towards different *sugars*,



and the results of all observers do not agree, so that only general statements can be made. Without going into the question of the particular strains to be placed in the two groups, we may say that, roughly, these may be classified into the Shiga-Kruse group and the Flexner group. All produce acid in peptone-glucose and in taurocholate peptone-glucose; none produce change in lactose or cane-sugar. The Shiga group do not produce acid in maltose or mannite, while the Flexner group do, and, generally speaking, the former do not produce *indol*, while the latter do. Forms intermediate between the two groups occur. There is never any evolution of gas observed in sugar media. In *litmus milk* there is developed at first a slight degree of acidity, which is followed by a phase of increased alkalinity; no coagulation of the milk ever occurs. On *potato* the organism forms a transparent or whitish layer, which, however, in the course of a few days assumes a brownish-red or dirty grey colour, with some discoloration of the potato at the margin of the growth.

*Relation to the Disease.*—The organism has been found in large numbers in the dejecta, especially in the acute cases, where it may be present in almost pure culture. In the thirty-six cases examined, Shiga obtained it in thirty-four from the dejecta, and in the two others *post mortem* from the intestinal mucous membrane. The organism does not appear to spread deeply or to invade the general circulation. In the more chronic cases it is difficult to obtain, on account of the large number of the bacillus coli and other bacteria present. Vedder and Duval found agar plates to be the best method of culture, these being incubated at the blood temperature. They also found that if the colonies which appeared at twelve hours were marked with a pencil, there was a greater probability of obtaining the bacillus of dysentery from those which appeared later, most of those appearing early being colonies of the bacillus coli. McConkey's agar medium with lactose added may be used for isolation from stools. A little of the faeces is rubbed up in broth and some of the mixture stroked on the medium. The formation of acid by the *b. coli* colonies enables them to be excluded, and therefore, as the *b. dysenteriae* is not a lactose fermenter, the colourless colonies which develop after twenty-four hours are picked out for further investigation.

As already stated, both acute and chronic cases are marked by the presence of this organism. In the former, where death may occur in from one to six days, the chief changes, according to Flexner, are a marked swelling and corrugation of the mucous membrane, with hæmorrhage and pseudo-membrane at places. There is extensive coagulation-necrosis with fibrinous exuda-



tion and abundance of polymorpho-nuclear leucocytes, and the structure of the mucous membrane, as well as that of the muscularis mucosæ, is often lost in the exudation. There is also great thickening of the sub-mucosa, with great infiltration of leucocytes, these being chiefly of the character of "plasma cells." In the more chronic forms the changes correspond, but are more of a proliferative character. The mucous membrane is granular, and superficial areas are devoid of epithelium, whilst ulceration and pseudo-membrane are present in varying degree. A feature of bacillary dysentery is the fact that abscess of the liver does not occur as a complication.

*Agglutination.*—All the above-mentioned observers agree regarding the agglutination of this bacillus by the serum—that is, in the cases of dysentery from which the organism can be cultivated. The reaction may appear on the second day, and is most marked after from six to seven days in the acute cases; it is usually given in a dilution of from one in twenty to one in fifty within an hour, though sometimes much higher dilutions give a positive result. In the more chronic cases the reaction is less marked, and here the sedimentation method is to be preferred. It is difficult to make any general statements with regard to the effects of dysenteric sera on the different strains of the bacilli, but it may be said that generally a serum agglutinates the strain which produced it and the other strains of the same group in higher dilutions than it does the strains of the other group. Many observers have found that the serum from a case associated with strains of the Shiga-Kruse group has not agglutinated strains of the Flexner group, and corresponding observations have been made in cases associated with the Flexner group. Often the sera of animals immunised with bacilli have been used for such tests, but apparently great care must be exercised in basing diagnoses on such observations, as the sera vary in different instances as regards their action on strains allied to that used for injection. Agglutination of the organism has not been obtained with serum from cases other than those of dysentery, nor has a similar bacillus been cultivated from such sources. The reaction is also absent in those cases of dysentery which are manifestly of amœbic nature.

*Pathogenic Properties.*—The organism is pathogenic in guinea-pigs and other laboratory animals, but, in these, characteristic changes in the intestine are often wanting. Shiga, however, obtained such effects by introducing the organism into the stomach of young cats and dogs, and confirmatory results were obtained by Flexner. Such attempts have been specially



successful when the virulence of the organism has been previously exalted by intraperitoneal *passage*. In two cases, apparently well authenticated, a dysenteric condition has followed in the human subject from ingestion of pure cultures of the organism.

It is probable that in the action of the bacillus a toxin is concerned. If the organism be grown for two or three weeks in an alkaline bouillon, there appears, probably by autolysis of the bacteria, a toxin in the culture medium separable by filtration in the ordinary way. The optimum alkalinity is achieved by adding .3 per cent. of soda to bouillon neutral to litmus, the resulting precipitate not being removed; free access of oxygen is permitted during growth. Apparently, the Shiga-Kruse strains yield the most toxic filtrates, and with the Flexner strain, the results of most observers show that soluble toxins cannot be obtained. The poison is very toxic to animals, especially rabbits, and however introduced into the body it causes after an incubation period hæmorrhagic enteritis with a diphtheritic-like exudate on the surface of the mucous membrane. Toxins isolated from different strains differ as regards the animals for which they are most toxic. The toxin is fairly resistant to heat, standing temperatures up to 70° C. without being injured.

It may be said that an aggressive reaction (*vide* p. 189) has also been described in the case of the dysentery bacillus.

*Immunisation Experiments.*—Both large and small animals have been immunised against the bacillus and also against its toxic filtrates. In the former case the immunisation has been commenced either with non-lethal doses of living cultures, or with cultures killed by heat. The nature of the immunisation is probably complex. When cultures have been used, a bactericidal serum, in which immune bodies and complements (*vide* Immunity) are concerned, is developed. When the toxin is used for immunisation, a serum protecting against the toxin is produced. According to some results, animals immunised with cultures are immune against the toxin, and *vice versa*. All races of animals do not lend themselves to immunisation.

Considerable work has been done in immunising large animals (horses, goats) against the soluble toxins of the dysentery bacillus with a view to obtaining therapeutic sera. Doerr, using his toxin from the Shiga-Kruse strain, produced in horses an antitoxic serum having protective and curative properties in animals. This serum has been used in a number of cases of bacillary dysentery in man with good results. Shiga



produced a polyvalent serum by injecting horses with agar cultures of different strains, and states that it has been used in Japan with good results. Further observation is necessary as to the therapeutic effects in cases associated with the Flexner strain of an antitoxin produced by the Shiga strain.

It will be seen that the evidence furnished is practically conclusive as to the causal relationship between this bacillus and one form of dysentery, a form, moreover, which is both widespread and embraces a large proportion of cases of the disease; and especially of importance is the fact that observations made independently in different countries have yielded practically identical results on this point.

**Bacillus Dysenteriae (Ogata).**—Ogata obtained this bacillus in an extensive epidemic in Japan in which no amœbæ were present. He found in sections of the affected tissues enormous numbers of small bacilli of about the same thickness as the tubercle bacillus, but very much shorter. These bacilli were sometimes found in a practically pure condition. They were actively motile, and could be stained by Gram's method. He also obtained pure cultures from various cases and tested their pathogenic effects. They grew well on gelatin, at the ordinary temperature producing liquefaction, the growth somewhat resembling that of the cholera spirillum. By injection into cats and guinea-pigs, as well as by feeding them, this organism was found to have distinct pathogenic effects; these were chiefly confined to the large intestine, hæmorrhagic inflammation and ulceration being produced. It still remains to be determined whether this organism has a causal relationship to one variety of dysentery.

#### BACILLUS ENTERITIDIS SPOROGENES.

This organism was first isolated by Klein from the evacuations in an outbreak of diarrhœa following the ingestion of milk which contained the microbe, and it was subsequently found by him in certain cases of infantile diarrhœa and of summer diarrhœa, in certain instances in milk, and as a constant inhabitant of sewage (see Chap. V.). In films made from the stools in diarrhœa cases where it is present, it can be microscopically recognised as a bacillus  $1.6\ \mu$  to  $4.8\ \mu$  in length and  $.8\ \mu$  in breadth, staining by ordinary stains and retaining the dye in Gram's method. It often contains a spore near one of the ends, or sometimes nearer the centre. It is slightly motile, and in cultures can be shown to possess a small number of terminal flagella. It grows well under anaerobic conditions in ordinary media, especially on those containing reducing agents. On agar the colonies are circular, grey, and translucent, and under a low power are seen to have a granular appearance. On this medium spore formation does not occur, but is easily obtained if the organism is grown on solidified blood serum, which, further, is liquefied during growth. On gelatin plates liquefaction commences after twenty-four hours at  $20^{\circ}\text{C}$ . It produces acid and gas in bile-salt glucose media, and in peptone-salt solution containing glucose or mannite. Spore formation can be seen to take place in 2 per cent. dextrose gelatin, but



the degree seems to be in inverse ratio to the amount of gas formation. Very typical is the growth in milk, and it is by this medium that isolation can be best effected. A small quantity of the material suspected to contain the bacillus is placed in 15 to 20 c.c. of sterile milk, which is then heated for ten minutes at 80° C. to destroy all vegetative bacteria; the tube is cooled, placed under anaerobic conditions, and incubated at 37° C. for from twenty-four to thirty-six hours. If the bacillus be present there is abundant gas formation, and almost complete separation of the curd from the whey takes place. The former adheres to the sides of the tube in shreds, and large masses gather with the cream on the top of the fluid, all being torn by the gas evolved. The whey is only slightly turbid, and contains numerous bacilli. The growth has an odour of butyric acid. If a small quantity (say 1 c.c.) of the whey be injected into a guinea-pig, the animal becomes ill in a few hours and dies in twenty-four hours. At the point of inoculation, the skin and subcutaneous tissues, and sometimes even the subjacent muscles, are green and gangrenous and evil-smelling, there is considerable œdema, and there may also be gas formation. The exudation is crowded with bacilli, which, however, are not generally distributed in any numbers throughout the body. These pathogenic properties of the bacillus enteritidis sporogenes are important in its recognition, for its culture reactions taken alone are very similar to those of the bacillus butyricus of Botkin.

#### SUMMER DIARRHŒA.

As has been already stated, the bacillus of dysentery, the *b. coli*, and the *b. enteritidis sporogenes* have been found associated with epidemics of this disease. This indicates that the condition may be originated by a variety of organisms, and it is further probable that the clinical features in different epidemics vary. This is to a certain extent illustrated by the condition of the stools. In Britain these are usually green, watery, slimy, and putrid, without blood or mucus, but in many outbreaks in America blood and mucus are present. The multiple origin of the disease has been illustrated by the work of Morgan, who, in a careful investigation of the disease in Britain, has been unable to find evidence of the dysentery bacillus being present. He has, however, very frequently (in 63 per cent. of the cases examined) found in the stools and intestine a bacillus ("Morgan's No. 1 bacillus") which is a motile Gram-negative organism producing acid and slight gas formation in glucose, lævulose, and galactose, and no change in mannite, dulcitol, maltose, dextrin, cane-sugar, lactose, inulin, amygdalin, salicin, arabinose, raffinose, sorbitol, or erythritol; it further causes indol formation, and in litmus milk slowly originates an alkaline reaction. It produces diarrhœa and death in young rabbits, rats, and monkeys when these animals are fed on cultures. It is thus possible that in this bacillus we have



still another cause of the disease. Morgan has found that in diarrhoea cases the lactose fermenters, so characteristic of normal fæces, are relatively less frequent and tend to be replaced by non-fermenters of lactose. His bacillus has been found in a certain proportion of normal children, but this especially during the epidemic season ; it has also been found in flies.

#### GENERAL REVIEW OF THE COLI-TYPHOID BACILLI.

A general view of the organisms belonging to the coli-typhoid group which we have now considered indicates a close alliance between the various members. All are microscopically indistinguishable from one another, and react negatively to the Gram stain. The chief sub-groups can be differentiated by culture reactions, of which the action on sugars is most important. Here important information is obtained by the study of the glucose and lactose reactions. The typhoid sub-group produces acid on glucose, but has no action on lactose. The dysentery sub-group is similar, but is chiefly marked off from the typhoid sub-group by its relative non-motility, by its tendency to form alkali after a preliminary acid development on litmus milk, and by the fact that it does not ferment sorbite. The food-poisoning sub-group is differentiated from the typhoid sub-group by forming acid and gas in glucose and from the coli-sub-group by its producing no change on lactose. The positive features of the coli sub-group are the formation of acid and gas in both glucose and lactose.

From work done not only with bacteria isolated from pathological conditions, but in connection with the bacteriology of water, milk, and fæces, it has been found that an enormous number of organisms exist, having the capacity of fermenting glucose and lactose, but which, when further investigated, present individual differences. Much has been done in attempting to differentiate these so-called "lactose fermenters" from one another. Here the work of McConkey may be taken as constituting one of the best attempts at such further classification, and it has the merit of simplifying a technique unduly complicated by the use of fermentation tests in a great series of sugars, on which the various sub-groups have all the same effect. McConkey is of opinion that certain of the tests applied to the lactose fermenters in reality give little information. These are, first, the growth on litmus whey, observation of which only corroborates what is observed with litmus milk ; second, observation of fluorescence on neutral-red lactose media (on account of



the inconstancy of the occurrence of this change in lactose fermenters, and from the fact that many other bacteria also produce it); third, the reduction of nitrates,—this appears to be a common property of nearly all the members of the group; fourth, observation of differences in the naked eye or low power appearances on gelatin; these are very inconstant, and different colonies of the same organism may show different appearances. On the other hand, important information may be obtained by the observation of the Voges and Proskauer reaction (p. 353). With regard to sugars, McConkey concludes that in the differentiation of the lactose fermenters, the only sugars necessary are lactose, saccharose, dulcitol, adonitol, inulin, inositol, and mannitol. Using these, a preliminary classification can be made from the actions on cane-sugar and dulcitol, and four groups are constituted: I. Organisms not affecting either cane-sugar or dulcitol. II. Organisms having no action on cane-sugar, but fermenting dulcitol. III. Organisms fermenting both cane-sugar and dulcitol. IV. Organisms fermenting cane-sugar but having no action on dulcitol. Of the first, the *bacillus acidi lactici* of Hüppe may be taken as a type; of the second, the *bacillus coli communis* of Escherich; of the third, *bacillus Friedländer*; of the fourth, the *bacillus lactis aerogenes* and the *bacillus cloacæ*. Group IV. is further sub-divided into sub-group 1, in which there is no liquefaction of gelatin and an absence of the Voges and Proskauer reaction; 2, with no liquefaction of gelatin, presence of Voges and Proskauer's reaction (*bacillus lactis aerogenes*); 3, with liquefaction of gelatin, presence of Voges and Proskauer's reaction (*bacillus cloacæ*); 4, with liquefaction of gelatin and production of a yellow pigment. Taking the properties named as type characteristics, the great mass of lactose fermenters can be further differentiated by the application of the other sugar tests. It is well to refer any organism found as belonging to one or other of the types, as in most cases no name has been assigned. Examples are constantly met with in work on water or faecal contents.

Although many of the named varieties were originally described in connection with other bacterial processes, all these bacteria are of frequent occurrence, especially in the human and animal intestine. As in the case of the members of the food-poisoning group, great difficulty has been experienced in identifying the types from mere description, and considerable complication has arisen from the fact that before the elaboration of the modern differentiation technique, different observers



identified organisms as belonging to a classical type, which have now been found not to conform in properties with the historic strains; here again, it is now customary during classification work to have at hand such historic strains in order that comparative parallel observations may be made.

With regard to the type strains, a few words may be added. The original *bacillus coli communis* of Escherich was isolated from the intestine of newly-born infants in connection with the first appearance of bacteria in the alimentary tract. About the same time, an organism now known as the *bacillus neapolitanus* was obtained by Emmerich in an outbreak of choleraic disease in Naples, and this organism was looked upon as identical with Escherich's *bacillus*, but it ferments saccharose, on which Escherich's has no effect. The *bacillus acidi lactici* of Hüppe was stated by this observer to be the chief cause of the souring of milk. It is now known that a large number of organisms of the same type, but differing slightly in cultural characters, are concerned in this process, and, as a matter of fact, McConkey found the presence of the classical strain to be relatively infrequent in milk. The *bacillus lactis aerogenes* was originally described by Escherich, in connection with his work on the bacteriology of the intestine in children, as an organism differing from the ordinary milk-souring bacteria by its producing gas from milk in the absence of air. Although it is a free gas-producer, this property is not specific for it, and within recent years it has attracted attention chiefly from its apparently being closely allied to the *bacillus pneumoniae* of Friedländer. Like the latter, this organism is stated when injected into animals to appear in a capsulated form. Another member of this group is *bacillus oxytocus perniciosus*, which is said originally to have been isolated from milk. This organism, along with the *bacillus vesiculosus* and an organism denominated No. 71, were found by McConkey to be of very common occurrence in human and animal faeces.

In work of the kind with which we are dealing, two other organisms are not infrequently observed which morphologically belong to the coli-typhoid group, but neither of which is a lactose fermenter. These are the *bacillus faecalis alcaligenes*, and the *bacillus coli anaerogenes*. The reactions of these will be found in the Table (p. 394). The latter *bacillus* somewhat resembles the typhoid *bacillus*, but produces acid in lactose and can be distinguished by agglutinating reactions.

When any question arises regarding the relationships of an organism isolated under saprophytic conditions and resembling



TABLE SHOWING CHARACTERS OF THE GRAM-NEGATIVE BACILLI OF THE COLI-TYPHOID GROUP.

Bacterium.	Motility.	Gelatin.	Glucose.	Lactose.	Saccharose.	Mannite.	Dulcite.	Adonite.	Sorbit.	Inosite.	Inulin.	Litmus Milk.			Indol.	Voges and Proskauer's Reaction.
												1 Day.	3 Days.	15 Days.		
<i>B. coli communis</i> . . .	+	-	A. G.	A. G.	-	A. G.	A. G.	-	A. G.	-	-	A. C.	A. C.	A. C.	+	-
<i>B. typhosus</i> . . .	+	-	A.	-	-	A.	-	-	A.	-	-	A.	A.	Alk. or A.	-	-
<i>B. paratyphosus</i> . . .	+	-	A. G.	-	-	A. G.	A. G.	-	A. G.	-	-	A.	Alk.	Alk.	-	-
<i>B. enteritidis</i> (Gaertner) .	+	-	A. G.	-	-	A. G.	A. G.	-	A. G.	-	-	A.	Alk.	Alk.	-	-
<i>B. dysenteriae</i> (Shiga) .	-	-	A.	-	-	-	-	-	-	-	-	A.	Alk.	Alk.	-	-
<i>B. dysenteriae</i> (Flexner) .	-	-	A.	-	-	A.	-	-	-	-	-	A.	Alk.	Alk.	+	-
<i>B. "Morgan's No. 1"</i> .	+	-	A. G.	-	-	-	-	-	-	-	-	0	0	Alk.	++	-
<i>B. lactis aerogenes</i> . . .	-	-	A. G.	A. G.	A. G.	A. G.	-	A. G.	A. G.	A. G.	-	A. C.	A. C.	A. C.	-	+
<i>B. acidilactici</i> (Hüppe) .	-	-	A. G.	A. G.	-	A. G.	-	A. G.	A. G.	-	-	A. C.	A. C.	A. C.	+	-
<i>B. cloacae</i> . . .	+	+	A. G.	A. G.	A. G.	A. G.	-	-	A. G.	A.	-	A. C.	A. C.	A. C.	+	+
<i>B. faecalis alcaligenes</i> .	+	-	-	-	-	-	-	-	-	-	-	Alk.	Alk.	Alk.	-	-
<i>B. coli anaerogenes</i> . . .	-	-	A.	A.	-	A.	-	-	-	-	-	A.	A.	A.	+	-
<i>B. oxytocus pernicius</i> *	-	+	A. G.	A. G.	A. G.	A. G.	A. G.	A. G.	A. G.	A. G.	A. G.	A. C.	A. C.	A. C.	+	+
<i>B. vesiculosus</i> * . . .	-	-	A. G.	A. G.	-	-	-	-	-	-	-	A. C.	A. C.	A. C.	+	-
<i>B. "McConkey's No. 71"</i> *	+	-	A. G.	A. G.	A. G.	-	A. G.	-	-	-	-	A. C.	A. C.	A. C.	+	-
<i>B. Friedländer</i> * . . .	-	-	A. G.	A. G. or A.	A. G.	A. G.	A. G.	A. G.	A. G.	-	-	A. C.	or	A.	+	-

+ in Motility column = presence of motility ; in Gelatin = liquefaction ; in Indol = presence of indol ; in Voges and Proskauer = presence of reaction.

- in Motility column = absence of motility ; in Gelatin = no liquefaction ; in Indol = absence of indol ; in Voges and Proskauer = absence of reaction ; in other columns = absence of change.

"A" = Acid production ; "G" = Gas ; "C" = Clot ; "Alk." = development of alkalinity. \* McConkey, *Journal of Hygiene* v. 333, ix. 86.



some definite pathogenic type, important information can often be obtained by studying its agglutinating reactions. In such a case the effect of sera produced by the pathogenic type upon the unknown organism, and of sera produced by injection into animals of the pathogenic type in question, ought to be studied.



## CHAPTER XVI.

### DIPHTHERIA.

THERE is no better example of the valuable contributions of bacteriology to scientific medicine than that afforded in the case of diphtheria. Not only has research supplied, as in the case of tubercle, a means of distinguishing true diphtheria from conditions which resemble it, but the study of the toxins of the bacillus has explained the manner by which the pathological changes and characteristic symptoms of the disease are brought about, and has led to the discovery of the most efficient means of treatment, namely, the anti-diphtheritic serum.

**Historical.**—The first account of the bacillus now known to be the cause of diphtheria was given by Klebs in 1883, who described its characters in the false membrane, but made no cultivations. It was first cultivated by Löffler from a number of cases of diphtheria, his observations being published in 1884, and to him we owe the first account of its characters in cultures and of some of its pathogenic effects on animals. The organism is for these reasons known as the Klebs-Löffler bacillus, or simply as Löffler's bacillus. By experimental inoculation with the cultures obtained, Löffler was able to produce false membrane on damaged mucous surfaces, but he hesitated to conclude definitely that this organism was the cause of the disease, for he did not find it in all the cases of diphtheria examined, he was not able to produce paralytic phenomena in animals by its injection, and, further, he obtained the same organism from the throat of a healthy child. This organism became the subject of much inquiry, but its relationship to the disease may be said to have been definitely established by the brilliant researches of Roux and Yersin, which showed that the most important features of the disease could be produced by means of the separated toxins of the organism. Their experiments were published in 1888-90. Further light has been thrown on the subject by the work of Sidney Martin, who has found that there can be separated from the organs in cases of diphtheria substances which act as nerve poisons, and also produce other phenomena met with in diphtheria.

**General Facts.**—Without giving a description of the pathological changes in diphtheria, it will be well to mention the outstanding features which ought to be considered in connection



with its bacteriology. In addition to the formation of false membrane, which may prove fatal by mechanical effects, the chief clinical phenomena are the symptoms of general poisoning, great muscular weakness, tendency to syncope, and albuminuria; also the striking paralyses which occur later in the disease, and which may affect the muscles of the pharynx, larynx, and eye, or less frequently the lower limbs (being sometimes of paraplegic type), all these being grouped together under the term "post-diphtheritic paralyses." It may be stated here that all these conditions have been experimentally reproduced by the action of the bacillus of diphtheria, or by its toxins. Other bacteria are, however, concerned in producing various secondary inflammatory complications in the region of the throat, such as ulceration, gangrenous change, and suppuration, which may be accompanied by symptoms of general septic poisoning. The detection of the bacillus of Löffler in the false membrane or secretions of the mouth is to be regarded as supplying the only certain means of diagnosis of diphtheria.

**Bacillus Diphtheriæ.**—*Microscopical Characters.*—If a film preparation be made from a piece of diphtheria membrane (in the manner described below) and stained with methylene-blue, the bacilli are found to have the following characters:—They are slender rods, straight or slightly curved, and usually about  $3\ \mu$  in length, their thickness being a little greater than that of the tubercle bacillus. The size, however, varies somewhat in different cases, and for this reason varieties have been distinguished as small and large, and even of intermediate size. It is sufficient to mention here that in some cases most are about  $3\ \mu$  in length, whilst in others they may measure fully  $5\ \mu$ . Corresponding differences in size are found in cultures. They stain deeply with the blue, sometimes being uniformly coloured, but often showing, in their substance, little granules more darkly stained, so that a dotted or beaded appearance is presented. Sometimes the ends are swollen and more darkly stained than the rest; often, however, they are rather tapered off (Fig. 111). In some cases the terminal swelling is very marked, so as to amount to clubbing, and with some specimens of methylene-blue these swellings and granules stain of a violet tint. Distinct clubbing, however, is less frequent than in cultures. There is a want of uniformity in the appearance of the bacilli when compared side by side. They usually lie irregularly scattered or in clusters, the individual bacilli being disposed in all directions. Some may be contained within leucocytes. They do not form chains, but occasionally forms



longer than those mentioned may be found, and these specially occur in the spaces between the fibrin as seen in sections.

**Distribution of the Bacillus.**—The diphtheria bacillus may be found in the membrane wherever it is formed, and may also occur in the secretions of the pharynx and larynx in the disease. It may be mentioned that distinctions formerly drawn between true diphtheria and non-diphtheritic conditions from the appear-

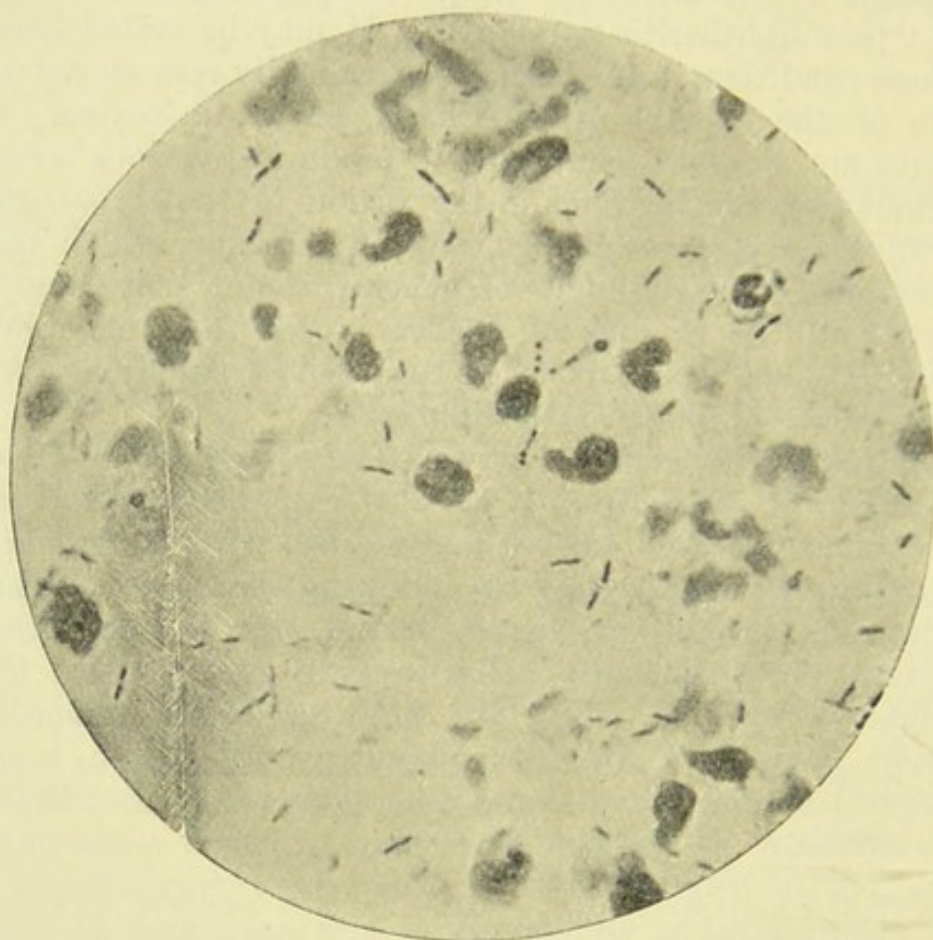


FIG. 111.—Film preparation from diphtheria membrane, showing numerous diphtheria bacilli. One or two degenerated forms are seen near the centre of the field. (Cultures made from the same piece of membrane showed the organism to be present in practically pure condition.)

Stained with methylene-blue.  $\times 1000$ .

ance and site of the membrane, have no scientific value, the only true criterion being the presence of the diphtheria bacillus. The occurrence of a membranous formation produced by streptococci has already been mentioned (p. 212).

In diphtheria the membrane has a somewhat different structure, according as it is formed on the surface covered with stratified squamous epithelium, as in the pharynx, or on a surface covered by ciliated epithelium, as in the trachea. In the former



situation necrosis of the epithelium occurs either uniformly or in patches, and along with this there is marked inflammatory reaction in the connective tissue beneath, attended by abundant fibrinous exudation. The necrosed epithelium becomes raised up by the fibrin, and its interstices are also filled by it. The fibrinous exudation also occurs around the vessels in the tissue beneath, and in this way the membrane is firmly adherent. In



FIG. 112.—Section through a diphtheritic membrane in trachea, showing diphtheria bacilli (stained darkly) in clumps, and also scattered amongst the fibrin. Some streptococci are also shown, towards the surface on the left side.

Stained by Gram's method and Bismarck-brown.  $\times 1000$ .

the trachea, on the other hand, the epithelial cells rapidly become shed, and the membrane is found to consist almost exclusively of fibrin with leucocytes, the former arranged in a reticulated or somewhat laminated manner, and varying in density in different parts. The membrane lies upon the basement membrane, and is less firmly adherent than in the case of the pharynx.

The position of the diphtheria bacilli varies somewhat in different cases, but they are most frequently found lying in oval



or irregular clumps in the spaces between the fibrin, towards the superficial, that is, usually, the oldest part of the false membrane (Fig. 112). There they may be in a practically pure condition, though streptococci and occasionally some other organisms may be present along with them. They may occur also in deeper parts, but are rarely found in the fibrin around the blood vessels. On the surface of the membrane they may be also seen lying in large numbers, but are there accompanied by numerous other organisms. Occasionally a few bacilli have been detected in the lymphatic glands. As Löffler first described, they may be found after death in pneumonic patches in the lung, these being due to a secondary extension by the air passages. They have also been occasionally found in the spleen, liver, and other organs after death. This occurrence is probably to be explained by an entrance into the blood stream shortly before death, similar to what occurs in the case of other organisms, *e.g.* the bacillus coli communis. The diphtheria bacillus may also infect other mucous membranes. It is found in true diphtheria of the conjunctiva, and may also occur in similar affections of the vulva and vagina; some of these cases have been treated successfully with diphtheria antitoxin. The pseudo-diphtheria bacillus, however, may also occur in these situations.

*Association with other Organisms.*—The diphtheria organism is sometimes present alone in the membrane, but more frequently is associated with some of the pyogenic organisms, the streptococcus pyogenes being the commonest. The staphylococci, and occasionally the pneumococcus or the bacillus coli, may be present in some cases. Streptococci are often found lying side by side with the diphtheria bacilli in the membrane, and also penetrating more deeply into the tissues. In some cases of tracheal diphtheria we have found streptococci alone at a lower level in the trachea than the diphtheria bacilli, where the membrane was thinner and softer, the appearance in these cases being as if the streptococci acted as exciters of inflammation and prepared the way for the bacilli. It is still a matter of dispute as to whether the association of the diphtheria bacillus with the pyogenic organisms is a favourable sign or the contrary, though on experimental grounds the latter is the more probable. We know, however, that some of the complications of diphtheria may be due to the action of pyogenic organisms. The extensive swelling of the tissues of the neck, sometimes attended by suppuration in the glands, and also various hæmorrhagic conditions, have been found to be associated with their presence;



in fact, in some cases the diphtheritic lesion enables them to get a foothold in the tissues, where they exert their usual action and may lead to extensive suppurative change, to septic poisoning or to septicæmia. In cases where a gangrenous process is superadded, a great variety of organisms may be present, some of them being anaerobic. Against such complications produced by other organisms anti-diphtheritic serum produces no favourable effect.

**Cultivation.** — The diphtheria bacillus grows best in cultures at the temperature of the body; growth still takes place at  $22^{\circ}\text{C}$ ., but ceases about  $20^{\circ}\text{C}$ . The best media are the following: Löffler's original medium (p. 41), solidified blood serum, alkaline blood serum (Lorrain Smith), blood agar, and the ordinary agar media. If inoculations be made on the surface of blood serum with a piece

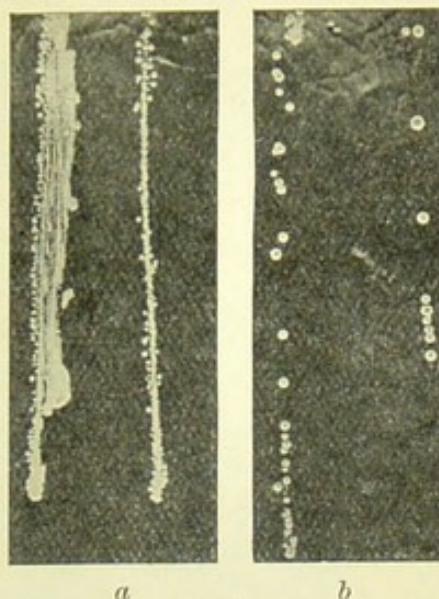


FIG. 113. — Cultures of the diphtheria bacillus on an agar plate; twenty-six hours' growth. (Natural size.)  
(a) Two successive strokes; (b) isolated colonies from the same plate.



FIG. 114. — Diphtheria colonies, two days old, on agar.  $\times 8$ .

reach 3 mm. in size, but when numerous they remain smaller. On the agar media the colonies have much the same appearance



(Fig. 113) but grow less quickly, and sometimes they may be



FIG. 115.—Diphtheria bacilli from a twenty-four hours' culture on agar. Stained with methylene-blue.  $\times 1000$ .

comparatively minute, so as rather to resemble those of the streptococcus pyogenes. In stroke cultures the growth forms a continuous layer of the same dull whitish colour, the margins of which often show single colonies partly or completely separated. On *gelatin* at  $22^{\circ}$  C. a puncture culture shows a line of dots along the needle track, whilst at the surface a small disc forms, rather thicker in the middle. In none of the media does any liquefaction occur. In *bouillon*

the organism produces a turbidity which soon settles to the bottom and forms a powdery layer on the wall of the vessel. If the growth is started on the surface and the flask is kept at rest, a distinct scum forms, and this is especially suitable for the development of toxin. Ordinary bouillon becomes acid during the first two or three days, and several days later again acquires an alkaline reaction. If, however, the bouillon is dextrose-free (p. 80) the acid reaction does not occur. The organism not only ferments glucose, but also galactose, lævulose, maltose, and usually also glycerine and lactose in



FIG. 116.—Diphtheria bacilli of larger size than in previous figure, showing also irregular staining of protoplasm. From a three days' agar culture. Stained with weak carbol-fuchsin.  $\times 1000$ .



older cultures; mannite and saccharose are not fermented (Graham-Smith).

In culture media the bacilli show the same characters as in the membrane, but the beading is a more marked feature, except in the very youngest cultures, and sometimes the stained protoplasm has a sort of septate appearance (Figs. 115, 116). They are at first fairly uniform in size and shape, but later involution forms may appear, especially on the less favourable media, such as agar. Many are swollen at their ends into club-shaped masses which stain deeply, and the protoplasm becomes broken up into globules with unstained parts between (Fig. 117). Some become thicker throughout, and segmented so as to appear like large cocci, and others show globules at their ends, the rest of the rod appearing as a faintly stained line. Occasionally branched forms are met with. The bacilli are non-motile, and do not form spores.

*Staining.*—They take up the basic aniline dyes, *e.g.* methylene-blue in watery solution, with great readiness, and stain deeply, the granules often giving the metachromatic reaction

as described. They also retain the colour in Gram's method, though they are more easily decolorised than the pyogenic cocci. By Neisser's stain (p. 115) the granules are stained almost black, the rest of the bacillary substance yellowish-brown, or by the new method, pink (Plate III., Fig. 12).

**Powers of Resistance, etc.**—In cultures the bacilli possess long duration of life; at room temperature they may survive for two months or longer. In the moist condition, whether in cultures or in membrane, they have a low power of resistance, being killed at 60° C. in a few minutes. On the other hand, in the dry condition they have great powers of endurance. In membrane which is perfectly dry, for example, they can resist a temperature of 98° C. for an hour. Dried diphtheria membrane,

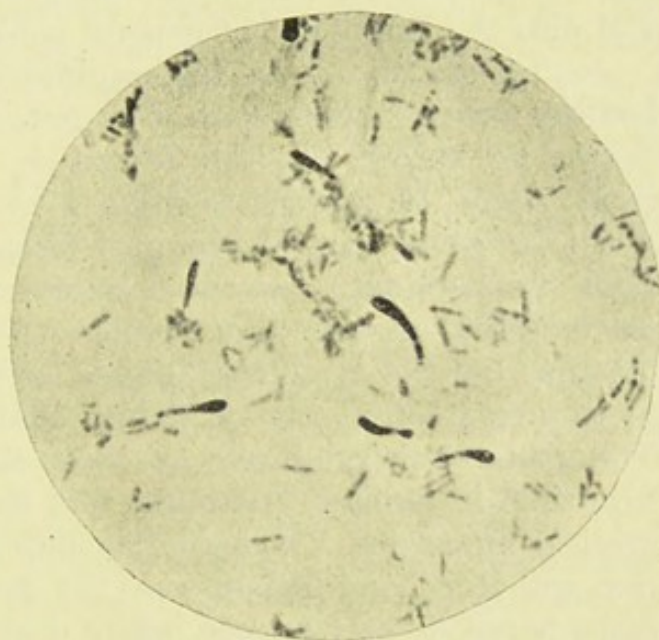


FIG. 117.—Involution forms of the diphtheria bacillus; from an agar culture of seven days' growth. See also Plate III., Fig. 13. Stained with carbol-thionin-blue.  $\times 1000$ .



kept in the absence of light and at the room temperature, has been proved to contain diphtheria bacilli still living and virulent at the end of several months. The presence of light, moisture, or a higher temperature, causes them to die out more rapidly. Corresponding results have been obtained with bacilli obtained from cultures and kept on dried threads. These facts, especially with regard to drying, are of great importance, as they show that the contagium of diphtheria may be preserved for a long time in the dried membrane.

**Effects of Inoculation.**—In considering the effects produced in animals by experimental inoculations of pure cultures, we have to keep in view the local changes which occur in diphtheria, and also the symptoms of general poisoning.

As Löffler stated in his original paper, inoculation of the healthy mucous membranes of various animals with pure cultures causes no lesion, but the formation of false membrane may result when the surface is injured by scarification or otherwise. A similar result may be obtained when the trachea is inoculated after tracheotomy has been performed. In this case the surrounding tissues may become the seat of a blood-stained oedema, and the lymphatic glands become enlarged, the general picture resembling pretty closely that of laryngeal diphtheria. The membrane produced by such experiments is usually less firm than in human diphtheria, and the bacilli in the membrane are less numerous. Rabbits inoculated after tracheotomy often die, and Roux and Yersin were the first to observe that in some cases paralysis may appear before death.

*Subcutaneous injection* in guinea-pigs of diphtheria bacilli in a suitable dose produces death within thirty-six hours. At the site of inoculation there is usually a small patch of greyish membrane, whilst in the tissues around there is extensive inflammatory oedema, often associated with hæmorrhages, and there is also some swelling of the corresponding lymphatic glands. The internal organs show general congestion, the suprarenal capsules being especially reddened and often hæmorrhagic. The renal epithelium may show cloudy swelling, and there is often effusion into the pleural cavities. After injection the bacilli increase in number for a few hours, but multiplication soon ceases, and at the time of death they may be less numerous than when injected. The bacilli remain practically local, cultures made from the blood and internal organs usually giving negative results, though sometimes a few colonies may be obtained. If a non-fatal dose of a culture be injected, a local necrosis of the skin and subcutaneous tissue may follow at the site of inoculation.



In rabbits, after subcutaneous inoculation, results of the same nature follow, but these animals are less susceptible than guinea-pigs, and the dose requires to be proportionately larger. Roux and Yersin found that after intravenous injection the bacilli rapidly disappeared from the blood, and when 1 c.c. of a broth culture had been injected no trace of the organisms could be detected by culture after twenty-four hours; nevertheless the animals died with symptoms of general toxæmia, nephritis also being often present (cf. Cholera, p. 455). The dog and sheep are also susceptible to inoculation with virulent bacilli, but the mouse and rat enjoy a high degree of immunity.

Klein found that cats also were susceptible to inoculation. The animals usually die after a few days, and *post-mortem* there is well-marked nephritis. He also found that after subcutaneous injection in cows, a vesicular eruption appeared on the teats of the udder, the fluid in which contained diphtheria bacilli. At the time of death the diphtheria bacilli were still alive and virulent at the site of injection. The most striking result of these experiments is that the diphtheria bacilli passed into the circulation and were present in the eruption on the udder. He considers that this may throw light on certain epidemics of diphtheria in which the contagion was apparently carried by the milk. Other observers, *e.g.* Abbott, have, however, failed to obtain similar results. Dean and Todd, in investigating an outbreak of diphtheria traceable to a milk supply, found a vesicular eruption on the teats of the udder in which diphtheria bacilli were present. They, however, came to the conclusion that these bacilli were not the cause of the eruption, but were the result of a secondary contamination, probably from the saliva of the milkers. The existence of a true diphtheria infection in cows must still be considered doubtful. A case of true diphtheria in the horse has been described by Cobbett.

**The Toxins of Diphtheria.**—As in the above experiments the symptoms of poisoning and ultimately a fatal result occur when the bacilli are diminishing in number, or even after they have practically disappeared, Roux and Yersin inferred that the chief effects were produced by toxins, and this supposition they proved to be correct. They showed that broth cultures of three or four weeks' growth freed from bacilli by filtration were highly toxic. The filtrate when injected into guinea-pigs and other animals produces practically the same effects as the living bacilli; locally there is fibrinous exudation but a considerable amount of inflammatory oedema, and, if the animal survive long enough, necrosis in varying degree of the superficial tissues may follow. The toxicity may be so great that .005 c.c. or even less may be fatal to a guinea-pig in five days.

After injection either of the toxin or of the living bacilli, when the animals survive long enough, paralytic phenomena



occasionally occur. The hind-limbs are usually affected first, the paralysis afterwards extending to other parts, though sometimes the fore-limbs and neck first show the condition. Sometimes symptoms of paralysis do not appear till two or three weeks after inoculation. After paralysis has appeared, a fatal result usually follows in the smaller animals, but in dogs recovery may take place. There is evidence that these paralytic phenomena are produced by toxone, as they specially occur when there is injected along with the toxin sufficient antitoxin to neutralise the more rapidly acting toxin proper. This toxone is supposed by Ehrlich to have a different toxic action, *i.e.* a different toxophorous group (p. 198), from that of the ordinary toxin; it produces the late nervous phenomena, while its local action on the tissues is very slight. It also has a weaker affinity for antitoxin, and thus much of it may be left unneutralised. It is to be noted in this connection that paralytic symptoms are of not uncommon occurrence in the human subject after treatment with antitoxin, the explanation of which occurrence is probably the same as that just given. One point of much interest is the high degree of resistance to the toxin possessed by mice and rats. Roux and Yersin, for example, found that 2 c.c. of toxin, which was sufficient to kill a rabbit in sixty hours, had no effect on a mouse, whilst of this toxin even  $\frac{1}{15}$  c.c. produced extensive necrosis of the skin of the guinea-pig.

*Preparation of the Toxin.*—The obtaining of a very active toxin in large quantities is an essential in the preparation of anti-diphtheritic serum. Certain conditions favour the development of a high degree of toxicity, namely, a free supply of oxygen, the presence of a large proportion of peptone or albumin in the medium, and the absence of substances which produce an acid reaction. In the earlier work a current of sterile air was made to pass over the surface of the medium, as it was found that by this means the period of acid reaction was shortened and the toxin formation favoured. This expedient is now considered unnecessary if an alkaline medium free from glucose is used, as in this no acid reaction is developed; it is then sufficient to grow the cultures in shallow flasks. The absence of glucose may be attained by the method described above (p. 80), or by using for the preparation of the meat extract flesh which is just commencing to putrefy (Spronck). L. Martin uses a medium composed of equal parts of freshly prepared peptone (by digesting pigs' stomachs with HCl at 35° C.), and glucose-free veal bouillon. By this medium he has obtained a toxin of which  $\frac{1}{500}$  c.c. is the fatal dose to a guinea-pig of 500 grms. Park



and Williams and also Dean find that the amount of glucose present in ordinary beef is not sufficient to interfere with toxin formation, provided that a considerable amount of peptone, 2 per cent., be added, and the medium be made sufficiently alkaline; after making it neutral to litmus they add to each litre of broth 7 c.c. of normal caustic soda solution. There is in all cases a period at which the toxicity reaches a maximum; Roux and Yersin found this period to be two to three weeks, but later observers find that in favourable conditions the greatest toxicity is reached about the tenth to twelfth day, sometimes even earlier. It may be added that the power of toxin formation varies much in different races of the diphtheria bacillus, and that many may require to be tested ere one suitable is obtained.

*Properties and Nature of the Toxin.*—The toxic substance in filtered cultures is a relatively unstable body. When kept in sealed tubes in the absence of light, it may preserve its powers little altered for several months, but, on the other hand, it gradually loses them when exposed to the action of light and air. As will be shown later (p. 527), the toxin probably does not become destroyed, but its toxophorous group suffers a sort of deterioration, so that a toxoid is formed which has still the power of combining with antitoxins. Heating at 58° C. for two hours destroys the toxic properties in great part, but not altogether. When, however, the toxin is evaporated to dryness, it has much greater resistance to heat. One striking fact, discovered by Roux and Yersin, is that after an organic acid, such as tartaric acid, is added to the toxin the toxic property disappears, but it can be in great part restored by again making the fluid alkaline.

Guinochet found that toxin was formed by the bacilli when grown in urine with no proteid bodies present. After growth had taken place he could not detect proteid bodies in the fluid, but, on account of the very minute amount of toxin present, their absence could not be excluded. Uschinsky also found that toxic bodies were produced by diphtheria bacilli when grown in a proteid-free medium.<sup>1</sup> It follows from this that if the toxin is a proteid, it may be formed by synthesis within the bodies of the bacilli. Brieger and Boer have separated from diphtheria cultures a toxic body which gives no proteid reaction (*vide p.*

<sup>1</sup> Uschinsky's medium has the following composition: water, 1000 parts; glycerin, 30-40; sodium chloride, 5-7; calcium chloride, .1; magnesium sulphate, .2-4; di-potassium phosphate, .2-25; ammonium lactate, 6-7; sodium asparaginate, 3-4.



193). Whether or not diphtheria toxin is of proteid nature must, however, be considered to be a question not yet settled.

Toxic bodies have also been obtained from the tissues of those who have died from diphtheria. Roux and Yersin, by using a filtered watery extract from the spleen from very virulent cases of diphtheria, produced in animals death after wasting and paralysis, and also obtained similar results by employing the urine. The subject of toxic bodies in the tissues has, however, been specially worked out by Sidney Martin. He has separated from the tissues, and especially from the spleen, of patients who have died from diphtheria, by precipitation with alcohol, chemical substances of two kinds, namely, albumoses (proto- and deuto-, but especially the latter), and an organic acid. The albumoses, when injected into rabbits, especially in repeated doses, produce fever, diarrhoea, paresis, and loss of weight, with ultimately a fatal result. He further found that this paresis is due to well-marked changes in the nerves. Substances obtained from diphtheria membrane have an action like that of the bodies obtained from the spleen, but in higher degree. Martin considers that this is due to the presence in the membrane of an enzyme which has a proteolytic action within the body, resulting in the formation of poisonous albumoses.

**Immunity.**—This is described in the general chapter on Immunity. It is sufficient to state here that a high degree of immunity, against both the bacilli and their toxins, can be produced in various animals by gradually increasing doses either of the bacilli or of their filtered toxins (*vide* Chapter XXI.).

**Variations in the Virulence of the Diphtheria Bacillus.**—In cultures on serum the diphtheria bacilli retain their virulence fairly well, but they lose it much more quickly on less suitable media, such as glycerin agar. Roux and Yersin found that, when the bacilli were grown at an abnormally high temperature, namely,  $39.5^{\circ}$  C., and in a current of air, the virulence diminished so much that they became practically innocuous. When the virulence was much diminished, these observers found that it could be restored if the bacilli were inoculated into animals along with streptococci, inoculation of the bacilli alone not being successful for this purpose. If, however, the virulence had fallen very low, even the presence of the streptococci was insufficient to restore it. The virulence is tested by the amount of living bacilli necessary to produce a fatal result on injection, and is to be distinguished from the power of producing toxin in a fluid medium; as pointed out by Dean, the two properties often do not correspond. It has been abundantly established



that, after the cure of the disease, the bacilli may persist in the mouth for weeks, though they often quickly disappear. Roux and Yersin found, by making cultures at various stages after the termination of the disease, that these bacilli in the mouth gradually become attenuated.

L. Martin, moreover, has shown that some races of diphtheria bacillus are so attenuated that 1 c.c. of a twenty-four hours' growth in bouillon does not cause death in a guinea-pig, yet their true nature is shown not only by their microscopical characters, etc., but also by the fact that on more prolonged growth they form small quantities of toxin, which is neutralisable by diphtheria antitoxin. The persistence of these non-virulent bacilli in the throats of those who have suffered from the disease, and their occasional presence in quite healthy individuals, may manifestly be of importance in relation to the continuance of the infection and the reappearance of epidemics of the disease.

#### BACILLI ALLIED TO THE DIPHTHERIA BACILLUS.

Bacteriological examinations carried on within recent times have shown that the diphtheria bacillus is merely a member of a group of organisms with closely allied characters which are of common occurrence and have a wide distribution. The terms "pseudo-diphtheria bacilli" and "diphtheroid bacilli" have been applied in a loose way to organisms which resemble the diphtheria bacillus microscopically, especially as regards the beaded appearance. Such bacilli have been obtained from the mouth, nose, skin, genital organs, and even from the blood in certain diseases. They are to be met with sometimes in conditions of health, and they have been obtained from many diverse morbid conditions—from skin diseases, from coryza, from leprosy, and even from general paralysis of the insane. As has been found with other groups, the differentiation is a matter of considerable difficulty. Some are practically identical with the diphtheria bacillus both morphologically and culturally, and a few even give the characteristic reaction with Neisser's stain; others, again, differ in essential particulars. The fermentative action on sugars<sup>1</sup> has also been called into requisition as a means of distinguishing them, but the results obtained cannot be said to be of a definite character, and further work is necessary. It may be stated, however, that most observers have found the diphtheria bacillus of all the members of the group to be the most active acid-producer, though here the

<sup>1</sup> *Vide* a paper by Graham-Smith, *Journal of Hygiene*, vi. 286.



difference seems to be one of degree rather than of kind. The absence of the power of fermenting certain sugars, notably glucose, may, however, be accepted in any particular case as sufficient to exclude the organism from being the diphtheria bacillus. From these facts, and from what has been stated with regard to attenuated diphtheria bacilli, it will be seen that an absolute decision as to the nature of a suspected organism may in some cases be a practical impossibility. It may be that some of the "diphtheroid" organisms cultivated have really been non-virulent diphtheria bacilli. The bearing of this on the practical means of diagnosis will be discussed below.

Ford Robertson and his co-workers have obtained from numerous cases of general paralysis of the insane cultures of a diphtheroid organism, which he considers is the chief agent in producing the condition of chronic intoxication underlying the disease. The organism has been obtained from various situations, including the central nervous system, but it seems to flourish specially in the respiratory and alimentary tracts. It closely resembles the diphtheria bacillus; the morphological and cultural characters are indeed practically identical, but the diphtheroid bacillus is non-pathogenic to the guinea-pig. Robertson and Shennan found that when administered to rats by the alimentary tract it produced certain nervous symptoms which were associated with changes in the brain of the same order as those in general paralysis. Further research on this subject is still necessary.

The term "pseudo-diphtheria bacillus" is often restricted by present writers to an organism frequently met with in the throat. This organism, which is also known as Hofmann's bacillus, merits a separate description.

**Hofmann's Bacillus — Pseudo-Diphtheria Bacillus.** — This organism, described by Hofmann in 1888, is probably the same as one observed by Löffler in the previous year, and regarded by him as being a distinct species from the diphtheria bacillus. The organism is a shorter bacillus than the diphtheria bacillus, with usually a single unstained septum running across it, though sometimes there may be more than one (Fig. 118). The typical beaded appearance is rarely seen, and the characteristic reaction with Neisser's stain is not given, though in old cultures a few granules which stain deeply may sometimes be found. It grows readily on the same media as the diphtheria bacillus, but the colonies are whiter and more opaque. It does not form acid from glucose or other sugars, and is non-pathogenic to the guinea-pig. Involution forms may sometimes be produced by



it. It is usually a relatively easy matter to distinguish this organism from the diphtheria bacillus.

Hofmann's bacillus is of comparatively common occurrence in the throat in normal as well as diseased conditions, including diphtheria; it seems to be specially frequent in poorly nourished children of the lower classes. Cobbett found it 157 times in an examination of 692 persons examined, of whom 650 were not suffering from diphtheria. Boycott's statistics show that the time of its maximum seasonal prevalence precedes that of the diphtheria bacillus. To what extent, if any, it is responsible for pathological changes in the throat, must be considered a question which is not yet settled. Hewlett and Knight have found evidence that a true diphtheria bacillus may assume the characters of Hofmann's bacillus, but attempts to effect the transformation have met with negative results in the hands of other observers. The general opinion is that the two organisms are distinct species with comparatively easily distinguished characters.

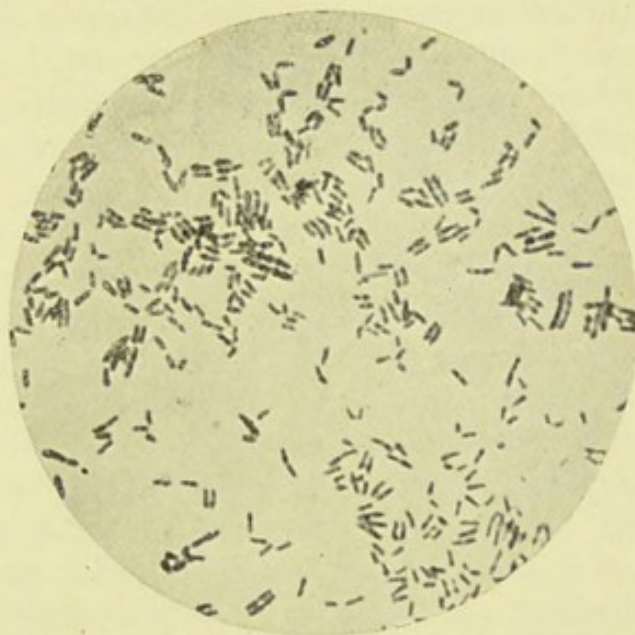


FIG. 118.—Pseudo-diphtheria bacillus (Hofmann's). Young agar culture. See also Plate III., Fig. 14.

Stained with thionin-blue.  $\times 1000$ .

**Xerosis Bacillus.**—This term has been given to an organism first observed by Kuschbert and Neisser in xerosis of the conjunctiva, and which has been since found in many other affections of the conjunctiva and also in normal conditions. Morphologically it is practically similar to the diphtheria bacillus, and even in cultures presents very minor differences; it, however, grows more slowly on serum, and its colonies have a tougher consistence and a more irregular margin. It is non-virulent to animals, and does not produce an acid reaction in glucose bouillon, or does so to only a slight extent; in this way it can be distinguished from the diphtheria bacillus. It is still doubtful whether it is pathogenic to the human subject. Its morphological characters are shown in Fig. 119.

**Action of the Diphtheria Bacillus — Summary.**—From a study of the morbid changes in diphtheria and of the results produced experimentally by the bacillus and its toxins, the



following summary may be given of its action in the body. Locally, the bacillus produces inflammatory change with fibrinous exudation, but at the same time cellular necrosis is also an outstanding feature. Though false membranes have not been produced by the toxins, a necrotic action may result when these are injected subcutaneously. The toxins also act upon the blood vessels, and hence œdema and tendency to hæmorrhage are produced; this action on the vessels is also exemplified by the general congestion of organs. The hyaline change in the walls of arterioles and capillaries so often met with in diphtheria is another example of the action of the toxin. The toxins have also a pernicious action on highly developed cells and on nerve

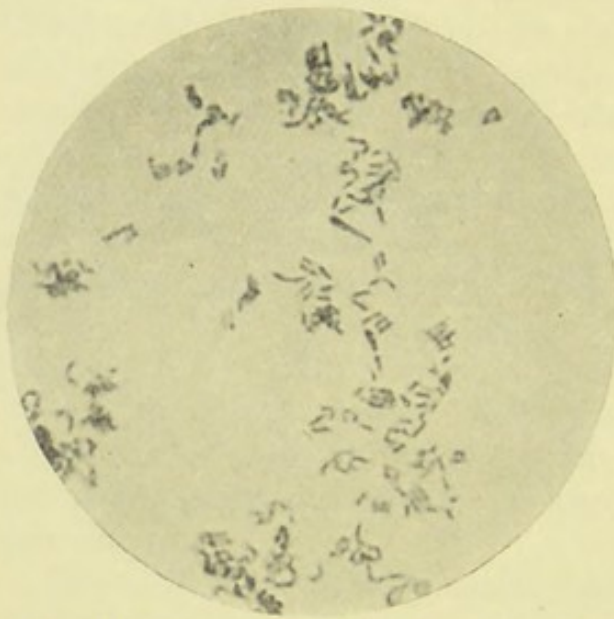


FIG. 119.—Xerosis bacillus from a young agar culture.  $\times 1000$ .

fibres. Thus in the kidney cloudy swelling occurs, which may be followed by actual necrosis of the secreting cells, and along with these changes albuminuria is present. The action is also well seen in the case of the muscle fibres of the heart, which may undergo a sort of hyaline change, followed by granular disintegration or by an actual fatty degeneration. These changes are of great importance in relation to heart failure in the disease.

Changes of a somewhat similar nature have been recently observed in the nerve cells of the central nervous system, those lying near the capillaries, it is said, being affected first. There is also the striking change in the peripheral nerves, which is shown first by the disintegration of the medullary sheaths as already described. It is, however, still a matter of dispute to what extent these nerve lesions are of primary nature or secondary to changes in the nerve cells.

**Methods of Diagnosis.**—The bacteriological diagnosis of diphtheria depends on the discovery of the bacillus. As the bacillus occurs in largest numbers in the membrane, a portion of this should be obtained whenever it is possible, and transferred to a sterile test-tube. (The tube can be readily sterilised by boiling some water in it.) If, however, membrane cannot be



obtained, a scraping of the surface with a platinum loop may be sufficient. Where the membrane is confined to the trachea the bacilli are often present in the secretions of the pharynx, and may be obtained from that situation by swabbing it with cotton-wool (non-antiseptic), the swab being put into a sterile tube or bottle for transport. A convenient method is to twist a piece of cotton-wool round the roughened end of a piece of very stout iron wire, 6 inches long, and pass the other end of the latter through a cotton plug inserted in the mouth of a test-tube (compare Fig. 46, the wire taking the place of the pipette), and sterilise. In use the wire and plug are extracted in one piece, and after swabbing are replaced in the tube for transit. A scraping may be made off the swab for microscopic examination, and the swab may be smeared over the surface of a serum tube to obtain a culture. This method of taking and treating swabs is that usually employed in routine public health work. The results obtained ordinarily suffice for the diagnosis of cases suspected to be diphtheritic in nature.

The means for identifying the bacillus are (a) *By Microscopical Examination*.—For microscopical examination it is sufficient to tease out a piece of the membrane with forceps and rub it on a cover-glass; if it be somewhat dry, a small drop of normal saline should be added. The films are then dried in the usual way, and stained with any ordinary basic stain, though methylene-blue is on the whole to be preferred, used either as a saturated watery solution or in the form of Löffler's solution. After staining for two or three minutes, the films are washed in water, dried, and mounted. As a rule no decolorising is necessary, as the blue does not overstain. Neisser's stain (p. 115) may also be used with advantage, although it is to be noted that sometimes in a secretion the diphtheria bacillus does not react typically to this stain. Any secretion from the pharynx or other part is to be treated in the same way. The value of microscopical examination alone depends much upon the experience of the observer. In some cases the bacilli are present in characteristic form in such numbers as to leave no doubt in the matter. In other cases a few only may be found, mixed with large numbers of other organisms, and sometimes their characters are not sufficiently distinct to render a definite opinion possible. The bacillus may be frequently obtained by means of cultures, when the result of microscopical examination is inconclusive. As already said, however, microscopical examination alone is more reliable after the observer has had experience in examining cases of diphtheria and making cultures from them.



(b) *By making Cultures.*—For this purpose a piece of the membrane should be separated by forceps from the pharynx or other part when that is possible. It should be then washed well in a tube containing sterile water, most of the surface impurities being removed in this way. A fragment is then fixed in a platinum loop by means of sterile forceps, and a series of stroke cultures is made on the surface of any of the media mentioned (p. 401), the same portion of the membrane being always brought into contact with the surface. The tubes are then incubated at 37° C., and, in the case of the serum media and blood-agar, the circular colonies of the diphtheria bacillus are well formed within twenty-four hours. A small portion of a colony is then removed by means of a platinum needle, stained, and examined in the usual way, Neisser's stain being also applied. When the material has been taken from the throat, an organism with all the morphological and cultural characters of the diphtheria bacillus may for *all practical purposes* be accepted as the diphtheria bacillus.

In cases where a suspicion arises that the organism found is a pseudo-diphtheria bacillus, bouillon containing a trace of glucose should be inoculated and incubated at 37° C. The reaction should be tested after one and after two days' growth. If it remains alkaline, the diphtheria bacillus may be excluded. If an acid reaction results, then all the microscopical and cultural characters must be carefully observed, and the virulence of the bacillus may be ascertained by inoculating a guinea-pig, say with 1 c.c. of a broth culture of two days' growth. (See also pp. 404, 410.) A fatal result with characteristic appearances may be taken as positive evidence; but if the animal survive there is still theoretically the possibility that the organism is an attenuated diphtheria bacillus (p. 408).



## CHAPTER XVII.

### TETANUS<sup>1</sup>: CONDITIONS CAUSED BY OTHER ANAEROBIC BACILLI.

**Introductory.** — Tetanus (German, *Wundstarrkrampf*) is a disease which in natural conditions affects chiefly man and the horse. Clinically it is characterised by the gradual onset of general stiffness and spasms of the voluntary muscles, commencing in those of the jaw and the back of the neck, and extending to all the muscles of the body. These spasms are of a tonic nature, and, as the disease advances, succeed each other with only a slight intermission of time. There are often, towards the end of a case, fever and rise of respiration and pulse-rate. The disease is usually associated with a wound received from four to fourteen days previously, and which has been defiled by earth or dung. The disease is, in the majority of cases, fatal.

**Historical.** — The general association of the development of tetanus with the presence of wounds, though these might be very small, suggested that some infection took place through the latter, but for long nothing was known as to the nature of this infection. Carle and Rattone in 1884 announced that they had produced the disease in a number of animals by inoculation with material from a wound in tetanus. They thus demonstrated the transmissibility of the disease. Nicolaier (1885) infected mice and rabbits with garden earth, and found that many of them developed tetanus. Suppuration occurred in the neighbourhood of the point of inoculation, and in this pus, besides other organisms, there was always present, when tetanus had occurred, a bacillus having certain constant microscopic characters. Inoculation of fresh animals with such pus reproduced the disease. Nicolaier's attempts at its isolation by the ordinary gelatin plate-culture method were, however, unsuccessful. He succeeded in getting it to grow in liquid blood serum, but always in

<sup>1</sup> This disease is not to be confused with the "tetany" of infants, which in its essential pathology probably differs from tetanus (*vide* Frankl-Hochwart, "Die Tetanie der Erwachsenen," Vienna, 1907). This remark of course does not exclude the possibility of the occurrence of true tetanus in very young subjects.



mixture with other organisms. Infection of animals with such a culture produced the disease. These results were confirmed by Rosenbach, who, though failing to obtain a pure culture, cultivated the other organisms present, and inoculated them, but with negative results. He further pointed out, as characteristic of the bacillus, its development of terminal spores. In 1889, Kitasato succeeded in isolating from the local suppuration of mice inoculated from a human case, several bacilli, only one of which, when injected in pure culture into animals, caused the disease, and which was now named the *b. tetani*. This organism is the same as that observed by Nicolaier and Rosenbach. Kitasato found that the cause of earlier culture failures was the fact that it could only grow in the absence of oxygen. The pathology of the disease was further elucidated by Faber, who, having isolated bacterium-free poisons from cultures, reproduced the symptoms of the disease.

**Bacillus Tetani.**—If in a case of tetanus naturally arising in man, there be a definite wound with pus formation or necrotic change, the bacillus tetani may be recognised in film preparations from the pus, if the characteristic spore formation has occurred (Fig. 120). If, however, the tetanus bacilli have not formed spores, they appear as somewhat slender rods, without presenting any characteristic features. There is usually present in such pus a great variety of other organisms—cocci and bacilli. The characters of the bacillus are, therefore, best studied in cultures. It is then seen to be a slender organism, usually about  $4\ \mu$  to  $5\ \mu$  in length and  $\cdot 4\ \mu$  in thickness, with somewhat rounded ends. Besides occurring as short rods it also develops filamentous forms, the latter being more common in fluid media. It stains readily by any of the usual stains and also by Gram's method. A feature in it is the uniformity with which the protoplasm stains. It is very slightly motile, and its motility can be best studied in an anaerobic hanging-drop preparation. When stained by the special methods already described, it is found to possess numerous delicate flagella attached both at the sides and at the ends (Fig. 121). These flagella, though they may be of considerable length, are usually curled up close to the body of the bacillus. The formation of flagella can be best studied in preparations made from surface anaerobic cultures (p. 68). As is the case with many other anaerobic flagellated bacteria, the flagella, on becoming detached, often become massed together in the form of spirals of striking appearance (Fig. 122). At incubation temperature *b. tetani* readily forms spores, and then presents a very characteristic appearance. The spores are round, and in diameter may be three or four times the thickness of the bacilli. They are developed at one end of a bacillus, which thus assumes what is usually described as the drumstick form (Figs. 120, 123). In



a specimen stained with a watery solution of gentian-violet or methylene-blue, the spores are uncoloured except at the periphery, so that the appearance of a small ring is produced; if a powerful stain such as carbol-fuchsin be applied for some time, the spores become deeply coloured like the bacilli. Further, especially if

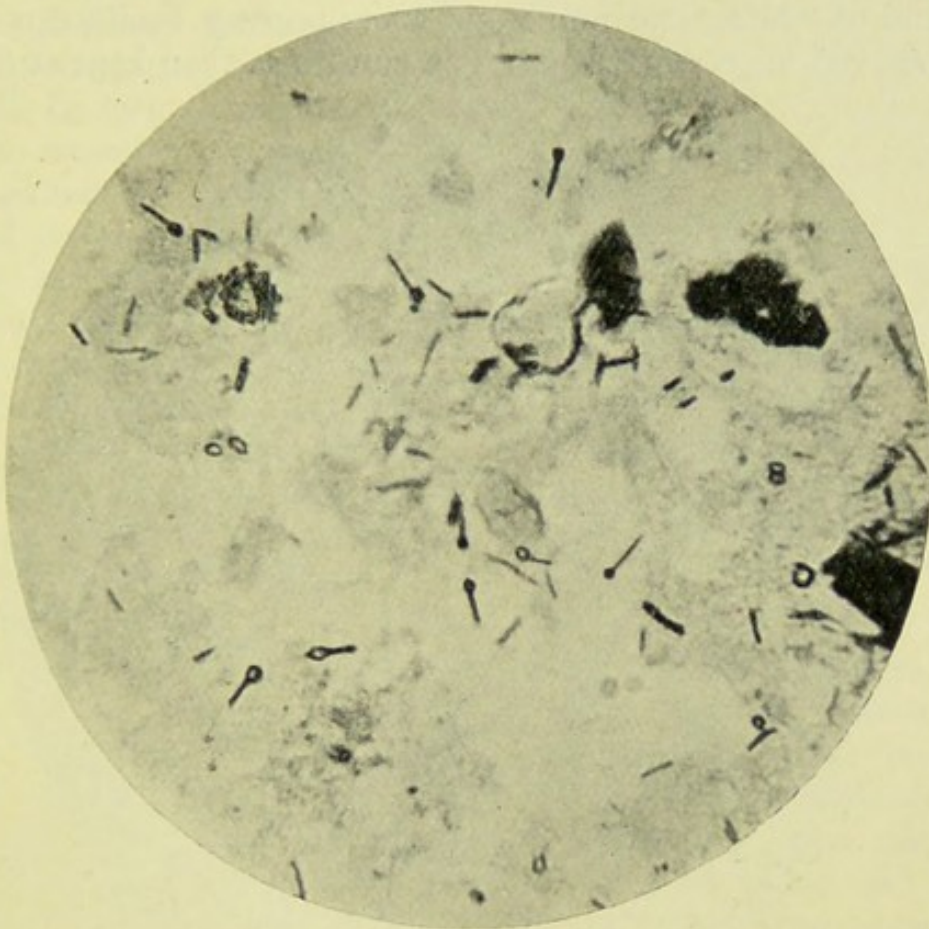


FIG. 120.—Film preparation of discharge from wound in a case of tetanus, showing several tetanus bacilli of "drumstick" form. (The thicker bacillus present is not a tetanus bacillus, but a putrefactive anaerobe which was obtained in pure culture from the wound.)

Stained with gentian-violet.  $\times 1000$ .

the preparation be heated, many spores may become free from the bacilli in which they were formed.

**Isolation.**—The isolation of the tetanus bacillus is somewhat difficult. By inoculation experiments in animals, its natural habitat has been proved to be garden soil, and especially the contents of dung-heaps, where it probably leads a saprophytic existence, though its function as a saprophyte is unknown. From such sources and from the pus of wounds in tetanus, occurring naturally or experimentally produced, it has been isolated by



means of the methods appropriate for anaerobic bacteria. The best methods for dealing with such pus are as follows:—

(1) The principle is to take advantage of the resistance of the spores of the bacillus to heat. A sloped tube of inspissated serum or a deep tube of glucose agar is inoculated with the pus and incubated anaerobically at 37° C. for forty-eight hours, at the end of which time numerous spore-bearing bacilli can often be observed microscopically. The culture is then kept at 80° C.

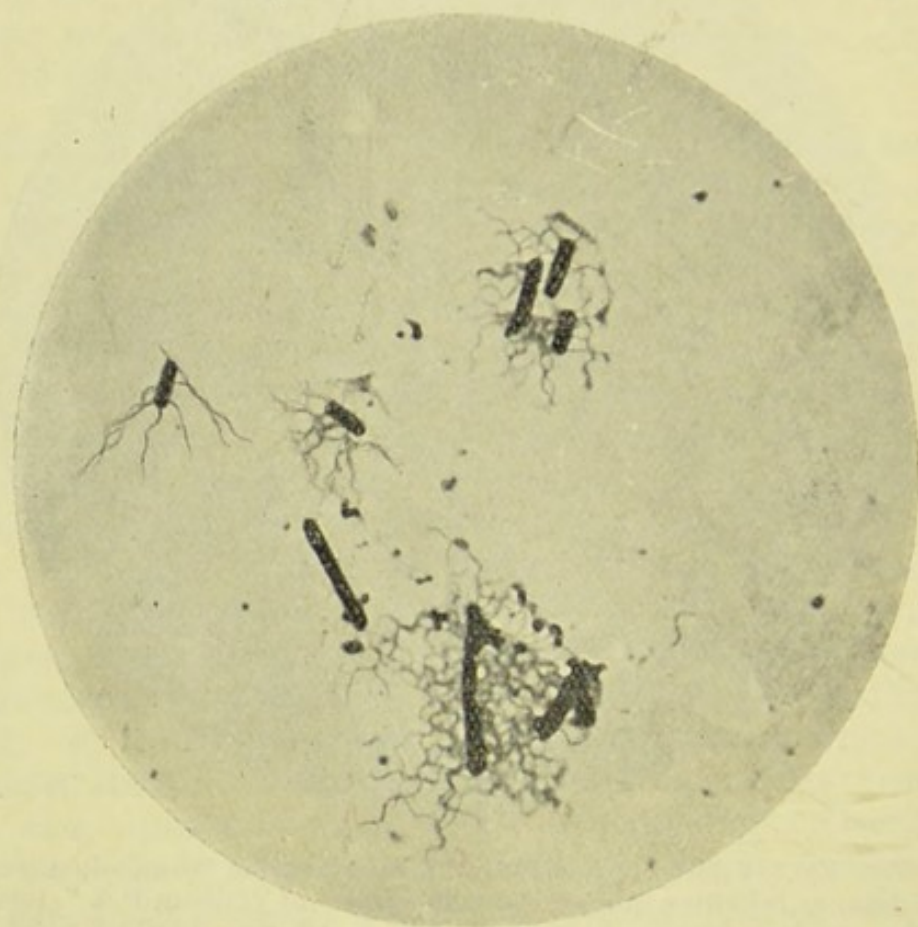


FIG. 121.—Tetanus bacilli, showing flagella.  
Stained by Rd. Muir's method.  $\times 1000$ .

for from three-quarters to one hour, with the view of killing all organisms except those which have spored. A loopful is then added to glucose gelatin, and roll-tube cultures are made in the usual way and kept in an atmosphere of hydrogen at 22° C.; after five days the plates are ready for examination. Kitasato compares the colonies in gelatin plates to those of the *b. subtilis*. They consist of a thick centre with shoots radiating out on all sides. They liquefy the gelatin more slowly than the *b. subtilis*. This method of isolation is not always successful, partly because along with the tetanus bacilli, both in its natural



habitats outside the body and in the pus of wounds, other spore-forming obligatory and facultative anaerobes occur, which grow faster than the tetanus bacillus, and thus overgrow it.

(2) If in any discharge the spore-bearing tetanus bacilli be seen on microscopic examination, then a method of isolation based on the same principle as the last may be adopted. Inoculations with the suspected material are made in half a dozen deep tubes of glucose bouillon, previously raised to a temperature of  $100^{\circ}\text{C}$ . After inoculation they are again placed in boiling water and kept for varying times, say

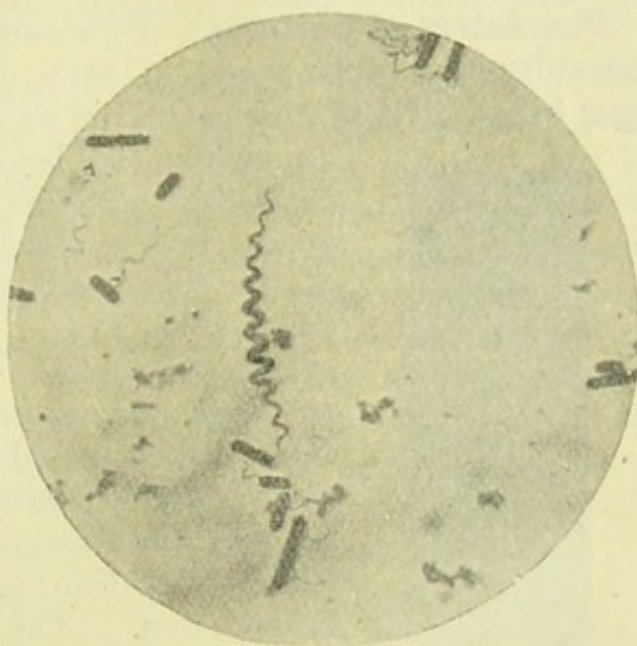


FIG. 122.—Spiral composed of numerous twisted flagella of the tetanus bacillus. Stained by Rd. Muir's method.  $\times 1000$ .



FIG. 123.—Tetanus bacilli; some of which possess spores. From a culture in glucose agar, incubated for three days at  $37^{\circ}\text{C}$ . See also Plate IV., Fig. 20.

Stained with carbol-fuchsin.  $\times 1000$ .

for half a minute, for one, three, four, five, and six minutes respectively. They are then plunged in cold water till cool, and thereafter placed in the incubator at  $37^{\circ}\text{C}$ ., in the hope that in one or other of the tubes all the organisms present will have been killed, except the tetanus spores which can develop in pure culture. A series of deep glucose agar tubes may also be inoculated from the series of bouillon tubes.

(3) Some method of anaerobically making plates, such as that of

Bullock, may be employed. The isolation of the tetanus



bacillus is in many cases a difficult matter, and several methods should always be tried.

**Characters of Cultures.**—Pure cultures having been obtained, sub-cultures can be made in deep upright glucose gelatin or agar tubes. On *glucose gelatin* in such a tube there commences, an inch or so below the surface, a growth consisting of fine straight threads, rather longer in the lower than in the upper parts of the tube, radiating out from the needle track (Fig. 124). Slow liquefaction of the gelatin takes place, with slight gas formation. In *agar* the growth is somewhat similar, consisting of small nodules along the needle track, with irregular short offshoots passing out into the medium (Fig. 128, A). There is slight formation of gas, but, of course, no liquefaction. On anaerobic agar plates colonies have under a low power a feathery outline (Fig. 125). Growth also occurs in *blood serum* and also in *glucose bouillon* under anaerobic conditions. The latter is the medium usually employed for obtaining the soluble products of the organism. There is in it at first a slight turbidity, and later a thin layer of a powdery deposit on the walls of the vessel. All the cultures give out a peculiar burnt odour of rather unpleasant character.

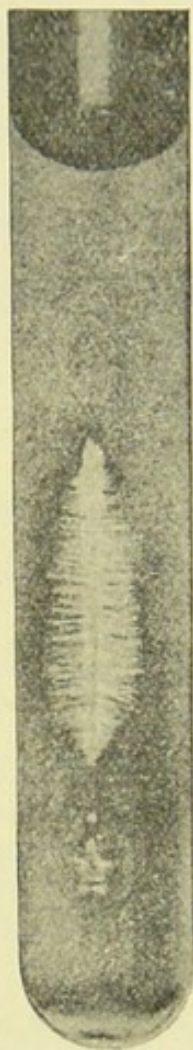


FIG. 124.—Stab culture of the tetanus bacillus in glucose gelatin, showing the lateral shoots (Kitasato).

Natural size.

**Conditions of Growth, etc.**—The *b. tetani* grows best at 37° C. The minimum growth temperature is about 14° C., and below 22° C. growth takes place very slowly. Growth takes place in the absence of oxygen, the organism being an *anaerobe*. Sporulation may commence at the end of twenty-four hours in cultures grown at 37° C.,—much later at lower temperatures. Like other spores, those of tetanus are extremely resistant. They can usually withstand boiling for five minutes, and can be kept in a dry condition for many months without being killed or losing their virulence. They have also high powers of resistance to antiseptics.

**Pathogenic Effects.**—The proof that the *b. tetani* is the cause of tetanus is complete. It can be isolated in pure culture, and



when re-injected in pure culture it reproduces the disease. It may be impossible to isolate it from some cases of the disease, but the cause of this very probably is the small numbers in which it sometimes occurs.

(a) *The Disease as arising naturally.*—The disease occurs naturally, chiefly in horses and in man. Other animals may, however, be affected. There is usually some wound, often of a ragged character, which has either been made by an object soiled with earth or dung, or which has become contaminated with these substances. There is often a purulent or foetid discharge, though this may be absent. In tetanus following

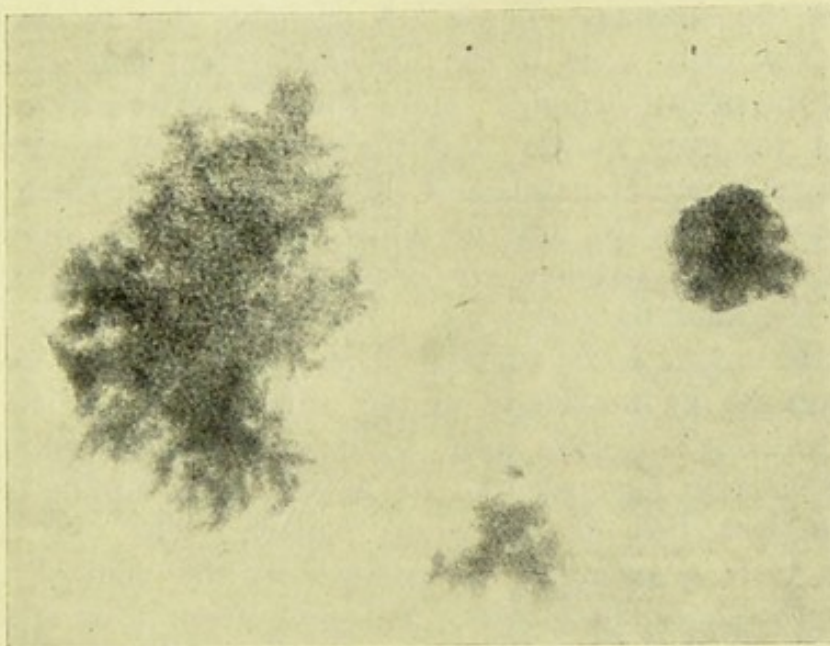


FIG. 125.—Colonies of the tetanus bacillus on anaerobic agar plates, seven days old.  $\times 50$ .

clean operation wounds, catgut ligatures may be the source of infection. Microscopic examination of sections may show at the edges of the infected wound necrosed tissue in which the tetanus bacilli may be very numerous. If a scraping from the wound be examined microscopically, bacilli resembling the tetanus bacillus may be recognised. If these have spored, there can be practically no doubt as to their identity, as the drumstick appearance which the terminal spore gives to the bacillus is not common among other bacilli. Care must be taken, however, to distinguish it from other thicker bacilli with oval spores placed at a short distance from their extremities, such forms being common in earth, etc., and also met with in contaminated wounds. It is important to note that the wound through which infection has



taken place may be very small, in fact, may consist of a mere abrasion. In some cases, especially in the tropics, it may be merely the bite of an insect. The absence of a definite channel of infection has given rise to the term "idiopathic" tetanus. There is, however, practically no doubt that all such cases are true cases of tetanus, and that in all of them the cause is the *b. tetani*. The latter has also been found in the bronchial mucous membrane in some cases of the so-called rheumatic tetanus, the cause of which is usually said to be cold; infection of the intestinal mucosa may also occur.

The pathological changes found *post mortem* are not striking. There may be hæmorrhages in the muscles which have been the subject of the spasms. These are probably due to mechanical causes. Naturally it is in the nervous system that we look for the most important lesions. Here there is ordinarily a general redness of the grey matter, and the most striking feature is the occurrence of irregular patches of slight congestion which are not limited particularly to grey or white matter, or to any tract of the latter. These patches are usually best marked in the grey matter of the medulla and pons. Microscopically there is little of a definite nature to be found. There is congestion, and there may be minute hæmorrhages in the areas noted by the naked eye. The ganglion cells may show appearances which have been regarded as degenerative in nature, and similar changes have been described in the white matter. The only marked feature is thus a vascular disturbance in the central nervous system, with a possible tendency to degeneration in its specialised cells. Both of these conditions are probably due to the action of the toxins of the bacillus. In the case of the cellular degenerations the cells have been observed to return to the normal under the curative influence of the antitoxins (*vide infra*). In the other organs of the body there are no constant changes.

We have said that the general distribution of pathogenic bacteria throughout the body is probably a relative phenomenon, and that bacteria usually found locally may occur generally, and *vice versa*. With regard to the tetanus bacillus, it is, however, probably the case that very rarely, if ever, are the organisms found anywhere except in the local lesion.

(b) *The Artificially-produced Disease*.—The disease can be communicated to animals by any of the usual methods of inoculation, but does not arise in animals fed with bacilli, whether these contain spores or not. Kitasato found that pure cultures, injected subcutaneously or intravenously, caused death in mice, rats, guinea-pigs, and rabbits. In mice, symptoms appear in a



day, and death occurs in two or three days, after inoculation with a loopful of a bouillon culture. The other animals mentioned require larger doses, and death does not occur so rapidly. Usually in animals injected subcutaneously the spasms begin in the limb nearest the point of inoculation. In the case of intravenous inoculation the spasms begin in the extensor muscles of the trunk, as in the natural disease in man. After death there is found slight hyperæmia without pus formation, at the seat of inoculation. The bacilli diminish in number, and may be absent at the time of death. The organs generally show little change.

Kitasato stated that in his earlier experiments the quantity of culture medium injected along with the bacilli already contained enough of the poisonous bodies formed by the bacilli to cause death. The symptoms came on sooner than by the improved method mentioned below, and were, therefore, due to the toxins already present. In his subsequent work, therefore, he employed splinters of wood soaked in cultures in which spores were present, and subsequently subjected for one hour to a temperature of 80° C. The latter treatment not only killed all the vegetative forms of the organism, but, as we shall see, was sufficient to destroy the activity of the toxins. When such splinters are introduced subcutaneously, death results by the development of the spores which they carry. In this way he completed the proof that the bacilli by themselves can form toxins in the body and produce the disease. Further, if a small quantity of garden earth be placed under the skin of a mouse, death from tetanus takes place in a great many cases. [Sometimes, however, in such circumstances death occurs without tetanic symptoms, and is not due to the tetanus bacillus but to the bacillus of malignant œdema, which also is of common occurrence in the soil (*vide infra*).] By such experiments, supplemented by the culture experiments mentioned, the natural habitats of the *b. tetani*, as given above, have become known.

**The Toxins of the Tetanus Bacillus.**—The tetanus bacillus being thus accepted as the cause of the disease, we have to consider how it produces its pathogenic effects.

Almost contemporaneously with the work on diphtheria was the attempt made with regard to tetanus to explain the general symptoms by supposing that the bacillus could excrete soluble poisons. The earlier results, in which certain bases, tetanin and tetanotoxin, were said to have been isolated, have only a historic interest, as they were obtained by faulty methods. In 1890, Brieger and Fraenkel announced that they had isolated a *toxalbumin* from tetanus cultures, and this body was independently discovered by Faber in the same year. Brieger and



Fraenkel's body consisted practically of an alcoholic precipitate from filtered cultures in bouillon, and was undoubtedly toxic. Within recent years such attempts to isolate tetanus toxins in a pure condition have practically been abandoned, and attention has been turned to the investigation of the physiological effects either of the crude toxin present in filtered bouillon cultures, or of the precipitate produced from the same by ammonium sulphate (cf. p. 195).

The toxic properties of bacterium-free filtrates of pure cultures of the *b. tetani* were investigated in 1891 by Kitasato. This observer found that when the filtrate, in certain doses, was injected subcutaneously or intravenously into mice, tetanic spasms developed, first in muscles contiguous to the site of inoculation, and later all over the body. Death resulted. He found that guinea-pigs were more susceptible than mice, and rabbits less so. In order that a strongly toxic bouillon be produced, it must originally have been either neutral or slightly alkaline. Kitasato further found that the toxin was easily injured by heat. Exposure for a few minutes at 65° C. destroyed it. It was also destroyed by twenty minutes' exposure at 60° C., and by one and a half hours' at 55° C. Drying had no effect. It was, however, destroyed by various chemicals such as pyrogallol and also by sunlight.

In anaerobic bouillon cultures the maximum toxicity is developed in from ten to fifteen days. Behring pointed out that after the filtration of cultures containing toxin, the latter may very rapidly lose its power, and in a few days may only possess  $\frac{1}{100}$ th of its original toxicity. This he attributed to such factors as temperature and light, and especially to the action of oxygen. Toxins should thus have a layer of toluol floated on the surface and be kept in a cool, dark place. The effect of harmful agents on the crude toxin is apparently to cause a degeneration of the true toxin so as to form what it is convenient at present to call toxoids similar to those produced in the case of diphtheria toxin, and it is also true here that the toxoids while losing their toxicity may still retain their power of producing immunity against the potent toxin. Further, altogether apart from the occurrence side by side in the crude toxin of strong and weak poisons, it has been shown that such crude toxin contains toxic substances of probably quite a different nature. Ehrlich has shown that besides the predominant spasm-producing toxin (called by him *tetanospasmin*), there exists in crude toxin a poison capable of producing the solution of certain red blood corpuscles. This hæmolytic agent he calls *tetanolysin*. It does not occur in all samples of crude tetanus toxin, nor is it found



when a bouillon culture of the bacillus is filtered through porcelain. To obtain it the fresh culture must be treated by ammonium sulphate, as described in the method of obtaining concentrated toxins (p. 195). This substance also has the power of originating an antitoxin, so that certain antitetanic sera can protect red blood corpuscles against its action. Madsen, studying the interactions of this anti-tetanolysin with the tetanolysin, has shown that phenomena can be demonstrated similar to those noted by Ehrlich as occurring with diphtheria toxin, and which the latter interpreted as indicating the presence of degenerated toxins (toxoids) in the crude poison. With tetanus as with diphtheria toxin the action of an acid is to cause an apparent disappearance of toxicity, but if before a certain time has elapsed the acid be neutralised by alkali, then a degree of the toxicity returns.

As with other members of the group, nothing is known of the nature of tetanus toxin. Uschinsky has found that the tetanus bacillus can produce its toxin when growing in a fluid containing no proteid matter. The toxin may thus be formed independently of the breaking up of the proteins on which the bacillus may be living, though the latter no doubt has a digestive action on such a protein as gelatin. There is, however, evidence that peptic digestion and toxin formation are due to different vital processes on the part of the tetanus bacillus.

Whatever the nature of the toxin is, it is undoubtedly one of the most powerful poisons known. Even with a probably impure toxalbumin Brieger found that the fatal dose for a mouse was  $\cdot 0005$  of a milligramme. If the susceptibility of man be the same as that of a mouse, the fatal dose for an average adult would have been  $\cdot 23$  of a milligramme, or about  $\frac{7}{2000}$ ths of a grain. Animals differ very much in their susceptibilities to the action of tetanus toxin. According to v. Lingelsheim, if the minimal lethal dose per gramme weight for a horse be taken as unity, that for the guinea-pig would be 6 times the amount, the mouse 12, the goat 24, the dog about 500, the rabbit 1800, the cat 6000, the goose 12,000, the pigeon 48,000, and the hen 360,000.

A striking feature of the action of tetanus toxin is the occurrence of a definite incubation period between the introduction of the toxin into an animal's body and the appearance of symptoms. The incubation period varies according to the species of animal employed, and the path of infection. In the guinea-pig it is from thirteen to eighteen hours, in the horse five days, and the incubation is shorter when the poison is introduced into



a vein than when injected subcutaneously. In man the period between the receiving of an injury and the appearance of tetanic symptoms is from two to fourteen days.

With regard to the *action* of the toxin, it has been shown to have no effect on the sensory or motor endings of the nerves. It acts solely as an exciter of the reflex excitability of the motor cells in the spinal cord. The motor cells in the pons and medulla are also affected, and to a much greater degree than those in the cerebral cortex. When injected subcutaneously the toxin is absorbed into the nerves, and thence finds its way to that part of the spinal cord from which these nerves spring. This explains the fact that in some animals the tetanic spasms appear first in the muscles of the part in which the inoculation has taken place. This is not the case with man, in whom usually the first symptoms appear in the neck. After subcutaneous injection of toxin, part finds its way into the blood stream, and if infected animals be killed during the incubation period there is often evidence of toxin in the blood and solid organs. In the guinea-pig there is little doubt that tetanus toxin has an affinity solely for the nervous system. In other animals, *e.g.* the rabbit, an affinity may exist in other organs, and the fixation of the poison in such situations may give rise to no recognisable symptoms. In such an animal as the alligator, it is possible that while some of its organs have an affinity for tetanus toxin its nervous system has none. These facts are of great scientific interest, and a possible explanation of them will be discussed in the chapter on Immunity. If tetanus toxin be introduced into the stomach or intestine, it is not absorbed, but to a large extent passes through the intestine unchanged. Evidence that any destruction takes place is wanting.

Within recent years some important light has been shed on the mode of action of tetanus toxin. Marie and Morax studied the path of absorption when the toxin was injected into the muscles of the hind limb. The sciatic nerve in a rabbit was cut near the spinal cord and toxin introduced into the muscles of the same side; after some hours the nerve was excised and introduced into a mouse—the animal died of tetanus. But if the nerve were cut near the muscles and the same procedure adopted, the mouse did not contract the disease, though no doubt the cut nerve had been surrounded by lymph containing toxin. If the same experiment were performed and an excess of toxin injected into the other limb, still only the nerve which was left in connection with the muscle showed evidence of the presence of toxin. From this it was deduced that the toxin was absorbed by the end-



plates in the muscle and not from the lymphatics surrounding the nerve. It was further shown that a nerve in the process of degeneration following section did not absorb toxin after the manner of a normal nerve. By a similar method it was shown that the absorption by the nerve was fairly rapid, as one hour after injection the toxin was present in it, and from other experiments the view was put forth that the toxin was centripetal in its flow and did not pass centrifugally in a nerve to which it artificially gained access. Further observations have been made on this subject by Meyer and Ransom. These observers found evidence that toxin is only absorbed by the motor filaments of a nerve, for while tetanus could be produced by injection into a mixed nerve like the sciatic, the introduction of a lethal dose into such a sensory nerve as the infra-orbital was not followed by disease symptoms. If a small dose of toxin be injected into the sciatic nerve, it reaches the corresponding motor cells of the cord, and a local tetanus of the muscles supplied by the nerve results. With a larger dose the poison passes across the commissure to the corresponding cells of the other side, and if still further excess is present it passes up the cord to higher centres. The affection of such higher centres can be prevented by section of the cord. Meyer and Ransom hold that when toxin is injected subcutaneously or intravenously, it only acts by being absorbed by the end-plates in muscles and thence passes to the cord, and they consider that the incubation period is to be explained by the time taken for this extended passage to occur. In this connection they point out that it is in the larger animals, where the nerve path is longest, that the incubation period is also long. Like Marie and Morax, they believe that absorption of toxin by its bathing the lateral aspects of uninjured nervous structures does not occur. In support of this they bring forward the observation that when intravenous injection is practised, the occurrence of tetanus in a part of the body can be precipitated by the injection of a drop of normal saline into the corresponding part of the cord,—sufficient injury being thus caused to allow the toxin in the surrounding lymph to obtain access to the nervous elements. With regard to the action of tetanus toxin, Meyer and Ransom believe that there is a double effect on the nerve cells—first, an exaggeration of the normal tonus, which accounts for the continuous stiffness of the muscles, and secondly, an increase in reflex irritability, which is a prominent factor in the recurring spasms. While no absorption of toxin takes place by sensory filaments, they have found evidence of affection of the sensory apparatus in the



occurrence of what they call *tetanus dolorosus*. This is a great hyperæsthesia and a paroxysmal hyperalgesia which can be caused by injecting toxin into the spinal cord or into a sensory root on the spinal side of the posterior root ganglion. These symptoms are unaccompanied by motor spasms, but the animal may die from exhaustion. The same observers have also made interesting observations on the action of antitoxin. They found that the injection of this substance into the course of a mixed nerve could prevent toxin from passing up to the cord, but that if antitoxin were injected even in great excess intravenously, and a short time thereafter toxin were introduced into a nerve, the death of the animal was not prevented. This they attribute to the fact that antitoxin can only neutralise the toxin which is still circulating in the blood. This is a very far-reaching conclusion, as it throws doubt on what has been held to be a possibility, namely, that toxin can be actually detached from cells in which it is already anchored. But a still more significant observation was made, for in one case of an animal actively immunised against tetanus, and which contained in its serum a considerable quantity of antitoxin, the injection of toxin into the sciatic nerve was followed by tetanus. This would appear to militate against Ehrlich's position that antitoxin is manufactured in the cells which are sensitive to the toxin (see Immunity).

Reference may here be made to the effects of injecting tetanus toxin into the brain itself, as investigated by Roux and Borrel. It was found that the ordinary type of the disease was not produced, but what these observers called "cerebral tetanus." This consisted of general unrest, symptoms of a psychic character (apparent hallucinations, fear, etc.), and epileptiform convulsions. Death occurred in from twelve to twenty hours without any true tetanic spasms. In this manifestation of tetanus the incubation period was much shorter than with subcutaneous injection, and the fatal dose was one twenty-fifth of the minimal subcutaneous dose. Further, the injection of antitoxin forty-eight to ninety-six hours previously did not prevent an animal from succumbing to the intracerebral inoculation. In the light of what has been already said, these results would seem to indicate a special effect of the toxin when brought into direct contact with the protoplasm of the brain cells.

We have seen that unless suitable precautions are adopted in experiments with tetanus cultures in animals, death results not from the multiplication of the bacilli, but from an intoxication with toxin previously existent in the fluid in which the



bacilli have been growing. According to Vaillard, if spores rendered toxin-free, by being kept for a sufficient time at 80° C., are injected into an animal, death does not take place. It was found, however, that such spores can be rendered pathogenic by injecting along with them such chemicals as lactic acid, by injuring the seat of inoculation so as to cause effusion of blood, by fracturing an adjacent bone, by introducing a mechanical irritant such as soil or a splinter of wood (as in Kitasato's experiments), or by the simultaneous injection of other bacteria such as the *staphylococcus pyogenes aureus*. These facts, especially the last, throw great light on the disease as it occurs naturally, for tetanus results especially from wounds which have been accidentally subjected to conditions such as those enumerated. Kitasato now holds that in the natural infection in man, along with tetanus spores, the presence of foreign material or of other bacteria is necessary. Spores alone or tetanus bacilli without spores die in the tissues, and tetanus does not result.

**Immunity against Tetanus.—Antitetanic Serum.**—The artificial immunisation of animals against tetanus has received much attention. The most complete study of the question is found in the work of Behring and Kitasato in Germany, and of Tizzoni and Cattani in Italy. The former observers found that such an immunity could be conferred by the injection of very small and progressively increasing doses of the tetanus toxin. The degree of immunity attained, however, was not high. Subsequent work has shown that the less rich a crude toxin is in modifications of the true toxin, the less useful it is for immunisation procedures. In fact it is doubtful if small animals can be immunised at all by fresh filtrates. In some cases it has been found that the injection of non-lethal doses instead of commencing an immunity actually increases the susceptibility of the animal. This observation has recently acquired fresh interest from its falling into line with the work on the development of supersensitiveness to proteids, which is a very common phenomenon (see "anaphylaxis" under Immunity). More successful in producing immunity are the methods of accompanying the early injections of crude toxin with the subcutaneous introduction of small doses of iodine terchloride, or of using toxin which has been acted on with iodine terchloride or with iodine itself. Tizzoni and Cattani also used the method of administering progressively increasing doses of living cultures attenuated in various ways, *e.g.* by heat. By any of these methods susceptible animals can be made to acquire great immunity, not



only against many times the fatal dose of tetanus toxin, but also against injections of the living bacilli. The degree of immunisation thus acquired remains in existence for a very long time. Not only so, but when a high degree of immunity has been produced by prolonged treatment, it is found that the serum of immune animals possesses the capacity, when injected into animals susceptible to the disease, of protecting them against a subsequent infection with a fatal dose of tetanus bacilli or toxin. Further, if injected subsequently to such infection, the serum can in certain cases prevent a fatal result, even when symptoms have begun to appear. The degree of success attained depends, however, on the shortness of the time which has elapsed between the infection with the bacilli or toxin and the injection of the serum. In animals where symptoms have fully manifested themselves only a small proportion of cases can be saved. As with other antitoxins, there is no evidence that the antitetanic serum has any detrimental effect on the bacilli. It only neutralises the effects of the toxin. The standardisation of the antitetanic serum is of the highest importance. Behring recommends that for protecting animals a serum should be obtained of which one gramme will protect 1,000,000 grammes weight of mice against the minimum fatal dose of the bacillus or toxin. A mouse weighing twenty grammes would thus require  $\cdot 00002$  gramme of the serum to protect it against the minimum lethal dose. In the injection of such a serum subsequent to infection, if symptoms have begun to appear, 1000 times this dose would be necessary; a few hours later 10,000 times, and so on.

As the result of his experiments, Behring aimed at obtaining a curative effect in the natural disease occurring in man. For this purpose, as for his later laboratory experiments, he obtained serum by the immunisation of such large animals as the horse, the sheep, and the goat, by the injection of toxin accompanied at first with the injection of iodine terchloride. It was found that the greater the degree of the natural susceptibility of an animal to tetanus, the easier was it to obtain a serum of a high antitetanic potency. The horse was, therefore, the most suitable animal. If now we take for granted that the relative susceptibilities of man and the mouse towards tetanus are nearly equal, a man weighing 100 kilogrm. would require  $\cdot 1$  grm. of the serum mentioned above to protect him from inoculation with the minimum lethal dose of bacilli or toxin. If symptoms had begun to appear, 100 c.c. at once would be necessary, and as the injection of such a quantity might be inconvenient,



Behring recommended that for man a more powerful serum should be obtained, namely, a serum of which one gramme would protect 100,000,000 grammes weight of mice.<sup>1</sup> The potency is maintained for several months if precautions are taken to avoid putrefaction, exposure to bright light, etc. To this end .5 per cent. carbolic acid is usually added, and the serum is kept in the dark. In a case of tetanus in man, 100 c.c. of such a serum should be injected within twenty-four hours in five doses, each at a different part of the body, and this followed up by further injections if no improvement takes place. Intravenous injection of the antitoxin has also been practised, and, in cases which we have seen treated in this way, has seemed to give better results than those obtained by the subcutaneous method. The serum is warmed to the body temperature and slowly introduced into a vein in the arm, the pulse and respiration being carefully watched during the proceeding. Ten to twenty c.c. can be injected every few hours, and in all 100 c.c. should be given in as short a time as possible. Henderson Smith has shown that when antitoxins to toxins of the tetanus group are injected intravenously a high concentration in the body fluid is maintained for some time, and the opportunity for neutralisation of toxin is thus great. He suggests that both intravenous and subcutaneous injections should be simultaneously practised. The former gives quickly the concentration which is desirable, and when the antitoxin injected intravenously is beginning to be eliminated, that introduced hypodermically comes into the circulation and the concentration is maintained. The antitoxin has also been introduced intracerebrally, very slow injection into the brain substance being practised, but no better results have been obtained than by the subcutaneous method.

Many cases of human tetanus have been thus treated, but the improvement in the death-rate has not been nearly so marked as that which has occurred in diphtheria under similar circumstances. As in the case of diphtheria, however, the results would probably be better if more attention were paid to the dosage of the serum. The great difficulty is that usually we have not the opportunity of recognising the presence of the tetanus bacilli till they have begun to manifest their gravest effects. In diphtheria we have a well-marked clinical feature,—sore throat,—which draws attention to the pro-

<sup>1</sup> The antitetanic serum sent out by the Pasteur Institute in Paris has a strength of 1 : 1,000,000,000. Of this it is recommended that 50 to 100 c.c. should be injected subcutaneously in one or two doses.



bable presence of the bacilli, and the curative agent can thus be early applied. In tetanus the wound in which the bacilli exist may be, as we have seen, of the most trifling character, and even when a well-marked wound exists, the search for the bacilli may be a matter of difficulty. Still it might be well, when practicable, that every ragged, unhealthy-looking wound, especially when contaminated with soil, should, as a matter of routine, be examined bacteriologically. In such cases, undoubtedly, from time to time the tetanus bacillus would be early detected, and treatment could be undertaken with more hope of success than at present. However, in the existing state of matters, whenever the first symptoms of tetanus appear, large doses, such as those above indicated, of a serum whose strength is known, should be at once administered. In giving a prognosis as to the probable result, the two clinical observations on which chief reliance ought to be placed are the presence or absence of interference with respiration, and the rapidity with which the groups of muscles usually affected are attacked. If dyspnoea or irregularity in respiration or rise of temperature comes on soon, and if group after group of muscles is quickly involved, then the outlook is extremely grave. In addition to these points, the duration of the incubation period is of high importance in forming a prognosis. The shorter the time between the infliction of a wound and the appearance of symptoms the graver is the outlook.

The theory as to the nature of antitoxic action will be discussed later in the chapter on Immunity.

**Methods of Examination in a case of Tetanus.**—The routine bacteriological procedure in a case presenting the clinical features of tetanus ought to be as follows :—

(a) *Microscopic.*—Though tetanus is not a disease in which the discovery of the bacilli is easy, still microscopic examination should be undertaken in every case. From every wound or abrasion from which sufficient discharge can be obtained, film preparations ought to be made and stained with any of the ordinary combinations, *e.g.* carbol-fuchsin diluted with five parts of water. Drumstick-shaped spore-bearing bacilli are to be looked for. The presence of such, having characters corresponding to those of the tetanus bacilli, though not absolutely conclusive proof of identification, is yet sufficient for all practical purposes. If only bacilli without spores resembling the tetanus bacilli are seen, then the identification can only be provisional.

The microscopic examination of wounds contaminated by soil,



etc., may, as we have said, in some cases lead to the anticipation that tetanus will probably result.

(b) *Cultivation*.—The methods to be employed in isolating the tetanus bacilli have already been described (p. 417). It may be added, however, that if the characteristic forms are not seen on microscopic examination of the material from the wound, they may often be found by inoculating a deep tube of one of the glucose media with such material, and incubating for forty-eight hours at 37° C. At the end of this period, spore-bearing tetanus bacilli may be detected microscopically, though of course mixed with other organisms.

(c) *Inoculation*.—Mice and guinea-pigs are the most suitable animals. Inoculation with the material from a wound should be made subcutaneously. A loopful of the discharge introduced at the root of the tail in a mouse will soon give rise to the characteristic symptoms, if tetanus bacilli are present. With suspicious organisms isolated by culture it is well to use the splinter method (p. 423), as some strains of the *b. tetani* tend to produce little toxin in artificial media, and may be injected without causing tetanic symptoms.

#### MALIGNANT ŒDEMA (*Septicémie de Pasteur*).

The organism now usually known as the bacillus of malignant œdema is the same as that first discovered by Pasteur and named *vibrion septique*. He described its characters, distinguishing it from the anthrax bacillus, which it somewhat resembles morphologically, and also the lesions produced by it. He found that it grew only in anaerobic conditions, but was able to cultivate it merely in an impure state. It was more fully studied by Koch, who called it the bacillus of malignant œdema, and pointed out that the disease produced by it is not really of the nature of a septicæmia, as immediately after death the blood is practically free from the bacilli.

“Malignant œdema” in the human subject is usually described as a spreading inflammatory œdema attended with emphysema, and ultimately followed by gangrene of the skin and subjacent parts. In many cases of this nature the bacillus of malignant œdema is present, associated with other organisms which aid its spread, whilst in others it may be absent. One of us has, however, observed a case in which the bacillus was present in pure condition. Here there occurred intense œdema with swelling and induration of the tissues, and the formation of vesicles on the skin. Those changes were attended with a



reddish discoloration afterwards becoming livid. Emphysema was not recognisable until the limb was incised, when it was detected, though in small degree. Further, the tissues had a peculiar heavy, but not putrid, odour. The bacillus, which was obtained in pure culture, was present in enormous numbers in the affected tissues, attended by cellular necrosis, serous exudation, and at places much leucocytic emigration. The

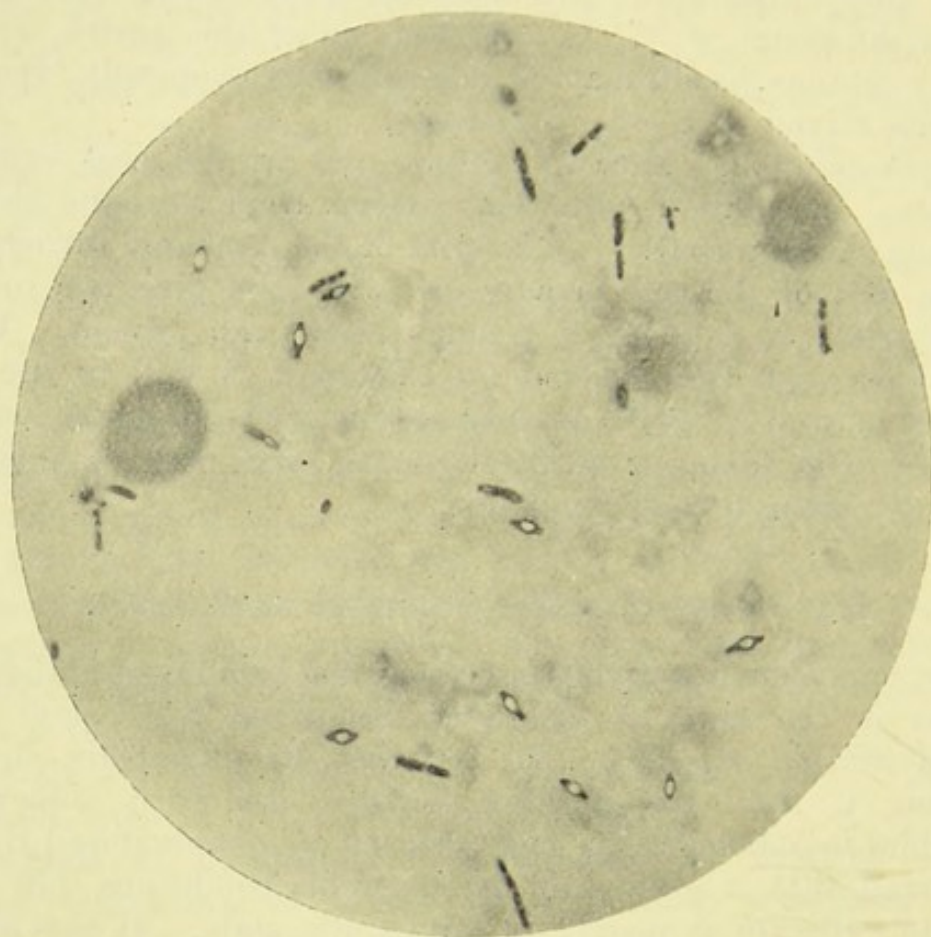


FIG. 126.—Film preparation from the affected tissues in a case of malignant œdema in the human subject, showing the spore-bearing bacilli.

Gentian-violet.  $\times 1000$ .

picture, in short, corresponded with that seen on inoculating a guinea-pig with a pure culture. The term "malignant œdema" should be limited in its application to cases in which the bacillus in question is present. In most of these there is a mixed infection ; in some the bacillus may be present alone.

This organism has a very widespread distribution in nature, being present in garden soil, dung, and various putrefying animal fluids ; and it is by contamination of lacerated wounds by such substances that the disease is usually set up in the



human subject. Malignant oedema can be readily produced by inoculating susceptible animals, such as guinea-pigs, with garden soil. The bacillus is also often present in the intestine of man and animals, and has been described as occurring in some gangrenous conditions originating in connection with the intestine in the human subject.

**Microscopical Characters.**—The bacillus of malignant oedema is a comparatively large organism, being slightly less than  $1\ \mu$  in thickness, that is, thinner than the anthrax bacillus. It occurs in the form of single rods 3 to  $10\ \mu$  in length, but both in the tissues and in cultures in fluids it frequently grows out into long filaments, which may be uniform throughout or segmented at irregular intervals. In cultures on solid media it chiefly occurs in the form of shorter rods with somewhat rounded ends. The rods are motile, possessing several laterally placed flagella, but in a given specimen, as a rule, only a few bacilli show active movement. Under suitable conditions they form spores, which are usually near the centre of the rods and have an oval shape, their thickness somewhat exceeding that of the bacillus (Figs. 126, 127). The bacillus can be readily stained by any of the basic aniline stains, but loses the colour in Gram's method, in this way differing from the anthrax bacillus.

**Characters of Cultures.**—This organism grows readily at ordinary temperature, but only under *anaerobic* conditions. In a puncture culture in a deep tube of glucose gelatin, the growth appears as a whitish line giving off minute short processes, the growth, of course, not reaching the surface of the medium. Soon liquefaction occurs and a long fluid funnel is formed, with turbid contents and flocculent masses of growth at the bottom. At the same time bubbles of gas are given off, which may split up the gelatin. The colonies in gelatin plates under anaerobic

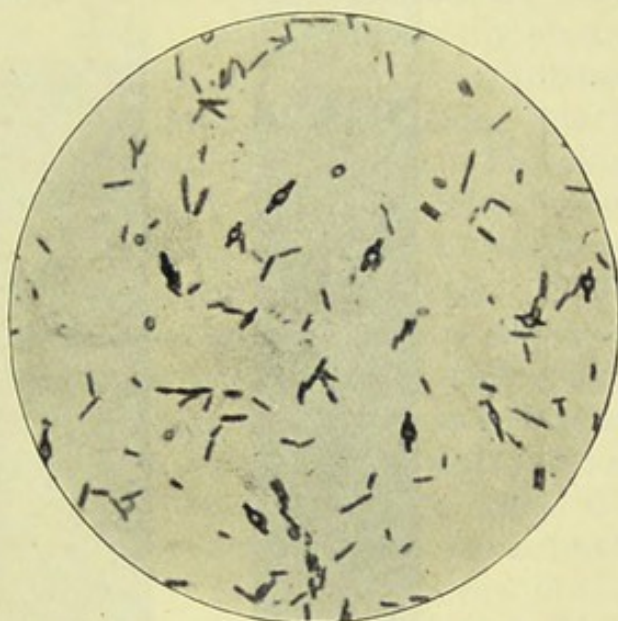


FIG. 127.—Bacillus of malignant oedema, showing spores. From a culture in glucose agar, incubated for three days at  $37^{\circ}\text{C}$ . Stained with weak carbol-fuchsin.  $\times 1000$ .



conditions appear first as small whitish points which under the microscope show a radiating appearance at the periphery, resembling the colonies of the bacillus subtilis. Soon, however, liquefaction occurs around the colonies, and spheres with turbid contents result; gas is developed around the colonies.

In deep tubes of glucose agar at 37° C. growth is extremely

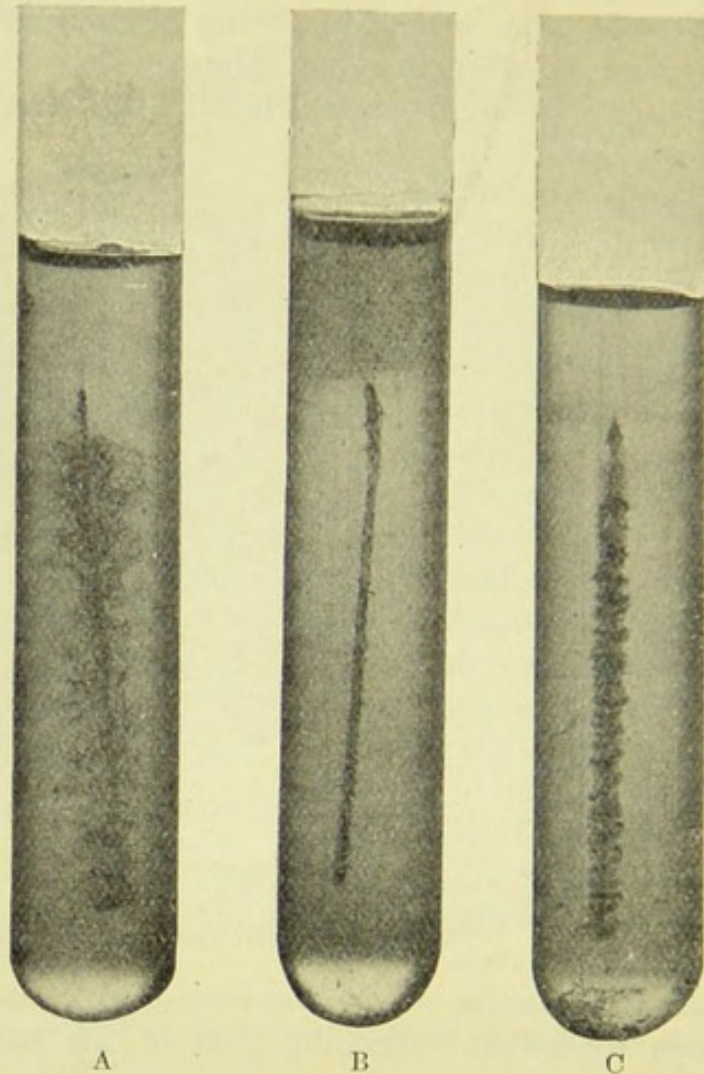


FIG. 128.—Stab cultures in agar, five days' growth at 37° C.  
Natural size.

A. Tetanus bacillus. B. Bacillus of malignant oedema. C. Bacillus of quarter-evil (Rauschbrand).

rapid. Along the line of puncture, growth appears as a somewhat broad white line with short lateral projections here and there (Fig. 128, B). Here also gas may be formed, but this is most marked in a shake culture, in which the medium becomes cracked in various directions, and may be pushed upwards so high as to displace the cotton-wool plug. The cultures possess a peculiar heavy, though not putrid, odour.



Spore formation occurs above 20° C., and is usually well seen within forty-eight hours at 37° C. The spores have the usual high powers of resistance, and may be kept for months in the dried condition without being killed.

**Experimental Inoculation.**—A considerable number of animals—the guinea-pig, rabbit, sheep, and goat, for example—are susceptible to inoculation with this organism. The ox is said to be quite immune to experimental inoculation, though it can, under certain conditions, contract the disease by natural channels. The guinea-pig is the animal most convenient for experimental inoculation. When the disease is set up in the guinea-pig by subcutaneous inoculation with garden soil, death usually occurs in about twenty-four to forty-eight hours. There is an intense inflammatory œdema around the site of inoculation, which extends over the wall of the abdomen and thorax. The skin and subcutaneous tissue are infiltrated with a reddish-brown fluid and softened; they contain bubbles of gas and are at places gangrenous. The superficial muscles are also involved. These parts have a very putrid odour. The internal organs are congested, the spleen soft but not much enlarged. In such conditions the bacillus of malignant œdema, both in short and long forms, will be found in the affected tissues along with various other organisms. Spores may be present, especially when the examination is made some time after the death of the animal. If the animal is examined immediately after death, a few of the bacilli may be present in the peritoneum and pleuræ, usually in the form of long motile filaments, but they are almost invariably absent from the blood. A short time after death, however, they spread directly into the blood and various organs, and may then be found in considerable numbers.

Subcutaneous inoculation with pure cultures of the bacillus of malignant œdema produces chiefly a spreading bloody œdema, the muscles being softened and partly necrosed; but there is little formation of gas, and the putrid odour is almost absent.

When the bacilli are injected into mice, however, they enter and multiply in the blood stream, and they are found in considerable numbers in the various organs, so that a condition not unlike that of anthrax is found. The spleen also is much swollen.

The virulence of the bacillus of malignant œdema varies considerably in different cases, and it always becomes diminished in cultures grown for some time. A smaller dose produces a fatal result when injected along with various other organisms (bacillus prodigiosus, etc.).



**Immunity.**—Malignant œdema was one of the first diseases against which immunity was produced by injections of toxins. The filtered cultures of the bacillus in sufficient doses produce death with the same symptoms as those caused by the living organisms, but a relatively large quantity is necessary. Chamberland and Roux (1887) found that if guinea-pigs were injected with several non-fatal doses of cultures sterilised by heat or freed from the bacilli by filtration, immunity against the living organism could be developed in a comparatively short time. They found that the filtered serum of animals dead of the disease is more highly toxic, and also gives immunity when injected in small doses. These experiments were confirmed by Sanfelice.

**Methods of Diagnosis.**—In any case of supposed malignant œdema, the fluid from the affected tissues ought first to be examined microscopically, to ascertain the characters of the organisms present. Though it is not possible to identify absolutely the bacillus of malignant œdema without cultivating it, the presence of spore-bearing bacilli with the characters described above is highly suspicious (Fig. 126). In such a case the fluid containing the bacilli should be first exposed to a temperature of 80° C. for half an hour, and then a deep glucose agar tube should be inoculated. In this way the spore-free organisms are killed off. Pure cultures may be thus obtained, or this procedure may require to be followed by the roll-tube method under anaerobic conditions. An inoculation experiment, if available, may also be made on a guinea-pig.

#### BACILLUS BOTULINUS.

The term "meat-poisoning" embraces a number of conditions produced by different agents, and the relation of the bacillus of Gaertner to one class of case has already been discussed. Another group was shown by van Ermengem in 1896 to be caused by an anaerobic bacillus to which he gave the name *bacillus botulinus*. He cultivated the organism from a sample of ham, the ingestion of which in the raw condition had produced a number of cases of poisoning, some of them followed by fatal result. The symptoms in these cases closely corresponded with those occurring in the so-called "sausage poisoning" met with from time to time in Germany and other countries where sausages and ham are eaten in an imperfectly cooked condition. Such cases form a fairly well-defined group, the symptoms in which are chiefly referable to an action on the medulla, and, as will be detailed



below, similar symptoms have been experimentally produced by means of the bacillus mentioned or its toxins. The chief symptoms of this variety of botulismus, as detailed by van Ermengem, are disordered secretion in the mouth and nose, more or less marked ophthalmoplegia, externa and interna (dilated pupil, ptosis, etc.), dysphagia, and sometimes aphagia with aphonia, marked constipation and retention of urine, and in fatal cases interference with the cardiac and respiratory centres. Along with these there is practically no fever and no interference with the intellectual faculties. The symptoms commence at earliest twelve to twenty-four hours after ingestion of the poison. From the ham in question, which was not decomposed in the ordinary sense, van Ermengem obtained numerous colonies of this bacillus, the leading characters of which are given below. It may be added that Römer obtained practically the same results as van Ermengem in a similar condition, and that the bacillus botulinus has been cultivated by Kempner from the intestine of the pig.

**Microscopical and Cultural Characters.**—The organism is a bacillus of considerable size, measuring 4 to 9  $\mu$  in length and  $\cdot 9$  to 1.2  $\mu$  in thickness; it has somewhat rounded ends and sometimes is seen in a spindle form. It is often arranged in pairs, sometimes in short threads. Under certain conditions it forms spores which are oval in shape, usually terminal in position, and a little thicker than the bacilli. It is a motile organism and has 4 to 8 lateral flagella of wavy form. It stains readily with the ordinary dyes, and also retains the colour in Gram's method, though care must be employed in decolorising.

The organisms can be readily cultivated on the ordinary media, but only under strictly anaerobic conditions. In glucose gelatin a whitish line of growth forms with lateral offshoots, but liquefaction with abundant gas formation soon occurs. In gelatin plates the colonies after four to six days are somewhat characteristic; they appear to the naked eye as small semi-transparent spheres, and these on examination under a low power of the microscope have a yellowish-brown colour and are seen to be composed of granules which show a streaming movement, especially at the periphery. Cultures in glucose agar resemble those of certain other anaerobes; there is abundant development of gas, and the medium is split up in various directions. The cultures have a rancid, though not foul, odour, due chiefly to the development of butyric acid. The optimum temperature is below that of the body, namely, between 20° and



30° C. ; at the body temperature growth is slower and less abundant and spore formation does not occur.

*Pathogenic Effects.*—Like the tetanus bacillus, the bacillus botulinus has little power of flourishing in the tissues, whereas it produces a very powerful toxin. Van Ermengem found that the characteristic symptoms could be produced in certain animals by administering watery extracts of the infected ham or cultures either by the alimentary canal or by subcutaneous injection. Here also there is a period of incubation of not less than six to twelve hours before the symptoms appear, and when the dose is small a somewhat chronic condition may result, in which local paralyses form a striking feature. The characteristic effects can also be produced by means of the filtered toxin by either of the methods mentioned, though in the case of administration by the alimentary canal the dose requires to be larger. Here also, as in the case of the tetanus poison, the potency of the toxin is remarkable, the fatal dose for a guinea-pig of 250 grm. weight being in some instances '0005 c.c. of the filtered toxin. In cases of poisoning in the human subject, the effects would accordingly appear to be produced by absorption of the toxin from the alimentary canal; it is only after or immediately before death that a few bacilli may enter the tissues. Van Ermengem obtained a few colonies from the spleen of a patient who had died from ham-poisoning. The properties of the botulinus toxin have been investigated, and have been found to correspond closely, as regards relative instability, conditions of precipitation, combination with sensitive cells (*i.e.* of brain and cord), etc., with the toxins of diphtheria and tetanus. An antitoxin has also been prepared by Kempner by the usual methods, and has been shown not only to have a neutralising property, but to have considerable therapeutical value when administered some hours after the toxin. The subject has been recently studied by Leuchs, and he has found that the combination toxin-antitoxin can be split up by the action of acids and the two components recovered, just as Morgenroth showed to occur in the case of diphtheria (p. 529). The direct combining affinity of the toxin for the central nervous system has been demonstrated by Kempner and Schepilewsky by the same methods as Wassermann and Takaki employed in the case of the tetanus toxin. The condition of the nerve cells in experimental poisoning with the botulinus toxin has been investigated independently by Marinesco and by Kempner and Pollack, and these observers agree as to the occurrence of marked degenerative changes, especially in the motor cells in the spinal cord and medulla. Marinesco also



observed hypertrophy and proliferation of the neuroglia cells around them.

These observations, therefore, show that in one variety of meat-poisoning the symptoms are produced by the absorption of the toxins of the bacillus botulinus from the alimentary canal, and, as van Ermengem points out, it is of special importance to note that the meat may be extensively contaminated with this bacillus, and may contain relatively large quantities of its toxins without the ordinary signs of decomposition being present. The production of an extracellular toxin by this organism, with extremely potent action on the nervous system, is a fact of great scientific interest, and has a bearing on the etiology of other obscure nervous affections.

QUARTER-EVIL (GERMAN, RAUSCHBRAND ; FRENCH, CHARBON SYMPTOMATIQUE).

The characters of the bacillus need be only briefly described, as, so far as is known, it never infects the human subject. The natural disease, which occurs especially in certain localities, affects chiefly sheep, cattle, and goats. Infection takes place by some wound of the surface, and there spreads in the region around, inflammatory swelling attended by bloody œdema and emphysema of the tissues. The part becomes greatly swollen, and of a dark, almost black, colour. Hence the name "black-leg" by which the disease is sometimes known. The bacillus which produces this condition is present in large numbers in the affected tissues, associated with other organisms, and also occurs in small numbers in the blood of internal organs. For the isolation of the bacillus, Grassberger and Schattenfroh recommend the use of anaerobic sugar agar plates containing pieces of sterile ox flesh.

The bacillus morphologically closely resembles that of malignant œdema. Like the latter, also, it is a strict anaerobe, and its conditions of growth as regards temperature are also similar. It is, however, somewhat thicker, and does not usually form such long filaments. Moreover, the spores, which are of oval shape and broader than the bacillus, are almost invariably situated close to one extremity, though not actually terminal (Fig. 129). The characters of the cultures, also, resemble those of the bacillus of malignant œdema, but in a stab culture in glucose agar there are more numerous and longer lateral offshoots, the growth being also more luxuriant (Fig. 128, c). This bacillus is actively motile, and possesses numerous lateral flagella. When cultures derived from disease conditions are continuously subcultured on sugar media, they tend to lose their capacities of motility and spore formation. The organism seems to occupy a position somewhat intermediate between the *b. saccharobutyricus* (v. Klecki), which is a free sugar fermenter, and the *b. putrificus* (Bienstock), which has great powers of splitting up albumins.

The disease can be readily produced in various animals, *e.g.* guinea-pigs, by inoculation with the affected tissues of diseased animals, and also by means of pure cultures, though an intramuscular injection of a considerable amount of the latter is sometimes necessary. The condition produced in this way closely resembles that in malignant œdema, though



there is said to be more formation of gas in the tissues. Rabbits are more resistant to this disease, whilst they are comparatively susceptible to malignant oedema. As in the case of tetanus, inoculation with living spores which have been deprived of adherent toxin by heat does not produce the disease. A toxin can be separated by filtration from cultures of bouillon containing 5 per cent. glucose and a thick emulsion of sterile calcium carbonate. It is fairly resistant to heat, withstanding two hours at 70–75° C. without being destroyed, and it is also very rapid in its action, being capable in appropriate dose of killing a horse in five minutes. It is to be noted as an important fact, that while freshly isolated cultures possess a high degree of virulence they may have little

capacity for *in vitro* toxin production. Grassberger and Schattenfroh state that there may be an antagonism between maximum virulence and maximum toxin production. One of the properties of the toxin is said to be a capacity for killing leucocytes.

The disease is one against which immunity can be produced in various ways, and methods of preventive inoculation have been adopted in the case of animals liable to suffer from it. This subject was specially worked out by Arloing, Cornevin, and Thomas, and later by others. Immunity may be produced by injection (especially by the intravenous and intraperitoneal routes) with a non-fatal dose of the virus (*i.e.*



FIG. 129.—Bacillus of quarter-evil, showing spores. From a culture in glucose agar, incubated for three days at 37° C. Stained with weak carbol-fuchsin.  $\times 1000$ .

the oedematous fluid found in the tissues of affected animals and which contains the bacilli), or by injection with larger quantities of the virus attenuated by heat, drying, etc. It can be produced also by cultures attenuated by heat and by the products of the bacilli obtained by filtration of cultures. An antitoxin has been produced against the toxins of the bacillus, and a method of protection in which the action of this antitoxin is combined with that of the virus has been used (*cf.* Anthrax, p. 348). The antitoxin is said to increase the chemiotactic properties of the leucocytes.

#### BACILLUS AEROGENES CAPSULATUS.

This bacillus, though sometimes aiding in the production of pathological changes, is chiefly of interest on account of the extensive gaseous development to which it gives rise in the tissues *post mortem*. It was described by Welch and Nuttall in 1892; it is now recognised as being identical with an organism found in gaseous phlegmon by E. Fraenkel, and called by him the *bacillus phlegmones emphysematosae*. The organism is a comparatively large one, measuring 3 to 6  $\mu$  in length and having a



thickness about the same as that of the anthrax bacillus ; its ends are square or slightly rounded (Fig. 130). It often occurs in pairs, sometimes in chains ; occasionally filamentous forms are met with. It usually shows a well-marked capsule, hence the name ; it is non-motile and does not form spores. It stains readily with the basic aniline dyes and retains the stain in Gram's method. It grows readily on the ordinary media, but only under anaerobic conditions ; the optimum temperature is that of the body, growth at the room temperature being comparatively slow. In a puncture culture in agar there is an abundant whitish line of growth, with somewhat indented margin ; the individual colonies are white and of rounded or oval form. There is practically no liquefaction of gelatin, though this medium becomes somewhat softened around the growth. In all cases there is a tendency to abundant evolution of gas in the cultures, and this is especially marked when fermentable sugars are present.

The organism appears to be the most frequent cause of rapid gaseous development in the blood and organs post-mortem, this depending upon an invasion of the blood immediately before death. In such cases, even within twenty-four hours under ordinary conditions, large bubbles of gas may be present in the veins, and the organs may be beset with gas-containing spheres of various sizes ; the liver is usually the organ most affected, and its appearance has been compared to that of Gruyère cheese. The invasion by this organism is met with from time to time in puerperal cases,

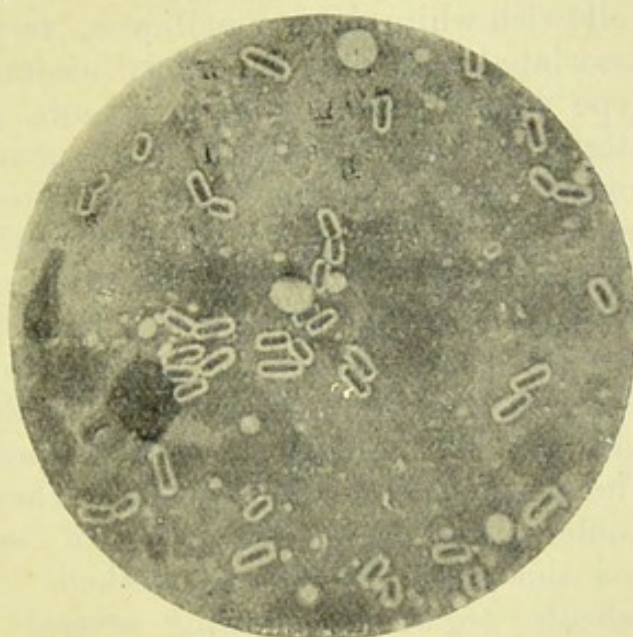


FIG. 130.—*Bacillus aerogenes capsulatus* ; film preparation from bone-marrow in a case where gas-cavities were present in the organs.  $\times 1000$ .

and also in connection with ulcerative or gangrenous conditions of the intestine ; the bacillus is also found not infrequently in the peritoneum in cases of perforation. Although the striking changes in the organs are due to a post-mortem development of the bacillus, there is no doubt that its entrance into the blood stream often hastens death, and may in some instances be the cause of it. As already stated, the organism is also met with in some cases of spreading oedema with emphysema as a leading feature.

When tested experimentally, the bacillus by itself is found to have little pathogenic action. Injection of pure cultures in rabbits and guinea-pigs may be followed by little result, but sometimes in the latter animals "gaseous phlegmon" is produced, without suppuration unless other organisms are present. If a small quantity of culture be injected intravenously, *e.g.* in a rabbit, and then the animal be killed, bubbles of gas are rapidly produced in the blood and organs, the picture corresponding with that in the human cases.



## FUSIFORM ANAEROBIC BACILLI PATHOGENIC TO MAN.

Babés in 1884 described organisms of this type in a diphtheria-like affection of the fauces, and since that time the presence of similar organisms has been noted in necrotic inflammations, ulcerative stomatitis, noma, and like affections. The association of fusiform bacilli with a form of angina has been specially recognised since the work of Vincent (1898-99); and this condition often goes now under the name of "Vincent's angina." He recognised two forms of the affection—(a) a diphtheroid type characterised by the formation of a firm yellowish-white false membrane, very like that of diphtheria, associated with only superficial ulceration; and (b) an ulcerative type where the membrane is soft, greyish, and foul-smelling, attended with ulceration and surrounding œdema. In the former type fusiform bacilli are present alone; in the latter, which is distinctly the commoner, there are also spirochætes. The fusiform bacilli are thin rods measuring on the average 10 to 14  $\mu$  in length, and less than 1  $\mu$  in thickness; they are straight or slightly curved and are tapered at their extremities. The central portion often stains less deeply than the extremities, and not infrequently shows unstained points (Plate I., Fig. 4). The organisms are non-motile. They stain fairly deeply with Löffler's methylene-blue or with weak carbol-fuchsin. They lose the stain in Gram's method. The spirochætes are long delicate organisms showing several irregular curves, and are motile; in appearance they resemble the spirochæte refringens and similar organisms found in gangrenous conditions. They stain less deeply than the bacilli. Sometimes they are numerous, sometimes scanty; they seem to be similar to spirochætæ found in the mouth in a variety of other conditions. In a section through the false membrane, when stained with methylene or thionin blue, there is usually to be seen a darkly stained band, a short distance below the surface, which is due to the presence of large masses of the fusiform bacilli closely packed together; neither they nor the spirochætes appear to pass deeply into the tissues. Vincent's results have been confirmed by others, and there is no doubt that fusiform bacilli, of which there are probably several species, are associated with various spreading necrotic conditions. Cultures of fusiform bacilli have been obtained by Ellermann and by Weaver and Tunnicliffe. They grow only under anaerobic conditions, and the best media are those consisting of a mixture of serum or blood and agar (1 : 3). The organisms form small rounded colonies of whitish or



yellowish colour, somewhat like those of a streptococcus, but rather felted in appearance on the surface. Injections of pure cultures in animals sometimes produce suppuration but never necrosis (Ellermann). Tunncliffe finds that the spirochaetes are only stages in the development of fusiform bacilli, as cultures which at an early stage show only fusiform bacilli afterwards contain spirochaetes, and intermediate forms can be found. These results, however, have not yet been confirmed. It is also to be noted that fusiform bacilli are sometimes present in the secretions of the mouth in normal conditions, and may occur in increased numbers in true diphtheria.



## CHAPTER XVIII.

### THE CHOLERA SPIRILLUM AND ALLIED ORGANISMS.

**Introductory.**—It is no exaggeration of the facts to say that previously to 1883 practically nothing of value was known regarding the nature of the virus of cholera. In that year Koch was sent to Egypt, where the disease had broken out, in charge of a Commission for the purpose of investigating its nature. In the course of his researches he discovered the organism now generally known as the “comma bacillus” or the “cholera spirillum.” He also obtained pure cultures of the organism from a large number of cases of cholera, and described their characters. The results of his researches were given at the first Cholera Conference at Berlin in 1884.

Since Koch’s discovery, and especially during the epidemic in Europe in 1892–93, spirilla have been cultivated from cases of cholera in a great many different localities, and though this extensive investigation has revealed the invariable presence in true cholera of organisms resembling more or less closely Koch’s spirillum, certain difficulties have arisen. For it has been found that the cultures obtained from different places have shown considerable variations in their characters, and, further, spirilla which closely resemble Koch’s cholera spirillum have been cultivated from sources other than cases of true cholera. There has therefore been much controversy, on the one hand, as to the signification of these variations,—whether they are to be regarded as indicating distinct species or merely varieties of the same species,—and, on the other hand, as to the means of distinguishing the cholera spirillum from other species which resemble it. These questions will be discussed below.

In considering the bacteriology of cholera, it is to be borne in mind that in this disease, in addition to the evidence of great intestinal irritation, accompanied by profuse watery discharge, and often by vomiting, there are also symptoms of general



systemic disturbance which cannot be accounted for merely by the withdrawal of water and certain substances from the system. Such symptoms include the profound general prostration, cramps in the muscles, extreme cardiac depression, the cold and clammy condition of the surface, the subnormal temperature, suppression of urine, etc. These, taken in their entirety, are indications of a general poisoning in which the circulatory and thermo-regulatory mechanisms are specially involved. In some, though rare, cases known as *cholera sicca*, general collapse occurs with remarkable suddenness, and is rapidly followed by a fatal result, whilst there is little or no evacuation from the bowel, though post-mortem the intestine is distended with fluid contents. As the characteristic organisms in cholera are found only in the intestine, the general disturbances are to be regarded as the result of toxic substances absorbed from the bowel. It is also to be noted that cholera is a disease of which the onset and course are much more rapid than is the case in most infective diseases,

such as typhoid and diphtheria; and also that recovery, when it takes place, does so more quickly. The two factors to be correlated to these facts are: (a) a rapid multiplication of organisms, (b) the production of rapidly acting toxins.

**The Cholera Spirillum.**—*Microscopical Characters.*—The cholera spirilla, as found in the intestines in cholera, are small organisms measuring about 1.5 to 2  $\mu$  in length, and rather less than .5 in thickness. They are distinctly curved in one direction, hence the appearance of a comma (Fig. 131); most occur singly, but some are attached in pairs and curved in opposite directions, so that an S-shape results. Longer forms are rarely seen in the intestine, but in cultures in fluids, as is especially well seen in hanging-drop preparations, they may grow into longer spiral filaments, showing a large number of turns. In

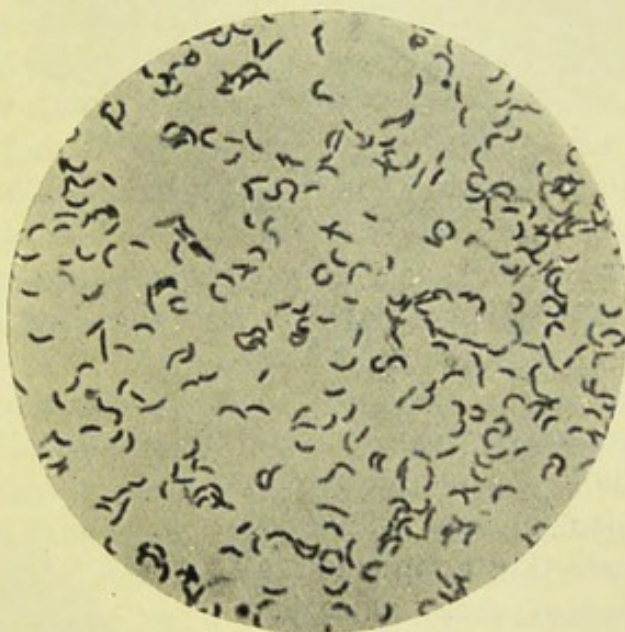


FIG. 131.—Cholera spirilla, from a culture on agar of twenty-four hours' growth. Stained with weak carbol-fuchsin.  $\times 1000$ .



film preparations made from the intestinal contents in typical cases, these organisms are present in enormous numbers in almost pure culture, most of the spirilla lying with their long axes in the same direction, so as to give the appearance which Koch compared to a number of fish in a stream.

They possess very active motility, which is most marked in the single forms. When stained by the suitable methods they are seen to be flagellated.

Usually a single terminal flagellum is present at one end only (Fig. 132).

It is very delicate, and

FIG. 132.—Cholera spirilla stained to show the terminal flagella. See also Plate IV., Fig 19.  $\times 1000$ .

measures four or five times the length of the organism. Cholera spirilla do not form spores. In old cultures the organisms may present great variety in size and shape. Some are irregularly twisted filaments, sometimes globose, sometimes clubbed at their extremities, and also showing irregular swellings along their course; others are short and thick, and may have the appearance of large cocci, often staining faintly. All these changes in appearance are to be classed together as *involution forms*.

*Staining.*—Cholera spirilla stain readily with the usual basic aniline stains, though Löffler's methylene-blue or



FIG. 133.—Cholera spirilla from an old agar culture, showing irregularities in size and shape, with numerous faintly-stained coccoid bodies—involution forms.

Stained with fuchsin.  $\times 1000$ .



weak carbol-fuchsin is specially suitable. They lose the stain in Gram's method.

**Distribution within the Body.**—The chief fact in this connection is that the spirilla are confined to the intestine, and are not present in the blood or internal organs. In cases in which there is the characteristic "rice-water" fluid in the intestines, they occur in enormous numbers—almost in pure culture. The lower half of the small intestine is the part most affected. Its surface epithelium becomes shed in great part, and the flakes floating in the fluid consist chiefly of masses of epithelial cells and mucus, amongst which are numerous spirilla. The spirilla also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial lining, which becomes loosened by their action. They are, however, rarely found in the connective tissue beneath, and never penetrate deeply. Along with these changes there is congestion of the mucosa, especially around the Peyer's patches and solitary glands, which are somewhat swollen and prominent. In some very acute cases the mucosa may show general acute congestion, with a rosy pink colour but very little desquamation of epithelium, the intestinal contents being a comparatively clear fluid containing the spirilla in large numbers. In other cases of a more chronic type, the intestine may show more extensive necrosis of the mucosa and a considerable amount of hæmorrhage into its substance, along with formation of false membrane at places. The intestinal contents in such cases are blood-stained and foul-smelling, there being a great proportion of other organisms present besides the cholera spirilla (Koch).

**Cultivation.**—(For methods, see p. 459).

The cholera spirillum grows readily on all the ordinary media, and, with the exception of that on potato, growth takes place at the ordinary room temperature. The most suitable temperature, however, is that of the body, and growth usually stops about 16° C., though in some cases it has been obtained at a lower temperature. Abundant growth occurs on media with sufficiently alkaline reaction to inhibit the growth of many intestinal bacteria, *e.g.* Dieudonné's medium, p. 44.

**Peptone Gelatin.**—On this medium the organism grows well and produces liquefaction. In puncture cultivations at 22° C. a whitish line appears along the needle track, at the upper part of which liquefaction commences, and as evaporation quickly occurs, a small bell-shaped depression forms, which gives the appearance of an air-bubble. On the fourth or fifth day we get the following appearance: there is at the surface the bubble-



shaped depression; below this there is a funnel-shaped area of liquefaction, the fluid being only slightly turbid, but showing at its lower end thick masses of growth of a more or less spiral shape (Fig. 134). The liquefied portion gradually tapers off downwards towards the needle track. (This appearance is, however, in some varieties not produced till much later, especially when the gelatin is very stiff, and, in other varieties which liquefy very slowly, may not be met with at all.) At a later stage, liquefaction spreads and may reach the side of the tube.



FIG. 134. — Puncture culture of the cholera spirillum in peptone gelatin — six days' growth. Natural size.

In *gelatin plates* the colonies are somewhat characteristic. They appear as minute whitish points, visible in twenty-four to forty-eight hours, the surface of which, under a low power of the microscope, is irregularly granular or furrowed (Fig. 135, A). Liquefaction occurs, and the colony sinks into the small cup formed, the plate then showing small sharply-marked rings around the colonies. Under the microscope the outer margin of the cup is circular and sharply marked. Within the cup the liquefied portion forms a ring which has a more or less granular appearance, whilst the mass of growth in the centre is irregular and often broken up at its margins (Fig. 135, B).

On the surface of the *agar* media a thin, almost transparent, layer forms, which presents no special characters. On solidified *blood serum* the growth has at first the same appearance, but afterwards liquefaction of the medium occurs. On *agar plates* the superficial colonies under a low power are circular discs of brownish-yellow colour, and more transparent than those of most other organisms. On *potato* at the ordinary temperature, growth does not take place, but on incubation at a temperature of from 30° to 37° C., a moist layer appears, which assumes a dirty brown colour, somewhat like that of the glanders bacillus; the appearance, however, varies somewhat in different varieties, and also on different sorts of potatoes.

In *bouillon* with alkaline reaction the organism grows very readily, there occurring in twelve hours at 37° C. a general



turbidity, while the surface shows a thin pellicle composed of spirilla in a very actively motile condition. Growth takes place under the same conditions equally rapidly in peptone solution (1 per cent. with .5 per cent. sodium chloride added).

In *milk* also the organism grows well, and produces no coagulation nor any change in its appearance, at least for several days.

On all the media the growth of the cholera spirillum is a relatively rapid one, and especially is this the case in peptone solution and in bouillon, a circumstance of importance in relation to its separation in cases of cholera (*vide* p. 459).

The cholera organism is one which grows much more rapidly in the presence of oxygen than in anaerobic conditions; in the complete exclusion of oxygen very little growth occurs.

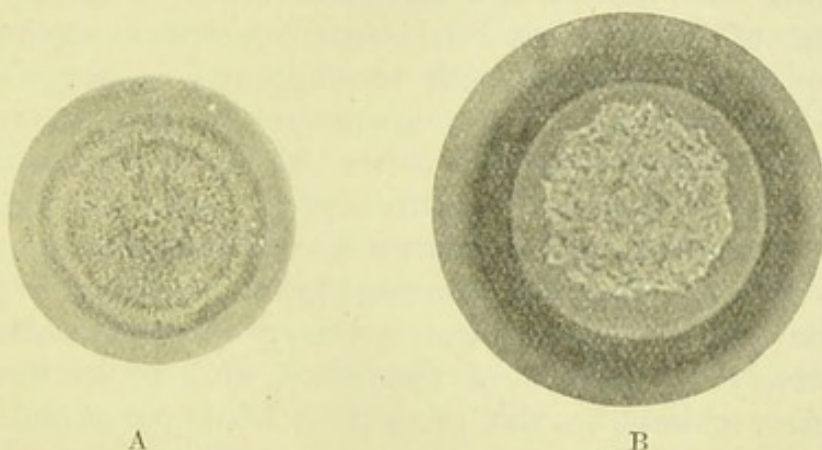


FIG. 135.—Colonies of the cholera spirillum in a gelatin plate—three days' growth. A shows the granular surface, liquefaction just commencing; in B liquefaction is well marked.

*Cholera-Red Reaction.*—This is one of the most important tests in the diagnosis of the cholera organism. It is always given by a true cholera spirillum, and though the reaction is not peculiar to it, the number of organisms which give the reaction under the conditions mentioned are comparatively few. The test is made by adding a few drops of pure sulphuric acid to a culture in bouillon or in peptone solution (1 per cent.) which has been incubated for twenty-four hours at 37° C.; in the case of the cholera spirillum a reddish-pink colour is produced. This is due to the fact that both indol and a nitrite are formed by the spirillum in the medium, and hence in applying the test for indol the addition of a nitrite is not necessarily the red colour. Here, as in testing for the production of indol by other bacteria, it is found that not every specimen of peptone is suitable, and it is advisable to select a peptone which gives the characteristic



reaction with a known cholera organism, and to use it for further tests. It is also essential that the sulphuric acid should be pure, for if traces of nitrites are present the reaction may be given by an organism which has not the power of forming nitrites.

*Hæmolytic Test.*—This method, introduced by Kraus, is performed by means of agar plates, a small quantity of sterile defibrinated blood being added to the agar and thoroughly diffused; if any organism has hæmolytic properties, a clear zone or areola forms around each colony by the diffusion of hæmoglobin. In no instance has an undoubted cholera organism been found to produce hæmolysis, whereas many species of spirilla closely resembling it possess marked hæmolytic action. This test may accordingly be applied along with the others in determining the identity of a supposed cholera organism.

**Powers of Resistance.**—In their resistance against heat, cholera spirilla correspond with most spore-free organisms, and are killed in an hour by a temperature of  $55^{\circ}$  C., and much more rapidly at higher temperatures. They have comparatively high powers of resistance against great cold, and have been found alive after being exposed for several hours to the temperature of  $-10^{\circ}$  C. They are, however, killed by being kept in ice for a few days. Against the ordinary antiseptics they have comparatively low powers of resistance, and Pfuhl found that the addition of lime, in the proportion of 1 per cent., to water containing the cholera organisms was sufficient to kill them in the course of an hour.

As regards the powers of resistance in ordinary conditions, the following facts may be stated: In cholera stools kept at the ordinary room temperature, the cholera organisms are rapidly outgrown by putrefactive bacteria, but in exceptional cases they have been found alive even after two or three months. In most experiments, however, attempts to cultivate them even after a much shorter time have failed. The general conclusion may be drawn from the work of various observers, that the spirilla do not multiply freely in ordinary sewage water, although they may remain alive for a considerable period of time. On moist linen, as Koch showed, they can flourish very rapidly. Though we can state generally that the conditions favourable for the growth of the cholera spirillum are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material, we do not know the exact circumstances under which it can flourish for an indefinite period of time as a saprophyte. The fact that the area in which cholera is an endemic disease is



so restricted, tends to show that the conditions for a prolonged growth of the spirillum outside the body are not usually supplied. Yet, on the other hand, there is no doubt that in ordinary conditions it can live a sufficient time outside the body and multiply to a sufficient extent to explain all the facts known with regard to the persistence and spread of cholera epidemics. During an epidemic at St. Petersburg the cholera organism was cultivated from the stools of a considerable number of people suffering from slight intestinal disturbance, and even quite healthy individuals. The latter may be regarded as "cholera-carriers," but the organisms were obtained from them over a comparatively short period of time, and it is unknown to what extent they maintain and spread the infection.

Cholera organisms are, as a rule, rapidly killed by being thoroughly dried, and it is inferred from this that they cannot be carried in the living condition for any great distance through the air, a conclusion which is well supported by observations on the spread of the disease. Cholera is practically always transmitted by means of water or food contaminated by the organism, and there is no doubt that contamination of the water supply by choleraic discharges is the chief means by which areas of population are rapidly infected. It has been shown that if flies are fed on material containing cholera organisms, the organisms may be found alive within their bodies twenty-four hours afterwards. And further, Haffkine found that sterilised milk might become contaminated with cholera organisms if kept in open jars to which flies had free access, in a locality infected by cholera. It is quite possible that infection may be carried by this agency in some cases.

**Experimental Inoculation.**—In considering the effects of inoculation with the cholera organism, we are met with the difficulty that none of the lower animals, so far as is known, suffer from the disease under natural conditions. And, further, attempts to produce the disease by feeding with cholera dejecta, as well as with cultures, have been unsuccessful. As the organisms are confined to the alimentary tract in the natural disease, attempts to induce their multiplication within the intestine of animals by artificially arranging favouring conditions, have occupied a prominent place in the experimental work. We shall give a short account of such experiments :—

Nikati and Rietsch were the first to inject the organisms directly into the duodenum of dogs and rabbits, and they succeeded in producing, in a considerable proportion of the animals, a choleraic condition of the intestine. These experiments were confirmed by other observers, in-



cluding Koch. Thinking that probably the spirillum, when introduced by the mouth, is destroyed by the action of the hydrochloric acid of the gastric secretion, Koch first neutralised this acidity by administering to guinea-pigs 5 c.c. of a 5 per cent. solution of carbonate of soda, and some time afterwards introduced a pure culture into the stomach by means of a tube. As this method failed to give positive results, he tried the effect of artificially interfering with the intestinal peristalsis by injecting tincture of opium into the peritoneum (1 c.c. per 200 grm. weight), in addition to neutralising as before with the carbonate of sodium solution. The result was remarkable, as thirty out of thirty-five animals treated died with symptoms of general prostration and collapse. Death occurs after a few hours. Post mortem the small intestine is distended, its mucous membrane congested, and it contains a colourless fluid with small flocculi and the cholera organisms in practically pure cultures. Koch, however, found that when the spirilla of Finkler and Prior, of Deneke, and of Miller (*vide infra*), were employed by the same method, a certain, though much smaller, proportion of the animals died from an intestinal infection. Though the changes in these cases were not so characteristic, they were sufficient to prevent the results obtained with the cholera organism from being used as a demonstration of the specific relation of the latter to the disease.

Within later years some additional facts of high interest have been established with regard to choleraic infection of animals. For example, Sabolotny found that in the marmot an intestinal infection readily takes place by simple feeding with the organism, there resulting the usual intestinal changes, sometimes with hæmorrhagic peritonitis—the organisms, however, being present also in the blood. And of special interest is the fact, discovered by Metchnikoff, that in the case of young rabbits shortly after birth a large proportion die of choleraic infection when the organisms are simply introduced along with the milk, as may be done by infecting the teats of the mother. Further, from these animals thus infected the disease may be transmitted to others by a natural mode of infection. In this affection of young rabbits many of the symptoms of cholera are present. Many of these experiments were performed with the vibrio of Massowah, which is now admitted not to be a true cholera organism, others with a cholera vibrio obtained from the water of the Seine.

It will be seen from the above account that the evidence obtained from experiments on intestinal infection of animals, though by no means sufficient to establish the specific relationship of the cholera organism, is on the whole favourable to this view, especially when it is borne in mind that animals do not in natural conditions suffer from the disease.

Experiments performed by direct inoculation also supply interesting facts. *Intraperitoneal* injection in guinea-pigs is followed by general symptoms of illness, the most prominent being distension of the abdomen, subnormal temperature, and, ultimately, profound collapse. There is peritoneal effusion, which may be comparatively clear, or may be somewhat turbid.



and contain flakes of lymph, according to the stage at which death takes place. If the dose is large, organisms are found in considerable numbers in the blood and also in the small intestine, but with smaller doses they are practically confined to the peritoneum. Kolle found that when the minimum lethal dose was used in guinea-pigs, the peritoneum might be free from living organisms at the time of death, the fatal result having taken place from an intoxication (cf. *Diphtheria*, p. 405). These and other experiments show that though the organisms undergo a certain amount of multiplication when introduced by the channels mentioned, still the tendency to invade the tissues is not a marked one. On the other hand, the symptoms of general intoxication are always pronounced.

**Experiments on the Human Subject.**—Experiments have also been performed in the case of the human subject, both intentionally and accidentally. In the course of Koch's earlier work, one of the workers in his laboratory shortly after leaving was seized with severe choleraic symptoms. The stools were found to contain cholera spirilla in enormous numbers. Recovery, however, took place. In this case there was no other possible source of infection than the cultures with which the man had been working, as no cholera was present in Germany at the time. Within recent years a considerable number of experiments have been performed on the human subject, which certainly show that in some cases more or less severe choleraic symptoms may follow ingestion of pure cultures, whilst in others no effects may result. The former was the case, for example, with Emmerich and Pettenkofer, who made experiments on themselves, the former especially becoming seriously ill. In the case of both, diarrhoea was well marked, and numerous cholera spirilla were present in the stools, though toxic symptoms were proportionately little pronounced. Metchnikoff also, by experiments on himself and others, obtained results which convinced him of the specific relation of the cholera spirillum to the disease. Lastly, we may mention the case of Dr. Örgel in Hamburg, who contracted the disease in the course of experiments with the cholera and other spirilla, and died in spite of treatment. It is believed that in sucking up some peritoneal fluid containing cholera spirilla, a little entered his mouth and thus infection was produced. This took place in September 1894, at a time when there was no cholera in Germany. On the other hand, in many cases the experimental ingestion of cholera spirilla by the human subject has given negative results. Still, as the result of observation of what takes place in a cholera epidemic and of what has been



established with regard to cholera carriers, we may consider that only a certain proportion of people are susceptible to cholera, and the facts just mentioned are, in our opinion, of the greatest importance in establishing the relation of the organism to the disease.

**Toxins.**—The general statement may be made that filtered cholera cultures as a rule have little toxic action; that is, comparatively little extracellular toxin is produced by the organism. It was, however, shown by R. Pfeiffer that the dead spirilla were highly toxic, and that, in fact, they produced, on injection into guinea-pigs, the same phenomena as living cultures, profound collapse with subnormal temperature being a prominent feature. Pfeiffer considers that the toxic substances are contained in the bodies of the organisms—that is, they are *endotoxins*,—and that they are only set free by the disintegration of the latter. He showed also that when an animal is inoculated intraperitoneally with the cholera organism, and then some time later anti-cholera serum which produces bacteriolysis is injected, rapid collapse with a fatal result may ensue, apparently due to the liberation of the intracellular toxins. The dead cultures administered by the mouth produce no effect unless the intestinal epithelium is injured, in which case poisoning may result. He considers that the desquamation of the epithelium is an essential factor in the production of the phenomena of the disease in the human subject. Pfeiffer found that the toxic bodies were to a great extent destroyed at 60° C., but even after heating at 100° C. a small proportion of toxin remained, which had the same physiological action. Later A. Macfadyen found that the product obtained by grinding up the spirilla frozen by means of liquid air had a very high degree of toxicity when injected intravenously. Like Pfeiffer, he found that the “endotoxin” was in great part destroyed at 60° C.

On the other hand, other observers (Petri, Ransom, Klein, and others) have obtained toxic bodies from *filtered cultures*. Metchnikoff, E. Roux, and Taurelli-Salimbeni have demonstrated the formation of such diffusible toxic bodies in fluid media. By means of cultures placed in collodion sacs in the peritoneum of animals, they found that the living organisms produce toxic bodies which diffuse through the wall of the sac and cause toxic symptoms. By greatly increasing the virulence of the organism, then growing it in bouillon and filtering the cultures on the third and fourth day, they obtained a fluid which was highly toxic to guinea-pigs (the fatal dose



usually being  $\frac{1}{2}$  c.c. per 100 grm. weight). The symptoms closely resemble those obtained by Pfeiffer. They found that the toxicity of the filtrate was not altered by boiling,—apparently this toxic substance is different from Pfeiffer's endotoxin. Within recent times numerous attempts have been made to procure toxic fluids by disintegrating the cholera spirilla, *e.g.* by methods of grinding, by solution by alkali, by autolysis, etc., and a certain measure of success has been reached. There has also been much discussion as to whether the toxic bodies obtained are merely liberated endotoxins or whether they also represent true extracellular toxins. The true relations of these bodies has not yet been determined, but it would seem that in all probability the greater part of the toxic substance is closely bound up with the bacterial protoplasm, and is only set free on its disintegration.

**Immunity.**—As this subject is discussed later, only a few facts will be here stated, chiefly for the purpose of making clear what follows with regard to the means of distinguishing the cholera spirillum from other organisms. The guinea-pig or any other animal may be easily immunised against the cholera organism by repeated injections (conveniently made into the peritoneum) of non-fatal doses of dead spirilla; later the living organisms may be used. In this way a high degree of immunity against the organism is developed; and further, the blood serum of an animal thus immunised (anti-cholera serum) has markedly protective power when injected, even in a small quantity, into a guinea-pig along with five or ten times the fatal dose of the living organism. Under these circumstances the spirilla undergo a granular transformation and, ultimately, solution; this phenomenon is generally known as Pfeiffer's reaction, and was applied by him to distinguish the cholera spirillum from organisms resembling it. The following are the details:—

*Pfeiffer's Reaction.*—A loopful (2 mgrm.) of a recent agar culture of the organism to be tested is added to 1 c.c. of ordinary bouillon containing .001 c.c. of anti-cholera serum. The mixture is then injected into the peritoneal cavity of a young guinea-pig (about 200 grm. in weight), and the peritoneal fluid of this animal (conveniently obtained by means of capillary glass tubes inserted into the peritoneum) is examined microscopically after a few minutes. If the spirilla injected have been cholera spirilla, it will be found that they become motionless, swell up into globules, and ultimately break down and disappear—*positive result*. If they are found active and motile, then the possibility of their being true cholera spirilla may be excluded—*negative result*. In the former case (positive result) there is, however, still the possibility that the organism is devoid of pathogenic properties and has been destroyed by



the normal peritoneal fluid. A control experiment should accordingly be made with .001 c.c. of normal serum in place of the anti-cholera serum. If no alteration of the organism occurs with its use, then the conclusion is that a true reaction has been given. Corresponding bacteriolytic effects may be obtained by *in vitro* methods, introduced since Pfeiffer's original method (p. 534).

The serum of an animal immunised by the above method has also marked agglutinative and other anti-bacterial properties (p. 541) against the cholera spirillum, and these properties closely correspond with Pfeiffer's reaction as regards specificity. Such a serum has, however, little protective effect against the toxic action of the dead spirilla, and Pfeiffer maintains that little or no antitoxin to the endotoxin can be produced. On the other hand, Macfadyen, by injecting the endotoxin derived from the spirilla by grinding, obtained a serum which had antitoxic as well as agglutinative and bacteriolytic properties (*vide* Immunity). Metchnikoff and others have also obtained antitoxic sera which act on the extracellular toxins obtained by filtration.

The *serum of cholera convalescents* has been found to possess protective and increased bactericidal action. These properties of the serum may be present eight or ten days after the attack of the disease, but are most marked four weeks after; they then gradually diminish. Specific agglutinative properties have, however, been detected in the serum of cholera patients at a much earlier date, in some cases even on the first day of the disease, though usually a day or two later. The dilutions used have been usually 1 : 15 to 1 : 120, and these had no appreciable effect on organisms other than the cholera spirillum (Achard and Bensande). In some cases, however, the agglutinative property may not appear after recovery. Variations in the opsonic index, analogous to those in other diseases, have recently been observed in cholera, a marked fall on the acute onset of the disease being a noteworthy feature.

Within recent times there have been introduced for therapeutic purposes several so-called anti-sera which are supposed to be antitoxic as well as anti-bacterial, and of these the two most extensively used are those of Kraus and Schurupoff. Reports regarding the effects of these sera during the Russian epidemic are of somewhat conflicting character, but in any case it cannot be said that they have a markedly beneficial action. They have further been critically examined by others, who deny to them any marked antitoxic action when tested experimentally. While, therefore, it may be admitted that antitoxins to some of the cholera toxins may be obtained, yet Pfeiffer's position, that



cholera anti-sera have little effect on at least most of the endotoxins, cannot be said to be shaken. It should be noted, however, that he disclaims having made the general statement, often ascribed to him, that no antitoxins are formed to endotoxins.

*Anti-Cholera Inoculation.*—Haffkine's method for inoculation against cholera exemplifies the above principles. It depends upon (a) attenuation of the virus—that is, the cholera organism, and (b) exaltation of the virus. The virulence of the organism is diminished by passing a current of sterile air over the surface of the cultures, or by various other methods. The virulence is exalted by the method of *passage*—that is, by growing the organism in the peritoneum in a series of guinea-pigs. By the latter method the virulence after a time is increased twenty-fold—that is, the fatal dose has been reduced to a twentieth of the original. Cultures treated in this way constitute the *virus exalté*. Subcutaneous injection of the *virus exalté* produces a local necrosis, and may be followed by the death of the animal, but if the animal be treated first with the attenuated virus, the subsequent injection of the *virus exalté* produces only a local oedema. After inoculation first by attenuated and afterwards by exalted virus, the guinea-pig has acquired a high degree of immunity, and Haffkine believed that this immunity was effective in the case of every method of inoculation—that is, by the mouth as well as by injection into the tissues. After trying his method on the human subject and finding it free from risk, he extended it in practice on a large scale in India in 1894. In the human subject two or sometimes three inoculations were formerly made with attenuated virus before the *virus exalté* was used; now, however, a single injection of the latter is usually practised. The results of preventive inoculation in India and also during the recent epidemic in Russia have been such as to establish its efficiency, both the case incidence and the mortality being reduced.

**Methods of Diagnosis.**—In the first place, the stools ought to be examined microscopically. Dried film preparations should be made and stained by any ordinary stains, though carbol-fuchsin diluted four times with water is specially to be recommended. Hanging-drop preparations, with or without the addition of a weak watery solution of gentian-violet or other stain, should also be made, by which method the motility of the organism can be readily seen. By microscopic examination the presence of spirilla will be ascertained, and an idea as to their number obtained. In some cases the cholera spirilla are so numerous in the stools that a picture is presented which is obtained in no other con-



dition, and a microscopic examination may be sufficient for practical purposes. According to Koch, a diagnosis was made in 50 per cent. of the cases during the Hamburg epidemic by microscopic examination alone. In the case of the first appearance of a cholera-like disease, however, all the other tests should be applied before a definite diagnosis of cholera is made. Dunbar has recently introduced a method for rapid diagnosis which depends on the properties of an anti-cholera serum. Two hanging-drop preparations are made, each consisting of a small portion of mucus from the suspected stool broken up in peptone solution. To one a drop of a 50-fold dilution of normal serum is added, to the other a drop of a 500-fold dilution of an active cholera serum. If the spirilla present are cholera organisms, they retain their motility in the first preparation, while they lose it and then become agglutinated in the second. By this method a diagnosis may sometimes be given in a few minutes.

If the organisms are very numerous, gelatin or agar plates may be made at once and pure cultures obtained.

If the spirilla occur in comparatively small numbers, the best method is to inoculate peptone solution (1 per cent.) and incubate for from eight to twelve hours. At the end of that time the spirilla will be found on microscopic examination in enormous numbers at the surface, and thereafter plate cultures can readily be made. If the spirilla are very few in number, or if a suspected water is to be examined for cholera organisms, the peptone solution which has been inoculated should be examined at short intervals till the spirilla are found microscopically. A second flask of peptone solution should then be inoculated, and possibly again a third from the second, and then plates may be made. In such circumstances Dieudonné's medium (p. 44) has been found of much service.

When a spirillum has been obtained in pure condition by these methods, the appearance of the colonies in plates should be specially noted, the test for the cholera-red reaction should be applied, and in many cases it is advisable to test the effects of intraperitoneal injection of a portion of a recent agar culture in a guinea-pig, the amount sufficient to cause death being also ascertained. The agglutinating or sedimenting properties of the serum of the patient should be tested against a known cholera organism, and against the spirillum cultivated from the case. The action of an anti-cholera serum, *i.e.* the serum of an animal immunised against the cholera spirillum, should be tested in a similar manner.

Up till recent times there had been cultivated, from sources



other than cholera cases, no organism which gave all the cultural and biological tests (agglutination and Pfeiffer's reaction) of the cholera spirillum. In, 1905, however, Gotschlich obtained six different strains of a spirillum which conformed in all these respects. The organisms were obtained at El Tor from the intestines of pilgrims who had died with dysenteric symptoms, and there were no cases of cholera in the vicinity. The organisms in question, however, differ from the cholera organism in having marked hæmolytic action, and also in producing a rapidly acting extracellular toxin. Kraus and others have found, on comparing anti-sera to the cholera and El Tor spirilla, that while the anti-bacterial properties are similar there is a difference in antitoxic action. The El Tor antitoxin neutralises the cholera toxin, but a cholera antitoxin has no effect on the El Tor toxin; the El Tor spirillum is thus peculiar as regards its toxic products. There is accordingly difference of opinion as to whether these organisms are to be regarded as a distinct species or as true cholera spirilla which had been carried by the patients, though no symptoms resulted from their presence. This instance exemplifies well the great difficulty which may surround the identification of a particular organism obtained from non-cholera cases, and one can hardly doubt that if cholera-like symptoms had been present in the El Tor cases, the spirilla would have been accepted as varieties of the cholera organism. None of the facts ascertained, however, really affect the question as to the causal relationship of Koch's spirillum to cholera, although they indicate the difficulties which may attend the bacteriological diagnosis in isolated cases of disease.

**General Summary.**—We may briefly summarise as follows the facts in favour of Koch's spirillum being the cause of cholera: *First*, there is the constant presence of spirilla in true cases of cholera, which on the whole conform closely with Koch's description, though variations undoubtedly occur. Moreover, the facts known with regard to their conditions of growth, etc., are in conformity with the origin and spread of cholera epidemics. *Secondly*, the experiments on animals with Koch's spirillum or its toxins give as definite results as one can reasonably look for in view of the fact that animals do not suffer naturally from the disease. *Thirdly*, the experiments on the human subject and the results of accidental infection by means of pure cultures are also strongly in favour of this view. *Fourthly*, the agglutinative and protective properties of the serum of cholera patients and convalescents constitute another point in its favour. *Fifthly*, bacteriological methods, which proceed on the assumption that



Koch's spirillum is the cause of the disease, have been of the greatest value in the diagnosis of the disease. And *lastly*, the results of Haffkine's method of preventive inoculation in the human subject, which are on the whole favourable, also supply additional evidence. If all these facts are taken together, we consider the conclusion must be arrived at that the growth of Koch's spirillum in the intestine is the immediate cause of the disease. This does not exclude the probability of an important part being played by conditions of weather and locality, though such are very imperfectly understood. Pettenkofer, for example, recognised two main factors in the causation of epidemics, which he designated  $x$  and  $y$ , and considered that these two must be present together in order that cholera may spread. The  $x$  is the direct cause of the disease—an organism which he admitted to be Koch's spirillum; the  $y$  includes climatic and local conditions, *e.g.* state of ground-water, etc.

**Other Spirilla resembling the Cholera Organism.**—These have been chiefly obtained either from water contaminated by sewage or from the intestinal discharge in cases with choleraic symptoms. Some of them differ so widely in their cultural and other characters (some, for example, are phosphorescent) that no one would hesitate to classify them as distinct species. Others, however, closely resemble the cholera organism.

The *vibrio berolinensis*, cultivated by Neisser from Berlin sewage water, differs from the cholera organism only in the appearance of its colonies in gelatin plates, its weak pathogenic action, and its giving a negative result with Pfeiffer's test. It, however, gives the cholera-red reaction. The *vibrio Danubicus*, cultivated by Heider from canal water, also differs in the appearance of its colonies in plates, and also reacts negatively to Pfeiffer's test; in most respects it closely resembles the cholera organism. Another spirillum (*v. Ivanoff*) was cultivated by Ivanoff from the stools of a typhoid patient after these had been diluted with water. The organism differed somewhat in the appearance of its colonies and in its great tendency to grow out in the form of long threads, but Pfeiffer found that it reacted to his test in the same way as the cholera organism, and he considered that it was really a variety of the cholera organism. No spirilla could be found microscopically in the stools in this case, and Pfeiffer is of the opinion that the organism gained entrance accidentally. These examples will show how differences of opinion, even amongst experts, might arise as to whether a certain spirillum were really the cholera organism or a distinct species resembling it.

A few examples may also be given of organisms cultivated from cases in which cholera-like symptoms were present.

The *vibrio* of *Massowah* was cultivated by Pasquale from a case during



a small epidemic of cholera. The organism so closely resembles Koch's spirillum that it was accepted by several authorities as the true cholera organism, and, as already stated, Metchnikoff produced by it cholera symptoms in the human subject, and also the cholera-like disease in young rabbits. It possesses four flagella, has a high degree of virulence, producing septicæmia both in guinea-pigs and pigeons, and its colonies in plates differ somewhat from the cholera organism. Moreover, it reacts negatively to Pfeiffer's test. Another organism, the *v. Gindha*, was cultivated by Pasquale from a well, and was at first accepted by Pfeiffer as the cholera organism, but afterwards rejected, chiefly because it failed to give the specific immunity reaction. It also differs somewhat from the cholera organism in its pathogenic effects, and it fails to give the cholera-red reaction, or gives it very faintly.

Pestana and Bettencourt also cultivated a species of spirillum from a number of cases during an epidemic in Lisbon—an epidemic in which there were symptoms of gastro-enteritis, although only in a few instances did the disease resemble cholera. They also cultivated the same organism from the drinking water. It differs from the cholera organism in the appearance of its colonies and of puncture cultures in gelatin. It has very feeble pathogenic effects, and gives a very faint, or no, cholera-red reaction. To Pfeiffer's test it also reacts negatively. Another spirillum (*v. Romanus*) was obtained by Celli and Santori from twelve out of forty-four cases where there were the symptoms of mild cholera. This organism does not give the cholera-red reaction, nor is it pathogenic for animals. They look upon it as a "transitory variety" of the cholera organism, though sufficient evidence for this view is not adduced.

We have mentioned these examples in order to show some of the difficulties which exist in connection with this subject. It is important to note that, on the one hand, spirilla which have been judged to be of different species from the cholera organism, have been cultivated from cases in which cholera-like symptoms were present; and, on the other hand, in cases of apparently true cholera considerable variations in the characters of the cholera organisms have been found. Such variations have especially been recorded by Colonel Cunningham in India. It is therefore quite an open question whether some of the organisms in the former class may not be cholera spirilla which have undergone variations as a result of the conditions of their growth. The great bulk of evidence, however, goes to show that Asiatic cholera always spreads as an epidemic from places in India where the disease is endemic, and that its direct cause is Koch's spirillum with the characters described above. It is sufficient to bear in mind that choleraic symptoms may be produced by other causes, and that in some of such cases spirilla which have a resemblance to Koch's organism may be present in the intestinal discharges, though rarely in large numbers.

A number of other spirilla have been cultivated, which are of interest on account of their points of resemblance to the cholera



organism, though probably they produce no pathological conditions in the human subject.

**Metchnikoff's Spirillum (vibrio Metchnikovi).**—This organism was obtained by Gamaleia from an epidemic disease of fowls in Odessa, and is of special interest on account of its close resemblance to the cholera organism.

Morphologically the organism is practically identical with Koch's spirillum (Fig. 136). It is actively motile, and has the same staining reactions. Its growth in peptone-gelatin also closely resembles that of the cholera organism, though it produces liquefaction more rapidly (Fig. 137, A). In gelatin plates the young colonies are, however, smoother and more

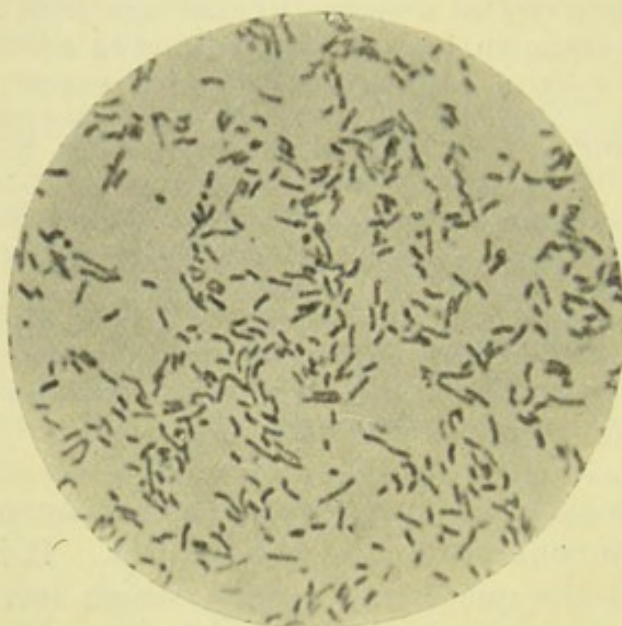


FIG. 136.—Metchnikoff's spirillum, both in curved and straight forms; from an agar culture of twenty-four hours' growth. Stained with weak carbol-fuchsin.  $\times 1000$ .

circular. After liquefaction occurs, some of the colonies are almost identical in appearance with those of the cholera vibrio, whilst others show more uniformly turbid contents. In puncture cultures the growth takes place more rapidly, but in appearance closely resembles that of the cholera organism a few days older. Its growth in peptone solution, too, is closely similar, and it also gives the cholera-red reaction.

This organism can, however, be readily distinguished from the cholera organism by the effects of inoculation on animals, especially on pigeons and guinea-pigs. Subcutaneous inoculation of small quantities of pure culture in pigeons is followed

by septicæmia, which produces a fatal result usually within twenty-four hours. Inoculation with the same quantity of cholera culture produces practically no result; even with large quantities death is rarely produced. The vibrio Metchnikovi produces somewhat similar effects in the guinea-pig to those in the pigeon, subcutaneous inoculation being followed by extensive hæmorrhagic œdema and a rapidly fatal septicæmia. Young fowls can be infected by feeding with virulent cultures. We have evidence from the work of Gamaleia that the toxins of this organism have somewhat the same action as those of the cholera organism.

The organism is therefore one which very closely resembles the cholera organism, the results on inoculating the pigeon offering the most ready means of distinction. It gives a negative reaction to Pfeiffer's test—that is, the properties of an anti-cholera serum are not exerted against it. It may also be mentioned that an organism which is apparently the same as the vibrio Metchnikovi was cultivated by Pfuhl from water, and named v. Nordhafen.

**Finkler and Prior's Spirillum.**—These observers, shortly after Koch's



discovery of the cholera organism, separated a spirillum, in a case of *cholera nostras*, from the stools after they had been allowed to decompose for several days. There is, however, no evidence that the spirillum has any causal relationship to this or any other disease in the human subject. Morphologically it closely resembles Koch's spirillum, and cannot be distinguished from it by its microscopical characters, although, on the whole, it tends to be rather thicker in the centre and more pointed at the ends (Fig. 138). In cultures, however, it presents marked differences. In puncture cultures on gelatin it grows much more quickly, and liquefaction is generally visible within twenty-four hours. The liquefaction spreads rapidly, and usually in forty-eight hours it has produced a funnel-shaped tube with turbid contents, denser below (Fig. 137, B). In plate cultures the growth of the colonies is proportionately rapid. Before they have produced liquefaction around them, they appear, unlike those of the cholera organism, as minute spheres with smooth margins. When liquefaction occurs, they appear as little spheres with turbid contents, which rapidly increase in size; ultimately general liquefaction occurs. On potatoes this organism grows well at the ordinary temperature, and in two or three days has formed a slimy layer of greyish-yellow colour, which rapidly spreads over the potato. On all the media the growth has a distinctly foetid odour. A growth in peptone solution fails to give the cholera-red reaction at the end of twenty-four hours, though later a faint reaction may appear.

An organism cultivated by Miller ("Miller's Spirillum") from the cavity of a decayed tooth in a human subject is almost certainly the same organism as Finkler and Prior's spirillum.

**Deneke's Spirillum**—This organism was obtained from old cheese, and is also known as the *spirillum tyrogenum*. It closely resembles Koch's spirillum in microscopic appearances, though it is rather thinner and smaller. Its growth in gelatin is also somewhat similar, but liquefaction proceeds more rapidly, and the bell-shaped depression on the surface is larger and shallower, whilst the growth has a more distinctly yellowish tint. The colonies in plates also show points of resemblance, though the youngest colonies are rather smoother and more regular on the surface, and liquefaction occurs more rapidly than in the case of the cholera

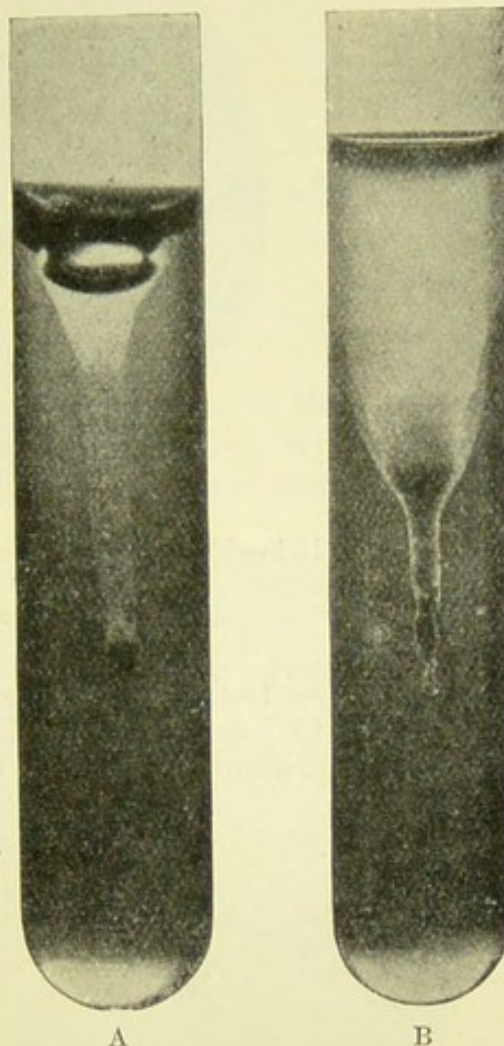


FIG. 137.—Puncture cultures in peptone-gelatin.

- A. Metchnikoff's spirillum. Five days' growth.
- B. Finkler and Prior's spirillum. Four days' growth. Natural size.



organism. The colonies have, on naked-eye examination, a distinctly yellowish colour. The organism does not give the cholera-red reaction, and on potato it forms a thin yellowish layer when incubated above

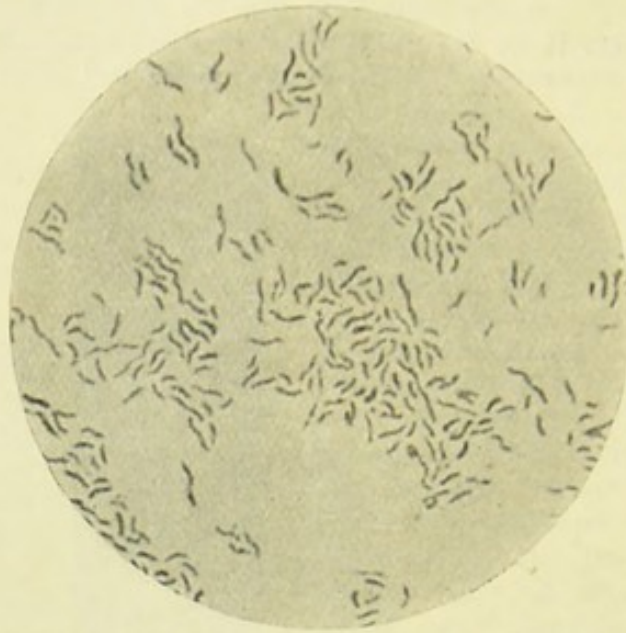


FIG. 138.—Finkler and Prior's spirillum ; from an agar culture of twenty-four hours' growth.  
Stained with carbol-fuchsin.  $\times 1000$ .

30° C. When tested by intraperitoneal injection and by other methods, it is found to possess very feeble, or almost no pathogenic properties. Deneke's spirillum is usually regarded as a comparatively harmless saprophyte.



## CHAPTER XIX.

### INFLUENZA, WHOOPING-COUGH, PLAGUE, MALTA FEVER.

#### INFLUENZA.

THE first accounts of the organism now known as the influenza bacillus were published simultaneously by Pfeiffer, Kitasato, and Canon, in January 1892. The two first-mentioned observers found it in the bronchial sputum, and obtained pure cultures, and Canon observed it in the blood in a few cases of the disease. It is, however, to Pfeiffer's work that we owe most of our knowledge regarding its characters and action. His results have been amply confirmed by those of others in various epidemics of the disease, and this organism has been generally accepted as the cause of the disease, although absolute proof is still wanting.

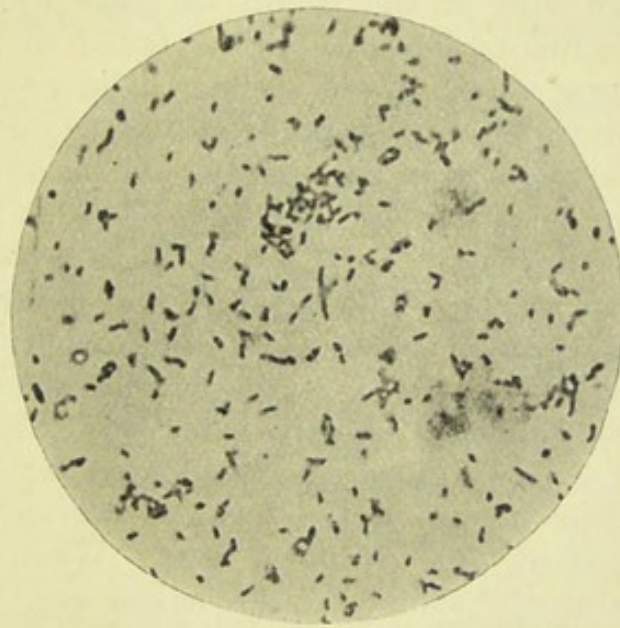


FIG. 139.—Influenza bacilli from a culture  
on blood agar.  
Stained with carbol-fuchsin.  $\times 1000$ .

**Microscopical Characters.**—The influenza bacilli as seen in the sputum are very minute rods not exceeding  $1.5 \mu$  in length and  $.3 \mu$  in thickness. They are straight, with rounded ends, and sometimes stain more deeply at the extremities (Fig. 139). The bacilli occur singly or form clumps by their aggregation, but do not grow into chains. They show no capsule. They take up the basic aniline stains somewhat feebly, and are best stained by a weak solution



(1 : 10) of carbol-fuchsin applied for five to ten minutes. They lose the stain in Gram's method. They are non-motile, and do not form spores.

In many cases of the disease, especially in the early stages of the more acute, influenza bacilli are present in large numbers and may be easily found. On the other hand, it is often difficult or impossible to find them, even when the symptoms are severe; this may be due to the restriction of the organisms to some part not readily accessible, or it may be that they actually die out in great part while the effects of their toxins persist. It has also been observed in recent epidemics, in which the disease has been less widespread and on the whole less severe, that the period during which the bacilli have been readily demonstrable in the secretions has been on the average shorter than in the previous epidemics.

**Cultivation.**—The best medium for the growth of the influenza bacillus is blood-smeared agar (see p. 43), which was introduced by Pfeiffer for this purpose. He obtained growths of the bacilli on agar which had been smeared with influenza sputum, but he failed to get any *sub*-cultures on the agar media or on serum. The growth in the first cultures he considered to be probably due to the presence of certain organic substances in the sputum, and accordingly he tried the expedient of smearing the agar with drops of blood before making the inoculations. In this way he completely succeeded in attaining his object. The blood of the lower animals is suitable, as well as human blood; and the favouring influences of the blood would appear to be due to the hæmoglobin, as a solution of this substance is equally effective. The colonies of the influenza bacilli on blood agar, incubated at 37° C., appear within twenty-four hours, in the form of minute circular dots almost transparent, like drops of dew. When numerous, the colonies are scarcely visible to the naked eye, but when sparsely arranged they may reach the size of a small pin's head. This size is generally reached on the second day. In cultures the bacilli may show considerable variations in size and in shape; they die out somewhat quickly, and in order to keep them alive sub-cultures should be made every four or five days. By this method the cultures may be maintained for an indefinite period. Even in sub-cultures growth on the ordinary agar media is slight and somewhat uncertain; there is, however, evidence that growth is more marked when other organisms are present, that is, is favoured by symbiosis. Neisser, for example, was able to cultivate the influenza bacillus on plain agar through several



generations by growing the xerosis bacillus along with it; dead cultures of the latter had not the same favouring effect. A very small amount of growth takes place in bouillon, but it is more marked when a little fresh blood is added. The growth forms a thin whitish deposit at the bottom of the flask. The limits of growth are from 25° to 42° C., the optimum temperature being that of the body. The influenza bacillus is a strictly aerobic organism.

The powers of resistance of this organism are of a low order. Pfeiffer found that dried cultures kept at the ordinary temperature were usually dead in twenty hours, and that if sputum were kept in a dry condition for two days, all the influenza bacilli were dead, or rather, cultures could be no longer obtained. Their duration of life in ordinary water is also short, the bacilli usually being dead within two days. From these experiments Pfeiffer concludes that outside the body in ordinary conditions they cannot multiply, and can remain alive only for a short time. The mode of infection in the disease he accordingly considers to be chiefly by means of fine particles of disseminated sputum, etc.

**Distribution in the Body.**—The bacilli are found chiefly in the respiratory passages in influenza. They may be present in large numbers in the nasal secretion, generally mixed with a considerable number of other organisms, but it is in the small masses of greenish-yellow sputum from the bronchi that they are present in largest numbers, in many cases almost in a state of purity. They occur in clumps which may contain as many as 100 bacilli, and in the early stages of the disease are chiefly lying free. As the disease advances, they may be found in considerable numbers within the leucocytes, and towards the end of the disease a large proportion have this position. It is a matter of considerable importance, however, that they may persist for weeks after symptoms of the disease have disappeared, and may still be detected in the sputum. Especially is this the case when there is any chronic pulmonary disease. They also occur in large numbers in the capillary bronchitis and catarrhal pneumonia of influenza, as Pfeiffer showed by means of sections of the affected parts. In these sections he found the bacilli lying amongst the leucocytes which filled the minute bronchi, and also penetrating between the epithelial cells and into the superficial parts of the mucous membrane. Other organisms also, especially Fraenkel's pneumococcus, may be concerned in the pneumonic conditions following influenza. In some cases influenza occurs in tubercular subjects, or is followed by tubercular



affection, in which cases both influenza and tubercle bacilli may be found in the sputum. In such a condition the prognosis is very grave. Regarding the presence of influenza bacilli in the other pulmonary complications following influenza, much information is still required. Occasionally in the foci of suppurative softening in the lung the influenza bacilli have been found in a practically pure condition. In cases of empyema the organisms present would appear to be chiefly streptococci and pneumococci; whilst in the gangrenous conditions, which sometimes occur, a great variety of organisms has been found.

Pfeiffer's observations on a large series of cases convinced him that the organism was very rarely present in the blood—that in fact its occurrence there must be looked upon as exceptional. The conclusions of other observers have, on the whole, confirmed this statement, and it is probable that the chief symptoms in the disease are due to toxins absorbed from the respiratory tract (*vide infra*). In a recent work, however, Ghedini was able to cultivate the organism from the blood and spleen during life in over 50 per cent. of the cases examined: he found that its occurrence in these situations was specially frequent during marked fever. The bacillus may be present in some of the lesions complicating influenza. Pfeiffer found it in inflammation of the middle ear, but in a case of meningitis following influenza Fraenkel's diplococcus was present. In a few cases of meningitis, however, the influenza bacillus has been found, sometimes alone, sometimes along with pyogenic cocci (Pfuhl and Walter, Cornil and Durante); Pfuhl considers that in these the path of infection is usually a direct one through the roof of the nasal cavity. This observer also found post mortem, in a rapidly fatal case with profound general symptoms, influenza bacilli in various organs, both within and outside of the vessels. In a few cases also the bacilli have been found in the brain and its membranes with little tissue change in the parts around.

Extensive observations on the bacteriology of the respiratory system show that influenza-like bacilli may be present in a great variety of conditions; we have, in fact, once more to do with a group of organisms with closely allied characters, of which Pfeiffer's influenza bacillus was the first recognised example. These "pseudo-influenza" bacilli have been obtained from the fauces, bronchi, and lungs in inflammatory conditions, and also in various specific fevers. To this group belongs the bacillus which has been cultivated from cases of whooping-cough by Spengler, Jochmann, Davis, and others, and which is present in considerable numbers in a large proportion of cases of this disease



(p. 472). Müller's "trachoma bacillus" (p. 219) is a member of the same group. All these organisms are very restricted in their growth, and require the addition of blood or hæmoglobin to the ordinary culture media; hence they are sometimes spoken of as hæmophilic bacteria. Some of the examples are a little larger than the influenza bacillus, and tend to form short filaments, but others are quite indistinguishable. All of them also seem to have very feeble pathogenic properties towards the lower animals. At present it can scarcely be claimed as possible to identify Pfeiffer's bacillus by its microscopic and cultural characters.

**Experimental Inoculation.**—There is no satisfactory evidence that any of the lower animals suffer from influenza in natural conditions, and accordingly we cannot look for very definite results from experimental inoculation. Pfeiffer, by injecting living cultures of the organism into the lungs of monkeys, in three cases produced a condition of fever of a remittent type. There was, however, little evidence that the bacilli had undergone multiplication, the symptoms being apparently produced by their toxins. In the case of rabbits, intravenous injection of living cultures produces dyspnoea, muscular weakness, and slight rise of temperature, but the bacilli rapidly disappear in the body, and exactly similar symptoms are produced by injection of cultures killed by the vapour of chloroform. Pfeiffer, therefore, came to the conclusion that the influenza bacilli contain toxic substances which can produce in animals some of the symptoms of the disease, but that animals are not liable to *infection*, the bacilli not having power of multiplying to any extent in their tissues.

Cantani succeeded in producing infection to some extent in rabbits, by injecting the bacilli directly into the anterior portion of the brain. In these experiments the organisms spread to the ventricles, and then through the spinal cord by means of the central canal, afterwards infecting the substance of the cord. An acute encephalitis was thus produced, and sometimes a purulent condition in the lateral ventricles. The bacilli were, however, never found in the blood or in other organs. Similar symptoms were also produced by injection of dead cultures, though in this case the dose required to be five or six times larger. Cantani therefore concludes that the brain substance is the most suitable nidus for their growth, but agrees with Pfeiffer in believing that the chief symptoms are produced by toxins resident in the bodies of the bacilli. He made control experiments by injecting other organisms, and also by injecting inert substances into the cerebral tissue.

The evidence, accordingly, that the influenza bacillus is the cause of the disease rests chiefly on the well-established fact that it is always present in the secretions of the respiratory tract in



true cases of influenza, and often in very large numbers. The observed relationships of the organism to lesions in the lungs and elsewhere leave no room for doubt that it is possessed of pathogenic properties, but we cannot yet maintain that its causal relationship to epidemic influenza is absolutely established.

**Methods of Examination.**—(a) *Microscopic.*—A portion of the greenish-yellow purulent material which often occurs in little round masses in the sputum should be selected, and film preparations should be made in the usual way. Films are best stained by Ziehl-Neelsen carbol-fuchsin diluted with ten parts of water, the films being stained for ten minutes at least. In sections of the tissues, such as the lungs, the bacilli are best brought out, according to Pfeiffer, by staining with the same solution as above for half an hour. The sections are then placed in alcohol containing a few drops of acetic acid, in which they are dehydrated and slightly decolorised at the same time. They should be allowed to remain till they have a moderately light colour, the time varying according to their appearance. They are then washed in pure alcohol, cleared in xylol, and afterwards mounted in balsam.

(b) *Cultures.*—A suitable portion of the greenish-yellow material having been selected from the sputum, it should be washed well in several changes of sterilised water. A portion should then be taken on a platinum needle, and successive strokes made on the surface of blood-agar tubes. The tubes should then be incubated at 37° C., when the transparent colonies of the influenza bacillus will appear, usually within twenty-four hours. These should give a negative result on inoculation on ordinary agar media.

#### WHOOPIING-COUGH.

Up to the year 1906, the chief result of bacteriological observations, of which those of Spengler, Krause and Jochmann, and Davis may specially be mentioned, had been to demonstrate the very frequent presence of minute influenza-like and hæmophilic bacilli in the sputum and also in the lesions in this disease. In the year mentioned, however, Bordet and Gengou published an account of another minute organism, and brought forward certain facts which gave strong support to its etiological relationship. A short description of this bacillus may accordingly be given.

**Characters of the Bacillus (Bordet-Gengou).**—The organism, as seen, for example, in the sputum, occurs in the form of



minute oval rods scarcely larger than the influenza bacillus. They stain rather faintly with ordinary stains, and their margin and extremities are often more deeply coloured than the centre, which may appear as an uncoloured spot; they are Gram-negative and do not form spores. In cultures they present the same characters and are less pleomorphous than the influenza bacillus (Fig. 140). They are specially numerous at the beginning of the disease, and they may be found in large numbers in almost pure culture in the opaque whitish sputum expectorated from the bronchi; as the disease advances they become scanty, and may disappear when the symptoms of the disease are still prominent. Bordet and Gengou succeeded in obtaining pure cultures on the blood-agar medium described on p. 44, and this was found to be the most suitable of all the media tried. In the first cultures growth is very scanty and may be invisible, but later it becomes much more abundant, and sub-cultures may also be readily made on ordinary serum-agar media. As compared with that of the influenza bacillus,

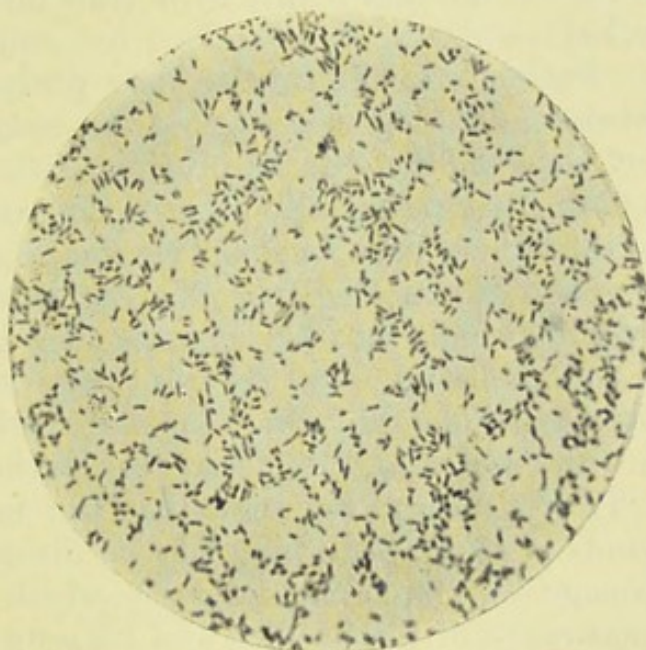


FIG. 140.<sup>1</sup>—Film preparation from a twenty-four hours' culture of the bacillus of whooping-cough. (Bordet-Gengou).

Stained with carbol-fuchsin.  $\times 1000$ .

growth is thicker and less transparent and the margins are more sharply marked off; the presence of hæmoglobin, though favouring the growth, is not so essential as in the case of the latter organism. The organism is a strict aerobe, and in the case of cultures in fluid media, *e.g.* serum bouillon, the tubes ought to be placed in a sloped position, in order to expose a greater surface to the air. Bordet and Gengou completely confirmed the observations mentioned above as to the very frequent, almost constant, presence of influenza-like bacilli. They obtained growths of these organisms, and on comparing them with their own bacillus found that distinct cultural

<sup>1</sup> We are indebted to Dr. Bordet for the culture from which this preparation was made.



differences could be made out. The most important distinctions were, however, obtained on studying the serum reactions of convalescents from the disease. They found that in many cases, though not invariably, such sera agglutinated their bacillus, but none of the influenza-like organisms. The most important result, however, was that in every case examined the serum of convalescents gave the deviation of complement reaction very markedly with the whooping-cough bacillus, but with none of the others. This means, of course, that a true anti-substance to the bacillus (immune-body or *substance sensibilisatrice*) was present in the serum, and points to a true infection with the organism (p.131).

**Pathogenic Effects.**—The general results obtained by Bordet and Gengou were that the ordinarily used animals were not susceptible to true infection with the bacillus, but that it contained a powerfully acting endotoxin, which produced both local and general effects. The injection of a small quantity of the bacillus into the eye of a rabbit produced a local necrosis, with little inflammatory change, and the introduction of dead, as well as living, cultures into the peritoneal cavity of a guinea-pig caused death from toxic action, there being great effusion into the cavity and numerous hæmorrhages in its lining.

They advanced the view that the bacillus is present in large numbers at the beginning of the disease, and inflicts some local damage on the bronchial tubes which may persist after the disappearance of the bacillus and keep up the irritation.

Similar results were obtained with an endotoxin prepared according to Besredka's method. It was not found possible to obtain an anti-toxin to this toxin. Very important results have, however, been since obtained by Klimenko, who succeeded in infecting monkeys and young dogs by intratracheal injection of pure cultures of the bacillus. After a period of incubation, there occurred an illness in which symptoms of pulmonary irritation and irregular pyrexia were outstanding features. Usually, in the case of the dogs, a fatal result followed after two or three weeks, and post mortem there were found symptoms of catarrh of the respiratory tract and sometimes patches of bronchopneumonia, from which the bacillus could be recovered in pure culture. The serum of the infected animals gave the deviation of complement reaction. A specially interesting fact is that a number of healthy young dogs contracted the disease by contact with the inoculated. Fraenkel also obtained positive results, closely similar to those of Klimenko, on inoculation with pure cultures of the bacillus.



The results of Bordet and Gengou have received general confirmation, although it is to be noted that Fraenkel and also Wollstein failed to obtain the deviation of complement reaction with the serum of convalescents. Bordet and Gengou have inquired into this discrepancy in the case of the former, and find that it depends on the nature of the culture medium used. At present it is not justifiable to make a definite pronouncement on the subject. We can only say that Bordet and Gengou have made out a strong case for the etiological relationship of their bacillus, and that their observations have been confirmed by those of others.

**Methods of Examination.**—A portion of sputum expectorated during a paroxysm of coughing should be obtained at as early as possible a stage of the disease; film preparations should be made in the usual way and stained by carbol-thionin or carbol-methylene blue. If the characteristic bacilli largely preponderate, tubes of the Bordet-Gengou medium may then be inoculated and incubated. If there are numerous colonies of other organisms in the tubes, a portion of the intervening agar should be scraped with a needle and fresh tubes inoculated. As already said, growth is at first very scanty but becomes more luxuriant in sub-cultures. On pure cultures being obtained, the deviation of complement test is to be applied by the method described (p. 130).

#### PLAGUE.

The bacillus of Oriental plague or bubonic pest was discovered independently by Kitasato and by Yersin during the epidemic at Hong Kong in 1894. They cultivated the organism from a large number of cases of plague, and reproduced the disease in susceptible animals by inoculation of pure cultures. It is to be noted that during an epidemic of plague, sometimes even preceding it, a high mortality has been observed amongst certain animals, especially rats and mice, and that from the bodies of these animals found dead in the plague-stricken district, the same bacillus was obtained by Kitasato and also by Yersin.

**Bacillus of Plague**—*Microscopical Characters.*—As seen in the affected glands or buboes in this disease, the bacilli are small oval rods, somewhat shorter than the typhoid bacillus, and about the same thickness (Fig. 141), though considerable variations in size occur. They have rounded ends, and in stained preparations a portion in the middle of the bacillus is often left uncoloured, giving the so-called "polar staining." In films from the tissues they are found scattered amongst the cells,



for the most part lying singly, though pairs are also seen. On the other hand, in cultures in fluids, *e.g.* bouillon, they grow chiefly in chains, sometimes of considerable length, the form known as a streptobacillus resulting (Fig. 143). In young agar cultures the bacilli show greater variation in size, and polar staining is less marked than in the tissues: sometimes forms of considerable length are present. After a time involution

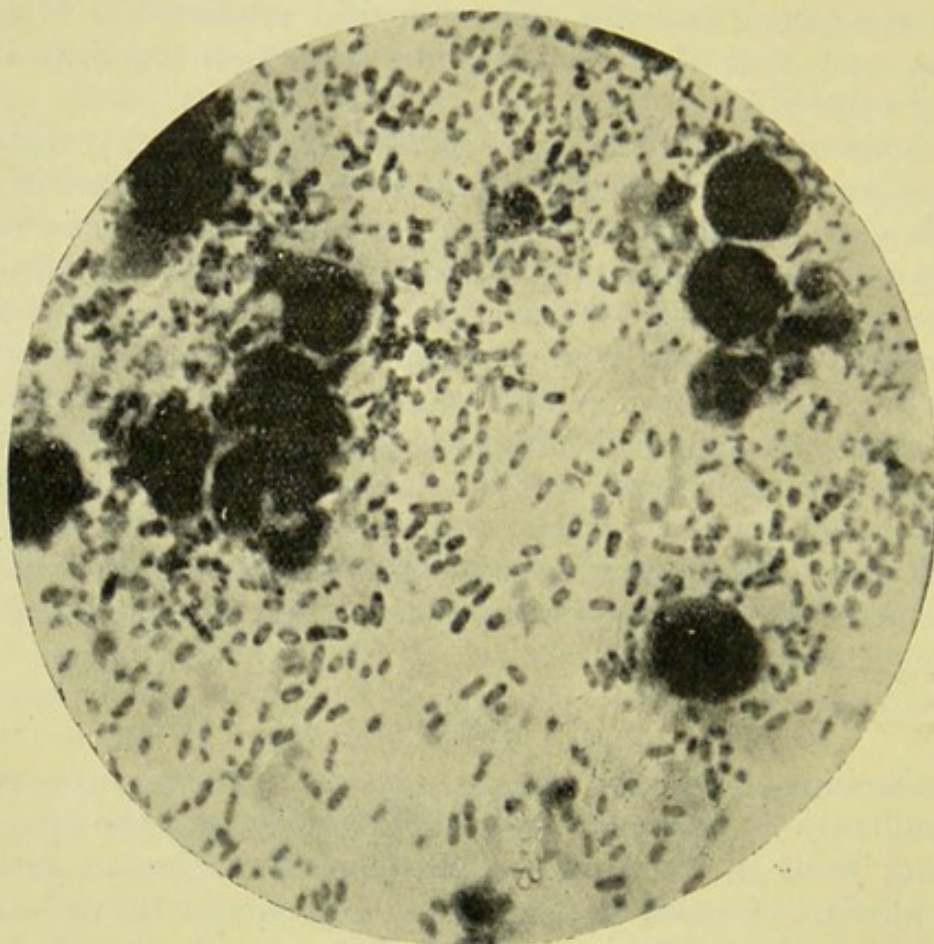


FIG. 141.—Film preparation from a plague bubo showing enormous numbers of bacilli, most of which show well-marked bipolar staining. Stained with weak gentian-violet.  $\times 1000$ .

forms appear, especially when the surface of the agar is dry; but the formation of these is much more rapid and more marked when 2 to 5 per cent. of sodium chloride is added to the medium, constituting the so-called "salt agar" (Hankin and Leumann). On this medium, especially with the higher percentage, the involution forms assume a great size and a striking variety of shapes, large globular, oval, or pyriform bodies resulting (Fig. 144); with about 2 per cent. sodium chloride, after twenty-four hours' incubation, the most striking feature is a general



enlargement of all the bacilli. Sometimes in the tissues they are seen to be surrounded by an unstained capsule, though this appearance is by no means common. They do not form spores. Gordon, who has found that they possess flagella which, however, stain with difficulty, states that they are motile. Most observers, however, and with these we agree, have failed to find evidence of true motility. They stain readily with the basic aniline stains, but are decolorised by Gram's method.

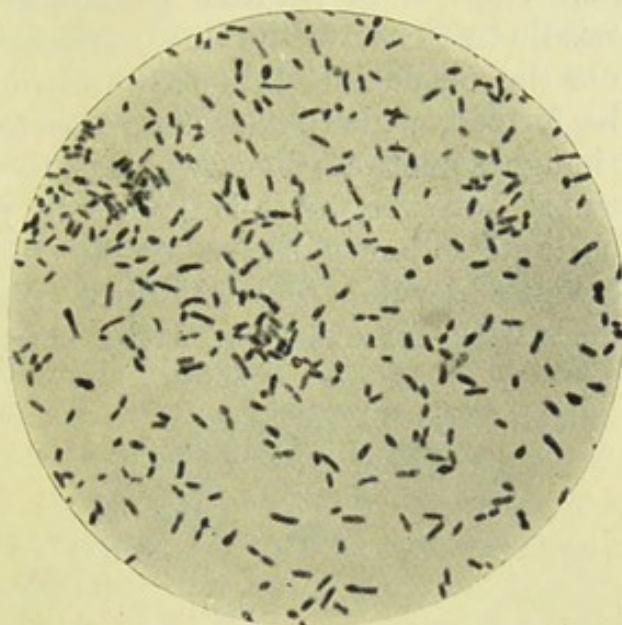


FIG. 142.—Bacillus of plague from a young culture on agar. Stained with weak carbol-fuchsin.  $\times 1000$ .

**Cultivation.**—From the affected glands, etc., the bacillus can readily be cultivated on the ordinary media. It grows best at the temperature of the

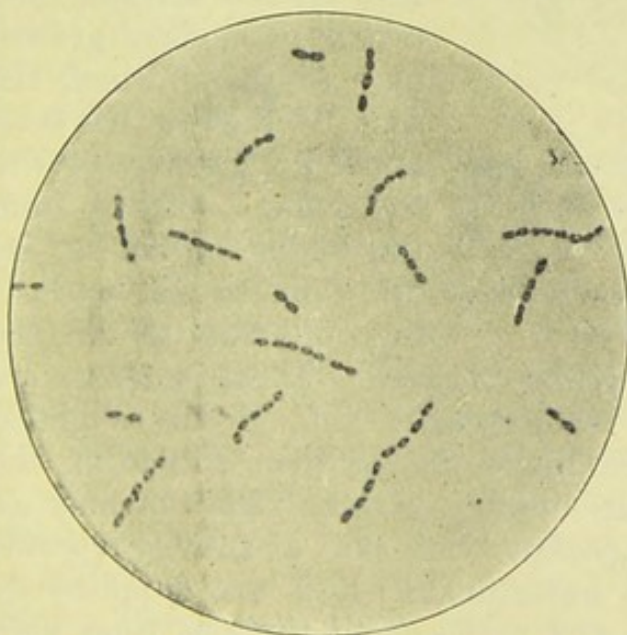


FIG. 143.—Bacillus of plague in chains showing polar staining. From a young culture in bouillon.

Stained with thionin-blue.  $\times 1000$ .

body, though growth occurs as low as  $18^{\circ}\text{C}$ . On agar and on blood serum the colonies are whitish circular discs of somewhat transparent appearance and smooth, shining surface. When examined with a lens, their borders appear slightly wavy. In stroke cultures on agar there forms a continuous line of growth with the same appearance, showing partly separated colonies at its margins. When agar cultures are kept at the room temperature, some of the colonies

may show a more luxuriant growth with more opaque appearance than the rest of the growth, the appearance in fact being often



such as to suggest the presence of impurities in the cultures. In stab cultures in peptone gelatin, growth takes place along the needle track as a white line, composed of small spherical colonies. On the surface of the gelatin a thin, semi-transparent layer may be formed, which is usually restricted to the region of puncture, though sometimes it may spread to the wall of the tube; sometimes, however, there is practically no surface growth. There is no liquefaction of the medium. In gelatin plates the superficial colonies develop first and form slightly raised semi-transparent discs with somewhat crenated margins; the deeper colonies are



FIG. 144.—Culture of the bacillus of plague on 4 per cent. salt agar, showing involution forms of great variety of size and shape. See also Plate IV., Fig. 17. Stained with carbol-thionin-blue.  $\times 1000$ .

smaller and of spherical shape, with smooth outline. In bouillon the growth usually forms a slightly granular or powdery deposit at the foot and sides of the flask, somewhat resembling that of a streptococcus. If oil or melted butter is added to the bouillon so that drops float on the surface, then a striking mode of growth may result, to which the term "stalactite" has been applied. This consists in the growth starting from the under surface of the fat globules and extending downwards in the form of pendulous, string-like masses. These masses are exceedingly delicate, and readily break off on the slightest shaking of the flask; accordingly during their formation the culture must be kept absolutely at rest. This manner of growth constitutes an important but not absolutely specific character of the organism; unfortunately it is not supplied by all races of the organism, and varies from time to time with the same race. The organism flourishes best in an abundant supply of oxygen; in strictly anaerobic conditions almost no growth takes place.

The organism in its powers of resistance corresponds with other spore-free bacilli, and is readily killed by heat, an exposure for an hour at  $58^{\circ}$  C. being fatal. On the other hand, it has



remarkable powers of resistance against cold ; it has been exposed to a temperature several degrees below freezing-point without being killed. Experiments on the effects of drying have given somewhat diverse results, but as a rule the organism has been found to be dead after being dried for from six to eight days, though sometimes it has survived the process for a longer period ; exposure to direct sunlight for three or four hours kills it. When cultivated outside the body the organism often loses its virulence, but some races remain virulent in cultures for a long period of time.

**Anatomical Changes and Distribution of Bacilli.**—The disease occurs in several forms, the *bubonic* and the *pulmonary* being the best recognised ; to these may be added the *septicæmic*. The most striking feature in the *bubonic* form is the affection of the lymphatic glands, which undergo intense inflammatory swelling, attended with hæmorrhage, and generally ending in a greater or less degree of necrotic softening if the patient lives long enough. The connective tissue around the glands is similarly affected. The bubo is thus usually formed by a collection of enlarged glands fused by the inflammatory swelling. True suppuration is rare. Usually one group of glands is affected first, constituting the primary bubo—in the great majority the inguinal or the axillary glands—and afterwards other groups may become involved, though to a much less extent. Along with these changes there is great swelling of the spleen, and often intense cloudy swelling of the cells of the kidneys, liver, and other organs. There may also occur secondary areas of hæmorrhage and necrosis, chiefly in the lungs, liver, and spleen. The bacilli occur in enormous numbers in the swollen glands, being often so numerous that a film preparation made from a scraping almost resembles a pure culture (Fig. 141). In sections of the glands in the earlier stages the bacilli are found to form dense masses in the lymph paths and sinuses (Fig. 145), often forming an injection of them ; they may also be seen growing as a fine reticulum between the cells of the lymphoid tissue. At a later period, when disorganisation of the gland has occurred, they become irregularly mixed with the cellular elements. Later still they gradually disappear, and when necrosis is well advanced it may be impossible to find any—a point of importance in connection with diagnosis. In the spleen they may be very numerous or they may be scanty, according to the amount of blood infection which has occurred ; in the secondary lesions mentioned they are often abundant. In the *pulmonary* form the lesion is the well-recognised “plague



pneumonia." This is of broncho-pneumonic type, though large areas may be formed by confluence of the consolidated patches, and the inflammatory process is attended usually by much hæmorrhage; the bronchial glands show inflammatory swelling. Clinically there is usually a fairly abundant frothy sputum often tinted with blood, and in it the bacilli may be found in large numbers. Sometimes, however, cough and expectoration may

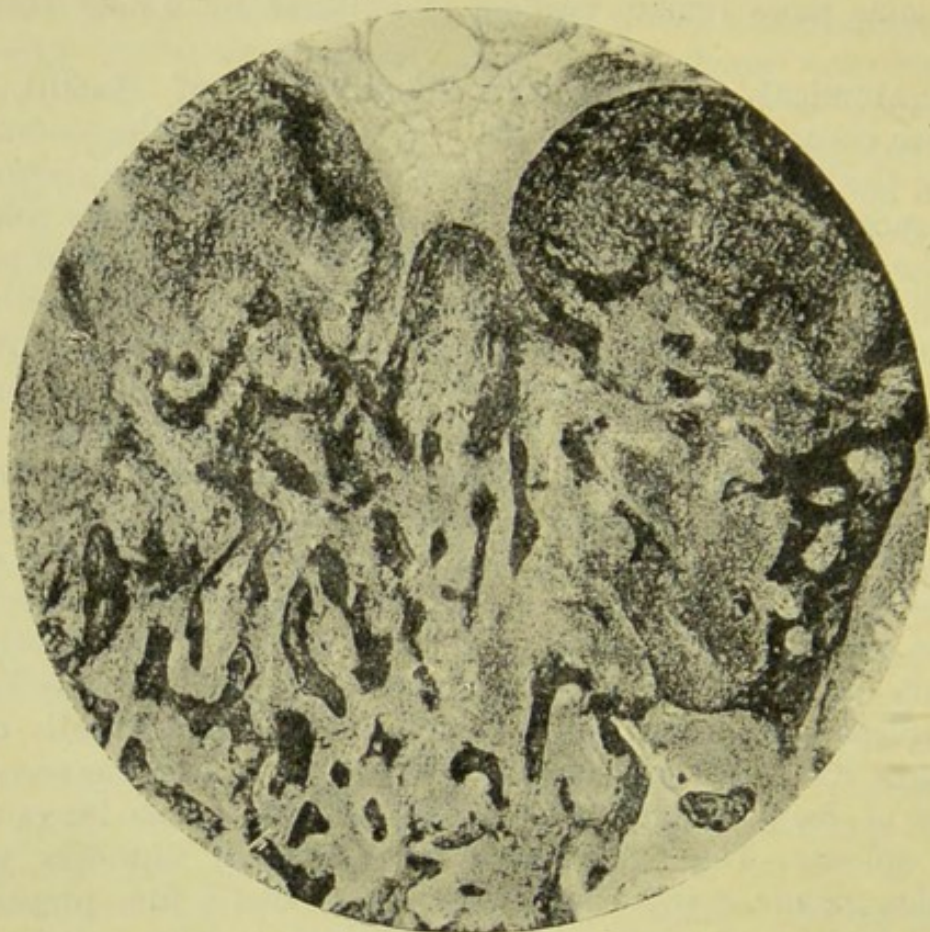


FIG. 145.—Section of a human lymphatic gland in plague, showing the injection of the lymph paths and sinuses with masses of plague bacilli—seen as black areas.

Stained with carbol-thionin-blue.  $\times 50$ .

be absent. The disease in this form is said to be invariably fatal. In the *septicæmic* form proper there is no primary bubo discoverable, though there is almost always slight general enlargement of lymphatic glands; here also the disease is of specially grave character. A bubonic case may, however, terminate with septicæmia; in fact all intermediate forms occur. An *intestinal* form with extensive affection of the mesenteric glands has been described, but it is exceedingly rare—so much



so that many observers with extensive experience have doubted its occurrence. In the various forms of the disease the bacilli occur also in the blood, in which they may be found during life by microscopic examination, chiefly, however, just before death in very severe and rapidly fatal cases. The examination of the blood by means of cultivation experiments is, however, a much more reliable procedure. For this purpose about 1 c.c. of blood may be withdrawn from a vein and distributed in flasks of bouillon (p. 72). It may be said from the results of different investigators that the bacillus may be obtained by culture in fully 50 per cent. of the cases, though the number will necessarily vary in different epidemics. The Advisory Committee, appointed by the Secretary of State for India in 1905, found that in some septicæmic cases the bacilli may be present in the blood in large numbers, two, or even three, days before death, though this is exceptional.

The above types of the disease are usually classified together under the heading *pestis major*, but there also occur mild forms to which the term *pestis minor* is applied. In these latter there may be a moderate degree of swelling of a group of glands, attended with some pyrexia and general malaise, or there may be little more than slight discomfort. Between such and the graver types, cases of all degrees of severity are met with.

**Experimental Inoculation.**—Mice, guinea-pigs, rats, and rabbits are susceptible to inoculation, the two former being on the whole most suitable for experimental purposes. After subcutaneous injection there occurs a local inflammatory œdema, which is followed by inflammatory swelling of the corresponding lymphatic glands, and thereafter by a general infection. The lesions in the lymphatic glands correspond in their main characters with those in the human subject, although usually at the time of death they have not reached a stage so advanced. By this method of inoculation mice usually die in 1 to 3 days, guinea-pigs and rats in 2 to 5 days, and rabbits in 4 to 7 days. Post mortem the chief changes, in addition to the glandular enlargement, are congestion of internal organs, sometimes with hæmorrhages, and enlargement of the spleen; the bacilli are numerous in the lymphatic glands and usually in the spleen (Fig. 146), and also, though in somewhat less degree, throughout the blood. Infection can also be produced by smearing the material on the conjunctiva or mucous membrane of the nose, and this method of inoculation has been successfully applied in cases where the plague bacilli are present along with other virulent organisms, *e.g.* in sputum along with pneumococci.



Rats and mice can also be infected by feeding either with pure cultures or with pieces of organs from cases of the disease, though in this case infection probably takes place through the mucous membrane of the mouth and adjacent parts, and only to a limited extent, if at all, by the alimentary canal. Monkeys also are highly susceptible to infection, and it has been shown in the case of these animals that when inoculation is made on the skin surface, for example, by means of a spine charged with the bacillus, the glands in relation to the part may show the characteristic lesion and a fatal result may follow without there

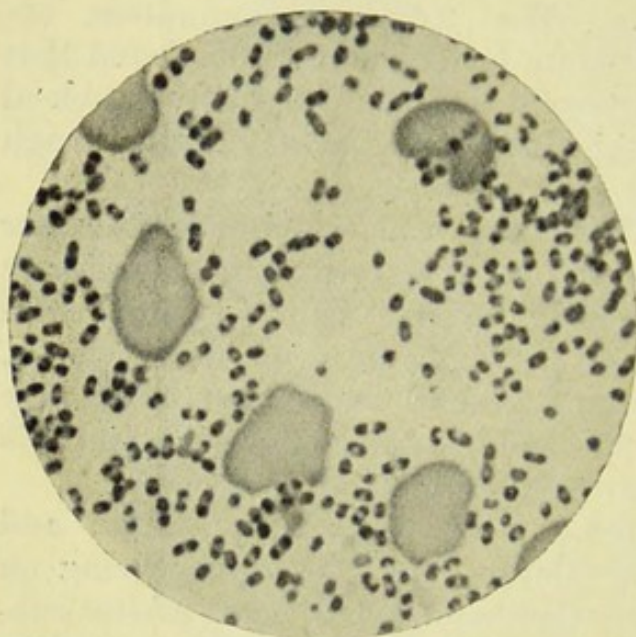


FIG. 146.—Film preparation of spleen of rat after inoculation with the bacillus of plague, showing numerous bacilli, most of which are somewhat plump. Stained with carbol-thionin-blue.  $\times 1000$ .

being any noticeable lesion at the primary seat. This fact throws important light on infection by the skin in the human subject. The disease may also extensively affect monkeys by natural means during an epidemic.

**Paths and Mode of Infection.**—Plague bacilli may enter the system by the skin surface through small wounds, cracks, abrasions, etc., and in such cases there is usually no reaction at the site of entrance. This last fact is in accordance with what

has been stated above with regard to experiments on monkeys. The path of infection is shown by the primary buboes, which are usually in the glands through which the skin is drained, those in the groin being the commonest site. Absolute proof of the possibility of infection by the skin is supplied by several cases in which the disease has been acquired at post mortem examinations, the lesions of the skin surface being in the majority of these of trifling nature; in only two was there local reaction at the site of inoculation. In most of these cases the period of incubation has been from two to three days; under natural conditions of infection the average period is within five days. While infection may occur by accidental inoculation through small wounds of the skin surface, it appears



in the majority of cases to take place by means of the bites of fleas. For some time it had been known that plague bacilli might be found for some time afterwards in the stomach of fleas allowed to feed on animals suffering from plague, and some observers, for example Simond, had succeeded in transmitting the disease to other animals by means of the infected insects. Most observers, however, had obtained negative results, and it was only by the work of the Advisory Committee referred to above,<sup>1</sup> that the importance of this means of infection was established. By carefully planned experiments, the Committee showed that the disease could be transmitted from a plague rat to a healthy rat kept in adjacent cages when fleas were present; whereas this did not occur when means were taken to prevent the access of fleas, though the facilities for aerial infection were the same. The disease can also be produced by fleas removed from plague rats and transferred directly to healthy animals, success having been obtained in fully 50 per cent. of experiments of this kind. When plague-infected guinea-pigs are placed amongst healthy guinea-pigs, comparatively few of the latter acquire the disease when fleas are absent or scanty; whereas all of them may die of plague when fleas are numerous. This result demonstrates the comparatively small part played by direct contact, even when of a close character. Important results were also obtained with regard to the mode of infection in houses where there had been cases of plague. It was found possible to produce the disease in susceptible animals by means of fleas taken from rats in plague houses. When animals were placed in plague houses and efficiently protected from fleas they remained healthy; whereas they acquired the disease when the cages were free to the access of fleas in the neighbourhood.

The following are some of the experiments which were conducted:—A series of six huts were built which only differed in the structure of their roofs. In two the roofs were made of ordinary native tiles in which rats freely lodge; in two others, flat tiles were used in which rats live, but in which they have not such facilities for movement as in the first set, and in the third pair the roof was formed of corrugated iron. Under the roof in each case was placed a wire diaphragm which prevented rats or their droppings having access to the hut, but which would not prevent fleas falling down on to the floor of the hut. The huts were left a sufficient time to become infected with rats, and then on the floor in each case healthy guinea-pigs mixed with guinea-pigs artificially infected with plague were allowed to run about together. In the first two sets of huts to which fleas had access the healthy guinea-pigs contracted plague, while in the third set they remained unaffected, though they were freely liable to contamination by contact with the bodies and excreta

<sup>1</sup> See *Journal of Hygiene*, vi. 421; vii. 323.



of the diseased animals. In the third set of huts no infection took place as long as fleas were excluded, but when accidentally these insects obtained admission, then infection of the uninoculated animals commenced. Other experiments were also performed. In one case healthy guinea-pigs were suspended in a cage two inches above a floor on which infected and flea-infested animals were running about. Infection occurred in the cage, but if the latter were suspended at a distance above the floor higher than a flea could jump, then no infection took place. Again, in a hut in which guinea-pigs had died of plague, and which contained infected fleas, two cages were placed, each containing a monkey. One cage was surrounded by a zone of sticky material broader than the jump of a flea. The monkey in this cage remained unaffected, but the other monkey contracted plague.

Other experiments showed that when plague bacilli were placed on the floors of houses, they died off in a comparatively short period of time. After forty-eight hours it was not found possible to reproduce plague by inoculation with material from floors which had been grossly contaminated with cultures of the bacillus. Afterwards, however, animals placed in such a house might become infected by means of fleas. In all these experiments the common rat-flea of India—*Pulex cheopis* (Rothschild)—was used, but it has been shown that this flea, when a rat is not available, will bite a man. Recent observations show that not only is plague transferable by means of fleas, but that this is practically the only method obtaining in natural conditions, with the exception that rats may become infected by eating the carcasses of other animals containing large numbers of plague bacilli. It is improbable from the experiments made that plague is transmitted by direct contact even when of a close nature; in fact, it has been shown that plague-infected guinea-pigs may suckle their young without the latter acquiring the disease. The general results show that in the human subject direct infection by dust and other material through small lesions of the skin plays, probably, a comparatively small part in the spread of the disease, fleas apparently being in nearly all cases the carriers of infection.

The more recent work of the Committee has supplied information of the highest value with regard to the epidemiology of the disease; it has shown, in short, that plague in its epidemic form is dependent on the epizootic among rats, and with regard to this some further facts may be given. Plague in Bombay occurs in two chief species of rats, the *mus rattus*, the black house-rat, and *mus decumanus*, the grey rat of the sewers. The former, owing to its presence in dwelling-houses, is chiefly responsible for the transmission of the disease to man; while the latter, on account of the large number of fleas which infest



it, is of special importance in maintaining the disease from season to season. The year may be divided into two portions—an epizootic season, from December to May inclusive, and a non-epizootic, from June to November. During the latter period there are few cases of plague in rats on account of fleas being scanty; especially is this so in the case of *mus rattus*. In fact, in certain villages where this species alone is present, the disease may actually die out at the end of the epizootic season, and accordingly when plague reappears in these places this is due to a fresh importation—a fact of great practical importance. A fresh epizootic first affects chiefly *mus decumanus*, and a little later spreads to *mus rattus*, while a little later still the disease attacks the human subject in the epidemic form; in each case fleas form the vehicle of transmission, and an interval of from ten to fourteen days intervenes between the outbreak of the epizootic and that of the epidemic. The proportion of cases of plague in *mus decumanus* is much higher than in *mus rattus*, for the reason mentioned. It has been further shown that the bacilli flourish in the stomach of the flea and are passed in a virulent condition in the fæces, that a large proportion of fleas removed from plague-infected rats contain plague bacilli, and that the fleas may remain infective for a considerable number of days, sometimes for a fortnight. The repeated contamination of flea-bites by means of the excrement of fleas seems to be the most likely means of infection of the human subject.

In primary plague pneumonia, from a consideration of the anatomical changes and the clinical facts, the disease may be said to be produced by the direct passage of the bacilli into the respiratory passages. Nevertheless there must be certain factors, still imperfectly understood, which determine the incidence of this form; as in some epidemics of the highest virulence, plague pneumonia has been practically absent, though opportunities for infection by inhalation must have been present. On the other hand, a case of plague pneumonia is of great infectivity in producing other cases of plague pneumonia. If we except infection through the respiratory passages in such cases, it may be said that direct infection from patient to patient is relatively uncommon. This is in accordance with the fact that in bubonic plague the bacilli are not discharged from the unbroken surface of the body, and are only present in the secretions in severe cases.

**Toxins, Immunity, etc.**—As is the case with most organisms which extensively invade the tissues, the toxins in plague



cultures are chiefly contained in the bodies of the bacteria. Injection of dead cultures in animals produces distinctly toxic effects; post mortem hæmorrhage in the mucous membrane of the stomach, areas of necrosis in the liver and at the site of inoculation, may be present. The toxic substances are comparatively resistant to heat, being unaffected by an exposure to 65° C. for an hour. By the injection of dead cultures in suitable doses, a certain degree of immunity against the living virulent bacilli is obtained, and, as first shown by Yersin, Calmette, and Borrel, the serum of such immunised animals confers a degree of protection on small animals such as mice. On these facts the principles of preventive inoculation and serum treatment, presently to be described, depend. It may also be mentioned that the filtrate of a plague culture possesses a very slight toxic action, and the Indian Plague Commission found that such a filtrate has practically no effect in the direction of conferring immunity.

1. *Preventive Inoculation—Haffkine's Method.*—To prepare the preventive fluid, cultures are made in flasks of bouillon with drops of oil on the surface (in India Haffkine employed a medium prepared by digesting goat's flesh with hydrochloric acid at 140° C. and afterwards neutralising with caustic soda). In such cultures stalactite growths (*vide supra*) form, and the flasks are shaken every few days so as to break up the stalactites and induce fresh crops. The flasks are kept at a temperature of about 25° C., and growth is allowed to proceed for about six weeks. At the end of this time sterilisation is effected by exposing the contents of the flasks to 65° C. for an hour; thereafter carbolic acid is added in the proportion of .5 per cent. The contents are well shaken to diffuse thoroughly the sediment in the fluid, and are then distributed in small sterilised bottles for use. The preventive fluid thus contains both the dead bodies of the bacilli and any toxins which may be in solution. It is administered by subcutaneous injection, the dose, which varies according to the "strength," being on an average about 7.5 c.c. Usually only one injection is made, sometimes two, though the latter procedure does not appear to have any advantage. The method has been systematically tested by inoculating a certain proportion of the inhabitants of districts exposed to infection, leaving others uninoculated, and then observing the proportion of cases of disease and the mortality amongst the two classes. The results of inoculation, as attested by the first Indian Commission, have been distinctly satisfactory. For although absolute protection is not afforded by inoculation,



both the proportion of cases of plague and the percentage mortality amongst these cases have been considerably smaller in the inoculated, as compared with the uninoculated. Protection is not established till some days after inoculation, and lasts for a considerable number of weeks, possibly for several months (Bannerman). In the Punjab during the season 1902-3 the case incidence among the inoculated was 1·8 per cent., among the uninoculated 7·7 per cent., while the case mortality was 23·9 and 60·1 per cent. respectively in the two classes, the statistics being taken from villages where 10 per cent. of the population and upwards had been inoculated.

2. *Anti-plague Sera*.—Of these, two have been used as therapeutic agents, namely, that of Yersin and that of Lustig. Yersin's serum is prepared by injections of increasing doses of plague bacilli into the horse. In the early stages of immunisation dead bacilli are injected subcutaneously, thereafter into the veins, and, finally, living bacilli are injected intravenously. After a suitable time blood is drawn off and the serum is preserved in the usual way. Of this serum 10 to 20 c.c. are used, and injections are usually repeated on subsequent days. Lustig's serum is prepared by injecting a horse with repeated and increasing doses of a substance derived from the bodies of plague bacilli, probably in great part nucleo-proteid. Masses of growth are obtained from the surface of agar cultures, and are broken up and dissolved in a 1 per cent. solution of caustic potash. The solution is then made slightly acid by hydrochloric acid, when a bulky precipitate forms; this is collected on a filter and dried. For use a weighed amount is dissolved in a weak solution of carbonate of soda and then injected. The serum is obtained from the animal in the usual way. Extensive observations with both of these sera show that neither of them can be considered a powerful remedy in cases of plague, though in certain instances distinctly favourable results have been recorded. The Indian Commission, however, came to the conclusion "that, on the whole, a certain amount of advantage accrued to the patients both in case of those injected with Yersin's serum and of those injected with Lustig's serum." It may also be mentioned that the Commission found, as the result of experiments, that Yersin's serum modified favourably the course of the disease in animals, whereas Lustig's serum had no such effect.

3. *Serum Diagnosis*.—Specific agglutinins may appear in the blood of patients suffering from plague, as also they do in the case of animals immunised against the plague bacillus. It is to be noted, however, that in clinical cases the reaction is not invariably present, the potency of the serum is not of high order, and the carrying out of the test is complicated by the natural tendency of the bacilli to cohere in clumps. For the last reason the macroscopic (sedimentation) method is to be preferred to the microscopic (p. 120). A suspension of plague bacilli is made by breaking up a young agar culture in '75 per cent. sodium chloride solution; the larger flocculi of growth are allowed to settle, and the fine, supernatant emulsion is employed in the usual way. According to the results of the German Plague Commission and the observations of Cairns, made during the Glasgow epidemic, it may be said that the reaction is best obtained with dilutions of the serum of from 1 : 10 to



1 : 50. Cairns found that the date of its appearance is about a week after the onset of illness, and that it usually increases till about the end of the sixth week, thereafter fading off. It is most marked in severe cases characterised by an early and favourable crisis, less marked in severe cases ultimately proving fatal, whilst in very mild cases it is feeble or may be absent. The method, if carefully applied, may be of service under certain conditions ; but it will be seen that its use as a means of diagnosis is somewhat restricted.

**Methods of Diagnosis.**—Where a bubo is present a little of the juice may be obtained by plunging a sterile hypodermic needle into the swelling. The fluid is then to be examined microscopically, and cultures on agar or blood serum should be made by the successive stroke method. The cultural and morphological characters are then to be investigated, the most important being the involution forms on salt agar and the stalactite growth in bouillon, though the latter may not always be obtained with the plague bacillus : the pathogenic properties should also be studied, the guinea-pig being on the whole most suitable for subcutaneous inoculation. In many cases a diagnosis may be made by microscopic examination alone, as in no known condition other than plague do bacilli with the morphological characters of the plague bacillus occur in large numbers in the lymphatic glands. The organism may be obtained in culture from the blood in a considerable proportion of cases by withdrawing a few cubic centimetres and proceeding in the usual manner. On the occurrence of the first suspected case, every care to exclude possibility of doubt should be used before a positive opinion is given.

In a case of suspected plague pneumonia, in addition to microscopic examination of the sputum, the above cultural methods along with animal inoculation with the sputum should be carried out ; subcutaneous injection in the guinea-pig and smearing the nasal mucous membrane of the rat may be recommended. Here a positive diagnosis should not be attempted by microscopic examination alone, especially in a plague-free district, as bacilli morphologically resembling the plague organism may occur in the sputum in other conditions.

#### MALTA FEVER.

*Synonyms*—*Mediterranean Fever : Rock Fever of Gibraltar : Neapolitan Fever, etc.*

This disease is of common occurrence along the shores of the Mediterranean and in its islands. Since its bacteriology has



been worked out, it has been found to occur also in India, China, South Africa, and in some parts of North and South America, its distribution being much wider than was formerly supposed. Although from its symptomatology and pathological anatomy it had been recognised as a distinct affection, and was known under various names, its precise etiology was unknown till the publication of the researches of Colonel Bruce in 1887. From the spleen of patients dead of the disease he cultivated a characteristic organism, now known as the *Micrococcus melitensis*, and by means of inoculation experiments established its causal relationship to the disease. Wright and Semple applied the agglutination test to the diagnosis of the disease, while within recent years the mode of spread of the disease has been fully studied by a Commission, and it has been demonstrated that goat's milk is the chief means of infection.

The duration of the disease is usually long—often two or three months, though shorter and much longer periods are met with. Its course is very variable, the fever being of the continued type with irregular remissions. In addition to the usual symptoms of pyrexia, there occur profuse perspiration, pains and sometimes swellings in the joints, occasionally orchitis, whilst constipation is usually a marked feature. The mortality is low—about 2 per cent. (Bruce).

In fatal cases the most striking post mortem change is in the spleen. This organ is enlarged, often weighing slightly over a pound, and in a condition of acute congestion; the pulp is soft and may be diffuent, and the Malpighian bodies are swollen and indistinct. In the other organs the chief change is cloudy swelling; in the kidneys there may be in addition glomerular nephritis. The lymphoid tissue of the intestines shows none of the changes characteristic of typhoid fever.

**Micrococcus melitensis.**—This is a small, rounded, or slightly oval organism about  $4\ \mu$  in diameter, which is specially abundant in the spleen. It usually occurs singly or in pairs, but in cultures short chains are also met with (Fig. 147). (Durham has shown that in old cultures kept at the room temperature bacillary forms appear, and we have noticed indications of such in comparatively young cultures; the usual form is, however, that of a coccus.) It stains fairly readily with the ordinary basic aniline stains, but loses the stain in Gram's method. It is generally said to be a non-motile organism. Gordon, however, is of a contrary opinion, and has recently demonstrated that it possesses from one to four flagella, which, however, are difficult to stain. In the spleen of a patient dead of the disease it



occurs irregularly scattered through the congested pulp; it may also be found in small numbers post mortem in the capillaries of various organs. It may be cultivated from the blood during life in a considerable proportion of cases; for this purpose 5 to 10 c.c. of blood should be withdrawn from a vein and distributed in small flasks of bouillon. The micrococcus was found by the members of the Commission in the urine of Malta fever patients in 10 per cent. of the cases examined; it was sometimes scanty, but sometimes present in large numbers. It has also occasionally been obtained from the fæces.

*Cultivation.*—This can usually readily be effected by making stroke cultures on agar tubes from the spleen pulp and incub-

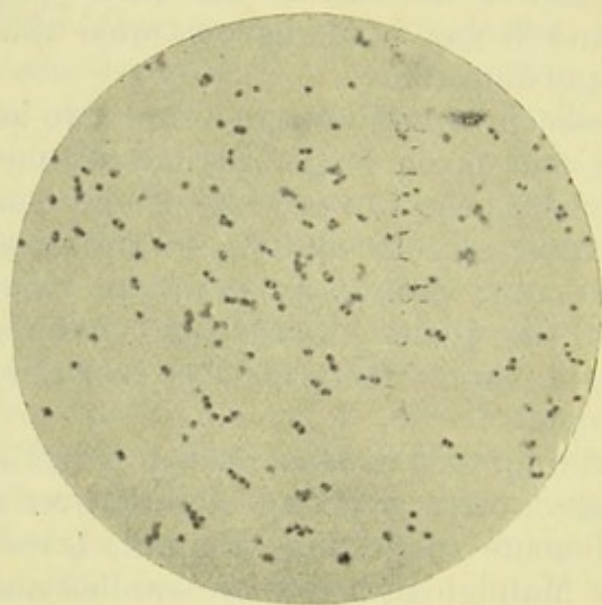


FIG. 147.—*Micrococcus melitensis*, from a two days' culture on agar at 37° C. Stained with fuchsin.  $\times 1000$ .

ating at 37° C. The colonies, which are usually not visible before the third or fourth day, appear as small round discs, slightly raised and of somewhat transparent appearance. The maximum size—2 to 3 mm. in diameter—is reached about the ninth day; at this period by reflected light they appear pearly white, while by transmitted light they have a yellowish tint in the centre, bluish white at the periphery. A stroke culture shows a layer of growth of similar appearance with

somewhat serrated margins. Old cultures assume a buff tint. The optimum temperature is 37° C., but growth still occurs down to about 20° C. On gelatin at summer temperature growth is extremely slow—after two or three weeks, in a puncture culture, there is a delicate line of growth along the needle track and a small flat expansion of growth on the surface. There is no liquefaction of the medium. In bouillon there occurs a general turbidity with flocculent deposit at the bottom; on the surface there is no formation of a pellicle. The reaction of the media ought to be very faintly alkaline, as marked alkalinity interferes with the growth; a reaction of + 10 (p. 34) has been found very suitable. On potatoes no visible growth takes place even at the body temperature, though the organism multiplies to a



certain extent. Outside the body the organism has considerable powers of vitality, as it has been found to survive in a dry condition in dust and clothing for a period of two months.

**Relations to the Disease.**—There is in the first place ample evidence, from examination of the spleen, both post mortem and during life, that this organism is always present in the disease. The experiments of Bruce and Hughes first showed that by inoculation with even comparatively small doses of pure cultures the disease could be produced in monkeys, sometimes with a fatal result. And it has now been fully established that inoculation with the minutest amount of culture, even by scarification, leads to infection both in monkeys and in the human subject.

Rabbits, guinea-pigs, and mice are insusceptible to inoculation by the ordinary method. Durham, by using the intracerebral method of inoculation, however, succeeded in raising the virulence, so that the organism is capable of producing in guinea-pigs on intraperitoneal injection illness with sometimes a fatal result many weeks afterwards. An interesting point brought out by these experiments is that, in the case of animals which survive, the micrococcus may be cultivated from the urine several months after inoculation.

**Mode of Spread of the Disease.**—The work of the recent Commission has resulted in establishing facts of the highest importance with regard to the spread of the disease. In the course of investigations Zammitt found that the blood of many of the goats agglutinated the micrococcus melitensis, and Horrocks obtained cultures of the organism from the milk. Further observations showed that agglutination was given in the case of 50 per cent. of the goats in Malta, whilst the organism was present in the milk in 10 per cent. Sometimes the organism was present in enormous numbers, and in these cases the animal usually appeared poorly nourished, whilst the milk had a somewhat serous character. In other cases, however, the organism was found when the animals appeared healthy, and there was no physical or chemical change in the milk. It was also determined that the organism might be excreted for a period of two to three months before any notable change occurred in the milk. Agglutination is usually given by the milk of infected animals, and this property was always present when the micrococcus was found in the milk. It was, moreover, found that monkeys and goats could be readily infected by feeding them with milk containing the micrococcus, the disease being contracted by fully 80 per cent. of the monkeys used. It was therefore rendered practically certain that the human subject was infected



by means of such milk, and the result of preventive measures by which milk was excluded as an article of dietary amongst the troops in Malta has fully borne out this view. After such measures were instituted, the number of cases in the second half of 1906 fell to 11 per thousand, as contrasted with 47 per thousand in the corresponding part of the preceding year; further successful results have followed. The various facts with regard to the epidemiology of the disease have thus been cleared up. For example, it is more prevalent in the summer months, when more milk is consumed, and there is a larger proportion of cases amongst those in good social position, the officers, for example, suffering more in proportion than the privates. Another interesting fact, pointed out by Horrocks, is that the disease has practically disappeared from Gibraltar since the practice of importing goats from Malta has stopped.

The work of the Commission, so far as it has gone, has been to exclude other modes of infection than the ingestion of infected milk as being of practical importance; if the disease is conveyed by contact at all, this is only when the contact is of an intimate character, and even then it is probably of rare occurrence. Although numerous patients suffering from the disease come to England, there is no known case of fresh infection arising under natural conditions.

There is distinct evidence that the disease may be acquired by inoculation through small lesions in the skin, and this method is probably not infrequent amongst those who handle infected milk. It has been shown that the organism may remain alive in the bodies of mosquitoes for four or five days, and possibly these insects may occasionally be the means of carrying the disease; there is no evidence, however, that this takes place to any extent.

*Agglutinative Action of Serum.*—The blood serum of patients suffering from Malta fever possesses the power of agglutinating the micrococcus melitensis in a manner analogous to what has been described in the case of typhoid fever. The reaction appears comparatively early, often about the fifth day, and may be present for a considerable time after recovery—sometimes for more than a year. Distinct agglutination with a 1:30 dilution of the serum in half an hour may be taken as a positive reaction, sufficient for diagnosis. The reaction is, however, usually given by much higher dilutions, *e.g.* 1:500, and even higher. It is to be noted that normal serum diluted 1:5 may produce some agglutination. As regards relation to prognosis, the observations of Birt and Lamb and of Bassett-Smith



have given results analogous to those obtained in typhoid (p. 372).

The Commission has found that vaccination with dead cultures of the micrococcus confers a certain degree of protection amongst those exposed to the disease. As a rule two injections were made, 200-300 million cocci being the dose of the first injection, and about 400 million the dose of the second. The use of vaccines has also been carried out in the treatment of the disease, but the observations are not sufficiently numerous to allow a definite statement to be made as to its value.

**Methods of Diagnosis.**—During life the readiest means of diagnosis is supplied by the agglutinative test just described (for technique, *vide* p. 118).

Cultures are most easily obtained from the spleen either during life or post mortem. Inoculate a number of agar tubes by successive strokes and incubate at 37° C. Film preparations should also be made from the spleen pulp and stained with carbol-thionin-blue or diluted carbol-fuchsin (1:10). Cultures may sometimes be obtained from the blood by the usual methods.



## CHAPTER XX.

### DISEASES DUE TO SPIROCHAETES—THE RELAPSING FEVERS, SYPHILIS, AND FRAMBÆSIA.

THE diseases produced by spirochaetes—spirilloses or spirochaetoses—fall into two main groups, one represented by the human spirillar fevers and the corresponding affections of various animals, and the second having as its two chief members syphilis and yaws, though to the organisms of these diseases various spirochaetes found in ulcerative and gangrenous conditions seem to be closely related. The members of the first group are essentially blood infections, and the organisms are in most, if not in all cases, transmitted by blood-sucking ectoparasites; in the second group the organisms are primarily tissue-parasites, blood infection when it occurs being a later phenomenon, and infection would appear to occur by direct contact. As regards general morphology, staining reactions, conditions of growth and culture, the various spirochaetes present certain common characters, and, as already stated, it is still uncertain whether they are to be regarded as bacteria or as protozoa, though the balance of opinion is now distinctly in favour of the latter.

#### RELAPSING FEVER AND AFRICAN TICK FEVER.

At a comparatively early date, namely in 1873, when practically nothing was known with regard to the production of disease by bacteria, a highly characteristic organism was discovered by Obermeier in the blood of patients suffering from relapsing fever. This organism is usually known as the *spirillum* or *spirochaete* *Obermeieri*, or the *spirillum* of *relapsing fever*. He described its microscopical characters, and found that its presence in the blood had a definite relation to the time of the fever, as the organism rapidly disappeared about the time of the crisis, and reappeared when a relapse occurred.



His observations were fully confirmed, and his views as to its causal relationship to the disease have been established as correct.

Within recent years relapsing fever has been carefully studied in different parts of the world, and the relationships of the organisms have been the subject of much investigation and discussion. This question will be referred to again below. Recently also it has been shown that the so-called "tick fever" prevalent in Africa is due to a spirochæte of closely similar character, and results of the highest importance have been established with regard to the part played by ticks in the transmission of the disease. As a matter of convenience, we shall give the chief facts regarding these diseases separately. It has also been shown that spirillar diseases or "spirillooses," as they are called, are widespread amongst vertebrates; they have been described, for example, in geese by Sacharoff, in fowls by Marchoux and Salimbeni, in oxen and sheep by Theiler, and in bats by Nicolle and Comte, and it is interesting to note that in the case of the spirillooses of oxen and fowls the infection is transmissible by means of ticks.

**Characters of the Spirochæte.**—The organisms as seen in the blood during the fever are delicate spiral filaments which have a length of from two to six times the diameter of a red blood corpuscle. They are, however, exceedingly thin, their thickness being much less than that of the cholera spirillum. They show several regular sharp curves or windings, of number varying according to the length of the organisms, and their extremities are finely pointed (Fig. 148). There are often to be seen in the spirals, portions which are thinner and less deeply stained than the rest, and which suggest the occurrence of transverse division. They are actively motile, and may be seen moving quickly across the microscopic field with a peculiar movement which is partly twisting and partly undulatory, and disturbing the blood corpuscles in their course.

They stain with watery solutions of the basic aniline dyes, though somewhat faintly, and are best coloured by the Romanowsky method or one of its modifications. When thus stained they usually have a uniform appearance throughout, or may be slightly granular at places, but they show no division into short segments. They lose the stain in Gram's method. There is no evidence that they form spores.

Novy found that the spirochæte of American relapsing fever remained alive and virulent in defibrinated rats' blood for forty days. He also succeeded, by Levaditi's method (p. 503),



in obtaining cultures in collodion sacs containing rats' blood which were placed in the peritoneum of rats. By this method cultures were maintained for many generations; the organisms were still virulent though the resulting infection was rather less intense than at first. The spirochaetes are readily killed at a temperature of 60° C., but may be exposed to 0° C. without being killed. Novy and Knapp have found that there is a single flagellum at one end of this organism.

**Relations to the Disease.**—In relapsing fever, after a period of incubation there occurs a rapid rise of temperature which lasts for about five to seven days. At the end of this time a

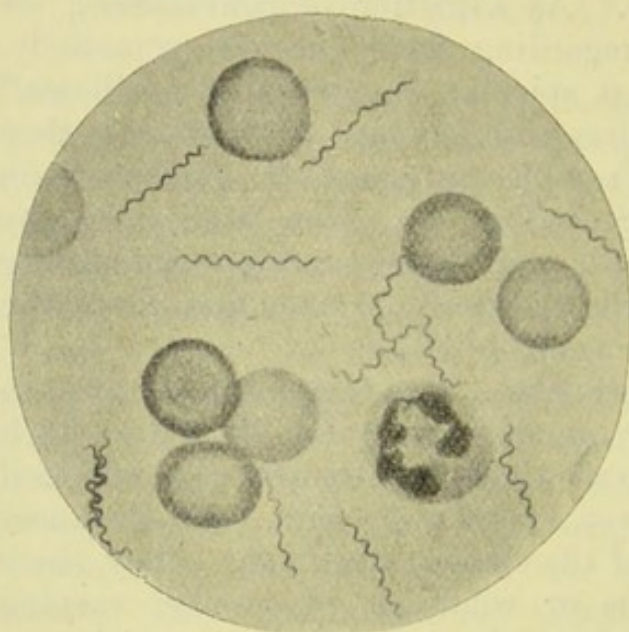


FIG. 148. —Spirochaetes of relapsing fever in human blood. Film preparation. (After Koch.) See also Plate IV., Fig. 18.  $\times$  about 1000.

crisis occurs, the temperature falling quickly to normal. In the course of about other seven days a sharp rise of temperature again takes place, but on this occasion the fever lasts a shorter time, again suddenly disappearing. A second or even third relapse may occur after a similar interval. The organisms begin to appear in the blood shortly before the onset of the pyrexia, and during the rise of temperature rapidly increase in number.

They are very numerous

during the fever, a large number being often present in every field of the microscope when the blood is examined at this stage. They begin to disappear shortly before the crisis: after the crisis they are entirely absent from the circulating blood. A similar relation between the presence of the organisms in the blood and the fever is found in the case of the relapses. Münch in 1876 produced the disease in the human subject by injecting blood containing the spirochaetes, and this experiment has been several times repeated with the same result. Additional proof that the organism is the cause of the disease has been afforded by experiments on animals. Carter in 1879 was the first to show that the disease could be readily produced in monkeys, and his experiments were confirmed by Koch. In such



experiments the blood taken from patients and containing the spirochaetes was injected subcutaneously. In the disease thus produced there is an incubation period which usually lasts about three days. At the end of that time the organisms rapidly appear in the blood, and shortly afterwards the temperature quickly rises. The period of pyrexia usually lasts for two or three days, and is followed by a marked crisis. As a rule there is no relapse, but occasionally one of short duration occurs.<sup>1</sup> White mice and

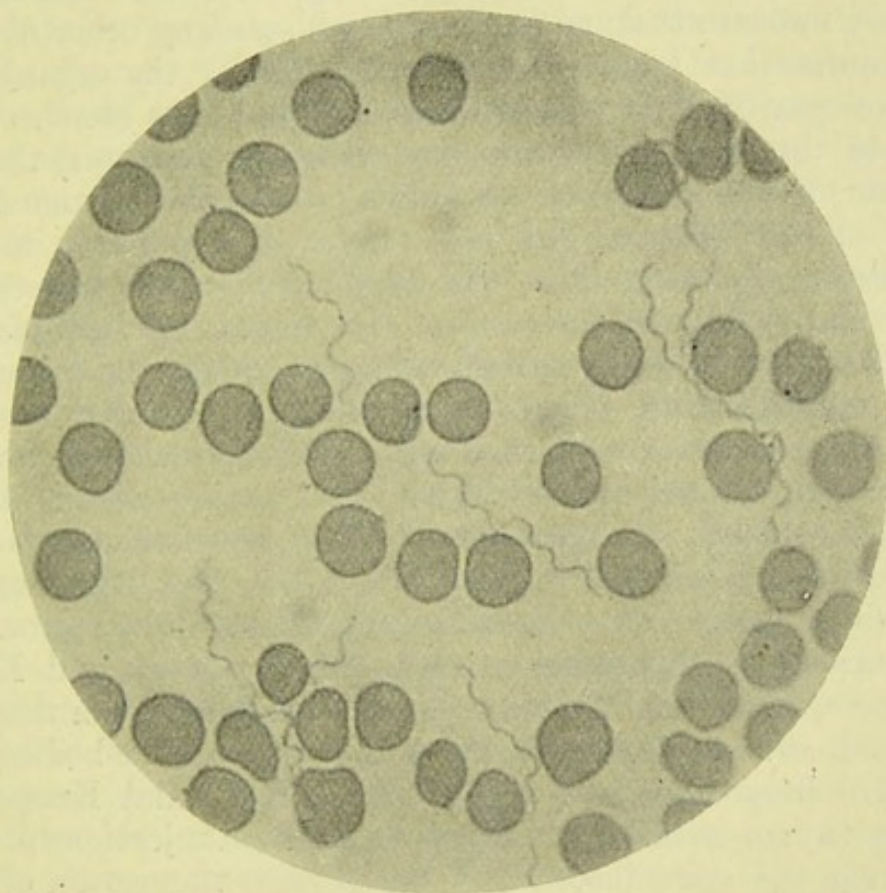


FIG. 149.—Spirochaete Obermeieri in blood of infected mouse.  
× 1000.

rats are also susceptible to infection. In the former animals the disease is characterised by several relapses, in the latter there is, however, no relapse.

**Immunity.**—Metchnikoff found that during the fever the spirochaetes were practically never taken up by the leucocytes in the circulating blood, but that at the time of the crisis, on disappearing from the blood, they accumulated in the spleen and were ingested in large numbers by the microphages or poly-

<sup>1</sup> Norris, Pappenheimer, and Flournoy, in their experiments on monkeys with the organism of American relapsing fever, found that several relapses occurred.



morphonuclear leucocytes. Within these they rapidly underwent degeneration and disappeared. It is to be noted in this connection that swelling of the spleen is a very marked feature in relapsing fever. These observations were confirmed by Soudakewitch, who also found that when the disease was produced in splenectomised monkeys (*cercopithecus fuliginosus*) the spirochætes did not disappear from the blood at the usual time, but rather increased in number, and a fatal result followed on the eighth and ninth days respectively. Recent observations, however, indicate that, as in the case of so many other diseases, the all-important factor in the destruction of the organisms is the development of antagonistic substances in the blood. Lamb found in the case of the monkey (*macacus radiatus*) that the removal of the spleen of an animal rendered immune by an attack of the disease did not render it susceptible to fresh inoculation, and attributed the immunity to the presence of bactericidal bodies in the serum. He found, for example, that *in vitro* the serum of an immune animal brought the movements of the spirilla to an end, clumped them, and caused their disintegration; and further, that when the spirochætes and the immune serum were injected in one case into a fresh monkey no disease developed. In opposition to Soudakewitch, Lamb found that with a monkey from which the spleen had been removed death did not occur after it was inoculated with the spirochætes. Observations by Sawtschenko and Milkich, Novy and Knapp, and others, also show that in the course of infection there are developed anti-substances of the nature of immune-bodies, with protective properties, and agglutinins. Novy and Knapp produced a "hyper-immunity" in rats by repeated injections of blood containing the spirochætes, and found that the serum of such animals had a markedly curative effect, and could cut short the disease in rats, mice, and monkeys. The course of events in the human disease might be explained by supposing that immunity of short duration is produced during the first period of pyrexia, but that it does not last until all the organisms have been destroyed, some still surviving in internal organs or in tissues where they escape the bactericidal action of the serum. With the disappearance of the immunity the organisms appear in the blood, the relapse being, however, of shorter duration and less severe than the first attack. This is repeated till the immunity lasts long enough to allow all the organisms to be killed.

**Varieties.**—As already stated, relapsing fever has been studied in different parts of the world, and, apart from the African tick fever, European, Asiatic, and American types have been dis-



tinguished. Differences have been made out with regard to clinical features, pathogenic effects, and immunity reactions. Of these the last mentioned are probably the most important. It has been shown, for example, by the work of Novy, Strong, and Mackie, that the American spirochæte is probably a distinct species, as animals immunised against it are still susceptible to infection by the European and Asiatic organisms, and *vice versa*. The relationship between the two latter is certainly closer, and no distinct immunity differences have been established. Relapsing fever in Asia is evidently a much more severe disease than in Europe; Mackie gives the mortality in Bombay at the comparatively high figure of 38 per cent. But differences in this respect, as well as in pathogenic effects, may simply depend on variations in virulence. At present no definite statement can be made on this point.

The fact that tick fever and other spirilloses are conveyed by the bites of insects makes it extremely probable that relapsing fever is transmitted in this way. At first the bed-bug was believed to be the vehicle of transmission, and the experiments of Karlinski and of Tictin, which showed that the spirochætes might remain alive and virulent in the body of this insect for some time after it had sucked the blood of a patient, lent some support to this view. Attempts to transmit the disease by means of the bites of bugs were, however, generally unsuccessful; Mackie produced the disease in only one out of six monkeys used for this purpose, though large numbers of bugs, which had bitten relapsing fever patients, were used. On investigating an epidemic of the disease, however, he obtained a considerable amount of evidence on epidemiological grounds that the disease was carried by the body louse. He also found that the spirochætes in the blood which had been sucked underwent great multiplication about three days afterwards, and formed large tangled masses in the stomach contents. The view that the louse is the agent of transmission of the human disease is strongly supported by the experiments of Manteufel, who was able to transmit infection from rat to rat in nearly 60 per cent. of the experiments made, whereas he obtained only negative results by means of bugs. Further observations are still necessary.

#### *African Tick Fever.*

The disease long known by this name as prevalent in Africa has also been shown to be caused by a spirillum or spirochæte,



*Sp. Duttoni*. Organisms of this nature had been seen in the blood of patients in Uganda by Greig and Nabarro in 1903, and Milne and Ross in the end of 1904 recorded a series of observations which led them to the conclusion that tick fever was due to a spirochæte. It is, however, chiefly owing to the work of Dutton and Todd in the Congo Free State, on the one hand, and of Koch in German East Africa, on the other, that our knowledge of this disease has been thoroughly established.

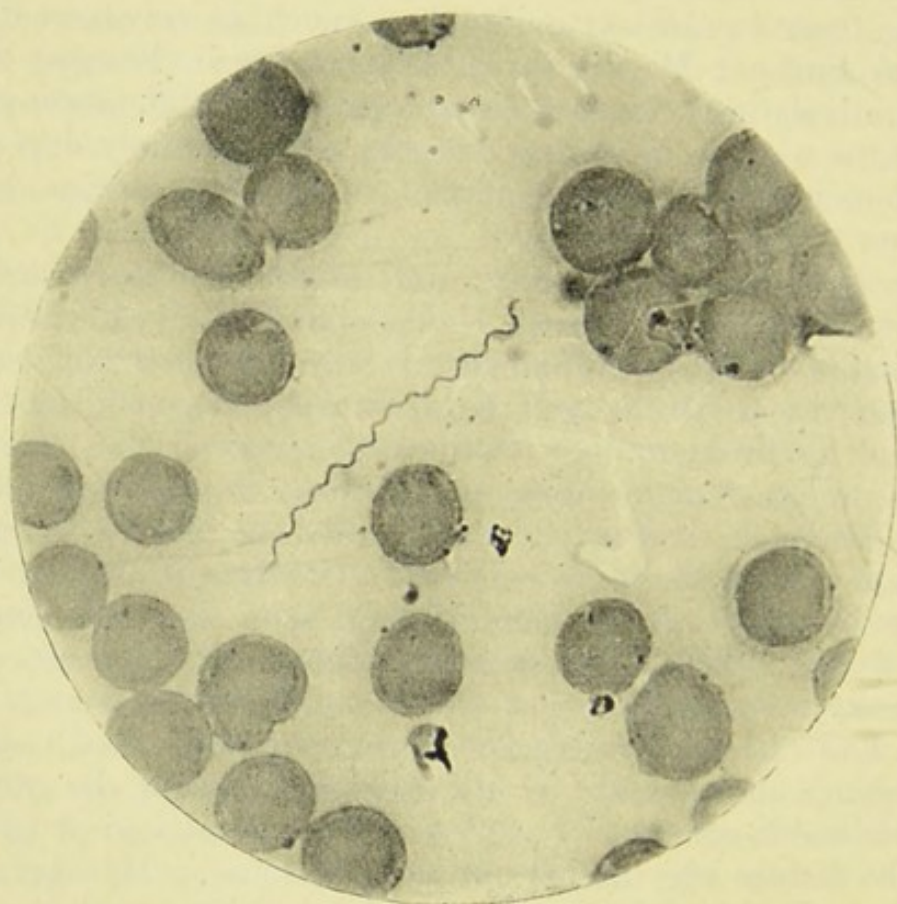


FIG. 150.—Film of human blood containing spirochæte of tick fever.  
× 1000.<sup>1</sup>

The following are the chief facts regarding this fever: Clinically the fever closely resembles relapsing fever, but the periods of fever are somewhat shorter, rarely lasting for more than two or three days. It is seldom attended with a fatal result unless in patients debilitated by other causes. The organisms are considerably fewer in the blood than in the European relapsing fever, and sometimes a careful search may be necessary before they are found. Morphologically, they are said to be

<sup>1</sup> We are indebted to Lieut.-Col. Sir William Leishman, R.A.M.C., for the preparations from which Figs. 149-151 were taken.



practically identical, although Koch thought that the organisms in tick fever tended on the whole to be slightly longer; the average length may be said to be 15 to 35  $\mu$ . Dutton and Todd showed that it was possible to transmit the disease to certain monkeys (*cercopithec*i) by means of ticks which had been allowed to bite patients suffering from the disease, the symptoms in these animals appearing about five days after inoculation. The disease thus produced is characterised by several relapses, and

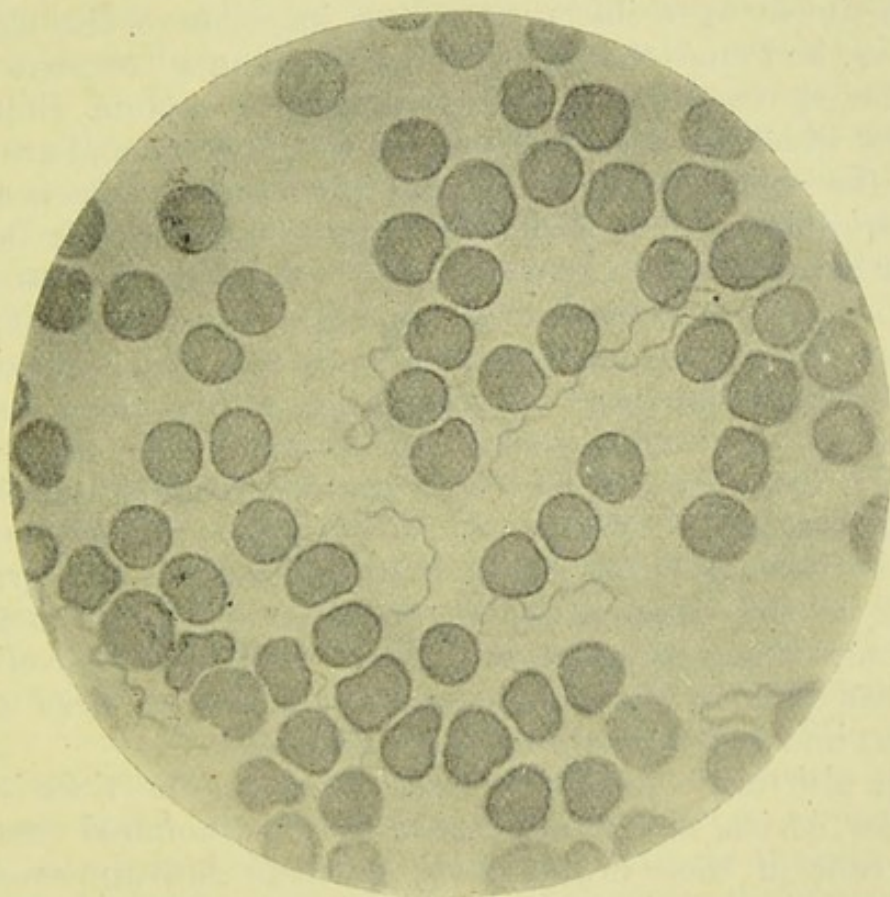


FIG. 151.—Spirillum of human tick fever (*Spirillum Duttoni*) in blood of infected mouse.  $\times 1000$ .

often leads to a fatal result. In one case they produced the disease by means of young ticks hatched from the eggs of ticks which had been allowed to suck the blood of fever patients, and they came to the conclusion that the spirochætes were not simply carried mechanically by the ticks, but probably underwent some cycle of development in the tissues of the latter. The species of tick concerned is the *ornithodoros moubata*. These results were confirmed and extended by Koch. He found that after the ticks had been allowed to suck the blood containing the organisms, these could be found for a day or two in the stomachs of the



insects. After this time they gradually disappeared from the stomach, but were detected in large numbers in the ovaries of the female ticks, where they sometimes formed felted masses. He also traced the presence of the spirochaetes in the eggs laid by the infected ticks, and in the young embryos hatched from them. On the other hand, Leishman has failed to find any evidence of spirochaetes in the tissues of ticks later than ten days after ingestion of blood containing them, or in the ova laid by them, or in the young ticks when hatched, though these were proved by experiment to be infective. After ingestion of the blood by the ticks, he found that morphological changes occurred in the spirochaetes, resulting in the formation of minute chromatin granules which traverse the walls of the intestine and are taken up by the cells of the Malpighian tubules; they also penetrate the ovaries and may be found in large numbers within the ova. Similar granules are to be seen in the Malpighian tubules of the embryo ticks, where they are also found in the subsequent stages of their life. He has abundantly proved that infection of animals may be produced by inoculation with crushed material containing the granules but no spirochaetes. He accordingly considers that the granules in question probably represent a phase in the life history of the parasite, and that infection probably occurs by inoculation of the skin with the chromatin granules voided in the Malpighian secretion and not by unaltered spirochaetes from the salivary glands. It is also interesting to note that Balfour has found similar granules in ticks (*argas persicus*) infected with *spirochaete gallinarum*.

Koch also made extensive observations on the ticks in German East Africa, and found that of over six hundred examined 11 per cent. of these insects along the main caravan routes contained spirilla, and in some localities almost half of the ticks were infected. In places removed from the main lines of commerce he still found them, though in smaller number. It has also been demonstrated that in some places the ticks are found to be infected with the spirilla although the inhabitants do not suffer from tick fever, a circumstance which is probably due to an acquired immunity against the disease.

It is now generally considered that the sp. Duttoni is a species distinct from, though closely allied to, the organisms of the relapsing fevers described above. We have mentioned some differences in the clinical characters of the diseases, and there are also differences in the pathogenic effects of the organisms on inoculation. The sp. Duttoni, for example, produces a much more severe disease in monkeys, and is pathogenic to more



species of the laboratory animals than the sp. *Obermeieri*. The most important differences are however brought out by immunity reactions. It was shown by Breinl that the immunity produced by the sp. *Obermeieri* did not protect against the sp. *Duttoni*, and that the converse also held good; and it has since been established that a similar difference obtains between the sp. *Duttoni* and the organisms of the Asiatic and American varieties of relapsing fever. Corresponding results are obtained on testing the various serum reactions *in vitro*.

Levaditi has succeeded in obtaining cultures of the spirochæte of tick fever by inoculating sacs filled with monkey's serum, heated at 70° C., and placing the sacs in the peritoneal cavity of a rat or rabbit; when opened at the end of five to seven days, the sacs were found to contain an abundant growth of spirochætes, some of which were of unusually great length. Growth was maintained in similar sub-cultures, and the virulence was well preserved.

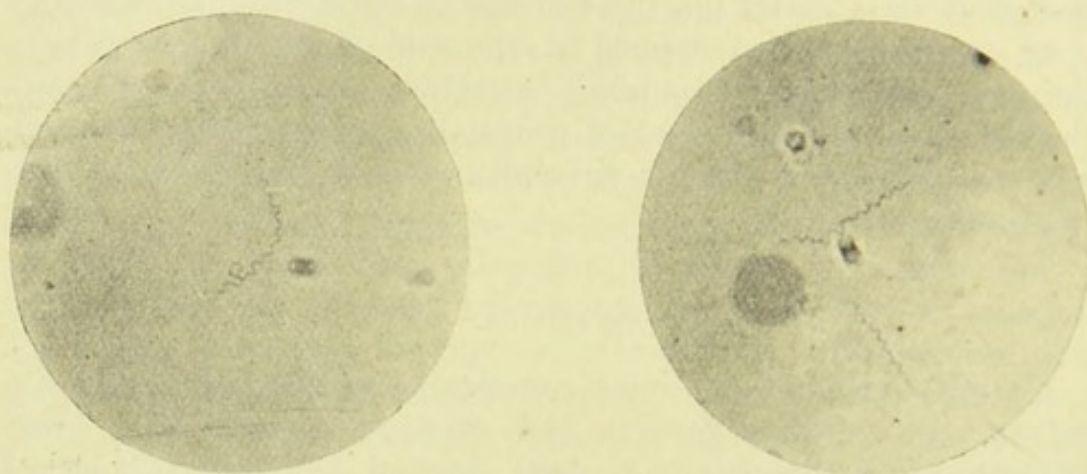
### SYPHILIS.

Up till quite recent times practically nothing of a definite nature was known regarding the etiology of syphilis. Most interest for a long time centred around the observations of Lustgarten, who in 1884 described a characteristic bacillus, both in the primary sore and in the lesions in internal organs. This organism occurred in the form of slender rods, straight, or slightly bent, 3 to 4  $\mu$  in length, often forming little clusters either within cells or lying free in the lymphatic spaces; it took up basic aniline dyes with some difficulty, but was much more easily decolorised by acids than the tubercle bacillus. The etiological relationship of the organism to the disease was, however, not generally accepted, and in view of the recent work on syphilis, the organism cannot be regarded as having any pathological importance.

**Spirochæte pallida.**—An entirely new light has been thrown on the etiology of the disease by the work of Schaudinn and Hoffmann which appeared in 1905. Since their first publication a great amount of work has been undertaken in order to test their conclusions, and the results have been of a confirmatory nature. These observers found in cases of syphilis an organism to which they gave the name *spirochæte pallida*; it now also goes by the name *spironema pallidum*. As described by them, it is a minute spiral-shaped organism, showing usually from six to eight curves, though longer forms are met with; the curves



are small, comparatively sharp, and regular (Figs. 152, 153). It may be said to measure 4 to 14  $\mu$  in length, while it is extremely thin, its thickness being only  $\cdot 25 \mu$ . In a fresh specimen, say a scraping from a chancre suspended in a little salt solution, the organism shows active movements, which are of three kinds—rotation about the long axis, gliding movements to and fro, and movements of flexion of the whole body. The ends are pointed and tapering. Its detection is comparatively difficult, as the organism is feebly refractile, and more difficult to see than most other organisms; the movement of small particles in the vicinity, however, is of assistance in finding it. The use of the



FIGS. 152 and 153.—Film preparations from juice of hard chancre showing spirochæte pallida,—Giemsa's stain.  $\times 1000$ . (From preparations by Dr. A. MacLennan.)

parabolic sub-stage condenser (p. 93) is of great service in searching for the organism.

In ulcerated syphilitic lesions other organisms are, of course, present, and not infrequently another spiral organism, to which the name *spirochæte refringens* has been given. This organism is usually somewhat longer, and is distinctly thicker than the spirochæte pallida. As the name implies, it is more highly refractile, and it is much more easily detected than the latter organism; its curves also are more open and much less regular, and they vary in their appearance during the movements. In stained films (see p. 115), the differences between the organisms come out more distinctly, as can be gathered from the accompanying photograph (Fig. 156). The spirochæte pallida by the Giemsa stain is coloured somewhat faintly, and of reddish tint, whilst the regular spiral twistings are preserved; the spirochæte refringens shows flatter, wave-like bends, and, like other organisms, is stained of a bluish tint. By using Löffler's stain for the



flagella of bacteria, Schaudinn was able to demonstrate a single delicate flagellum at each pole of the spirochæte pallida, while no undulating membrane could be detected; on the other hand, several other species, including the spirochæte refringens, showed a distinct undulating membrane. Two flagella at one pole of the spirochæte pallida were also seen, an appearance which Schaudinn thought might represent the commencement of longitudinal fission.

The number of publications with regard to the distribution of the spirochæte pallida is already very large, and a summary of

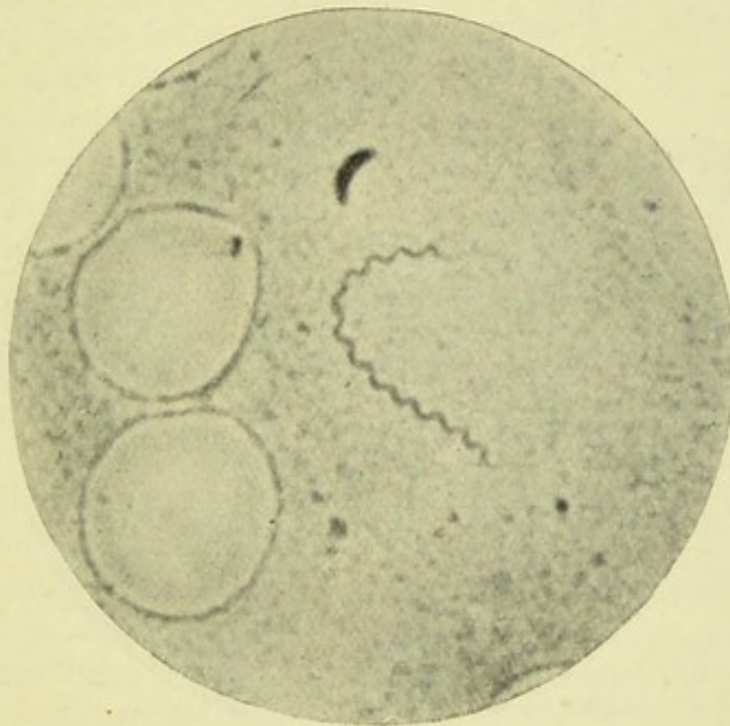


FIG. 154.—Film preparation from juice of hard chancre showing spirochæte pallida,—Giemsa's stain.  $\times 2000$ . (From a preparation by Dr. Haswell Wilson.)

the results may be given. In the primary sore and in the related lymphatic glands, the juice of which can be conveniently obtained by means of a hypodermic syringe, the organism has been found in a very large majority of cases. It has been also obtained in the papular and roseolar eruptions, in condylomata and mucous patches—in fact, one may say generally, in all the primary and secondary lesions. It has been obtained from the spleen during life, and on a few occasions, *e.g.* by Schaudinn, also from the blood during life in secondary syphilis. In the congenital form of the disease the organism may be present in large numbers (Plate II., Fig. 6), as was first shown by Buschke and Fischer, and by Levaditi. In the pemphigoid bullæ, in



the blood, in the internal organs, the liver, lungs, spleen, suprarenals, and even in the heart its detection may be comparatively easy, owing to the large numbers present (Fig. 155). It has been generally supposed that tertiary syphilitic lesions are non-infective, and the results of the earlier observations on the spirochæte pallida were apparently in accordance with this view, as they gave negative results. More prolonged search has, however, shown that the organism may occur in tertiary lesions also. It has been found to be present in the peripheral parts of gummata, especially at an early stage of their forma-

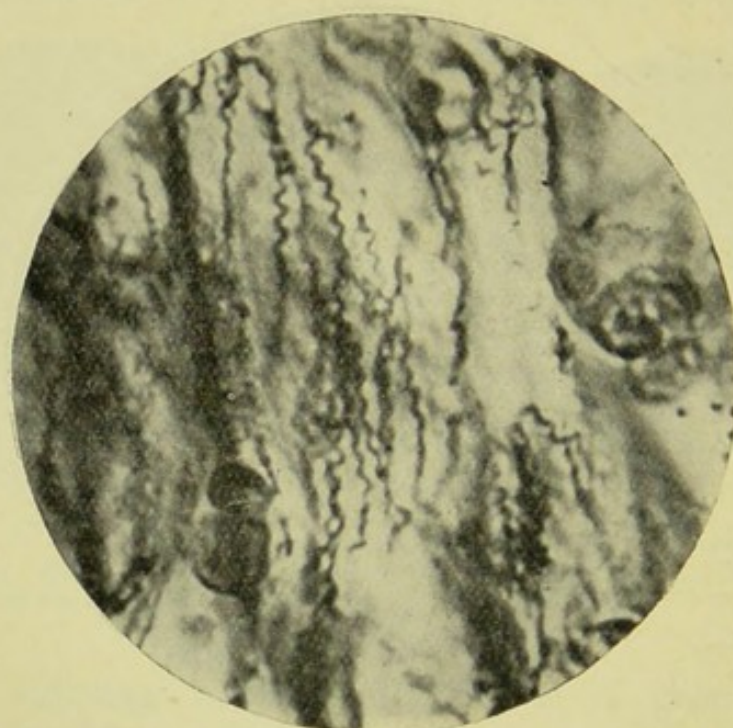


FIG. 155.—Section of spleen from a case of congenital syphilis, showing several examples of spirochæte pallida; Levaditi's method.  $\times 2000$ .

tion; and the observations of Schmorl, Benda, J. H. Wright and others show that it is often to be found in syphilitic aortitis, sometimes occurring in considerable numbers in the thickened patches. That the spirochæte may persist in the body for a very long time after infection, has been abundantly shown by different observers; in one case, for example, its presence was demonstrated sixteen years after the primary lesion. It can readily be demonstrated in sections of the organs by the method described on p. 112. In such preparations large numbers of spirochætes, chiefly extra-vascular in position, can be seen, and many may occur in the interior of the more highly specialised cells, for example, liver-cells; in many cases examina-



tion has been made within so short a period after the death of the child as to practically exclude the possibility of contamination from without. It also abounds sometimes on mucous surfaces, *e.g.* of the bladder and intestine in cases of congenital syphilis. The enormous numbers of the organism which may be present in a well preserved condition in macerated fœtuses render it probable that the organism may multiply in the dead tissues under anaerobic conditions. Although various organisms may be associated with it in the lesions of the skin or mucous membranes, there is agreement amongst observers that this organism occurs alone in syphilitic lesions where the entrance of bacteria, etc., from outside is excluded. The high percentage of cases in which it is found would, in view of the difficulty in detecting it, point to its invariable presence, and, as a matter of fact, Schaudinn in his last series of cases, numbering over seventy, found it in all. Shortly after the discovery of the organism, Metchnikoff was able to detect it in the lesions produced in monkeys by inoculation with material derived from syphilitic sores, and his observations have since been confirmed. Another question of considerable importance is, as to whether this organism has been found in other conditions. Observations show that in various conditions, such as ulcerated carcinomata, balanitis, etc., spirochætes are of comparatively common occurrence. There is no doubt whatever that the great majority of these are readily distinguishable by their appearance from the spirochæte pallida, but others resemble it closely. Hoffmann, however, who has seen many of these spirochætes from other sources, considers that even by their microscopic appearance they are capable of being distinguished, though with considerable difficulty. It must, of course, be borne in mind that the finding of an organism in non-syphilitic lesions with the same microscopical characters does not show that it is the same organism as the spirochæte pallida.

**Cultivation.**—Although numerous attempts have been made, it has not yet been found possible to obtain pure cultures of the spirochæte pallida outside the body. Levaditi and McIntosh inoculated with syphilitic material sacs of collodion containing

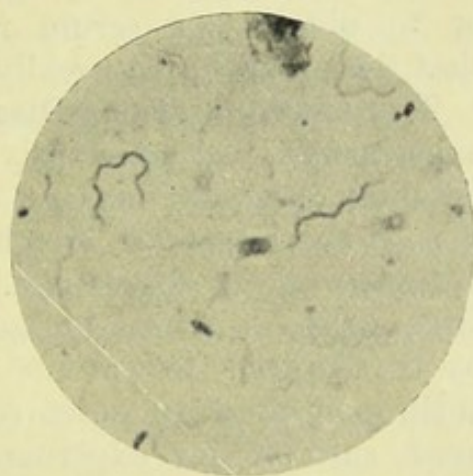


FIG. 156.—*Spirochæte refringens* in film preparation from a case of balanitis.  $\times 1000$ .



human serum, heated at 60°C., and placed them in the peritoneal cavity of a monkey (*macacus cynomolgus*). After an interval of about three weeks, they found in the sacs an abundant growth of spirochaetes morphologically identical with spirochaete pallida, along with various anaerobic bacteria. They were able to continue such cultures in like conditions, but were unable to obtain any pathogenic effects on inoculating animals with the material from the sacs, and considered that the organisms had become avirulent owing to their conditions of growth. Recently Schereschewsky claims to have obtained impure cultures of the organism in test tubes. He used for this purpose horse serum inspissated at 58° C., and then allowed to undergo autolysis for three days at 37° C. Although abundant growth of spirochaetes was obtained, he was unable to infect animals by means of them, or to obtain any serum reactions which would go to show that the organism was really the spirochaete pallida.

**Transmission of the Disease to Animals.**—Although various experiments had previously been from time to time made by different observers, in some cases with reported successful result, it is to the papers of Metchnikoff and Roux (1903-5) that we owe most of our knowledge. These observers have carried on a large series of observations, and have shown that the disease can be transmitted to various species of monkey. Of those the anthropoid apes are most susceptible, the chimpanzee being the most suitable for experimental purposes. Their results have been confirmed by Lassar, Neisser, Kraus, and others. The number of experiments on these animals is now very great, and the general result is that the disease has been transmitted by material from all the kinds of syphilitic lesions in which spirochaetes have been demonstrated, including even the blood in secondary syphilis and tertiary lesions. Inoculation is usually made by scarification on the eyebrows or genitals; the subcutaneous and other methods of inoculation give negative results. The primary lesion is in the form of an indurated papule or of papules, in every respect resembling the human lesion. Along with this there is a marked enlargement and induration of the corresponding lymphatic glands. The primary lesion appears on an average about thirty days after inoculation, and secondary symptoms develop in rather more than half of the cases after a further period of rather longer duration. These are of the nature of squamous papules on the skin, mucous patches in the mouth, and sometimes palmar psoriasis. As a rule, the secondary manifestations are of a somewhat mild degree, and in no instance up to the present has any tertiary



lesion been observed. By re-inoculation from the lesions, the disease may be transferred to other animals. The disease may also be produced in baboons and macaques (*macacus sinicus* is one of the most susceptible), but these animals are less susceptible, and secondary manifestations do not appear. The severity of the affection amongst apes would in fact appear to be in proportion to the nearness of the relationship of the animal to the human subject.

As shown first by Hänsell, and more recently by Bertarelli, the eye of the rabbit is susceptible to inoculation from syphilitic lesions. The material used is introduced in a finely divided state either into the tissue of the cornea or into the anterior chamber, and syphilitic keratitis or iritis, or both, may result, there being a period of incubation of at least two weeks. Levaditi and Yamanouchi have recently studied the stages in detail, and find that the spirochaetes remain in the inoculated material unchanged for a time; then organisation occurs and the spirochaetes multiply, and later still there is a more rapid multiplication and invasion by them of the tissues of the eye. The period of incubation is thus not due to the organism passing through some cycle of development, but simply to its requiring certain conditions for multiplying which are not supplied for some time.

The production of the disease, experimentally, has supplied us with some further facts regarding the nature of the virus. It has been shown repeatedly that the passage of fluid containing the virus through a Berkefeld filter deprives it completely of its infectivity: In other words, the virus does not belong to the ultra-microscopic group of organisms. The virus is also readily destroyed by heat, a temperature of 51° C. being fatal. With regard to the production of immunity, very little of a satisfactory nature has so far been established. It has been found that the virus from a macaque monkey produces a less severe disease in the chimpanzee than the virus from the human subject, inasmuch as secondary lesions do not follow; the virus would thus appear to have undergone a certain amount of attenuation in the tissues of that monkey. The effects of injecting emulsions of tertiary lesions or of serum from syphilitic patients, at the time of inoculation with the virus, appear to be nil; so also the employment of the virus rendered inactive by heating has apparently no influence in acting as a vaccine. There is some evidence that the serum from a patient suffering from the disease when mixed with the virus before inoculation modifies the disease to a certain extent, but further evidence on



this point is necessary. As mentioned above, the spirochæte *pallida* has been found in the lesions in monkeys, Metchnikoff and Roux obtaining positive results in more than 75 per cent. of the cases, and it is to be noted that here also the organism has been found deep in the substance of the papules, unaccompanied by any other organisms. It is also to be noted that the blood of infected apes after a time gives the Wassermann reaction.

**Serum Diagnosis—Wassermann Reaction.**—The method of applying this test has already been given (p. 131); we have now to consider the results of its application. There is general agreement amongst workers at the subject that the test affords by far the most reliable means of diagnosis of the disease; and on comparing the results obtained it will not be an overestimate to say that a positive result may be obtained in at least 90 per cent. of cases where there is evidence of active general infection. The reaction generally appears first on the fifteenth to thirtieth day after appearance of the sore, and then gradually becomes more marked; during the period of secondary manifestations it is practically always present; in the tertiary stage with active manifestations a positive result is only a little less frequent. As the disease becomes inactive or is cured the reaction may disappear, but it is to be noted that disappearance of the reaction after being present does not necessarily imply cure of the disease. It may only have become latent, and on its becoming once more active the reaction may re-appear. Energetic treatment with mercury may also diminish or annul the reaction; in fact, its presence and intensity would appear to be definitely related to the activity of the syphilitic lesions. A positive reaction is also present in the large majority of cases of general paralysis and of tabes, and may be given by the cerebro-spinal fluid as well as by the blood serum in such cases. As regards other diseases, a positive reaction has been recorded as occurring in leprosy (p. 304) and sleeping-sickness and also in yaws, but apart from these diseases it is practically never met with. At present little can be said in explanation of the Wassermann reaction. It seems to depend on the interaction of lipoidal substances in the extract with proteins in the serum, which are apparently contained in the globulin fraction; but we know nothing as to why this peculiar modification of the serum should be present in syphilis. It is now generally considered that it does not depend on the presence of an anti-substance (immune-body), which in association with the antigen (the spirochæte) fixes complement.



## FRAMBOESIA OR YAWS.

Framboesia is a contagious disease of the tropics, occurring in the west coast of Africa, Ceylon, the West Indies, and other parts. It is characterised by a peculiar cutaneous eruption, and it is markedly contagious. Its resemblance in many respects to syphilis has been noted, and the relation of the two diseases has been the subject of much controversy. It is accordingly a matter of great interest that an organism of closely similar characters to the spirochæte pallida has been found in the lesions of framboesia. This organism was discovered by Castellani, who gave to it the name *spirochæte pertenuis* or *pallidula*. Morphologically, it is practically identical with the spirochæte pallida; when ulceration has occurred other spirochætes of less regular form may be present as contaminations. In the skin lesions it has been shown by Levaditi's method to be present in considerable numbers, especially in the epidermis and also amongst the leucocytic infiltration, which comprises more polymorpho-nuclear leucocytes than is seen in the case of syphilis. Castellani showed that the disease could be transferred to monkeys (*semnopithecus* and *macacus* being used for this purpose), and that the organism could be demonstrated in the unbroken skin lesions. The lesions are as a rule confined to the site of inoculation, but the infection is general, as is shown by the presence of spirochætes in the lymphatic glands and the spleen. These results with regard to the presence of spirochæte pallidula in the lesions and the inoculation of apes have been confirmed by other workers, and the etiological relationship of the organism to the disease may now be regarded as practically established. The immunity reactions in monkeys infected with syphilis and framboesia, as experimentally studied by Castellani and by Neisser, Baermann, and Halberstädter, go to show that the two diseases are distinct. On the other hand, Levaditi and Nattan-Larrier found that, although monkeys infected with syphilis were refractory to framboesia (*Fr. pian*), monkeys infected with framboesia were susceptible to syphilis: they therefore concluded that framboesia is a modified or mild form of syphilis. The exact relationship of the two diseases cannot be yet accurately defined, but they are undoubtedly closely related, and probably have a common parentage.



## CHAPTER XXI.

### IMMUNITY.

**Introductory.**—By immunity is meant non-susceptibility to a given disease or to a given organism, either under natural conditions or under conditions experimentally produced. The term is also used in relation to the toxins of an organism. Immunity may be possessed by an animal naturally, and is then usually called *natural* immunity, or it may be *acquired* by an animal either by its passing through an attack of the disease, or by means of artificial inoculation. It is to be noted that man and the lower animals may be exempt from certain diseases under natural conditions, and yet the causal organisms of these diseases may produce pathogenic effects when injected in sufficient quantity. Immunity is, in fact, of very varying degrees, and accordingly the use of the term has a correspondingly relative significance. This is not only true of infection by bacteria, but of toxins also :—when the resistance of an animal to these is of high degree, the resistance may in certain cases be overcome by a very large dose of the toxic agent. On the other hand, in cases where the natural powers of resistance are very high, these can be still further exalted by artificial means, that is, the natural immunity may be artificially intensified.

**Acquired Immunity in the Human Subject.**—The following facts are supplied by a study of the natural diseases which affect the human subject. First, in the case of certain diseases, one attack protects against another for many years, sometimes practically for a lifetime, *e.g.* smallpox, typhoid, scarlet fever, etc. Secondly, in the case of other diseases, *e.g.* erysipelas, diphtheria, influenza, and pneumonia, a patient may suffer from several attacks. In the case of the diseases of the second group, however, experimental research has shown that in many of them a certain degree of immunity does follow; and, though we cannot definitely state it as a universal law, it must be considered highly probable that the passing through an attack



of an acute disease produced by an organism, confers immunity for a longer or shorter period. The immunity is not, however, to be regarded as the result of the disease *per se*, but of the bacterial products introduced into the system; as will be shown below, by suitable gradation of the doses of such products, or by the use of weakened toxins, a high degree of immunity may be attained without the occurrence of any symptoms whatever.

The facts known regarding vaccination and smallpox exemplify another principle. We may take it as practically proved that vaccinia is variola or smallpox in the cow, and that when vaccination is performed, the patient is inoculated with a modified variola (*vide* Smallpox, in Appendix). Vaccination produces certain pathogenic effects which are of trifling degree as compared with those of smallpox, and we find that the degree of protection is less complete and lasts a shorter time than that produced by the natural disease. Again, inoculation with lymph from a smallpox pustule produces a form of smallpox less severe than the natural disease but a much more severe condition than that produced by vaccination, and it is found that the degree of protection or immunity resulting occupies an intermediate position.

*Immunity and Recovery from Disease.*—Recovery from an acute infective disease shows that in natural conditions the virus may be exhausted after a time, the period of time varying in different diseases. How this is accomplished we do not yet fully know, but it has been found in the case of diphtheria, typhoid, cholera, pneumonia, etc., that in the course of the disease certain substances (called by German writers *Antikörper*) appear in the blood, which are antagonistic either to the toxin or to the vital activity of the organism. In such cases a process of immunisation would appear to be going on during the progress of the disease, and when this immunisation has reached a certain height, the disease naturally comes to an end. It cannot, however, be said as yet that such antagonistic substances are developed in all cases; though the results already obtained make this probable.

#### ARTIFICIAL IMMUNITY.

**Varieties.**—According to the means by which it is produced, immunity may be said to be of two kinds, to which the terms *active* and *passive* are generally applied, or we may speak of immunity *directly*, or *indirectly*, *produced*. We shall first give an account of the established facts, and afterwards discuss some



of the theories which have been brought forward in explanation of these facts.

*Active immunity* is obtained by (a) injections of the organisms either in an attenuated condition or in sub-lethal doses, or (b) by sub-lethal doses of their products, *i.e.* of their "toxins," the word being used in the widest sense. By repeated injections at suitable intervals the dose of organisms or of the products can be gradually increased; or, what practically amounts to the same, an organism of greater virulence or a toxin of greater strength may be used. A degree of resistance or immunity can thus be developed, and this in course of time may reach a very high level. Such methods constitute the means of *preventive inoculation* or *vaccination*. Immunity of this kind is comparatively slowly produced and lasts a considerable time, the duration varying in different cases. The principles of vaccination have within recent years been extended by Wright to the *treatment* of disease.

*Passive immunity* depends upon the fact that if an animal be immunised to a very high degree by the previous method, its serum may have distinctly antagonistic or neutralising effects when injected into another animal along with the organisms, or with their products, as the case may be. Such a serum, generally known as an *anti-serum*, may exert its effects if introduced into an animal at the same time as infection occurs or even a short time afterwards; it can, therefore, be employed as a *curative* agent. The serum is also preventive, *i.e.* protects an animal from subsequent infection, but the immunity thus conferred lasts a comparatively short time. These facts form the basis of serum therapeutics. When such a serum has the power of neutralising a toxin it is called *antitoxic*; when, with little or no antitoxic power, it protects against the living bacterium in a virulent condition, it is called *antimicrobial* or *antibacterial* (*vide infra*).

In the accompanying table a sketch of the chief methods by which an immunity may be artificially produced is given. It has been arranged merely for purposes of convenience and to aid subsequent description; the principles underlying all the methods are the same.

#### ARTIFICIAL IMMUNITY.

- A. Active Immunity—*i.e.* produced in an animal by an injection, or by a series of injections, of non-lethal doses of an organism or its toxins.



1. *By injection of the living organisms.*

(a) Attenuated in various ways. Examples:—

- (1) By growing in the presence of oxygen, or in a current of air.
- (2) By passing through the tissues of one species of animal (becomes attenuated for another species).
- (3) By growing at abnormal temperatures, etc.
- (4) By growing in the presence of weak antiseptics, or by injecting the latter along with the organism, etc.

(b) In a virulent condition, in non-lethal doses.

2. *By injection of the dead organisms.*3. *By injection of filtered bacterial cultures, i.e. toxins; or of substances derived from such filtrates.*

These methods may also be combined in various ways.

B. Passive Immunity, *i.e.* produced in one animal by injection of the serum of another animal highly immunised by the methods of A.

1. *By antitoxic serum, i.e.* the serum of an animal highly immunised against a particular toxin.
2. *By antibacterial serum, i.e.* the serum of an animal highly immunised against a particular bacterium in the living and virulent condition.

A. *Active Immunity.*

1. **By Living Cultures.**—(a) *Attenuated.*—In the earlier work on immunity in the case of anthrax, chicken cholera, swine plague, etc., the investigators had to deal with organisms of high virulence, which had accordingly to be reduced before the organisms could be injected in the living state. It is now found most convenient as a rule to start the process of active immunisation with the injection of dead cultures. The principle is the same as that of vaccination, and both attenuated cultures and also the dead cultures used for injection are often spoken of as *vaccines*. The virulence of an organism may be diminished in various ways, of which the following examples may be given:—

(1) In the first place, practically every organism, when cultivated for some time outside the body, loses its virulence, and in the case of some this is very marked indeed, *e.g.* the pneumococcus. Pasteur found in the case of chicken cholera, that



when cultures were kept for a time in ordinary conditions, they gradually lost their virulence, and that when sub-cultures were made the diminished virulence persisted. Such attenuated cultures could be used for protective inoculation. He considered the loss of virulence to be due to the action of the oxygen of the air, as he found that in tubes sealed in the absence of oxygen the virulence was not lost. Haffkine attenuated cultures of the cholera spirillum by growing them in a current of air (p. 459).

(2) The virulence of an organism for a particular animal may be lessened by passing the organism through the body of another animal. Duguid and Burdon Sanderson found that the virulence of the anthrax bacillus for bovine animals was lessened by its being passed through guinea-pigs, the disease produced in the ox by inoculation from the guinea-pig being a non-fatal one. This discovery was confirmed by Greenfield, who showed that the bacilli cultivated from guinea-pigs preserved their property in cultures, and could therefore be used for protective inoculation of cattle. A similar principle was applied in the case of swine plague by Pasteur, who found that if the organism producing this disease was inoculated from rabbit to rabbit, its virulence was increased for rabbits but was diminished for pigs. The method of vaccination against small-pox depends upon the same principle. There is also evidence to show that the virulence of the tubercle bacillus becomes modified according to its host, being often diminished for other animals.

(3) Many organisms become diminished in virulence when grown at an abnormally high temperature. The method of Pasteur, already described (p. 346), for producing immunity in sheep against anthrax bacilli, depends upon this fact. A virulent organism may also be attenuated by being exposed to an elevated temperature which is insufficient to kill it, as was found by Toussaint in the case of anthrax.

(4) Still another method may be mentioned, namely, the attenuation of the virulence by growing the organism in the presence of weak antiseptics. Chamberland and Roux, for example, succeeded in attenuating the anthrax bacillus by growing it in a medium containing carbolic acid in the proportion of 1 : 600.

These examples will serve to show the principles underlying attenuation of the virulence of an organism. There are, however, still other methods, most of which consist in growing the organism in conditions somewhat unfavourable to its growth, *e.g.* under compressed air, etc.

(b) *Immunity by living Virulent Cultures in Non-lethal*



*Doses.*—Immunity may also be produced by employing virulent cultures in small, that is non-lethal, doses. In subsequent inoculations the doses may be increased in amount. For example, immunity may thus be obtained in rabbits against the bacillus pyocyaneus. Such a method, however, has had only a limited application, as it has been found more convenient to commence the process by dead or attenuated cultures, and then to continue with virulent cultures.

*Exaltation of the Virulence.*—The converse process to attenuation, *i.e.* the exaltation of the virulence, is obtained chiefly by the method of cultivating the organism from animal to animal—the method of *passage* discovered by Pasteur (first, we believe, in the case of an organism obtained from the saliva in hydrophobia, though having no causal relationship to that disease). This is most conveniently done by intraperitoneal injections, as there is less risk of contamination. The organisms in the peritoneal fluid may be used for the subsequent injection, or a culture may be made between each inoculation. The virulence of a great number of organisms can be increased in this way, the animals most frequently used being rabbits and guinea-pigs. This method can be applied to the organisms of typhoid, cholera, pneumonia, to streptococci and staphylococci, and in fact to those organisms generally which invade tissues.

The virulence of an organism, especially when in a relatively attenuated condition, can also be raised by injecting along with it a quantity of a culture of another organism either in the living or dead condition. A few examples may be mentioned. An attenuated diphtheria culture may have its virulence raised by being injected into an animal along with the streptococcus pyogenes; an attenuated culture of the bacillus of malignant oedema by being injected with the bacillus prodigiosus; an attenuated streptococcus by being injected with the bacillus coli, etc. A culture of the typhoid bacillus may be increased in virulence, as already stated, by being injected along with a dead culture of the bacillus coli. In such cases the accompanying injection enables the attenuated organism to gain a foothold in the tissues, and it may be stated as a general rule that the virulence of an organism for a particular animal is raised by its growing in the tissues of that animal.

*Combination of Methods.*—The above methods may be combined in various ways. By repeated injections of cultures at first in the dead condition, then living and attenuated and afterwards more virulent, and by increasing the doses, a high degree of immunity may be obtained.



**2. Immunity by Dead Cultures of Bacteria.**—In some cases a high degree of immunity against infection by a given microbe may be developed by repeated and gradually increasing doses of the dead cultures, the cultures being killed sometimes by heat, sometimes by exposure to the vapour of chloroform. In this method the so-called endotoxins will be injected along with the other substances in the bacterial protoplasm, but the resulting immunity is chiefly directed against the vital activity of the organisms—is antibacterial rather than antitoxic (*vide infra*). The cultures when dead produce, of course, less effect than when living, and this method may be conveniently used in the initial stages of active immunisation,—to be afterwards followed by injections of the living cultures. The method is extensively used for experimental purposes, and is that adopted in anti-plague and anti-typhoid inoculations, and in the *treatment* of infections by means of vaccines.

**3. Immunity by the Separated Bacterial Products or Toxins.**—The organisms in a virulent condition are grown in a fluid medium for a certain time, and the fluid is then filtered through a Chamberland or other porcelain filter. The filtrate contains the toxins, and it may be used unaltered, or may be reduced in bulk by evaporation, or may be evaporated to dryness. The process of immunisation by the toxin is started by small non-lethal doses of the strong toxin, or by larger doses of toxin the power of which has been weakened by various methods (*vide infra*). Afterwards the doses are gradually increased. This method was carried out with a great degree of success in the case of diphtheria, tetanus, malignant oedema, etc. It appears capable of general application in the case of organisms where it is possible to get an active toxin from the filtered cultures. It has also been applied in the case of snake venoms by Calmette and by Fraser, and a high degree of immunity has been produced.

The following may be mentioned as some of the most important examples of the practical application of the principles of active immunity, *i.e.* of protective inoculation:—(1) Inoculation of sheep and oxen against anthrax (Pasteur) (p. 346); (2) Jennerian vaccination against smallpox (p. 565); (3) Anti-cholera inoculation (Haffkine) (p. 459); (4) Anti-plague inoculation (Haffkine) (p. 486); (5) Anti-typhoid inoculation (Wright and Semple) (p. 375); (6) Pasteur's method of inoculation against hydrophobia, which involves essentially the same principles (p. 579).

*Vaccines as a Method of Treatment.*—Up till recently the



principles of active immunity had not been directly applied in the treatment of an existing disease except in the case of tuberculosis. The work of Wright, however, shows that active immunisation in such circumstances is not only possible, but is also probably capable of wide application. From his study of the part played by phagocytosis in the successful combat of bacteria by the body, he was led to advocate the treatment of bacterial infections by carrying on an active immunisation against the causal agents by the injection of dead cultures of the latter. The justification for such a procedure lies in his contention that in many cases infections are to be looked on as practically localised, *e.g.* the cases of an acne pustule, or a boil. The reason for the local growth of bacteria in the part of the body affected is that there is for unknown causes a deficiency of the opsonic power (*vide* p. 291) of the body fluids, which is essential for the phagocytosis of the invading bacteria. Still more marked in such cases is the deficiency in the opsonic qualities of the fluids in the actual site of infection. Any procedure which will raise the opsonic power of the body fluids as a whole, and therefore of the fluids in the focus of infection, will aid the destruction of the bacteria by sensitising them to phagocytic action. Such a procedure is found in the active immunisation which results from the injection of a vaccine consisting of a dead culture of the causal bacteria. The application of a vaccine of this kind can be controlled by observation of the opsonic index of the patient's serum during the treatment. When a local infection is present the general opsonic index is usually found to be below unity. If dead bacteria be injected into the individual there may occur during the following few days a further fall in the opsonic index,—what Wright calls the occurrence of a negative phase. In a case where the treatment is successful, this negative phase is succeeded by a rise in the opsonic index above its original level,—occurrence of positive phase,—and with this reaction there is an improvement in the local condition. Usually in such cases repeated injections are required to effect a cure, and the important point, according to Wright, is to avoid giving an injection when a negative phase is in progress. If this point is not attended to an aggravation of symptoms may occur (*vide* p. 291). With regard to the details of the preparation of the vaccines see p. 133. The number of bacteria employed for a vaccination varies from 5,000,000 to 500,000,000 or more. Such vaccines have been used extensively in the treatment of acne, boils, sycosis, tuberculosis, infections of the genito-urinary tract by the



b. coli, infections of joints by the gonococcus, and in many cases considerable success has followed the treatment.

**Active Immunity by Feeding.**—Ehrlich found that mice could be gradually immunised against ricin and abrin by feeding them with increasing quantities of these substances (*vide* p. 199). In the course of some weeks' treatment in this way the resulting immunity was of so high a degree that the animals could tolerate on subcutaneous inoculation 400 times the dose originally fatal. Fraser also found, in the case of snake venom that rabbits could, by feeding with the poison, be immunised against several times the lethal dose of venom injected into the tissues.

By feeding animals with dead cultures of bacteria or with their separated toxins, a degree of immunity may in some cases be gradually developed. But this method is so much less certain in results, and so much more tedious than the others, that it has obtained no practical applications.

Active immunity of high degree developed by the methods described may be regarded as *specific*, that is, is exerted only towards the organism or toxin by means of which it has been produced. A certain degree of immunity, or rather of increased general resistance of parts of the body (for example the peritoneum), can, however, be produced by the injection of various substances—bouillon, blood serum, solution of nuclein, etc. (Issaëff). Also increased resistance to one organism can be thus produced by injections of another organism. Immunity of this kind, however, never reaches a high degree.

### B. *Passive Immunity.*

**Action of the Serum of Highly Immunised Animals.**—1. The serum of an animal A, treated by repeated and gradually increased doses of a toxin of a particular microbe, may protect an animal B against a certain amount of the same toxin when injected along with the latter, or a short time before it. As might be expected, it has less effect when injected some time afterwards, but even then within certain limits it has a degree of curative or palliative power. Seeing that the serum of animal A appears to neutralise the toxin, the term *antitoxic* has been applied to it.

2. The serum of an animal A, highly immunised against a bacterium by repeated and gradually increasing doses of the organism, may protect an animal B against an infection by the living organism when injected under conditions similar to the above. This serum is therefore *antimicrobial*, or *antibacterial*,



*i.e.* preventive against invasion by a particular organism. (In addition to the preventive or protective action *in vivo*, such a serum may exert certain recognisable effects on the corresponding organism *in vitro*. Thus (a) it may lead to the death or solution of the organism—*bactericidal* or *lysogenic action*; when no such effect follows, the presence of an immune-body (p. 128) may be shown by the *deviation of complement* method; (b) it may produce an increased susceptibility to ingestion by phagocytes—*opsonic action*; (c) it may lead to the clumping of the organism—*agglutinative action*, or to *precipitation* with an extract of a culture of the corresponding bacterium.)

*Anti-substances and their Specificity.*—The fundamental fact in passive immunity, namely, that immunity can be transferred to another animal, shows that the serum in question differs from the serum of a normal animal in containing antagonistic substances to the toxin or bacterium as the case may be,—these being generally spoken of as anti-substances. The development of these bodies, first observed in the case of the injection of toxins, is found to occur when a great many different substances are introduced into the tissues of the living body. We can, in fact, divide organic molecules into two classes—those which give rise to the production of anti-substances, and are thus known as *antigens*, and those which have not this property. Amongst the former are various toxins, ferments, molecules of tissue cells, bacteria, red corpuscles, etc. They are all probably of proteid nature, though their true constitution is not known, and none of them have been obtained in a pure condition. Amongst the latter may be placed the various poisons of known constitution, glucosides, alkaloids, etc. We may also state at present that the anti-substance forms a chemical or physical union with the particular antigen which has led to its development, and we shall discuss the evidence for this later. Furthermore, the anti-substance has apparently a specific combining group which fits, as it were, a group in the corresponding antigen, the two groups having been compared to a lock and key. It is, however, to be noted that this specificity is a chemical one rather than a biological one. An anti-serum, for example, developed by the injection of bacterium A may also have some effect on bacterium B, and thus appear not to be specific. We have, however, evidence to show that the antigens in bacterium A are not all identical, and that some of them may be present though in smaller proportion in bacterium B; thus the theory of combining specificity is not invalidated. The number of different anti-substances, as judged by their combining properties, would appear to be almost



unlimited, a fact which throws new light on the complexity of the structure of living matter. When anti-substances are studied as regards their action on the substances with which they combine, they may be conveniently arranged in three classes corresponding to Ehrlich's three classes of receptors (*vide* p. 549). In the first place, the anti-substance may simply combine with the substance without, so far as we know, producing any change in it, and to this group the anti-toxins and anti-ferments belong. In the second place, the anti-substance, in addition to combining, may produce some recognisable physical alteration. In other words, it possesses an active or zymotoxic group as well as a combining group. The agglutinins may be mentioned as examples of this group. In the third place, the anti-substance after combination may lead to the combination of another body normally present in serum called complement or alexine, and this latter, which has a constitution very similar to that of a toxin, may lead to physical change, for example, death or solution of a cell. Anti-substances of this class are known as immune-bodies or amboceptors (Ehrlich) or as sensitising substances—*substances sensibilisatrices* of French writers. Their essential feature is that they lead to the combination or fixation of complement, which may or not produce some recognisable change such as bacteriolysis, etc. If no such effect follows, however, the union of complement may be demonstrated by the indirect or deviation method (p. 130).

After this preliminary statement in explanation, we shall consider the actual properties of the two classes of serum, and later we shall resume the theoretical consideration.

**Antitoxic Serum.**—In a previous chapter (p. 188) a distinction has been drawn between extra- and intra-cellular toxins, and with regard to these the general statement may be made that while antitoxins are, as a rule, comparatively easily obtained in the case of the former, the matter is quite otherwise in the case of the latter. In fact some writers have gone so far as to say that antitoxins to endotoxins cannot be obtained. Such an extreme view is in our opinion unjustifiable in the light of the recent work on antitoxins to the typhoid, cholera, and dysentery endotoxins (pp. 366, 456, 388). Nevertheless we have the important fact that in many cases by the injection of dead cultures an active anti-bacterial serum can be obtained which has no neutralising action on the endotoxins, and we must conclude either that a large proportion of the endotoxin does not lead to the production of antitoxin or does so only with great slowness, the latter alternative being on general grounds rather improbable.



The best examples of antitoxic sera are those of diphtheria and tetanus, though similar principles and methods are involved in the case of the anti-sera to ricin and abrin, and to snake poison. We shall here speak of diphtheria and tetanus. The steps in the process of preparation may be said to be the following: First, the preparation of a powerful toxin; second, the estimation of the power of the toxin; third, the development of antitoxin in the blood of a suitable animal by gradually increasing doses of the toxin; fourth, the estimation from time to time of the antitoxic power of the serum of the animal thus treated.

1. *Preparation of the Toxin.*—The mode of preparation and the conditions affecting the development of diphtheria toxin have already been described (p. 406). In the case of tetanus the growth takes place in glucose bouillon under an atmosphere of hydrogen (*vide* p. 66). In either case the culture is filtered through a Chamberland filter when the maximum degree of toxicity has been reached. The term "toxin" is usually applied for convenience to the filtered (*i.e.* bacterium-free) culture.

2. *Estimation of the Toxin.*—The power of the toxin is estimated by the subcutaneous injection of varying amounts in a number of guinea-pigs, and the minimum dose which will produce death is thus obtained. This, of course, varies in proportion to the weight of the animal, and is expressed accordingly. In the case of diphtheria, in Ehrlich's standard, the minimum lethal dose—known as M.L.D.—is the smallest amount which will certainly cause death in a guinea-pig of 250 grms. within four days. The testing of a toxin directly is a tedious process, and in actual practice, where many toxins have to be dealt with, it is found more convenient to test them by finding how much will be neutralised by a certain amount of a standard antitoxic serum, namely, an "immunity unit" (p. 524).

3. *Development of Antitoxin.*—The earlier experiments on tetanus and diphtheria were performed on small animals, such as guinea-pigs, but afterwards the sheep and the goat were used, and finally horses. In the case of the small animals it was found advisable to use in the first stages of the process either a weak toxin or a powerful toxin modified by certain methods. Such methods are the addition to the toxin of terchloride of iodine (Behring and Kitasato), the addition of Gram's iodine solution in the proportion of one to three (Roux and Vaillard), and the plan, adopted by Vaillard in the case of tetanus, of using a series of toxins weakened to varying degrees by being exposed to different temperatures, namely, 60°, and 55°, and 50° C. In the case of large animals immunisation is sometimes started



with small doses of unaltered toxin; and the doses are gradually increased. The toxin is at first injected into the subcutaneous tissues, later into a vein. Ultimately 300 c.c., or more, of active diphtheria toxin thus injected may be borne by a horse, such a degree of resistance being developed after the treatment has been carried out for two or three months. The antitoxin content of the serum is estimated from time to time, the object being, of course, to raise it to as high a figure as possible. It is found that each injection produces a certain amount of fall in the antitoxin value, and this, in favourable cases, is followed by a rise to a higher level than before, the former event being due in part to the combination of a portion of the antitoxin with the toxin introduced. (Similar phenomena are observed in the development of all other classes of anti-substances.) In all cases of immunising the general health of the animal ought not to suffer. If the process is pushed too rapidly the antitoxic power of the serum may diminish instead of increasing, and a condition of marasmus may set in and may even lead to the death of the animal. After a sufficiently high degree of antitoxic power has been reached, the animal is bled under aseptic precautions, and the serum is allowed to separate in the usual manner. It is then ready for use, but some weak antiseptic, such as .5 per cent carbolic acid, is usually added to prevent its decomposing. Other antitoxic sera are prepared in a corresponding manner. Some further facts about antitetanic serum are given on p. 429. (In immunisation of small animals an indication of their general condition may be obtained by weighing them from time to time.)

4. *Estimating the Antitoxic Power of, or "standardising," the Serum.*—This is done by testing the effect of various quantities of the serum of the immunised animal against a certain amount of toxin. Various standards have been used, of which the two chief are that of Ehrlich and that of Roux. Ehrlich has adopted as the *immunity unit* the amount of antitoxic serum which will neutralise 100 times the minimum lethal dose of toxin, serum and toxin being mixed together, diluted up to 4 c.c. and injected subcutaneously into a guinea-pig of 250 grms. weight, the prevention of the death of the animal within four days being taken as the indication of neutralisation. 1 c.c. of a serum, of which .02 c.c. will protect against a hundred times the lethal dose, will possess 50 immunity units, and 20 c.c. of this serum 1000 immunity units. Sera have been prepared of which 1 c.c. has the value of 800 units or even more. As a standard in testing, Ehrlich employs quantities of serum of known antitoxic



power in a dry condition, preserved in a vacuum in a cool place, and in the absence of light. A thoroughly dry condition is ensured by having the glass bulb containing the dried serum connected with another bulb containing anhydrous phosphoric acid. With such a standard test-serum any newly prepared serum can readily be compared.

Roux has adopted a standard which represents the animal weight in grammes protected by 1 c.c. of serum against the dose of virulent bacilli lethal to a control guinea-pig in thirty hours, the serum being injected twelve hours previously. Thus, if .01 c.c. of a serum will protect a guinea-pig of 500 grms. against the lethal dose, 1 c.c. (1 grm.) will protect 50,000 grms. of guinea-pig, and the value of the serum will be 50,000.

*Use of Antitoxic Sera.*—In all cases the antitoxic serum ought to be injected as early in the disease as possible, and in large doses. In the case of diphtheria 1500 immunity units of antitoxic serum was the amount first recommended for the treatment of a bad case, but the advisability of using larger doses has gradually become more and more evident. Sidney Martin recommends that as much as 4000 units should be administered at once, and that if necessary this quantity should be repeated. A strong serum prepared by Behring contains 3000 units in 5 to 6 c.c., but even stronger sera may be obtained. Even very large doses of antitoxic serum are without any harmful effects beyond the occasional production of urticarial and erythematous rashes (p. 563). Where large quantities of serum require to be administered, as is always the case with antitetanic serum, injections must be made at different parts of the body; preferably not more than 20 c.c. should be injected at one place. In recent times intravenous injection has been introduced, the advantage being greater rapidity of action. The immunity conferred by injection of antitoxic serum lasts a comparatively short time, usually a few weeks at longest.

*Sera of Animals immunised against Vegetable and Animal Poisons.*—It was found by Ehrlich in the case of the vegetable toxins, ricin and abrin, and also by Calmette and Fraser in the case of the snake poisons, that the serum of animals immunised against these respective substances had a protective effect when injected along with them into other animals. Ehrlich found, for example, that the serum of a mouse which had been highly immunised against ricin by feeding as described above, could protect another mouse against forty times the fatal dose of that substance. He considered that in the case of the two poisons, antagonistic substances—"anti-ricin" and "anti-abrin"—were



developed in the blood of the highly immunised animals. A corresponding antagonistic body, to which Fraser gave the name "antivenin," appears in the blood of animals in the process of immunisation against snake poison.

These investigations are specially instructive, as such vegetable and animal poisons, both as regards their local action and the general toxic phenomena produced by them, present, as we have seen, an analogy to various toxins of bacteria.

*Nature of Antitoxic Action.*—This subject is only part of the general question with regard to the relation of anti-substances to their corresponding antigens, but it is with regard to antitoxic action that most of the work has been done. We have to consider here two points, namely, (*a*) the relation of antitoxin to toxin, and (*b*) the source of the antitoxin. With regard to the former subject there is now no doubt that the antagonism between toxin and antitoxin is not a physiological one but that the two bodies unite *in vitro* to form a compound inert towards the living tissues, there being in the toxin molecule an atom group which has a specific affinity for the antitoxin molecule or part of it. We shall consider the facts in favour of this view, and in doing so we must also take into account the anti-sera of the vegetable toxins, of snake poisons, etc.

When toxin and antitoxin are brought together *in vitro*, it can be proved that their behaviour towards each other resembles what is observed in chemical union. Thus it has been found that a definite period of time elapses before the neutralisation of the toxin is complete, that neutralisation takes place more rapidly in strong solutions than in weak, and that it is hastened by warmth and delayed by cold. C. J. Martin and Cherry, and also Brodie, have shown, that in the case of diphtheria toxin and in that of an Australian snake poison the toxin molecules will pass through a colloid membrane (p. 193), whilst those of the corresponding antitoxin will not. Now, if a mixture of equivalent parts of toxin and antitoxin is freshly prepared and at once filtered, a certain amount of toxin will pass through, but the longer such a mixture is allowed to stand before filtration the less toxin passes, till a time is reached when no toxin is found in the filtrate. Further, if the portion of fluid which at this stage has not passed through the filter be injected into an animal no symptoms take place; this shows that after a time neutralisation is complete. Again, in cases where the toxin has some definite physical effect, demonstrable *in vitro*, e.g. lysis, agglutination, coagulation, or the prevention of coagulation, its action can be annulled by the antitoxin; in such circumstances



manifestly no physiological action of antitoxin through the medium of the cells of the body can come into play. These facts are practically conclusive in favour of antitoxin action depending upon a direct union of the two substances concerned, and Morgenroth has recently shown that the combination toxin-antitoxin can be broken up by the action of hydrochloric acid and the two constituents recovered.

Although authorities are now agreed as to the direct combination of toxin and antitoxin, there is still much uncertainty as to the exact nature of this union. Regarding this subject there may be said to be three chief views—(a) that of Ehrlich, according to which there is a firm chemical union of toxin and antitoxin, and the former is not homogeneous but has a complex structure; (b) that of Arrhenius and Madsen, who consider that the phenomena correspond to the behaviour of two substances in weak chemical union; and (c) that of Bordet, who regards the combination not to be of chemical, but of physical nature, corresponding to a process of adsorption. Controversy on this question may be said to date from the important work of Ehrlich on the neutralisation of diphtheria toxin. Using an immunity unit of antitoxin (the equivalent of 100 doses of toxin) he determined with any example of crude toxin the largest amount of toxin which could be neutralised completely, so that no symptoms resulted from an injection of the mixture. This amount he called the *limes null* dose, expressed as  $L_0$ . He then investigated the effects of adding larger amounts of toxin to the immunity unit and observed the quantity which was first sufficient to produce a fatal result, that is, which contained one M.L.D. of free toxin; this amount he called the *limes tödtlich*, fatal limit, expressed as  $L_t$ . Now if, as he supposed, the union of toxin and antitoxin resembled that of a strong acid and base,  $L_t - L_0$  ought to be the equivalent of a minimum lethal dose of the toxin alone. This, however, was never found to be the case, the difference being always considerably more than one M.L.D. For example, in the case of one toxin, M.L.D. = .0165 c.c.,  $L_t = 1.26$  c.c.,  $L_0 = .9$  c.c.; difference = .36 c.c., i.e. 21.9 M.L.D. This, in brief, is what is known as the "Ehrlich phenomenon," and it has been explained by him as the result of the presence of toxoids (*vide* p. 198), i.e. toxin molecules in which the toxoporous group has become degenerated. He distinguishes three possible varieties of such bodies according to the affinity of the haptoporous group, namely, *prototoxoid* with more powerful affinity than the toxin molecule, *epitoxoid* with less powerful affinity, and *syntoxoid*



with equal affinity. The presence of epitoxoids would manifestly explain the above phenomenon. The  $L_0$  dose would represent toxin + epitoxoid molecules all united to antitoxin molecules and the addition of another M.L.D. of toxin would not result in there being a free fatal dose, but in the added toxin taking the place of epitoxoid. Several lethal doses would need to be added before the mixture was sufficient to produce a fatal result; that is,  $L_t - L_0$  would equal several M.L.D.s. Ehrlich observed another fact strongly in favour of the existence of toxoids, namely, that in the course of time the toxin might become much weakened, so that in one case observed the M.L.D. came to be three times the original fatal dose, and still the amount of antitoxin necessary to neutralise it completely was the same as before. Ehrlich also investigated the effects of partial neutralisation of the  $L_0$  amount of toxin, that is, he added to this amount different fractions of an immunity unit and estimated the toxicity of the mixture. He found by this method that the neutralisation of the toxin did not take place gradually, but as if there were distinct bodies present with different combining affinities—the graphic representation of the effects of the mixture not being a curve but a step-stair line. Thus he distinguished proto-, deutero-, and trito-toxins (with corresponding toxoids). It will thus be seen that Ehrlich regards the combination toxin-antitoxin to be a firm one, and that the neutralisation phenomena are to be explained by the complicated constitution of the crude toxin.

The chief criticism of Ehrlich's views has come from the important work of Madsen and Arrhenius. Their main contention is that the toxin-antitoxin combination is not a firm one but a reversible one, and is governed by the laws of physical chemistry. For example, in the case of a mixture of ammonia and boracic acid (*i.e.* of a weak base and a weak acid) in solution, there is a constant relation between the amounts of each of the substances in the free condition and the amounts in combination,—the combination is reversible, so that if some of the free ammonia were removed a certain amount of the combined ammonia would become dissociated to take its place; further, if to the mixture, in a state of equilibrium, more ammonia or more boracic acid were added, part would remain free while part would combine. Accordingly, if toxin and antitoxin behaved in a similar manner an explanation of the Ehrlich phenomenon would be afforded. Madsen and Arrhenius have worked out the question in the case of a great many toxins, and find that the graphic representation of neutralisation is in every case a curve which can be represented by a formula. It



should be noted in connection with this controversy that there are two questions which may be independent of each other, namely, (1) does the "toxin" in any particular case represent a single substance or several? (2) What is the nature of the combination of any one constituent substance and its anti-substance—is it reversible or is it not? It may be said that it is practically impossible to explain the facts with regard to diphtheria toxin on the hypothesis of a single substance, even if this should have its combining and toxic actions equally weakened; "toxoids" in Ehrlich's sense must in our opinion be supposed. Then there is an important fact established by Danysz and by v. Dungern, namely that the amount of toxin neutralisable by a given amount of antitoxin is different according as the toxin is added in several moieties or all at once—in the latter case the amount of toxin neutralisable is greater. There seems no explanation of this according to the view of Madsen and Arrhenius, as the same state of equilibrium ought to be reached in the two cases, that is, the amounts of toxin neutralised should be the same.

An important factor in the union of toxin and antitoxin is the time necessary for the union to be complete. Morgenroth has shown that in the case of diphtheria toxin this is considerable,—about twenty-four hours. Up to this time, mixtures of toxin and antitoxin, when injected intravenously, show decreasing degrees of toxicity according to the time they have kept. On the other hand, when the subcutaneous method of injection is used the time interval has no effect, and this he considers to be due to a catalytic action of the tissues which accelerates the union of the two substances. A striking phenomenon, which apparently points to the reversibility of the combination, was noted by Behring in the case of diphtheria toxin, and afterwards studied by Madsen and by Otto and Sachs in the case of botulismus toxin, namely, that when a mixture of toxin and antitoxin was found to be neutral on injection, a fraction of the mixture might produce toxic phenomena or even death. This was apparently due to dissociation of the toxin in the greater dilution, and in favour of this being the case Otto and Sachs found that when the mixture was allowed to stand for twenty-four hours, so that combination was complete, the phenomenon no longer occurred. It was shown by Morgenroth and by Muir independently that the union of a hæmolytic immune-body with the corresponding red corpuscle was of reversible nature, and the latter observer found that in this case the union was not increased in firmness after twenty-four hours. There is little doubt that there are varying degrees of firmness of union of an



antigen and its anti-substance and varying periods necessary for the combination to become complete; and it is quite evident that if there should be several toxic bodies in a "toxin," and that if the union of some of these with antitoxin should be reversible, the problem becomes one of extreme complexity.

There has recently been a tendency on the part of some authorities to consider that the union of toxin-antitoxin does not correspond to what takes place in ordinary chemical union, but is a physical interaction of bodies in a colloidal state, the action being one of the so-called adsorption phenomena. The smaller toxin molecule becomes entangled, as it were, in the larger antitoxin one, very much as a dye becomes attached to the structure of a thread. Bordet has long maintained a theory of this nature, and gives reasons for believing that there is no definite quantitative relationship in the combination of the molecules of the two substances, different amounts of antitoxin affecting in varying degree all the molecules of a given amount of toxin. A statement on the general question is at present impossible; we can only say that *direct combination of the two bodies does occur; that sometimes, probably often, the "toxin" contains different toxic bodies with varying affinity; and that in a few instances the combination has been proved to be reversible, but to what extent this is generally true remains still to be determined.*

The next question to be considered is the *source* of antitoxin. The following three possibilities present themselves: (a) antitoxin may be formed from the toxin, *i.e.* may be a "modified toxin"; (b) antitoxin may be the result of an increased formation of molecules normally present in the tissues; (c) antitoxin may be an entirely new product of the cells of the body. It can now be stated that antitoxin is not a modified toxin. It has been shown, for example, that the amount of antitoxin produced by an animal may be many times greater than the equivalent of toxin injected; and further, that when an animal is bled the total amount of antitoxin in the blood may some time afterwards be greater than it was immediately after the bleeding, even although no additional toxin is introduced. This latter circumstance shows that antitoxin is *formed* by the cells of the body. If antitoxin is a product of the cells of the body, we are almost compelled, on theoretical grounds, to conclude that it is not a newly-manufactured substance, but a normal constituent of the living cells which is produced in increased quantity. We have, however, direct evidence of the presence of antitoxin under normal conditions,—the presence of such being shown by its uniting with



toxin and rendering it inert. Normal horse serum, to mention an example, may have a varying amount of antitoxic action to the diphtheria poison, ox-bile has a similar action to snake poison, whilst in the case of other anti-substances—such as agglutinins, bacteriolysins, hæmolysins, etc.—whose production is governed by the same laws, numerous examples might be given. It is, however, rather to the protoplasm of living cells than to the serum that we must look for the source of antitoxins. In the first place, we have evidence that in the living body bacterial toxins enter into combination with, or, as it is often expressed, are fixed by the tissues—presumably by means of certain combining affinities. This has been shown by the experiments of Dönitz and of Heymans with tetanus toxin. We have, in such cases, however, no evidence as to where the toxin is fixed beyond that supplied by the occurrence of symptoms. Another line of research which has been followed is to bring emulsions of various organs into contact with a given toxin and observe whether any of the toxicity is removed. This was first carried out by Wassermann and Takaki, who investigated the action of emulsions of the central nervous system of the susceptible guinea-pig on tetanus toxin. They found in this way that the nervous system contained bodies which had a neutralising effect on the toxin. For example, it was shown that 1 c.c. of emulsion of brain and spinal cord was capable of protecting a mouse against ten times the fatal dose of toxin. These observations have been confirmed, though their significance has been variously interpreted: and in view of recently ascertained facts with regard to processes of physical adsorption, it is quite possible that this neutralisation of toxin does not represent a specific union as in the case of antitoxin action. We may note, however, that it is not a serious objection that in certain animals other tissues than that of the central nervous system can combine with tetanus toxin—this might take place with or without resulting symptoms.

It will be seen from what has been stated with regard to the relation of toxin and antitoxin, that the fixation of toxin by the tissues leads up theoretically to the possible production of antitoxin. In other words, the substance which, when forming part of the cells, fixes the toxin and thus serves as the means of poisoning, may act as an antitoxin when free in the blood. This will be discussed below in connection with Ehrlich's theory of passive immunity. We may conclude by saying *that antitoxin is probably represented by molecules normally present in the cells or (more rarely) in the fluids of the body.*

Of the *chemical nature of antitoxins* we know little. From



their experiments C. J. Martin and Cherry deduced that while toxins are probably of the nature of albumoses, the antitoxins probably have a molecule of greater size, and may be allied to the globulins. Such a supposed difference in the sizes of the molecules might explain the fact, observed by Fraser and also by C. J. Martin, that antitoxin is much more slowly absorbed when introduced subcutaneously than is the case with toxin. Hiss and Atkinson also came to the conclusion that antitoxin belongs to the globulins. They found that the precipitate with magnesium sulphate from anti-diphtheria serum contained practically all the antitoxin, and that any substance obtained which had an antitoxic value gave all the reactions of a globulin; and this result has been confirmed by others. They also found that the percentage amount of globulin precipitated from the serum of the horse increased after it was treated in the usual way for the production of antitoxin. Ledingham observed an increase of globulin during the process of immunisation of a horse which yielded a high-grade antitoxic serum, and he ascertained that while this increase was more on the part of the euglobulin than of the pseudoglobulin fraction, most of the antitoxin was contained in the latter.

Antitoxin, when present in the serum, leaves the body by the various secretions, and in these it has been found, though in much less concentration than in the blood. It is present in the milk, and a certain degree of immunity can be conferred on animals by feeding them with such milk, as has been shown by Ehrlich, Klemperer, and others. Klemperer also found traces of antitoxin in the yolk of eggs of hens whose serum contained antitoxin. Bulloch also found in the case of hæmolytic sera (*vide infra*) that the anti-substance ("immune-body") is transmitted from the mother to the offspring.

**Antibacterial Serum.**—The stages in the preparation of antibacterial sera correspond to those in the case of antitoxic sera, but living, or, in the early stages, dead cultures are used instead of toxin separated by filtration, and in order to obtain a serum of high antibacterial power a very virulent culture in large doses must be ultimately tolerated by the animal. For this purpose a fairly virulent culture is obtained fresh from a case of the particular disease, and its virulence may be further increased by the method of *passage*. This method of obtaining a high degree of immunity against the microbe is specially applicable in the case of those organisms which invade the tissues and multiply to a great extent within the body, and of which the toxic effects, though always existent, are proportionately small



in relation to the number of organisms present. The method has been applied in the case of the typhoid and cholera organisms, the bacillus of bubonic plague, the bacillus coli communis, the pneumococcus, streptococcus (Marmorek), and many others. In fact, it seems capable of very general application.

The important result obtained by such experiments is, that if an animal be highly immunised by the method mentioned, the development of the immunity is accompanied by the appearance in the blood of *protective* substances, which can be transferred to another animal. The law enunciated by Behring regarding immunity against toxins thus holds good in the case of the living organisms, as was first shown by Pfeiffer. The latter found, for example, that in the case of the cholera organisms, so high a degree of immunity could be produced in the guinea-pig, that .002 c.c. of its serum would protect another guinea-pig against ten times the lethal dose of the organisms, when injected along with them. Here again is presented the remarkable potency of the antagonising substances in the serum, which in this case lead to the destruction of the corresponding microbe.

The *anti-streptococcic serum* of Marmorek may be briefly described, as it has come into extensive practical use. This observer found that he could intensify the virulence of a streptococcus by growing it alternately in the peritoneal cavity of a guinea-pig and in a mixture of human blood serum and bouillon (*vide* p. 41). The virulence became so enormously increased by this method, that when only one or two organisms were introduced into the tissues of a rabbit a rapidly fatal septicæmia was produced. Streptococci of this high degree of virulence were used first by subcutaneous, afterwards by intravenous injection, to develop a high degree of resistance in the horse. Injections were continued over a considerable period of time, and the protective power of the serum was tested by mixing it with a certain dose of the virulent organisms, and then injecting into a rabbit. The serum of a horse highly immunised in this way constitutes the anti-streptococcic serum which has been extensively used in many cases of streptococcic invasion in the human subject. Marmorek, however, found that this serum had little antitoxic power, that is, could only protect from a comparatively small dose of toxin obtained by filtration of cultures.

Anti-typhoid, anti-cholera,<sup>1</sup> anti-pneumococcic, anti-plague, and other sera are all prepared in an analogous manner.

**Properties of Antibacterial Serum.**—We have here to consider the three main actions mentioned above, namely, (a) bactericidal and lysogenic action, (b) opsonic action, and (c) agglutinative and the closely allied precipitating action. Of

<sup>1</sup> A true *antitoxic* cholera serum has been prepared by Metchnikoff, E. Roux, and Taurelli-Salimbeni.



these the two first are concerned with the protective property of an anti-bacterial serum.

(a) *Bactericidal and Lysogenic Action*.—Pfeiffer found that if certain organisms, *e.g.* the cholera spirillum, were injected into the peritoneal cavity of a guinea-pig highly immunised against these organisms, they lost their motility almost immediately, gradually became granular, swollen, and then disappeared in the fluid—these changes constitute “Pfeiffer’s phenomenon.” Further, he showed that the same phenomenon was witnessed if a minute quantity of the anti-serum was added to a certain quantity of the corresponding organisms, and the mixture injected into the peritoneal cavity of a non-treated animal. Pfeiffer found that the serum of convalescent cholera patients gave the same reaction as that of immunised animals. He obtained the same reaction also in the case of the typhoid bacillus and other organisms. From his observations he concluded that the reaction was specific, and could be used as a means of distinguishing organisms which resemble one another. He accordingly considered that a specific substance was developed in the process of immunisation, and that this was rendered actively bactericidal by the aid of the living cells of the body. It was subsequently shown, however, by Metchnikoff and by Bordet that lysogenesis might occur outside the body by the addition of fresh peritoneal fluid or normal serum to the heated immune-serum. Pfeiffer also found that an anti-serum heated to 70° C. for an hour produced the reaction when injected with the corresponding organisms into the peritoneum of a fresh animal. The outcome of these and subsequent researches is to show that when an animal is immunised against a bacterium a substance appears in its serum with combining affinity for that particular organism. This substance, which is generally known as *the immune-body*, *amboceptor* (Ehrlich), or *substance sensibilisatrice* (Bordet) is comparatively stable, resisting usually a temperature of 70° C. for an hour. It cannot produce the destructive effect alone, but requires the addition of a substance normally present in the serum, which is spoken of under various names—*complement* (Ehrlich), *alexine* or *cytase* (French writers). The complement is relatively unstable, being rapidly destroyed by a temperature of 60° C., and it is not increased in amount during the process of immunisation. Though ferment-like in its instability, it differs from a ferment in being fixed or used up in definite quantities.

Recent observations show that complement is not a single substance, but is really made up of two components. Ferrata, who was the first to establish this fact, employed the following method: Fresh guinea-pig’s



serum is dialysed against running water for twenty-four hours; the precipitate which has formed at the end of that time is separated by the centrifuge, washed several times in distilled water, and then dissolved in normal salt solution. The separated fluid is passed through thick filter paper. The component in the solution of the precipitate unites directly with sensitised corpuscles—and then that in the separated fluid enters into combination; hence they have been called by Brand “middle-piece” and “end-piece” respectively. The separation by such a method is, however, far from being a complete one. Sachs and Altmann have introduced the following method: To .5 c.c. of fresh guinea-pig's serum is added 4.1 c.c. of a  $\frac{1}{250}-\frac{1}{300}$  normal solution of hydrochloric acid in distilled water. The sediment is centrifuged off after the mixture has been allowed to stand at room temperature for an hour, and, after being washed, is made up with a suitable amount of distilled water. The separated fluid is neutralised and made isotonic with .4 c.c. of a  $\frac{1}{25}-\frac{1}{30}$  normal soda solution containing 10 per cent. sodium chloride.

The phenomenon of lysogenesis is, however, only seen in the case of certain organisms when an animal is highly immunised against them; the typhoid and cholera group are outstanding examples. It is also to be noted that it sometimes is seen in the case of a normal serum (*vide* Natural Immunity). In other cases the bactericidal effect of a serum may occur without the rapid dissolution characteristic of lysogenesis though other structural changes may be produced. In still other instances, *e.g.* the anti-sera to staphylococci, streptococci, plague bacilli, etc., a bactericidal effect may be wanting; nevertheless it may be shown that an immune-body is developed in the process of immunisation. This may be done by observing the increased amount of complement which is fixed through the medium of the anti-serum (immune-body), sensitised red corpuscles being used as the test for the presence of free complement. The method is described on pp. 128–131.

The all-important action of the immune-body is thus to bring an increased amount of complement into union with bacteria; whether death of the bacteria will result or not will depend ultimately on their sensitiveness to the action of the particular complement.

It is to be noted that in the case of a bactericidal serum there is an optimum amount of immune-body which gives the greatest bactericidal effect with a given amount of complement. If this amount of immune-body be exceeded, the bactericidal action becomes diminished and may be practically annulled. This result, which is generally known as the Neisser-Wechsberg phenomenon, has been the subject of much controversy, and cannot yet be said to be satisfactorily explained. It would accordingly be out of place to discuss here the different views



with regard to it. (Regarding some theoretical considerations as to the therapeutic applications of antibacterial sera, *vide* p. 534.)

The laws of lysogenesis are, however, not peculiar to the case of solution of bacteria by the fluids of the body, but, as has been shown within the last few years, hold also in the case of other organised substances, red corpuscles, leucocytes, etc., when these are introduced into the tissues of an animal as in a process of immunisation. Of such sera the hæmolytic have been most fully studied, and, owing to the delicacy of the reaction and the ease with which it can be observed, have been the means of throwing much light on the process of lysogenesis, and thus on one part of the subject of immunity. A short account of their properties may now be given.

*Hæmolytic and other Sera.*—It has been known for some time that in some instances the blood serum of one animal has, in a certain degree, the power of dissolving the red corpuscles of another animal of different species; in other instances, however, this property cannot be detected. Bordet showed that if one animal were treated with repeated injections of the corpuscles of another of different species, the serum of the former acquired a marked hæmolytic property towards the corpuscles of the latter, the property being demonstrated when the serum is added to the corpuscles. He also found that the hæmolytic property disappeared when the hæmolytic serum was heated at  $55^{\circ}$  C., but, as in the case of a bacteriolytic serum, was regained on the subsequent addition of some serum from a fresh (*i.e.* non-treated) animal. These observations have been fully confirmed and greatly extended. Ehrlich and Morgenroth analysed the phenomena in question, and showed that the specially developed and heat-resisting substance, "immune-body," entered into combination with the red corpuscles at a comparatively low temperature, namely, at  $0^{\circ}$  C.; whereas complement does not combine at this temperature. In this way a method is supplied by which the immune-body can be removed from a hæmolytic serum while the complement is left. They came to the conclusion that immune-body combined with the complement though the combination was less firm and only occurred at a higher temperature—best about  $37^{\circ}$  C. They therefore consider that the immune-body acts as a sort of connecting-link between the red corpuscle and the complement, hence the term "amboceptor" which Ehrlich afterwards applied. It may be stated, however, that the direct union of complement and immune-body has not been conclusively demonstrated. Muir and Browning,



for example, found that when a fresh serum is passed through a Berkefeld filter, complement is largely retained in the pores of the filter, whereas immune-body passes through practically unchanged; and that if a mixture of complement and immune-body be made and filtered at a temperature of 37° C., the amount of immune-body which passes through is not diminished, whereas it would be if it had united with the retained complement. Accordingly by this method there was obtained no evidence of the direct union of immune-body and complement. Bordet holds that the immune-body acts merely as a sensitising agent—hence the term *substance sensibilisatrice*—and allows the ferment-like complement to act. It is quite evident from his writings, however, that he does not mean, as is often assumed, that the immune-body causes some lesion in the corpuscle which allows the complement to act, but simply that it produces in the molecules (receptors) of the red corpuscles an avidity for complement. All that we can say definitely at present is that the combination of receptor + immune-body takes up complement in firm union while neither does so alone; whether the immune-body acts as a link between the two or not must be left an open question. Even after the corpuscles are laked with water the receptors are not destroyed. Muir and Ferguson have shown that they can still take up immune-body and, through its medium, complement, just as the intact corpuscles do. Ehrlich and Morgenroth showed that in some cases the red corpuscles can take up much more immune-body than is necessary for their lysis, and Muir found in one case studied, that each further dose of immune-body led to the fixation of more complement, so that as many as ten times the hæmolytic dose of complement might thus be used up. It is a matter of considerable importance that the union of immune-body and red corpuscles can be shown to be a reversible action. If, as was found by Morgenroth and Muir independently, corpuscles treated with several doses of immune-body and then repeatedly washed in salt solution be mixed with untreated corpuscles and allowed to remain for an hour, then sufficient immune-body will pass from the former to the latter, so that all become lysed on the addition of sufficient complement. The combination of complement, on the other hand, is usually of very firm nature. It has been a disputed point whether there are several distinct complements in a normal serum with different relations to different immune-bodies, for which Ehrlich and his co-workers have brought forward a certain amount of evidence, or whether, as Bordet holds, there is a single comple-



ment which may, however, show slight variations in behaviour towards different immune-bodies. There is at least no doubt that all the complement molecules in a serum are not the same. For example, Muir and Browning have shown that the treatment of a normal serum with a small amount of emulsion of a bacterium will remove the bactericidal action for another bacterium, whereas the amount of complement as tested by hæmolysis is practically unchanged. They accordingly consider that there is a moiety of complement, "bacteriophilic complement," which is specially concerned in bactericidal action. On the other hand, many of the arguments adduced by Ehrlich and his co-workers in favour of a multiplicity of complements are open to another interpretation; the truth probably lies between Ehrlich's and Bordet's views. Workers of the French school also hold that complement does not exist in the free condition in the blood, but is liberated from the leucocytes when the blood is shed. This cannot be held as proved. On the contrary, there are facts which are strongly in support of the view that complement exists in the free condition in the circulating blood. There is, however, evidence that the amount of free complement increases after the blood is shed and some time later gradually diminishes.

The hæmolytic action of a *normal* serum can be shown in many cases to be of the same nature as that of an immune-serum, that is, complement and the homologue of an immune-body can be distinguished. For example, guinea-pig's serum is hæmolytic to ox's corpuscles; if a portion of serum be heated at  $55^{\circ}$  C. the complement will be destroyed; if another portion be treated with ox's corpuscles at  $0^{\circ}$  C., the natural immune-body will be removed and only complement will be left. Neither portion is in itself hæmolytic, but this property becomes manifest again when the two portions are mixed. Hæmolytic sera are of great service in the study of the question of specificity. Each is specific in the sense already explained (p. 521), but the serum developed against the corpuscles of an animal may have some action on those of an allied species, that is, some receptors are common to the two species. This fact can be readily shown by the usual absorption tests, for example, in the case of an anti-ox serum tested on sheep's corpuscles. A close analogy holds to what has been established in the case of agglutinins. It is further of great interest to note that by the injection of red corpuscles into an animal its serum not only becomes hæmolytic, but in many cases when heated at  $55^{\circ}$  C. possesses also agglutinating and opsonic properties towards the red corpuscles used. And further, it would appear that in some cases at least the immune-body, hæmagglutinin, and hæmopsonin are distinct substances. These facts abundantly show how close an analogy obtains between anti-bacterial and hæmolytic sera, and how important a bearing hæmolytic studies have on the questions of immunity in general.

In addition to hæmolytic sera, anti-sera have been obtained by the



injection of leucocytes, spermatozoa, ciliated epithelium, liver cells, nervous tissue, etc. The laws governing the production and properties of these are identical, that is, each serum exhibits a specific property towards the body used in its production—*i.e.* dissolves leucocytes, immobilises spermatozoa, etc. The specificity is, however, not so marked as in the case of sera produced against red blood corpuscles; thus a serum produced against tissue cells is often hæmolytic; this is probably due to various cells of the body having the same receptors. Here again, when the anti-serum produces no destructive effect on the corresponding cells, the presence of an immune-body may be demonstrated by the increased amount of complement which is taken up through its medium. It may also be mentioned that each anti-serum usually exhibits toxic properties towards the animal whose cells have been used in the injections, *e.g.* a hæmolytic serum may produce a fatal result, with signs of extensive blood destruction, hæmoglobinuria, etc., *i.e.* it is hæmotoxic for the particular animal; a serum prepared by injection of liver cells has been found to produce on injection necrotic changes in the liver in the species of animal whose liver cells were used. These are mentioned as examples of a very large group of specific activities.

With regard to the sites of origin of immune-bodies our information is still very deficient. Pfeiffer and Marx brought forward evidence in the case of typhoid, and Wassermann in the case of cholera, that the immune-bodies are chiefly formed in the spleen, lymphatic glands, and bone-marrow. According to certain workers of the French school, the chief source of anti-substances acting on cells such as red blood corpuscles is the large mononucleated leucocytes, whilst those acting on bacteria are chiefly derived from the polymorpho-nuclear leucocytes (*vide* p. 182). Another view is that immune-bodies are chiefly formed by the large mononucleated leucocytes, whilst complements are products of the polymorphs. That these cells are concerned in the production of antagonistic and protective substances is almost certain, though another possible source of wide extent, namely, the endothelium of the vascular system, has been largely overlooked. 'As yet, definite statements cannot be made on this point.

(b) *Opsonic Action*.—The presence of a substance in an immune-serum which makes the corresponding organism sensitive to phagocytosis was first demonstrated by Denys and Leclef in 1895, in the case of an anti-streptococcal serum. They also showed that the serum produced this effect by acting on the organism, not on the leucocytes. It is, however, chiefly to the researches of Wright and his co-workers that this subject has come into special prominence. Wright and Douglas in their first paper showed that the phagocytosis of staphylococci by leucocytes depended on a body in the normal serum which became fixed to the cocci and made them a prey to the



phagocytes. To this they gave the name of "opsonin" (*vide* pp. 122, 519). There is no phagocytosis of cocci by leucocytes washed in salt solution; normal serum heated to 55° C. is also without effect in inducing this phenomenon. They could not demonstrate any effect of the opsonin on the leucocytes. On the other hand, if bacteria be exposed to the fresh serum, and they be freed from the excess of serum and then exposed to phagocytes also washed free from serum, they will be readily taken up by the cells. It has been abundantly shown that the opsonic action of the serum is increased by the process of immunisation against an organism, and the opsonic index represents the degree of immunity in one of its aspects as already explained (p. 122). The matter has, however, become complicated by the circumstance that in an immune-serum an opsonin may still be present after the serum is heated at 55° C., as has been shown by Dean and others; and Muir and Martin have shown that this thermostable immune-opsonin (bacteriotropin of Neufeld) has all the specific characters of anti-substances in general. On the other hand, they have found that the thermolabile opsonin of a normal serum has quite different properties. For example, when a normal serum is tested on a particular bacterium, the opsonic effect on that bacterium may be removed by treating the serum with other bacteria; in other words, the thermolabile opsonin of normal serum does not possess the specific character of the opsonin developed in the process of immunisation. They have also found that various substances or combinations of substances which act as "complement absorbers" also remove the opsonic property from a normal serum, while they have no effect on an immune-opsonin.

That this thermolabile normal opsonin can act in a non-specific way is shown by the fact that particles of carmine and other substances become opsonised by the action of normal serum. It is, however, to be noted that in certain cases there have been found in a normal serum traces of substances which can be activated by thermolabile opsonin after the manner of immune-body and complement (as seen in the hæmolytic action of a normal serum (p. 538); to this extent the opsonic effect of a normal serum may have some degree of specificity. From this and other facts some observers have attempted to explain the whole of opsonic action according to the scheme of immune-body and complement as seen in hæmolysis. This, however, is not justifiable, since normal thermolabile opsonin can, as we have seen, act by itself, as can also the specific immune-opsonin after normal opsonin has been



destroyed by heating, and we know of no corresponding action in the case of an immune-body. The subject is one of considerable complexity, but it may be said that the most important variations in the opsonic content observed in infections depend on the specific immune-opsonins, though the presence of immune-body may play a part in raising the index by leading to the union of more normal-complement-opsonin.

Further study will be necessary before the exact relationships of these substances are fully understood, and other questions with regard to them have as yet scarcely been touched upon. Increased phagocytic action had long been known by the work of Metchnikoff to be associated with the development of active immunity, and the theory of stimulation of leucocytes was supported by many. The work on opsonins has caused a swing of the pendulum in the other direction, and points to the development of anti-substances in the serum as the all-important factor. It remains to be determined to what extent the opsonic and directly bactericidal properties taken together will explain the phenomena of natural and acquired immunity.

(c) *Agglutination*.—Charrin and Roger in 1889 observed that when the bacillus pyocyaneus was grown in the serum of an animal immunised against this organism, the growth formed a deposit at the foot of the vessel; whereas a growth in normal serum produced a uniform turbidity. Grüber and Durham, in investigating Pfeiffer's reaction, found that when a small quantity of an anti-serum is added to an emulsion of the corresponding bacterium, the organisms become agglutinated into clumps, this phenomenon depending upon the presence of definite bodies in the serum called *agglutinins*.

It had been already found that the serum of convalescents from typhoid fever could protect animals to a certain extent against typhoid fever, and, in view of the facts experimentally established, it appeared a natural proceeding to inquire whether such serum possessed an agglutinative action and at what stage of the disease it appeared. The result, obtained independently by Grünbaum and Widal, but first published by the latter, was to show that the serum possessed this specific action shortly after infection had taken place; in other words, the development of this variety of anti-substance can be demonstrated at an early stage of the disease. Agglutination may be said to be observed generally in bacterial infections, though the degree of the phenomenon and the facility with which it can be noted vary greatly in different cases. Details will be found in the chapters dealing with the individual diseases, etc. Furthermore, the



phenomenon is not peculiar to bacteria; it is seen, for example, when an animal is injected with the red corpuscles of another species, *hæmagglutinins* appearing in the serum, which have a corresponding specificity.

The physical changes on which agglutination depends cannot as yet be said to be fully understood. Grüber and Durham considered that the agglutinin produced a change in the envelope of the bacterium, causing it to swell up and become viscous, but the facts since established show that this is not the true explanation. For example, it has been shown by Nicolle and by Kruse that if an old bacterial culture be filtered through porcelain, the addition of some of the corresponding anti-serum produces a sort of granular precipitate in it; and that when, as in the agglutination of bacteria, minute inorganic particles are added to the mixture, they become aggregated into clumps. The phenomenon would thus appear to be the result of the interaction of the agglutinin and some substance in the bacterial cell which is known as the agglutinable substance or as the agglutininogen. Joos has found in the case of the typhoid bacillus that there are two agglutinable substances, which differ in their resistance to heat— $\alpha$  and  $\beta$  agglutininogen, and that they give rise to corresponding agglutinins. Further, as the result of a comparative study of the agglutinins of a motile and a non-motile variety of the hog cholera bacillus, Theobald Smith has come to the conclusion that there is an agglutinin which is produced by and acts on the flagella and another which is similarly related to the bacterial bodies. The former acts in very much higher dilutions than the latter, and this is regarded as an explanation of the fact that in the case of non-motile organisms the agglutinating serum acts only in proportionately high concentration as compared with the case of most motile forms. Another factor necessary for the phenomenon of agglutination is a proper salt content. Bordet showed that if the clumps of agglutinated bacteria are freed from salt by washing in distilled water they become resolved, and that on the addition of some sodium chloride they are formed again, and Joos has also brought forward striking confirmatory evidence as to the necessity for the presence of salts. It is thus probable that in the phenomenon of agglutination as ordinarily understood more than one factor is concerned, and it is possible that in part it may depend on some altered molecular relationship of the bacteria to the surrounding fluid analogous to altered surface tension.

In the phenomenon of agglutination we have to distinguish two factors, namely, the combination of agglutinin and agglu-



tinable substance (agglutininogen) and the actual clumping of the bacteria, and it is to be noted that whether or not the latter event follows depends on the physical condition of each of the two substances concerned. For example, in some cases when the bacteria are heated at a temperature of 65° C., for some time, they may lose the faculty of being agglutinated while they may still retain the property of combining with or binding agglutinin. Dreyer and Jex Blake have observed the remarkable fact that in certain instances on being heated to a still higher temperature they may once more become agglutinable. Another point of practical importance is that bacteria when freshly grown from the tissues are very often less agglutinable than they afterwards become when sub-cultured for some time. As stated above, the agglutinins are usually placed in the second order of anti-substances, and are regarded as possessing a combining group and an active or agglutinating group. The constitution would thus be analogous to that of a toxin, and in conformity with this view Eisenberg and Volk consider that the agglutinating group may be destroyed while the combining group remains, the result being an *agglutinoid*. The evidence for this lies in the fact that when an agglutinating serum is heated to a certain temperature, not only does it lose its agglutinating action, but when the bacteria are treated with such a serum their agglutination by active serum is interfered with, a sort of plugging up of the combining molecules having apparently taken place. Again, with agglutinating sera partially inactivated by heat or other means, what are known as "zone phenomena" occur; that is, when agglutination occurs with a given dilution of such a serum a lower dilution may fail to agglutinate, and this they suppose to be due to the interference of the union of agglutinin by agglutinoid in the greater concentration of serum. On the other hand, there are facts which cannot be brought into harmony with this view. For example, Dreyer and Jex Blake have shown that the inhibition zone may be slight when there has been much destruction of agglutinin, and on the other hand may be well marked when no weakening of the agglutinating power has resulted from the heating. The physical changes underlying such phenomena are still very obscure, but we may say at present that the existence of agglutinoids has not yet been proved.

Like immune-bodies, agglutinins are not destroyed at 55° C. (a temperature sufficient to annul bactericidal action), but different agglutinins show variations in this respect, some being affected by a temperature little above that named. The resist-



ance to heat also varies when the serum is diluted with salt solution, and it has been shown that conditions which interfere with the coagulation of the proteins increase their resistance. Like antitoxins, agglutinins seem to be chiefly contained in the globulin fraction. Discussion has taken place as to the relation of agglutinins to immune-bodies and as to how far agglutination is an indication of immunity. It may be said that in the case of certain sera investigated it has been shown that the immune-body and the agglutinin are separate substances, but it would not be justifiable to say this is always the case. And while the agglutinative power cannot in itself be taken as the measure of the degree of immunity, agglutinins and immune-bodies are the products of corresponding reactive processes, and their formation is governed by corresponding laws. Agglutinins become fixed in definite proportion by the receptors of the bacteria; that is, the agglutinin becomes used up in the process of agglutination, and it has been shown that bacteria may take up many times the amount necessary to their agglutination—a corresponding fact to what has been established with regard to immune-bodies of hæmolytic sera. The agglutinins are specific in the sense which has been explained above (p. 521). It can be shown by the method of absorption that in an agglutinating serum there may be several agglutinins with different combining groups, some of which may be taken up by organisms allied to that which has given rise to the anti-serum.

Besides those stated above, other phenomena have been observed in the interaction of anti-sera and the corresponding bacteria. For example, it has been shown that when certain bacteria—*e.g.* the typhoid bacillus, *b. coli*, and *b. proteus*—are grown in bouillon containing a small proportion of the homologous serum, their morphological characters may be altered, growth taking place in the form of threads or chains which are not observed in ordinary conditions. In other instances a serum may inhibit some of the vital functions of the corresponding bacterium.

**Precipitins.**—Shortly after the discovery of agglutinins, Kraus showed in the case of the organisms of typhoid, cholera and plague, that the anti-serum not only caused agglutination, but when added to a filtrate of a culture of the corresponding bacterium produced a cloudiness and afterwards a precipitate. To the substance in the immune-serum which brought about this effect he gave the name of *precipitin*. Subsequent study has shown that this phenomenon is closely related to agglutination; in fact several authorities consider that they represent the



same reaction under different conditions, that is, that the substances which when present in the bacterial bodies give rise to agglutination, on the addition of the anti-serum, produce a precipitate when free in a fluid. To test the reaction it is accordingly necessary to have as far as possible the substance of the bacteria in solution, and for this purpose there have been introduced various methods, of which the two following may be given :—

(a) It is well known that in an old bouillon culture the bacteria undergo disintegration and their constituents go into solution. Accordingly, if such a culture which has been kept in the incubator for several weeks be filtered through a porcelain filter, the filtrate will contain the interacting substance or precipitinogen.

(b) The growth from a recent agar culture is scraped off and suspended in normal salt solution, the mixture is made feebly alkaline with soda solution and boiled for a few minutes. The mixture is then neutralised, when a precipitate forms, and is filtered through filter-paper; the filtrate contains the precipitinogen.

The test is carried out by placing in a number of small test-tubes a given amount of the bacterial filtrate along with varying quantities of the homologous anti-serum. (The latter may be obtained in the usual way by the repeated injection of dead cultures or of bacterial filtrate.) As the precipitate forms slowly the tubes should be placed in the incubator for twenty-four hours, .5 per cent. carbolic acid being added to prevent the growth of bacteria. This precipitin reaction has now been observed in a great many bacterial diseases when the patient's serum is added to the corresponding bacterial filtrate, and has even been applied by some observers as a means of diagnosis. It is, however, less delicate and more restricted in its application than the agglutination methods.

**Serum Precipitins.**—This subject does not strictly belong to bacteriology, but the general phenomena are so closely allied to those just described, that some reference may be made to it. When the serum of an animal is injected in repeated doses into another animal of different species, after the type of an immunisation, there appears in the serum of the animal treated a substance called precipitin, which causes a cloudiness or precipitate when added to the serum (precipitinogen) used. (In the case of rabbits doses of 3 to 4 c.c. of the serum may be injected intraperitoneally at intervals of four to five days, a precipitin usually appearing at the end of about three weeks.) The reaction, which is a very delicate one, is conveniently observed by adding a given amount of the anti-serum, say .05 c.c., to varying amounts of the homologous serum .1, .01, etc., c.c., in a series of small test tubes, the volume being then made up with salt solution to 1 c.c. In this way a definite reaction may be observed with .001 c.c. of the serum or even less. Here again zone



phenomena, as in the case of agglutination, are met with. If the anti-serum be heated to a temperature of  $75^{\circ}$  C. for some time it acquires inhibitory properties, so that when added to a mixture of serum and anti-serum which would otherwise give a precipitate, this no longer occurs. Some observers consider that this is due to the presence of "precipitoid" in the heated anti-serum; but the observations of Welsh and Chapman show that this view is not in accordance with the facts, and indicate that the inhibition is related to a specific solvent action which the heated anti-serum has on the precipitate. They have also shown that the main mass of the precipitate is furnished by the anti-serum (precipitin), and not as is usually supposed by the precipitin throwing down the protein of the homologous serum; this result is of high importance in connection with the action of anti-substances in general. The precipitin reaction is specific in the sense explained above. It is always most marked towards the serum of the species used in the immunisation; but while this is so, there may also be a slight reaction towards animals of allied species. An anti-human serum, for example, gives the maximum reaction with human serum, but also a slight reaction with the serum of monkeys, especially of anthropoid apes; it, however, gives no reaction with the serum of other animals. The precipitin test has thus come to be employed as a means of differentiating human from other bloods. Another interesting phenomenon is what is known as the "deviation of complement," which is produced by the combination of the two substances in the serum and anti-serum respectively. If mixtures be made according to the above method, and then a small quantity of complement, say fresh guinea-pig serum, be added, it will be found that the complement becomes absorbed, as may be shown by subsequently adding a test amount of sensitised red blood corpuscles. This deviation phenomenon is even a more delicate reaction than the precipitin test, it being often possible to demonstrate by its use from a tenth to a hundredth of the smallest amount of serum which will give a perceptible precipitate; it also is specific within the same limits.<sup>1</sup>

**Therapeutic Effects of Anti-Sera.**—As will have been gathered, the chief human diseases treated by anti-sera are diphtheria, tetanus, streptococcus infection, pneumonia, dysentery, plague, and snake bite. Of the results of such treatment most is known in the case of diphtheria. Here a very great diminution in the mortality has resulted. The diphtheria antitoxin came into general use about October 1894, and the statistics published by Behring towards the end of 1895 indicated results which have since been confirmed. In the Berlin Hospitals the average mortality for the years 1891–93 was 36.1 per cent., in 1894 it was 21.1 per cent., and in January–July 1895, 14.9 per cent. The objection that in some epidemics a very mild type of disease prevails is met by the fact that similar diminutions of mortality have occurred all over the world. Loddo collected the results of 7000 cases in Europe, America, Australia, and Japan, in

<sup>1</sup> For an account of precipitins, *vide* Nuttall, "Blood Immunity and Relationships," Cambridge, 1904; and of complement deviation, Muir and Martin, *Journ. of Hyg.* (1906), vi. p. 265.



which the mortality was 20 per cent. as compared with a former mortality in the same hospitals of 44 per cent. It has also been observed that if during an epidemic the supply of serum fails, the mortality at once rises; and in two instances recorded it was doubled. It must here be remembered that from the spread of bacteriological knowledge the diagnosis of diphtheria is now much more accurate than formerly. Another effect of the antitoxic treatment has been that when tracheotomy is necessary the percentage of recoveries is now much higher, being 73 per cent. instead of 27 per cent. in a group of cases collected by the American Pediatric Society. In the London fever hospitals, since 1894 the recoveries after tracheotomy have been 56·4 as compared with 32·1 per cent. previous to the introduction of antitoxin. One of the most striking results obtained in the same hospitals is a reduction of the death-rate in post-scarlatinal diphtheria from 50 per cent. to between 4 per cent. and 5 per cent. As the disease here occurs while the patient is under observation, the treatment is nearly always begun on the first day. It is a matter of prime importance that the treatment should be commenced whenever the disease is recognised. Behring showed that in cases treated on the first and second days of the disease the mortality was only 7·3 per cent., and this has been generally confirmed, whilst after the fifth day it was of little service to apply the treatment. In order to obtain such results, it cannot be too strongly insisted on that attention should be given to the dosage. When bad results are obtained, it may be strongly suspected that this precaution has not been observed. In the treatment of acute tetanus by the antitoxin the improvement in results has not been marked, but some chronic cases have been benefited, and, as already stated (p. 431) better results are obtained in acute cases if intravenous injection be practised. In the case of Yersin's anti-plague serum, though some benefit has appeared to follow its use, this has been of quite a limited nature. The same may be said to be true of the anti-streptococcic and anti-pneumonic sera, though in the case of the first mentioned numerous cases of apparently successful result have been recorded. With regard to anti-venin, Lamb has shown that, if a cobra with full glands bites a man, many times the minimal lethal dose are probably injected. In cases of slight bite, however, benefit may accrue from the use of the anti-serum.

As has been shown above, antibacterial sera require for their bactericidal action a sufficiency of complement, and as this diminishes in amount when a serum is kept, the unsatisfactory



results with this class of sera may be due to a deficiency of complement. Or it may be as Ehrlich has suggested, that the complement naturally existing in human serum does not suit the immune-body in the anti-serum—that is, is not taken up through the medium of the latter and brought into combination with the bacterium. And there is still the further possibility that even though the complement should be taken up, the zymotoxic group of the latter is not sufficiently active towards the bacterium to effect its death. In both cases it will appear that an extracellular bactericidal action cannot be produced by the particular immune-body in association with the complement of the animal in question. There is no doubt that this question of complements is one of high importance, and that both combining affinity and toxic action of complements must be considered in each case.

#### *Theories as to Acquired Immunity.*

The advances made within recent years in our knowledge regarding artificial immunity, and the methods by which it may be produced, have demonstrated the insufficiency of various theories which had been propounded. Only a short reference need be made to these. The *theory of exhaustion*, with which Pasteur's name is associated, supposed that in the body of the living animal there are substances necessary for the existence of a particular organism, which become used up during the sojourn of that organism in the tissues; this pabulum being exhausted, the organisms die out. Such a supposition is, of course, quite disproved by the facts of passive immunity. According to the *theory of retention*, the bacteria within the body were considered to produce substances which are inimical to their growth, so that they die out, just as they do in a test-tube culture before the medium is really exhausted. Such a theory only survives now in the view that antitoxins are modified toxins, the evidence against which has already been discussed (p. 530). There then came the *humoral theory* and the *theory of phagocytosis*, but neither of these is tenable in its pure form, and the distinction between them need not be maintained. For, on the one hand, any substance with specific property in the serum must be the product of cellular activity, and, on the other hand, the facts with regard to passive immunity go far beyond the ingestive and digestive properties of phagocytes, though these cells may be in part the source of important bodies in the serum. At the present time interest centres around two theories, namely, Ehrlich's



side-chain theory and Metchnikoff's phagocytic theory as further developed. These will now be discussed, and it may be noted that the ground covered by each is not coextensive. For the former deals chiefly with the production of anti-substances and its biological significance, the latter deals with the defensive properties of cells, either directly by their phagocytic activity or indirectly by substances produced by them after the manner of digestive ferments. It will be seen, however, that each has a normal process as its basis, namely, that of nutrition.

1. **Ehrlich's Side-Chain Theory.**—This may be said to be an application of his views regarding the nourishment of protoplasm. A molecule of protoplasm (in the general sense) may be regarded as composed of a central atom group or executive centre (*Leistungskern*) with a large number of side-chains (*Seitenketten*), *i.e.* atom groups with combining affinity for food-stuffs. It is by means of these latter that the living molecule is increased in the process of nutrition, and hence the name *receptors* given by Ehrlich is on the whole preferable. These receptors are of three chief kinds corresponding to the classes of anti-substances described (p. 521); the first has a single unsatisfied combining group, and merely fixes molecules of relatively simple constitution—receptor of the first order; the second has a combining group for the food molecule, and another active or zymotoxic group, which leads to some physical change in it—receptor of the second order; the third has two combining groups, one for the food molecule and another which fixes a ferment (or complement) in the fluid medium around—receptor of the third order or *amboceptor*. These latter receptors come into action in the case of larger food molecules which require to be broken up by ferment-action for the purposes of the cell economy. In considering the application of this idea to the facts of acquired immunity, it must be kept in view that all the substances to which anti-substances have been obtained are, like proteids, of unknown but undoubtedly of very complex chemical constitution, and that in apparently every case the anti-substance enters into combination with its corresponding substance antigen. The dual constitution of toxins and kindred substances, as already described (p. 198), is also of importance in this connection. Now, to take the case of toxins, when these are introduced into the system they are fixed, like food-stuffs, by their haptophorous groups to the receptors of the cell protoplasm, but are unsuitable for assimilation. If they are in sufficiently large amount, the toxophorous part of the toxin molecule produces that disturbance of the protoplasm which



is shown by symptoms of poisoning. If, however, they are in smaller dose, as in the early stages of immunisation, fixation to the protoplasm occurs in the same way; and as the combination of receptors with toxin is supposed to be of firm nature, the receptors are lost for the purposes of the cell, and the combination R.-T. (receptor + toxin) is shed off into the blood. The receptors thus lost become replaced by new ones, and when additional toxin molecules are introduced, these new receptors are used up in the same manner as before. As a result of this repeated loss the regeneration of the receptors becomes an over-regeneration, and the receptors formed in excess appear in the free condition in the blood stream and then constitute antitoxin molecules. There are thus three factors in the process, namely, (1) fixation of toxin, (2) over-production of receptors, (3) setting free of receptors produced in excess. Accordingly these receptors which, when forming part of the cell protoplasm, anchor the toxin to the cell, and thus are essential to the occurrence of toxic phenomena, in the free condition unite with the toxin, and thus prevent the toxin from combining with the cells and exerting a pathogenic action. The three orders of receptors, when separated from the cells, thus give the three kinds of anti-substances. Ehrlich does not state what cells are specially concerned in the production of anti-substances, but from what has been stated it is manifest that any cell which fixes a toxin molecule, for example, is potentially a source of antitoxin. Cells, to whose disturbance, resulting from the fixation of toxin, characteristic symptoms of poisoning are due, will thus be sources of antitoxin, *e.g.* cells of the nervous system in the case of tetanus, though the cells not so seriously affected by toxin fixation may act in the same way. The experimental investigation of the source of antitoxins has, however, yielded little result, and no definite statement can be made on the subject.

When we come to consider how far Ehrlich's theory is in harmony with known facts, we find that there is much in its favour. In the first place, it explains the difference between active and passive immunity, *e.g.* difference in duration, etc.; in the former the cells have acquired the habit of discharging anti-substances, in the latter the anti-substances are simply present as the result of direct transference. It is also in harmony with the action of antitoxins, etc., as detailed above, and especially it affords an explanation of the multiplicity of anti-substances. For, if we take the case of antitoxins, we see that this depends upon the combining affinity of the toxin for certain of the cells of the body, and this again is referred back to the complicated



constitution of living protoplasm. Furthermore, the biological principle involved is no new one, being simply that of over-regeneration after loss. It would appear likely that the integrity of the executive centres of the protoplasm molecules would be essential to the satisfactory production of side-chains, and this would appear in accordance with the fact that antitoxin formation occurs most satisfactorily when there is no marked disturbance of the health of the animal.

It is to be noted, however, that it does not explain active immunity apart from the presence of anti-substances in the serum. For example, an animal may be able to withstand a much larger amount of toxin than could be neutralised by the total amount of antitoxin in its serum. This might theoretically be explained by supposing a special looseness of the cell receptors so that the toxin-receptor combination became readily cast off. The question, however, arises whether there may not be really an increased resistance of the cells to the toxophorous affinities. An observation made by Meyer and Ransom (*vide* p. 427) is also difficult of explanation, according to the view that antitoxin is formed by the cells with which the toxin combines and on which it acts. They found that in an animal actively immunised against tetanus and with antitoxin beginning to appear in its blood, the injection of a single M.L.D. of tetanus toxin into a peripheral nerve brought about tetanus with a fatal result. On the other hand, the injection of antitoxin into the sciatic nerve above the point of injection of toxin prevented the latter from reaching the cells of the cord. One can scarcely imagine an explanation of these facts if antitoxin molecules were in process of being shed off by the cells of the nervous system. Further, when the serum of an animal contains a large amount of antitoxin, how does the additional toxin injected reach the cells in order to influence them as we know it does? This also is difficult to understand, unless the toxin has a greater affinity for the receptors in the cells than for the free receptors (antitoxin) in the serum. A supersensitiveness of the nerve-cells of an animal to tetanus toxin, sometimes observed even when there is a large amount of antitoxin in the serum, has been often brought forward as an objection. But this also may perhaps be explained by there having occurred a partial damage of the cell protoplasm by the toxic action in the process of immunisation — an explanation which, of course, demands that in some way the freshly introduced toxin may reach the cells in spite of the antitoxin in the blood, or it may belong to the group of anaphylactic



phenomena described below (p. 558). Further investigation alone will settle these and various other disputed points, and may remove many of the apparent objections. At present we may say, however, that Ehrlich's theory is the only one which even attempts to explain the cardinal facts of this aspect of immunity.

**2. The Theory of Phagocytosis.**—This theory, brought forward by Metchnikoff to explain the facts of natural and acquired immunity, has been of enormous influence in stimulating research on the subject. Looking at the subject from the standpoint of the comparative anatomist, he saw that it was a very general property possessed by certain cells throughout the animal kingdom, that they should take up foreign bodies into their interior and in many cases digest and destroy them. On extending his observations to what occurred in disease, he came to the conclusion that the successful resistance of an animal against bacteria depended on the activity of certain cells called phagocytes. In the human subject he distinguished two chief varieties, namely—(a) the microphages, which are the "polymorpho-nuclear" finely granular leucocytes of the blood; and (b) the macrophages, which include the larger hyaline leucocytes, endothelial cells, connective tissue corpuscles, and, in short, any of the larger cells which have the power of ingesting bacteria. Insusceptibility to a given disease is indicated by a rapid activity on the part of the phagocytes, different varieties being concerned in different cases,—an activity which may rapidly destroy the bacteria and prevent even local damage. If the organisms are introduced into the tissues of a moderately susceptible animal, there occurs an inflammatory reaction with local leucocytosis, which results in the intracellular destruction of the invading organisms. Phagocytosis is regarded by Metchnikoff as the essence of inflammation. He also showed that the bacteria may be in a living and active state when they are ingested by leucocytes. On the other hand, he found that in a susceptible animal phagocytosis did not occur or was only imperfect. He also showed that when a naturally susceptible animal was immunised, the process was accompanied by the appearance of an active phagocytosis. The ingestion of bacteria by phagocytes is undoubtedly a phenomenon of the greatest importance in the defence of the organism. It is known that amœbæ and allied organisms have digestive properties which are specially active towards bacteria, and from what can be directly observed, as well as indirectly inferred, there can be no doubt that such a faculty is also possessed by the phagocytes of the body. Thus bacteria within these cells are in a position



favourable to their destruction, and do in many instances become destroyed. In fact, observations on phagocytosis *in vitro* show that such destruction may in the case of some organisms occur so rapidly that the actual number observable in the leucocytes is no indication of the activity of the process. In other instances, *e.g.* in gonorrhœa, the ingested organisms would appear to survive a considerable time without undergoing change. Undoubtedly phagocytosis is of the highest importance in active immunity, as by its means organisms which would not undergo an extra-cellular death may be killed off. In the process of immunisation of a susceptible animal we see a negative or neutral chemiotaxis becoming replaced by positive chemiotaxis. This has been explained by Metchnikoff as due to an education or stimulation of the phagocytes. The recent work on opsonins shows, however, that this is not the case, as leucocytes from an immunised animal are not more active in this direction than those of a normal animal, the all-important factor being the development of an opsonin in the immune animal. Thus this phase of immunity comes to be merely a part of the subject of anti-substances in general.

The digestive ferments of phagocytes or *cytases* are, according to Metchnikoff, retained within the cells under normal conditions, but are set free when these cells are injured, for example, when the blood is shed. They then become free in the serum by the breaking up of the cells—the process known as phagolysis—and they then constitute the alexines, or complements of Ehrlich. Of these, as has already been said, Metchnikoff thinks there are probably two kinds—one called *macrocytase*, contained in the macrophages; which is specially active toward the formed elements of the animal body, protozoa, etc.; and the other, *microcytase*, contained within the polymorpho-nuclear leucocytes, which has a special digestive action on bacteria. It is the microcytase which gives blood serum its bactericidal properties. It appears to us, however, that Metchnikoff has gone too far in distinguishing the activities of the two classes of cells so much as he has done.

When the properties of antibacterial sera, as above described, are considered in relation to phagocytosis, Metchnikoff gives the following explanation. He admits that the immune-body is fixed by the bacteria (or red corpuscles, as the case may be), though he does not state that a chemical combination takes place; hence he calls it a fixative (*fixateur*). The immune-bodies are to be regarded as auxiliary ferments (*ferments adjuvants*) which aid the action of the alexine. Unlike the latter, however,



they are formed in excess during immunisation and set free in the serum. He compares their action to that of enterokinase, a ferment which is produced in the intestine and which aids the action of trypsin. Thus, when the bacteria have fixed the immune-body, their digestion is facilitated either within the phagocytes, or outside of them when the alexine has been set free by phagolysis. He, however, maintains that extracellular digestion or lysogenesis does not take place without the occurrence of phagolysis. The source of immune-bodies is, in all probability, also the leucocytes, as these substances are specially abundant in organs rich in such cells—spleen, lymphatic glands, etc.; here again the mono-nuclear leucocytes are probably the source of the immune-bodies concerned in hæmolysis, the polymorpho-nuclear leucocytes the source of those concerned in bacteriolysis. Although the immune-bodies are usually set free in the serum, this is not always the case; sometimes they are contained in the cells, and this probably occurs when there is a high degree of active immunity against bacteria without a serum having an antibacterial action, the powers of intracellular digestion being in such cases increased. In this way the facts of immunity can be explained so far as these concern the destruction of bacteria.

Metchnikoff's work has less direct bearing on the production of antitoxins. He admits the fixation of the toxin by the antitoxin to form a neutral compound, and he apparently considers that leucocytes may also be concerned in the production of antitoxins. Apart, however, from antitoxin formation, he considers the acquired resistance of the cells themselves of high importance in toxin immunity.

When we consider Metchnikoff's theory as thus extended to cover recently established facts, it must be admitted that it affords a rational explanation of a considerable part of the subject, though the elucidation of the chemiotactic phenomena during immunisation as explained above detracts from the importance which he attached to the leucocyte. It, however, does not afford explanation of the multiplicity and specificity of antitoxins as Ehrlich's does; on the other hand, it is more concerned with the cells of the body as destroyers or digesters of bacteria. As regards the subject of antibacterial sera, the results of these two workers may be said to be in harmony in some of the fundamental conceptions. And it is of interest to note that Metchnikoff, starting with the phenomena of intracellular digestion, has arrived at the giving off of specific ferments by phagocytes; whilst Ehrlich, from his first investigations on the



constitution of toxins, has arrived at an explanation of antitoxins and immune-bodies also with a theory of cell-nutrition as its basis. Within the last few years marked progress has thus been made towards the establishment of the fundamental laws of immunity.

#### NATURAL IMMUNITY.

We have placed the consideration of this subject after that of acquired immunity, as the latter supplies facts which indicate in what direction an explanation of the former may be looked for. There may be said to be two main facts with regard to natural immunity. The first is, that there is a large number of bacteria—the so-called non-pathogenic organisms—which are practically incapable, unless perhaps in very large doses, of producing pathogenic effects in any animal; when these are introduced into the body they rapidly die out. This fact, accordingly, shows that the animal tissues generally have a remarkable power of destroying living bacteria. The second fact is, that there are other bacteria which are very virulent to some species of animals, whilst they are almost harmless to other species; the anthrax bacillus may be taken as an example. Now it is manifest that natural immunity against such an organism might be due to a special power possessed by an animal of destroying the organisms when introduced into its tissues. It might also possibly be due to an insusceptibility to, or power of neutralising, the toxins of the organism. For the study of the various diseases shows that the toxins (in the widest sense) are the weapons by which morbid changes are produced, and that toxin-formation is a property common to all pathogenic bacteria. There is, moreover, no such thing known as a bacterium multiplying in the living tissues without producing local or general changes, though, theoretically, there might be. As a matter of fact, however, natural immunity is in most cases one against *infection*, i.e. consists in a power possessed by the animal body of destroying the living bacteria when introduced into its tissues: such a power may exist though the animal is still susceptible to the separated toxins. We shall now look at these two factors separately.

1. *Variations in Natural Bactericidal Powers.*—The fundamental fact here is that a given bacterium may be rapidly destroyed in one animal, whereas in another it may rapidly multiply and produce morbid effects. The special powers of destroying organisms in natural immunity have been ascribed to (a) phagocytosis, and (b) the action of the serum.

(a) The chief factors with regard to phagocytosis have been



given above. The bacteria in a naturally immune animal, for example, the anthrax bacillus in the tissues of the white rat, are undoubtedly taken up in large numbers and destroyed by the phagocytes, whereas in a susceptible animal this only occurs to a small extent; and Metchnikoff has shown that they are taken up in a living condition, and are still virulent when tested in a susceptible animal. Variations in phagocytic activity are found to correspond more or less closely with the degree of immunity present, but are probably in themselves capable of explanation. The fundamental observations of Wright and Douglas show that in many cases at least, leucocytes do not ingest organisms in normal saline solution, and that this is not due to the medium in which they are, is readily shown by subjecting the organisms to the action of fresh serum and then washing them; thereafter, they are rapidly taken up by the leucocytes in salt solution. In most cases this result is due to the labile opsonin of normal serum which has combining affinities for a great many organisms, as already stated. In other cases more specific substances may be concerned. But the all-important fact is that whether phagocytosis occurs or not, appears to depend upon certain bodies in the serum. As yet we cannot say whether the phagocytosis in a given serum, observed according to the opsonic technique, always runs parallel with phagocytosis in the tissues of the animal from which the serum has been taken. This is a subject on which extended observations are necessary. But whether or not phagocytosis *in vivo* corresponds with that *in vitro* it is probably to be explained in the same way; that is, it probably depends upon the content of the serum. The composition of the latter, no doubt, is the result of cellular activity, and in this the leucocytes themselves are in all probability concerned, but the movements and phagocytic activity of these cells seem to be chiefly if not entirely controlled by their environments. Ingestion is, however, only the first stage in the process; intracellular destruction is the second, and is of equal importance. What may be called intracellular bactericidal action probably varies in the case of leucocytes of different animals, but regarding this our knowledge is deficient, and, further, bacteria may sometimes survive the cells which have ingested them.

(b) When it had been shown that normal serum possessed bactericidal powers against different organisms, the question naturally arose as to whether this bactericidal power varied in different animals in proportion to the natural immunity enjoyed by them. The earlier experiments of Behring appeared to give grounds for the belief that this was the case. He found, for



example, that the serum of the white rat, which has a remarkable immunity to anthrax, had greater bactericidal powers than that of other animals investigated. Further investigation, however, has shown that this is not an example of a general law, and that the bactericidal action of the serum does not vary *pari passu* with the degree of immunity. In many cases, however, non-pathogenic and also attenuated pathogenic bacteria can be seen to undergo rapid solution and disappear when placed in a drop of normal serum. The bactericidal action of the serum was specially studied by Nuttall, and later by Buchner and Hankin, who believe that the serum owes its power to certain substances in it derived from the spleen, lymphatic glands, thymus, and other tissues rich in leucocytes. To these substances Buchner gave the name of *alexines*; as already explained, they correspond with Metchnikoff's *cytases* and Ehrlich's complements described above. They can be precipitated by alcohol and by ammonium sulphate, and in this respect and in their relative lability correspond with enzymes or unorganised ferments. Variations in bactericidal power of the serum as tested *in vitro*, however, do not explain the presence or absence of natural immunity against a living bacterium. In some cases, for example, it has been found to be considerable, while the organisms flourish in the body, and the animal has no immunity. In such a case Metchnikoff says that there occurs in the living body no liberation of alexines by the phagocytes, and hence no bactericidal action such as occurs when the blood is shed. In the case of the hæmolytic action of a normal serum, it has been shown in many instances that in addition to complement a natural immune-body is also concerned (p. 534), and this would appear to be the rule; the process being analogous to what is seen in the case of an artificially developed hæmolytic serum. In certain instances an analogous condition appears to obtain in a normal bactericidal serum. For example the dog's serum heated at 58° C. contains a natural immune-body to anthrax which can be activated by the addition of normal guinea-pig's serum so as to produce a bactericidal action, though the latter is by itself without any such effect. At present, however, the possibility of bactericidal action by complement alone cannot be excluded, as it appears to combine with many bacteria without any intermediary. Further work is necessary to determine whether all the facts regarding natural immunity are explainable by the opsonic and bactericidal properties of the serum.

2. *Variations in Natural Susceptibility to Toxins.*—We must here start with the fundamental fact, incapable of explanation,



that toxicity is a relative thing, or, in other words, that different animals have different degrees of resistance or non-susceptibility to toxic bodies. In every case a certain dose must be reached before effects can be observed, and up to that point the animal has resistance. This natural resistance is found to present very remarkable degrees of variation in different animals. The great resistance of the common fowl to the toxin of the tetanus bacillus may be here mentioned (*vide* p. 425), and large amounts of this poison can be injected into the scorpion without producing any effects whatever; the high resistance of the pigeon to morphia is a striking example in the case of vegetable poisons. This variation in resistance to toxins applies also to those which produce local effects, as well as to those which cause symptoms of general poisoning. Instances of this are furnished, for example, by the vegetable poisons ricin and abrin, by the snake poisons, and by bacterial toxins such as that of diphtheria. We must take this natural resistance for granted, though it is possible that ere long it will be explained.

According to Ehrlich's view of the constitution of toxins, it might be due to the want of combining affinity between the tissue cells and the haptophorous group of the toxin; or, on the other hand, supposing this affinity to exist, it might be due to an innate non-susceptibility to the action of the toxophorous group. Certain investigations have been made in order to determine the combining affinity of the nervous system of the fowl with tetanus toxin, as compared with that obtaining in a susceptible animal, but the results have been somewhat contradictory. Accordingly, a general statement on this point cannot at present be made, though in all probability variations in the susceptibility to the toxophorous group will be found to play a very important part. It has been shown by Muir and Browning by means of hæmolytic tests that the toxic activity of complement, after it has been fixed to the corpuscles, varies very much; in some instances an amount of complement, which would rapidly produce complete lysis of one kind of corpuscle, may have practically no effect on another, even though it enters into combination. These results are of importance in demonstrating how the corresponding molecules of different animals may vary in sensitiveness to toxic action.

#### *Supersensitiveness or Anaphylaxis.*

Under this heading are to be grouped a number of phenomena which in their character and results afford a striking contrast to the state of immunity. The common feature is that repeated in



jections of certain substances in sub-toxic or non-toxic doses,—a suitable interval of time elapsing between the injections,—may be followed by markedly toxic or even fatal symptoms, and a similar result may follow repeated injections of substances which are practically non-toxic in a single dose. In such cases, then, a condition of supersensitiveness to the particular substance has been established. The substances which have been found to have the property of calling forth this condition are of various kinds, including bacteria and their toxins, animal poisons, and a great many foreign proteins, *e.g.* those of serum, milk, egg albumin, etc., and it is to be noted that they belong to the group of substances which can act as antigens. Probably no body of known chemical constitution develops supersensitiveness; and, just as tolerance, say to drugs, is to be distinguished from immunity, so accumulative action is to be distinguished from supersensitiveness. Of the latter condition the earliest example observed was probably the special susceptibility of tubercular patients to the action of tuberculin, to which reference has already been made (p. 284). At a comparatively early date also it was found, in the case of diphtheria and tetanus toxins, that in certain instances the injection of a minute dose followed by another at a suitable interval might be attended by serious results; and that this was not an example of accumulative action, was shown by the fact that the sum of the doses might amount to only a fraction of a lethal dose. Richet investigated a similar phenomenon in the case of a toxic substance obtained from the tentacles of actiniae, to which from its action he gave the name of “congestin.” He found that a certain time-interval between the injections was necessary; that after the second injection the symptoms occurred with remarkable suddenness, and that they appeared to be practically independent of the size of the first dose. He applied the term *anaphylaxis* to the supersensitive condition, and this has passed into general use; he found also that the condition lasted several weeks. Arthus found that after repeated injections of horse serum in rabbits a stage was reached at which an additional subcutaneous injection produced marked œdema and even necrosis, while an intravenous injection, harmless to an untreated animal, brought about a fatal result. The period of active research on the subject, however, may be said to date from the discovery of what is now known as the “phenomenon of Theobald Smith.” This observer found that guinea-pigs which had been treated with a neutral mixture of diphtheria toxin and antitoxin might, after a certain interval of time, succumb on being injected with a quantity of normal horse



serum. It was afterwards shown—especially by the researches of Otto and of Rosenau and Anderson—that the sensitising agent had really nothing to do with the toxin or antitoxin, but was contained in the normal serum.

After this brief review we may consider some of the phenomena of serum anaphylaxis, as it is now called. In its study horse serum has been chiefly employed, but other sera are also efficient, and guinea-pigs are the most suitable test animals, though rabbits have also been used; in the case of mice it is difficult if not impossible to bring about serum anaphylaxis. There is first of all the sensitising injection; a guinea-pig is injected subcutaneously with a minute quantity, *e.g.* .001 c.c. of horse serum, though even smaller amounts may be sufficient and other methods of injection may also be employed. After a certain number of days, usually twelve as a minimum, anaphylaxis has been established, and the test for this is usually made by injecting subcutaneously 5 c.c. of horse serum. In the anaphylactic animal severe symptoms occur; restlessness and hyperalgesia are followed by evidence of collapse, the temperature falls markedly, the heart's action becomes weak and the respiration embarrassed; finally death may occur. The intravenous injection of a smaller amount of serum brings about the same result more rapidly. It is to be noted that anaphylaxis has the character of specificity, apparently within corresponding limits to immunity (p. 521); that is, it is manifested only on the re-injection of the same protein substance as that used in the first instance. There is also a passive anaphylaxis, as is shown by the fact that if a certain amount of the serum of an anaphylactic guinea-pig be injected into a normal one, the latter becomes anaphylactic, so that the characteristic symptoms appear in it when the test amount of horse serum is injected. In most instances an interval of about twenty-four hours must, however, elapse between the injections (Otto); if the two injections are made at the same time there is usually no result. Another interesting observation has been made, namely, that the young of anaphylactic mothers may also be anaphylactic, and the condition may last for some time after birth. It is also possible to produce a condition of anti-anaphylaxis, that is, to vaccinate against the supersensitive condition. If, for example, the sensitising dose of horse serum is injected, and then before anaphylaxis is established (*i.e.* sometime before the twelfth day) another injection of a considerable quantity is made, anaphylaxis does not appear, and the animal is non-susceptible to further injections of small doses for a considerable period of time.



With regard to the mechanism underlying the phenomena described, practically all observers are agreed that there is a profound affection of the nervous system; but it is still an open question as to whether the severe and practically simultaneous affections of the other systems are merely secondary, or whether they are independently produced by some change common to all. A great fall in the blood-pressure is an important phenomenon, and is due chiefly to a general vaso-dilatation; and it has been pointed out by Auer and Lewis that in the case of guinea-pigs there occurs a spasm of the muscle fibres in the fine bronchi and alveolar passages, the chest-wall being fixed in full inspiration at the time of death. Besredka has shown that the fatal symptoms are more rapidly produced in an anaphylactic animal and with a smaller dose of serum, when the injection is made directly into the brain, than by any other method. Further, seeing that a single dose of horse serum is not toxic to the guinea-pig, and that an interval of several days must elapse before anaphylaxis is established, the majority of observers consider that at least two substances are concerned, one of which is contained in normal horse serum, whilst the other is developed in the guinea-pig in response to the presence of the first or of some other substance after the manner of an anti-substance. To this newly developed substance the name of "anaphylactic reaction-body" is often given. The phenomena thus depend upon the co-operation of the reaction-body with a substance or substances in the horse serum, and a rapid union of the two, probably within the nerve-cells, brings about the anaphylactic shock. Passive anaphylaxis would thus be due to the transference of the reaction-body to a fresh animal, and the interval necessary before the second injection might depend upon the time required for the reaction-body to accumulate in sufficient quantity within the nerve-cells.

Besredka considers that the sensitising and the toxic factors in the horse serum are not one and the same. He finds that serum heated to a certain temperature may still have the power of inducing the condition of anaphylaxis, but has lost the power of bringing about the toxic phenomena when injected into an anaphylactic animal. Gay and Adler similarly find that the sensitising substance (anaphylactin) is contained in the euglobulin fraction of the serum while the other is not. Besredka accordingly puts forward the view that in the horse serum there are two substances or rather factors, namely, *sensibilisinogen*, which is thermostable, and *anti-sensibilisin*, which is thermolabile. When the serum is injected the former



gives rise to *sensibilisin* as an anti-substance; and when, after a suitable time, fresh serum is injected, the anti-sensibilisin combines with the sensibilisin, and thus the anaphylactic shock results. In view, however, of the specific nature of the phenomena, it would appear that both sensibilisinogen and anti-sensibilisin must have the same special combining group for sensibilisin, and it is accordingly difficult to see why the latter should not also act as an antigen. He has also found that when an animal is anæsthetised with ether the anaphylactic shock may be averted. Other workers at this subject hold that there are only two substances concerned, and some consider that the phenomena depends on a process of precipitation. Friedberger, for example, considers that the real toxic agent is formed by the action of complement on serum-precipitate (antigen + precipitin). To this substance he gives the name "anaphylatoxin," and in support of his view he has shown that guinea-pig's complement, after it has been allowed to act for some time on such a precipitate and then removed by the centrifuge, has acquired toxic properties, and produces the symptoms of anaphylaxis when injected into a normal guinea pig. He also points out that during anaphylactic phenomena, especially in the case of passive anaphylaxis, there is a great fall of complement in the blood of the animal, and Scott has brought forward facts which indicate that there is a close relationship between this fall in complement and the occurrence of the symptoms in anaphylaxis. On the other hand, Gay and Southard do not believe in the theory of a reaction-body. They consider that the condition depends on the presence of a substance in the serum which they call anaphylactin, and which persists in the blood of the guinea-pig for a long period of time. This acts as a slight irritant to the nerve-cells, and produces in them an increased affinity for certain molecules in the serum. Accordingly, when the second injection is made, the rapid combination of these molecules with the cells results in the disturbances described. This view has, however, received little support, and there are various facts against it, especially in relation to the transference of anaphylaxis. Others, again, *e.g.* Citron, consider that supersensitiveness is so closely allied to immunity as to really represent the earliest stage in its development. At present it is impossible to express an opinion with regard to the real nature of the phenomena. Manifestly, however, if they depend upon the existence of an anti-substance in the nerve-cells and the cells of other organs, the injection of fresh serum, before the anti-substance is fully formed, say on the ninth day after



the first injection, will lead to its combination and thus to its being used up, and thus the condition of anti-anaphylaxis will be established.

It is still an open question as to what extent the phenomena of anaphylaxis just described are of the same nature as the supersensitiveness manifested by patients suffering from disease to the products of the corresponding organism, *e.g.* to tuberculin, mallein, etc. (pp. 284, 314); though in all probability they are at least similar in essence. It was held for some time as a distinction that this supersensitiveness in infections to bacterial products could not be transferred to another animal, but recent observations show that in certain circumstances this is possible in the case of tuberculin. There is no doubt that the supersensitive condition must play an important part in the clinical manifestations of many diseases. For example, the sensitiveness of tubercular patients to tuberculin shows that the symptoms in this disease are evidently produced by the absorption from the tubercular foci of a smaller amount of toxin than would be necessary to produce effects in a normal individual. And the sensitiveness of the conjunctiva in typhoid fever to the products of the bacillus suggests that in this disease also supersensitiveness plays an important part. It is also possible that the repeated absorption of proteins, harmless in single doses, may lead to toxic symptoms, and in a similar way may possibly be explained the relative non-toxicity of the products of certain bacteria when tested in the usual manner. But with regard to all these questions, which are of the highest importance, much further research is still necessary.

**The Serum Disease in Man.**—This condition, which is intimately related to supersensitiveness, includes the phenomena which have been observed after the injection of anti-diphtheric and other sera. The real factor is the introduction of foreign sera into the human tissues. As in the case of anaphylaxis, as above described, there is here also a period of incubation, of eight to twelve days on the average; after which, in a certain proportion of cases (in about 20 per cent. after the injection of a fairly large amount of horse serum, a group of characteristic symptoms appear. There may be as prodromal symptoms, swelling and tenderness at the site of injection, and in the corresponding lymphatic glands, and thereafter general exanthemata appear. These are usually of an urticarial type, but may be erythematous or morbilliform. There is usually moderate pyrexia of a remittent type, and sometimes oedema and slight albuminuria are present; occasionally there are pains in the joints; there is



also often leucopenia due to a fall in the number of polymorpho-nuclear leucocytes. These symptoms last for a few days and then disappear. Such are the phenomena of the serum disease after a single injection of the foreign serum. There are, however, two other types of reaction described by v. Pirquet and Schick, namely, the *immediate* and the *accelerated* reactions. The immediate reaction is seen when a large dose of serum has been administered and then after a certain interval of time another dose of serum is injected. This interval is usually from twelve days to eight weeks, though sometimes as long as six months. The symptoms of the immediate reaction, which appear shortly after the injection, or at least within twenty-four hours, are an intense œdema locally, general exanthemata and pyrexia, though the general phenomena are often little marked. The symptoms pass off comparatively quickly, usually within twenty-four hours. The accelerated reaction is also seen after a second injection, and it may occur from six weeks up to many months after the first injection. In the case of the accelerated reaction there is an incubation period, but it is shorter than in the case of the first injection, being usually five to seven days; the symptoms resemble those in the ordinary reaction as described above, but are of rather more acute onset and last a shorter time. In the interval from about the sixth week to the sixth month, there may occur both the immediate reaction, and also a few days later an accelerated reaction.

The nature of the serum disease is not yet fully understood, but in all probability depends upon the development of a reaction-body or anti-substance, and the combination of this with a substance in the serum, probably an antigen, leads to the symptoms. We suppose that the substances in the serum gradually disappear from the body after the injection; from about the eighth day onward anti-substances appear in the blood in large amount, and if antigens are still present, the combination of the two brings about the phenomena described. Manifestly, if the antigens have disappeared before the anti-substances appear in quantity, there will be no symptoms. At a later period anti-substances will be present alone in the serum, and then the injection of fresh antigens brings about an immediate reaction. After the anti-substances have disappeared, the injection of fresh serum causes no immediate reaction, but the mechanism of reaction has been stimulated by the first injection; anti-substances thus appear more quickly after the second injection, hence the reaction is accelerated as compared with the reaction after the first injection.



## APPENDIX A.

### SMALLPOX AND VACCINATION.

SMALLPOX is a disease to which much study has been devoted, owing, on the one hand, to the havoc which it formerly wrought among the nations of Europe,—a havoc which at the present day it is difficult to realise,—and, on the other hand, to the controversies which have arisen in connection with the active immunisation against it introduced by Jenner. Though there is little doubt that a *contagium vivum* is concerned in its occurrence, the etiological relationship of any particular organism to smallpox has still to be proved; and with regard to Jennerian vaccination, it is only the advance of bacteriological knowledge which is now enabling us to understand the principles which underlie the treatment, and which is furnishing methods whereby the vexed questions concerned will probably be satisfactorily settled. We cannot here do more than touch on some of the results of investigation with regard to the disease.

**Jennerian Vaccination.**—Up to Jenner's time the only means adopted to mitigate the disease had been by inoculation (by scarification) of virus taken from a smallpox pustule, especially from a mild case. By this means it was shown that in the great majority of cases a mild form of the disease was originated. It had previously been known that one attack of the disease protected against future infection, and that the mild attack produced by inoculation also had this effect. This inoculation method had long been practised in various parts of the world, and had considerable popularity all over Europe during the eighteenth century. Its disadvantage was that the resulting disease, though mild, was still infectious, and thus might be the starting-point of a virulent form among unprotected persons. Jenner's discovery was published when inoculation was still considerably practised. It was founded on the popular belief that those who had contracted cowpox from



an affected animal were insusceptible to subsequent infection from smallpox. In the horse there occurs a disease known as horsepox, especially tending to arise in wet, cold springs, which consists in an inflammatory condition about the hocks, giving rise to ulceration. Jenner believed that the matter from these ulcers, when transferred by the hands of men who dressed the sores to the teats of cows subsequently milked by them, gave rise to cowpox in the latter. This disease was thus identical with horsepox in epidemics of which it had its origin. Jenner was, however, probably in error in confounding horsepox with another disease of horses, namely, grease. Cowpox manifests itself as a papular eruption on the teats; the papules become pustules; their contents dry up to form scabs, or more or less deep ulcers occur at their sites. From such a lesion the hands of the milkers may become infected through abrasions, and a similar local eruption occurs, with general symptoms in the form of slight fever, malaise, and loss of appetite. It is this illness which, according to Jenner, gives rise to immunity from smallpox infection. He showed experimentally that persons who had suffered from such attacks did not react to inoculation with smallpox; and further, that persons to whom he communicated cowpox artificially were similarly immune. The results of Jenner's observations and experiments were published in 1798 under the title, *An Inquiry into the Causes and Effects of the Variola Vaccinæ*. Though from the first Jennerian vaccination had many opponents, it gradually gained the confidence of the unprejudiced, and became extensively practised all over the world, as it is at the present day.

The evidence in favour of vaccination is very strong. There is no doubt that inoculation with lymph properly taken from a case of cowpox, can be maintained with very little variation in strength for a long time by passage from calf to calf, and such calves are now the usual source of the lymph used for human vaccination. When lymph derived from them is used for the latter purpose, immunity against smallpox is conferred on the vaccinated individual. It has been objected that some of the lymph which has been used has been derived from calves inoculated, not with cowpox, but with human smallpox. It is possible that this may have occurred in some of the strains of lymph in use shortly after the publication of Jenner's discovery, but most of the strains at present in use have probably been derived originally from cowpox. The most striking evidence in favour of vaccination is derived from its effects among the staffs of smallpox hospitals, for here, in numerous instances, it is only



the unvaccinated individuals who have contracted the disease. While vaccination is undoubtedly efficacious in protecting against smallpox, Jenner was wrong in supposing that a vaccination in infancy afforded protection for more than a certain number of years thereafter. It has been noted in smallpox epidemics which have occurred since the introduction of vaccination, that whereas young unprotected subjects readily contract the disease, those vaccinated as infants escape more or less till after the thirteenth to the fifteenth years. It has become, therefore, more and more evident that revaccination is necessary if immunity is to continue ; and where this is done in any population, smallpox becomes a rare disease, as has happened in the German army, where the mortality is practically nil. The whole question of the efficacy of vaccination was investigated in this country in 1896 by a Royal Commission, whose general conclusions were as follows :—Vaccination diminishes the liability to attack by smallpox, and when the latter does occur, the disease is milder and less fatal. Protection against attack is greatest during nine or ten years after vaccination. It is still efficacious for a further period of five years, and possibly never wholly ceases. The power of vaccination to modify an attack outlasts its power wholly to ward it off. Revaccination restores protection, but this operation must be from time to time repeated. Vaccination is beneficial according to the thoroughness with which it is performed.

**The Relationship of Smallpox (Variola) to Cowpox (Vaccinia).**—This is the question regarding which, since the introduction of vaccination the greatest controversy has taken place ; a subsidiary point has been the inter-relationships within the group of animal diseases which includes cowpox, horsepox, sheep-pox, and cattle-plague. With reference to smallpox and cowpox the problem has been, Are they identical or not ? There is no doubt that cowpox can be communicated to man, in whom it produces the eruption limited to the point of inoculation, and the slight general symptoms which vaccination with calf lymph has made familiar. Apparently against the view that cowpox is a modified smallpox are the facts that it never reproduces in man a general eruption, and that the local eruption is only infectious when matter from it is introduced into an abrasion. The loss of infectiveness by transmission through the body of a relatively insusceptible animal is a condition of which we have already seen many instances in other diseases, and the uniformity of the type of the affection resulting from vaccination with calf lymph finds a parallel in such a disease as hydrophobia, where,



after passage through a series of monkeys, a virus of attenuated but constant virulence can be obtained. We have seen there are good grounds for believing that the virus of calf lymph confers immunity against human smallpox. In considering the relationships of cowpox and smallpox, this is an important though subsidiary point; for at present it is questionable whether there are any well-authenticated instances of one disease having the capacity of conferring immunity against another. The most difficult question in this connection is what happens when inoculations of smallpox matter are made on cattle. Chauveau denies that in such circumstances cowpox is obtained. He, however, only experimented on adult cows. The transformation has been accomplished by many observers, including, in this country, Simpson, Klein, Hime, and Copeman. The general result of these experiments has been that if a series of calves is inoculated with variolous matter, in the first there may not be much local reaction, though redness and swelling appear at the point of inoculation, and some general symptoms manifest themselves. On squeezing some of the lymph from such lesion as occurs, and using it to continue the passages through other calves, after a very few transfers a local reaction indistinguishable from that caused by cowpox lymph generally takes place, and the animals are now found to be immune against the latter. Not only so, but on using for human vaccination the lymph from such variolated calves, results indistinguishable from those produced by vaccine lymph are obtained, and the transitory illness which follows, unlike that produced in man by inoculation with smallpox lymph, is no longer infectious. In fact, many of the strains of lymph in use in Germany at present have been derived thus from the variolation of calves. The criticism of these experiments which has been offered, namely, that since many of them were performed in vaccine establishments, the calves were probably at the same time infected with vaccinia, is not of great weight, as in all the recent cases at least, very elaborate precautions have been adopted against such a contingency. And at any rate it would be rather extraordinary that this accident should happen in every case. We can, therefore, say that at present there is the very strongest ground for holding not only that vaccinia confers immunity against variola, but that variola confers immunity against vaccinia. The *experimentum crucis* for establishing the identity of the two diseases would of course be the isolation of the same micro-organism from both, and the obtaining of all the results just detailed by means of pure



cultures or the products of such. In the absence of this evidence we are at present justified in considering that there is strong reason for believing that vaccinia and variola are the same disease, and that the differences between them result from the relative susceptibilities of the two species of animals in which they occur naturally.

With regard to the relation of cowpox to horsepox, it is extremely probable that they are the same disease. Some epidemics of the former have originated from the horse, but in other cases such a source has not been traced. Cattle-plague from the clinical standpoint, and also from that of pathological anatomy, resembles very closely human smallpox. Though each of the two diseases is extremely infectious to its appropriate animal, there is no record of cattle-plague giving rise to smallpox in man or *vice versa*. When matter from a cattle-plague pustule is inoculated in man, a pustule resembling a vaccine pustule occurs, and further, the individual is asserted to be now immune to vaccination; but vaccination of cattle with cowpox lymph offers no protection against cattle-plague, though some have looked on the latter as merely a malignant cowpox. Sheep-pox also has many clinical and pathological analogies with human smallpox, and facts as to its relation to cowpox vaccination similar to those observed in cattle-plague have been reported. Smallpox, cowpox, cattle-plague, horsepox, and sheep-pox, in short, constitute an interesting group of analogous diseases, of the true relationships of which to one another we are, however, still ignorant.

**Micro-organisms associated with Smallpox.**—Burdon Sander-son and other observers early pointed out that in matter derived from variolous and vaccine pustules (especially the later stages of the latter), pyogenic organisms are always present, e.g. *staphylococcus aureus* and *staphylococcus cereus flavus*, and many of the ordinary skin saprophytes also are often present, but no organism has ever been isolated which on transference to animals has been shown to have any specific relationship to the disease. Streptococci have also been described as agglutinable by the sera of smallpox patients and of vaccinated persons; such sera, it may be said, had no effect on other strains of streptococci. Calmette and Guérin have described very minute granules in the lymph which could not be cultivated, but which persisted after all the bacteria had been removed. (The method by which the latter was accomplished was by exciting a leucocytosis in a rabbit's peritoneum and then introducing the vaccinal lymph; the leucocytes phagocyted the bacteria so that the lymph no longer



gave cultures on ordinary media. It was, however, still potent to produce vaccinia.)

Klein and also, independently, Copeman, have observed an organism in lymph taken from a vaccine pustule in a calf on the fifth and sixth days, in human vaccine lymph on the eighth day, and in lymph from a smallpox pustule on the fourth day. To demonstrate the bacilli, cover-glass films are dried and placed for five minutes in acetic acid (1 in 2), washed in distilled water, dried, and placed in alcoholic gentian-violet for from twenty-four to forty-eight hours, after which they are washed in water and mounted. Copeman and Kent also found the bacilli in sections of vaccine pustules stained by Löffler's methylene-blue, or by Gram's method. The organisms are  $\cdot 4$  to  $\cdot 8 \mu$  in length, and one-third to a half of this in thickness. They are generally thinner and stain better at the ends than at the middle. They occur in groups of from three to ten in both the lymph and the tissues. In the centre of their protoplasm there is often a clear globule, which is looked on as a spore. They have hitherto resisted the ordinary isolation methods, a fact which is rather in favour of their non-saprophytic nature. By inoculating fresh eggs with the crusts of smallpox pustules Copeman has, however, obtained a growth of a bacillus resembling that found by him in the tissues. Though subcultures on ordinary media have been obtained, the pathogenic effects of these have not been fully investigated, and thus the identity of this bacillus with that seen in the tissues is not proved.

Various observers have described structures in the epithelial cells in the neighbourhood of the smallpox or vaccine pustules, which they have interpreted as being protozoa. Thus Ruffer and Plimmer describe as occurring in clear vacuoles in the cells of the rete Malpighii at the edge of the pustule (in paraffin sections of vaccine and smallpox pustules carefully hardened in alcohol, and stained by the Ehrlich-Biondi mixture) small round bodies of about four times the size of a staphylococcus pyogenes, coloured red by the acid fuchsin, sometimes with a central part stained by the methyl-green. These are described as multiplying by simple division, and in the living condition exhibiting amoeboid movement. Similar bodies have been described by Reed in the blood of smallpox patients and of vaccinated children and calves.

These are probably the bodies described by Guarnieri and to which considerable attention has been paid. They are from 1 to  $8 \mu$  in diameter, are round, oval, or sickle-shaped, and stain by ordinary nuclear dyes. They lie in the cells in spaces often near the nucleus, and are readily demonstrable in vaccine pustules and also in the experimental lesions which can be produced in the rabbit's cornea, the larger bodies being defined in the cells towards the centre of the lesion. These bodies



have been looked on by many as protozoa, and Guarnieri himself stated that multiplication could be seen occurring in them in fresh lymph, but Ewing and also Prowazek have brought forward strong evidence for the appearances being due to nuclear changes, though the latter observer considers them to be the effect of a specific reaction of epithelial cells against the variolous virus. Here it may be said Wasielewski has shown that they persist through 46 transfers on the cornea of the rabbit and, further, no similar appearances have been found in other skin lesions. Prowazek examined material fixed in a hot mixture of two-thirds saturated perchloride of mercury and one-third 98 per cent. alcohol, washed in 40 per cent. iodine alcohol and stained in Grenacher's hæmatoxylin, and found bodies in the epithelial cells 1 to 4  $\mu$  in size, sharply contoured and having ragged edges as if made up of massed chromosomes. These were often broader at one end than at the other, and appearances have been seen which suggest longitudinal division. Prowazek has also seen these "lymph-bodies," as he has called them, in the lymph, and he inclines to the idea that they may be protozoa. Bonhoff and also Carini have described spirochætes as occurring in variolous lesions, but this has not been confirmed. Volpino states that in the epithelial cells in corneal infection in rabbits, minute motile bodies can be discerned which do not occur in other corneal inflammations. Future investigations must show what significance is to be attached to these various observations.

The causal organism of smallpox is probably very small, as, though there has been some difference in opinion on this point, there is little doubt that it will pass through the coarser porcelain filters.

**The Nature of Vaccination.**—From the facts known regarding vaccination we are justified in supposing that the principle underlying the efficacy of this prophylactic is the establishment of an active immunity against the causal organism, which is sufficiently lasting to protect the vaccinated individual for a considerable time. Although the virus of smallpox is unknown, several attempts have been made by indirect methods to establish the existence of reactions similar to those occurring in other immunisations. Thus, in cases of human smallpox and in animals intravenously injected with the vaccine lymph, it is stated that the serum when mixed with vaccine lymph acquires the property of deviating complement, and evidence has also been obtained by Prowazek that the serum of monkeys infected subcutaneously contains substances of the nature of anti-bodies, for, when it is mixed with the lymph, the mixture is not



capable of originating a vaccine pustule in children. Phenomena of hypersensitiveness on revaccination have also been described.

Considerable attention has been devoted to the study of the effects of corneal and cutaneous infection in the rabbit and monkey. Here it has been found that the infection of one cornea protects that eye against re-inoculation but not the other eye. Further, it is stated that while cutaneous vaccination causes the general skin surface after about ten days to become insusceptible, the cornea may still in the monkey be sensitive (this last fact is said not to be true for the rabbit). Again, intraperitoneal infection with lymph is said not to be followed by cutaneous immunity. Such facts have led some to suppose that smallpox is essentially a disease of the cutaneous tissues. In it we would have another example of local infection such as is found in tubercular leprosy, lupus, and certain other skin infections. Prowazek strongly holds that in cutaneous vaccinal infection there is never a distribution of the virus throughout the organs, but this result has been disputed by other workers. He also states that when the virus is injected intraperitoneally it is soon taken up by leucocytes and is not absorbed into the body fluids.



## APPENDIX B.

### HYDROPHOBIA.

SYNONYMS.—RABIES : *FRENCH*, LA RAGE : *GERMAN*, LYSSA,  
DIE HUNDWUTH, DIE TOLLWUTH.

**Introductory.**—Hydrophobia is an infectious disease which in nature occurs epidemically chiefly among the carnivora, especially in the dog and the wolf. Infection is carried by the bite of a rabid animal or by a wound or abrasion being licked by such. The disease can be transferred to other species, and when once started can be spread from individual to individual by the same paths of infection. Thus it occurs epidemically from time to time in cattle, sheep, horses, and deer, and can be communicated to man. It is questionable whether infection can take place from man to man, as the saliva of a person suffering from hydrophobia appears not to contain the virus. It is to be noted that the virus is apparently extremely potent, as cases of infection taking place through an unabraded mucous membrane by the licking of a rabid animal are on record, and the experimental applications of the virus to such surfaces as the mucous membrane of the nose or the conjunctiva is often followed by infection.

In Western Europe the disease is most frequently observed in the dog; but in Eastern Europe, especially in Russia, epidemics among wolves constitute a serious danger both to other animals and to man. All the manifestations of the disease point to a serious affection of the nervous system; but inasmuch as symptoms of excitement or of depression may predominate, it is customary to describe clinically two varieties of rabies,—(1) rabies proper, or furious rabies (*la rage vraie, la rage furieuse: die rasende Wuth*); and (2) dumb madness or paralytic rabies (*la rage mue: die stille Wuth*). The disease, however, is essentially the same in both cases. In the dog the furious form is the more common. After a period of incubation of from three to



six weeks, the first symptom noticed is a change in the animal's aspect ; it becomes restless, it snaps at anything which it touches, and tears up and swallows unwonted objects ; it has a peculiar high-toned bark. Spasms of the throat muscles come on, especially in swallowing, and there is abundant secretion of saliva ; its supposed special fear of water is, however, a myth, —it fears to swallow at all. Gradually convulsions, paralysis, and coma come on ; and death supervenes usually about five days after the appearance of symptoms. In the paralytic form, the early symptoms are the same, but paralysis appears sooner. The lower jaw of the animal drops, from implication of the elevator muscles, all the muscles of the body become more or less weakened, and death ensues without any very marked irritative symptoms.

In man the incubation period after infection varies from fifteen days to seven or eight months, or even longer, but is usually about forty days. When symptoms of rabies are about to appear, certain prodromata, such as pains in the wound and along the nerves of the limb in which the wound has been received, may be observed. To this succeeds a stage of nervous irritability, during which all the reflexes are augmented—the victim starting at the slightest sound, for example. There are spasms, especially of the muscles of deglutition and respiration, and cortical excitement evidenced by delirium may occur. On this follows a period in which all the reflexes are diminished, weakness and paralysis are observed, convulsions occur, and finally coma and death supervene. The duration of the acute illness is usually from four to eight days, and death invariably results. The existence of paralytic rabies in man has been denied by some, but it undoubtedly occurs. This is usually manifested by paralysis of the limb in which the infection has been received, and of the neighbouring parts ; but while in such cases this is often the first symptom observed, during the whole of the illness the occurrence of widespread and progressive paralysis is the outstanding feature. In man there also occur cases where the cerebellum and also the sympathetic system seem to be specially affected.

**The Pathology of Hydrophobia.**—In hydrophobia as in tetanus, to which it bears more than a superficial resemblance, the appearances discoverable by an ordinary examination of the nervous system, to which all symptoms are naturally referred, are comparatively unimportant. On naked-eye examination, congestions, and, it may be, minute hæmorrhages are the only features noticeable. Microscopically, leucocytic exudation into the peri-



vascular lymphatic spaces in the nerve centres has been observed, and in the cells of the anterior cornua of the grey matter in the spinal cord, and also in the nuclei of the cranial nerves, various degenerations have been described. Round the nerve cells in the grey matter of the cord and medulla Babés described accumulations of newly-formed cells, and Van Gehuchten observed a phagocytosis of the cells in the posterior root ganglia and also in the sympathetic ganglia. Both of these conditions were at one time thought to be specific of rabies, but this has been found not to be the case. In the white matter, especially in the posterior columns, swelling of the axis cylinders and breaking up of the myeline sheaths have been noted, and similar changes occur also in the spinal nerves, especially of the part of the body through which infection has come. In the nervous system also some have seen minute bodies which they have considered to be cocci, but there is no evidence that they are really of this nature. The changes in the other parts of the body are unimportant.

Experimental pathology confirms the view that the nervous system is the centre of the disease by finding in it a special concentration of what, from want of a more exact term, we must call the hydrophobic virus. Pasteur's first contribution to the subject was to show that the most certain method of infection was by inserting the infective matter beneath the dura mater. He found that in the case of any animal or man dead of the disease, injection, by this method, of emulsions of any part of the central nervous system, of the cerebro-spinal fluid, or of the saliva, invariably gave rise to rabies, and also that the natural period of incubation was shortened. Further, the identity of the furious and paralytic forms was proved, as sometimes the one, sometimes the other, was produced, whatever form had been present in the original case. Inoculation into the anterior chamber of the eye is nearly as efficacious as subdural infection. Infection with the blood or solid organs of rabid animals does not reproduce the disease. There is evidence, however, that the poison also exists in such glands as the pancreas and mamma. *Subcutaneous* infection with part of the nervous system of an animal dead of rabies may or may not give rise to the disease.

In consequence of the introduction of such reliable inoculation methods, further information has been acquired regarding the spread and distribution of the virus in the body. Gaining entrance by the infected wound, it early manifests its affinity for the nervous tissues. It reaches the central nervous system chiefly by spreading up the peripheral nerves. This can be



shown by inoculating an animal subcutaneously in one of its limbs, with virulent material. If now the animal be killed before symptoms have manifested themselves, rabies can be produced by subdural inoculation from the nerves of the limb which was infected. Further, rabies can often be produced from such a case by subdural infection with the part of the spinal cord into which these nerves pass, while the other parts of the animal's nervous system do not give rise to the disease. This explains how the initial symptoms of the disease (pains along nerves, paralysis, etc.) so often appear in the infected part of the body, and it probably also explains the fact that bites in such richly nervous parts as the face and head are much more likely to be followed by hydrophobia than bites in other parts of the body. Again, injection into a peripheral nerve, such as the sciatic, is almost as certain a method of infection as injection into the subdural space, and gives rise to the same type of symptoms as injection into the corresponding limb. Intravenous injection of the virus, on the other hand, differs from the other modes of infection in that it more frequently gives rise to paralytic rabies. This fact Pasteur explained by supposing that the whole of the nervous system in such a case becomes simultaneously affected. In certain animals the virus seems to have an elective affinity for the salivary glands, as well as for the nervous system. Roux and Nocard found that the saliva of the dog became virulent three days before the first appearance of symptoms of the disease.

**The Virus of Hydrophobia.**—While a source of infection undoubtedly occurs in all cases of hydrophobia, and can usually be traced, all attempts to determine the actual morbid cause have been unsatisfactory. In this connection various organisms (yeasts, diphtheroid bacilli) have been described as being associated with the disease, but none of these have been shown to possess the capacity of producing immunity against the ordinary hydrophobic virus.

In 1903 Negri described certain bodies as occurring in the nervous system in animals dying of rabies to which much attention has since been devoted, and regarding the significance of which opinion is still divided. As Negri's observations have been generally confirmed, and as it is probable that the occurrence of the bodies is specific to the disease, and that their recognition is of value for diagnosis, we shall describe the methods for their demonstration.

The method of Williams and Lowden is to take a piece of the brain tissue, to squeeze it between a slide and cover-glass, and, sliding off the



latter, to make a smear which is then fixed in methyl alcohol for five minutes and stained by Giemsa's stain (p. 115) for half an hour to three hours; the preparation is then washed in tap water for 2-3 min. and dried. For rapid work, after fixation, equal parts of distilled water and stain are used instead of the more dilute mixture.

For sections the tissues are left in Zenker's fluid<sup>1</sup> for 3-4 hours, then placed in tap water for five minutes, 80 per cent. alcohol with enough iodine added to give it a port wine colour for 24 hours; 95 per cent. alcohol and iodine, 24 hours; absolute alcohol, 4-6 hours; cleared with cedar oil and embedded in paraffin of melting point 52° C.; sections should be 3 to 6  $\mu$  thick. For staining, Mallory's methylene-blue eosin is recommended; the steps are as follows: xylol; absolute alcohol; 95 per cent. alcohol and iodine,  $\frac{1}{4}$  hour; 95 per cent. alcohol,  $\frac{1}{2}$  hour; absolute alcohol,  $\frac{1}{2}$  hour; eosin solution (5-10 per cent. aqueous solution), 20 minutes; rinse in tap water; Unna's polychrome methylene-blue solution diluted 1-4 with distilled water, 15 minutes; differentiation in 95 per cent. alcohol for 1-5 minutes (the preparation being kept in motion and its progress watched with a low power); rapid and careful dehydration and clearing.

Frothingham recommends a method of making "impression preparations" of the brain. The part (*e.g.* hippocampus) is laid on a piece of wood whose porosity causes it to adhere; a clean slide is then lowered upon the tissue and slight pressure applied; on raising the slide a thin film of cells preserving their original arrangement is lifted off, and this can be fixed and stained like a smear, van Gieson's method being used by this author.

The Negri bodies (Plate IV., Fig. 16)<sup>2</sup> vary much in size, measuring from .5 to 25  $\mu$ . They are round, oval, or angular in outline. They are found in the protoplasm of the nerve cells and of their processes. When examined in unstained preparations, they are seen to have a sharply defined outline, and some of the features of the internal structure presently to be described can be noted. With regard to staining reactions, they are frankly eosinophil for certain combinations containing eosin, *e.g.* alcoholic eosin-methylene-blue, Mann's eosin mixture, and, in certain circumstances, Leishman's stain. For the finer differentiation of the internal structure, Negri employed Giemsa's stain. With this stain and under high magnification the groundwork of the body is a pale blue; in it there appear certain round or oval, multiple or single formations, of varying size, stained pink, sometimes occupying nearly the whole of the body, sometimes being relatively small (*grosse Innenformationen*). In addition,

<sup>1</sup> Zenker's fluid is of the following composition: potassium bichromate 2.5 gr., sodium sulphate 1 gr., perchloride of mercury 5 gr., glacial acetic acid 5 c.c., water to 100 c.c. Dissolve the perchloride of mercury and the bichromate of potassium in the water with the aid of heat and add the acetic acid.

<sup>2</sup> For the material from which this preparation was made we are indebted to Capt. W. F. Harvey, I.M.S.



both inside the larger formations and in the general protoplasm of the body are smaller red or violet-red granules, occurring singly or in clumps (*kleine Innenformationen*). With the eosin dyes named above, and magnifications of 800 to 1000, the smaller bodies appear a homogeneous reddish pink, and in the larger bodies the outlines of the larger internal formations can be recognised (see Plate). With Mallory's stain they present similar appearances with a bluish stippling of the protoplasm.

The Negri bodies have been found in practically 98 per cent. of cases of street rabies examined by many observers in different parts of the world. Numerous control observations on other toxic conditions of the nervous system, especially where these are characterised by spasms, have been made, and although occasionally, *e.g.* in tetanus, a somewhat similar appearance has been seen, at present the consensus of opinion is in favour of an experienced observer being able to recognise the Negri bodies as a specific appearance in nerve cells. The bodies occur in all parts of the nervous system, but are most common in the Purkinje cells of the cerebellum, and especially in the cells of the cornu Ammonis (hippocampus major). It is in the last situation, therefore, that they are generally looked for. They are apparently not so readily found, and may be altogether absent, in animals dying from inoculation with the exalted fixed virus. Hitherto they have not been found in the salivary glands or saliva of a rabid animal.

While there is a general tendency to recognise the Negri bodies as being specific to rabies, great difference of opinion exists as to their true nature and as to their possessing any etiological significance in the disease. Negri himself looks upon them as protozoa, and the organism has been named by Calkins *neuroryctes hydrophobice*. The chief arguments advanced in favour of this position have been the constancy of the occurrence of the bodies in the brains of animals suffering from the natural disease, and their peculiar structure which, such authorities as Golgi state, does not correspond to any cellular degeneration. Against their protozoal nature has been urged their absence from the virulent brains of animals dying from fixed virus, their non-discovery in the infected saliva, and the fact that the virus can pass through a coarse filter. These objections have been met with the argument that the smaller internal formations may be the infective agent in its essential form, and a modification of this view is that the Negri body is a cellular reaction against an invasion with these ultimate forms. The whole question must be looked upon as *sub judice*.



There is no doubt that between rabies and the bacterial diseases we have studied there are at every point analogies, the most striking being the protective inoculation methods, the discovery of which constitutes the great work of Pasteur ; and everything points to a micro-organism being the cause. The organism, whatever it is, is, in its infective form, probably very small, as it can pass through the coarser Berkefeld filters, and also occasionally through the coarser Chamberland candles. Evidence that it is the organism itself which passes through, is found in the fact that when an animal dies from infection with the filtrate, a small portion of its central nervous system will originate the disease in a fresh animal. Judging from our knowledge of similar diseases, we would strongly suspect that it is actually present in a living condition in the central nervous system, the saliva, etc., which yield what we have called the hydrophobic virus, for by no mere toxin could the disease be transmitted through a series of animals, as we shall presently see can be done. A toxin may, however, be concerned in the production of the pathogenic effects. Remlinger found that death with paralytic symptoms followed the injection of filtered virus, but that the nervous system of the dead animals sometimes did not reproduce rabies. He explains this occurrence by supposing that the filtrate contained a toxin but not the actual infective agent. The resistance of the virus to external agents varies. Thus a nervous system containing it is virulent till destroyed by putrefaction ; it can resist the prolonged application of a temperature of from  $-10^{\circ}$  to  $-20^{\circ}$  C., but, on the other hand, it is rendered non-virulent by one hour's exposure at  $50^{\circ}$  C. Again, its potency probably varies in nature according to the source. Thus, while the death-rate among persons bitten by mad dogs is about 16 per cent., the corresponding death-rate after the bites of wolves is 80 per cent. Here, however, it must be kept in view that, as the wolf is naturally the more savage animal, the number and extent of the bites, *i.e.* the number of channels of entrance of the virus into the body and the total dose, are greater than in the case of persons bitten by dogs. As we shall see, alterations in the potency of the virus can certainly be effected by artificial means.

**The Prophylactic Treatment of Hydrophobia.**—Until the publication of Pasteur's researches in 1885, the only means adopted to prevent the development of hydrophobia in a person bitten by a rabid animal had consisted in the cauterisation of the wound. Such a procedure was undoubtedly not without effect. It has been shown that cauterisation within five minutes



of the infliction of a rabic wound prevents the disease from developing, and that if done within half an hour it saves a proportion of the cases. After this time, cauterisation only lengthens the period of incubation; but, as we shall see presently, this is an extremely important effect.

The work of Pasteur has, however, revolutionised the whole treatment of wounds inflicted by hydrophobic animals. Pasteur started with the idea that, since the period of incubation in the case of animals infected subdurally from the nervous systems of mad dogs is constant in the dog, the virus has been from time immemorial of constant strength. Such a virus, of what might be called natural strength, is usually referred to in his works as the virus of *la rage des rues*,<sup>1</sup> in the writings of German authors as the virus of *die Strasswuth*. Pasteur found on inoculating a monkey subdurally with such a virus, and then inoculating a second monkey from the first, and so on with a series of monkeys, that it gradually lost its virulence, as evidenced by lengthened periods of incubation on subdural inoculation of dogs, until it wholly lost the power of producing rabies in dogs, when introduced subcutaneously. When this point had been attained, its virulence was not diminished by further passage through the monkey. On the other hand, if the virus of *la rage des rues* were similarly passed through a series of rabbits or guinea-pigs, its virulence was increased till a constant strength (the *virus fixe*) was attained,—constancy of strength being indicated by the unvarying recurrence of paresis on the sixth day. Pasteur had thus at command three varieties of virus—that of natural strength, that which had been attenuated, and that which had been exalted. He further found that, commencing with the subcutaneous injection of a weak virus, and following this up with the injection of the stronger varieties, he could ultimately, in a very short time, immunise dogs against subdural infection with a virus which, under ordinary conditions, would certainly have caused a fatal result. He also elucidated the fact that the exalted virus contained in the spinal cords of rabbits such as those referred to, could be attenuated so as no longer to produce rabies in dogs by subcutaneous injection. This was done by drying the cords in air over caustic potash (to

<sup>1</sup> While Pasteur's original statements regarding the constancy of the virulence of the street-virus were probably accurate for the street dogs of Paris, it has been found that if the general virulence of virus derived from animals in nature be studied, considerable variation occurs. It is now usual to apply the term street-virus to any virus derived from an animal becoming rabid under natural conditions of infection.



absorb the moisture), the diminution of virulence being proportional to the length of time during which the cords were kept. Accordingly, by taking a series of such spinal cords kept for various periods of time, he was supplied with a series of vaccines of different strengths. Pasteur at once applied himself to find whether the comparatively long period of incubation in man could not be taken advantage of to "vaccinate" him against the disease before its gravest manifestation took place. The following is the record of the first case thus treated. The technique was to rub up in a little sterile bouillon a small piece of the cord used, and inject it under the skin by means of a hypodermic syringe. The first injection was made with a very attenuated virus, *i.e.* a cord fourteen days old. In subsequent injections the strength of the virus was gradually increased, as shown in the table:—

July	7,	1885,	9 A.M.,	cord of June 23,	<i>i.e.</i>	14 days old.
"	7	"	6 P.M.	"	25	" 12 "
"	8	"	9 A.M.	"	27	" 11 "
"	8	"	6 P.M.	"	29	" 9 "
"	9	"	11 A.M.,	cord of July	1	" 8 "
"	10	"	"	"	3	" 7 "
"	11	"	"	"	5	" 6 "
"	12	"	"	"	7	" 5 "
"	13	"	"	"	9	" 4 "
"	14	"	"	"	11	" 3 "
"	15	"	"	"	13	" 2 "
"	16	"	"	"	15	" 1 day old.

The patient never manifested the slightest symptom of hydrophobia. Other similarly favourable results followed; and this prophylactic treatment of the disease quickly gained the confidence of the scientific world, which it still retains.

An important modification in the method which further experience led Pasteur to make was in the treatment of serious cases, such as multiple bites from wolves, extensive bites about the head, especially in children, cases which come under treatment at a late period of the incubation stage, and cases where the wounds have not cicatrised. In such cases the stages of the treatment are condensed. Thus on the first day, say at 11 A.M. and 4 P.M. and 9 P.M., cords of 12, 10, and 8 days respectively are used; on the second day, cords of 6, 4, and 2 days; on the third day, cords of 1 day; on the fourth day, cords of 8, 6, and 4 days; on the fifth, cords of 3 and 2 days; on the sixth, cords of 1 day; and so on for ten days. In each case the average dose is about 2 c.c. of the emulsion.

The details of the prophylactic treatment with regard to dosage and virulence of material used vary in different Pasteur institutes. The most important modification which has within



recent times taken place is the substitution by Högyes of increasing concentrations of a fairly fresh virulent rabbit's cord for emulsions of cords subjected to decreasing periods of drying. Equally good results apparently are obtained by this method, and it is stated that in cases so treated certain symptoms sometimes following the ordinary treatment, the gravest of which may be the occurrence of temporary paralyses, are not so frequently observed. This, according to Harvey and McKendrick, who have studied the subject very fully, may be due to the fact that a smaller amount of nerve tissue is injected under the Högyes system.

The success of the treatment has been very marked. The statistics of the cases treated in Paris are published quarterly in the *Annales de l'Institut Pasteur*, and general summaries of the results of each year are also prepared. As we have said, the ordinary mortality formerly was 16 per cent. of all persons bitten. During the ten years 1886-95, 17,337 cases were treated, with a mortality of 48 per cent. It has been alleged that many people are treated who have been bitten by dogs that were not mad. This, however, is not more true of the cases treated by Pasteur's method than it was of those on which the ordinary mortality of 16 per cent. was based, and care is taken in making up the statistics to distinguish the cases into three classes. Class A includes only persons bitten by dogs proved to have had rabies, by inoculation in healthy animals of parts of the central nervous system of the diseased animal. Class B includes those bitten by dogs that a competent veterinary surgeon has pronounced to be mad. Class C includes all other cases. During 1895, 122 cases belonging to Class A were treated, with no deaths; 940 belonging to Class B, with two deaths; and 449 belonging to Class C, with no deaths. Besides the Institute in Paris, similar institutions exist in other parts of France, in Italy, and especially in Russia, as well as in other parts of the world; and in these similar success has been experienced. It may be now taken as established, that a very grave responsibility rests on those concerned, if a person bitten by a mad animal is not subjected to the Pasteur treatment. Sometimes during or after treatment there appear slight paralytic symptoms with obstinate constipation and it may be retention of urine, but these usually pass off within a few weeks and leave behind no ill effects.

The principles underlying the prophylactic treatment of rabies raise questions of the highest interest from the standpoint of immunity. The prime fact is, as has been stated, the taking advantage of the long period of incubation of the disease in man to neutralise an infection which may be supposed to be gradually gathering force. We have here again to deal with an example of the reinforcement of the natural powers of resistance of the body in order to enable it to cope with a local pathological change, the locus in this case being the nervous system. We are thus unable at present to give a rational explanation of the



efficacy of the treatment, but again attention may be directed to the bearing which the development of hypersensitiveness may have to the occurrence of the phenomena of infective disease, and Harvey and McKendrick draw attention to the fact that some of the concurrent symptoms associated with the treatment closely resemble anaphylactic phenomena.

*Antirabic Serum.*—In the early part of the nineteenth century an Italian physician, Valli, showed that immunity against rabies could be conferred by administering through the stomach progressively increasing doses of hydrophobic virus. Following up this observation, Tizzoni and Centanni have attenuated rabic virus by submitting it to peptic digestion, and have immunised animals by injecting gradually increasing strengths of such virus. This method is usually referred to as the Italian method of immunisation. The latter workers showed from this that the serum of animals thus immunised could give rise to passive immunity in other animals; and further, that if injected into animals from seven to fourteen days after infection with the virus, it prevented the latter from producing its fatal effects, even when symptoms had begun to manifest themselves. They further succeeded in producing in the sheep and the dog an immunity equal to from 1-25,000 to 1-50,000 (*vide* p. 525), and they recommended the use, in severe cases, of the serum of such animals in addition to the treatment of the patient by the Pasteur method. A like serum has been obtained from animals treated by the ordinary Pasteur method.

**Methods.**—(1) *Diagnosis.*—Recent work with regard to the specificity of the Negri bodies for rabies has led to a modification in the procedure to be adopted. Formerly it was advisable if possible to keep an animal suspected of rabies alive for the observation of symptoms. While the clinical history of the animal ought to be carefully obtained, greater information will be obtained by examination of its hippocampus. The animal should therefore be killed and the brain removed after reflecting the scalp and cutting through the calvarium with a sharp chisel. The brain is laid down, vertex uppermost, and the upper parts of one hemisphere are removed in thin horizontal slices till the anterior part of the lateral ventricle is reached. The roof of the ventricle is then cut away with a probe-pointed bistoury, and the hippocampus will be recognised as the laterally arched ridge which forms the floor of the ventricle. This may be transversely incised and parts removed for the making of smears and sections (p. 576).

In addition to microscopic examination, a small piece of the



medulla or cord of the suspected animal must be taken, with all aseptic precautions, rubbed up in a little sterile .75 per cent. sodium chloride solution, and injected by means of a syringe beneath the dura mater of a rabbit, the latter having been trephined over the cerebrum by means of the small trephine which is made for the purpose. In rabies in the rabbit symptoms of paresis usually occur in from six to twenty-three days and death in fifteen to twenty-five days. When the material for inoculation has to be sent any distance, this is best effected by packing the head of the animal in ice. The virulence of organs is not lost, however, if they are simply placed in sterile water or glycerin in well-stoppered bottles.

(2) *Treatment*.—Every wound inflicted by a rabid animal ought to be cauterised with the actual cautery as soon as possible. By such treatment the incubation period will at any rate be lengthened, and therefore there will be better opportunity for the Pasteur inoculation method being efficacious. The person ought then to be sent to the nearest Pasteur Institute for treatment. It is of great importance that in such a case the nervous system of the animal should also be sent, in order that the diagnosis may be certainly verified.



## APPENDIX C.

### MALARIAL FEVER.

It has now been conclusively proved that the cause of malarial fever is a protozoon of which there are several species. They belong to the hæmosporidia (a sub-class of the sporozoa) which are blood parasites, infecting the red corpuscles of mammals, reptiles, and birds. The parasite was formerly known as the *hæmatozoon* or *plasmodium malaricæ*, although the use of the latter form is incorrect; the term *hæmamœba* is, however, now generally employed. The parasite was first observed by Laveran in 1880, and his discovery received confirmation from the independent researches of Marchiafava and Celli, and later from the researches of many others in various parts of the world. Golgi supplied valuable additional information, especially in relation to the sporulation of the organism and the varieties in different types of malarial fever. In this country valuable work on the subject was done by Manson, and to him specially belongs the credit of regarding the exflagellation of the organism as a preparation for an extra-corporeal phase of existence. By induction he arrived at the belief that the cycle of existence outside the human body probably took place in the mosquito. It was specially in order to discover, if possible, the parasite in this insect, that Ross commenced his long series of observations, which were ultimately crowned with success. After patient and persistent search, he found rounded pigmented bodies in the wall of the stomach of a dapple-winged mosquito (a species of *Anopheles*) which had been fed on the blood of a malarial patient. The pigment in these bodies was exactly similar to that in the malarial parasite, and he excluded the possibility of their representing anything else than a stage in the life cycle of the organism. He confirmed this discovery and obtained corresponding results in the case of the proteosoma infection of birds, where the parasite is closely related to that of malaria. In birds affected with this organism, he was able to trace all



stages of its development, from the time it entered the stomach along with the blood, till the time when it settled in a special form in the salivary glands of the insect. Ross's results were published in 1898. Exactly corresponding stages were afterwards found in the case of the different species of the human parasite, by Grassi, Bignami, and Bastianelli; and these with other Italian observers also supplied important information regarding the transmission of the disease by infected mosquitoes. Abundant additional observations, with confirmatory results, were supplied by Koch, Daniels, Christophers, Stephens, and others. Wherever malaria has been studied the result has been the same. Lastly, we may mention the striking experiment carried out by Manson by means of mosquitoes fed on the blood of patients in Italy suffering from mild tertian fever. The insects, after being thus fed, were taken to London and allowed to bite the human subject, Manson's son, Dr. P. Thurburn Manson, offering himself for the purpose. The result was that infection occurred; the parasites appeared in the blood, and were associated with an attack of tertian fever. Ross's discovery has not only been a means of elucidating the mode of infection, but, as will be shown below, has also supplied the means of successfully combating the disease.

From the zoological point of view the mosquito is regarded as the definitive host of the parasite, the human subject as the intermediate host. But in describing the life history, it will be convenient to consider, first, the cycle in the human body, and, secondly, that in the mosquito. Various terms have been applied to the various stages, but we shall give those now generally used.

**The Cycle in the Human Subject.**—With regard to this cycle (Plate V., Fig. 21 *a-l*), it may be stated that the parasite is conveyed by the bite of the mosquito in the form of a small filamentous cell—*sporozoite* or *exotospore*, which penetrates a red corpuscle and becomes a small amœboid organism or amœbula. There is then a regularly repeated asexual cycle of the parasite in the blood, the length of which cycle determines the type of the fever. During this cycle there is a growth of the amœbulæ or trophozoites within the red corpuscles up to their complete development; schizogony (formerly called sporulation) then occurs. The onset of the febrile attack corresponds with the stage of schizogony and the setting free of the merozoites or enhæmospores, *i.e.* with the production of a fresh brood of parasites. These soon become attached to, and penetrate into, the interior of the red corpuscles, becoming intra-corpuscular



amœbulæ; the cycle is thus completed. The parasites are most numerous in the blood during the development of the pyrexia, and, further, they are also much more abundant in the internal organs than in the peripheral blood; in the malignant type, for example, the process of schizogony is practically confined to the former.

In addition to these forms which are part of the ordinary asexual cycle, there are derived from the amœbulæ other forms, which are called *gametocytes*, or sexual cells. These remain unaltered during successive attacks of pyrexia, and undergo no further change until the blood is removed from the human body. In the simple tertian and quartan fevers (*vide infra*) the gametocytes resemble somewhat in appearance the fully developed amœbulæ before sporulation, whereas in the malignant type they have a characteristic crescent-like or sausage-shaped form; hence they are often spoken of as "crescentic bodies" (Plate V., Fig. 22, *f, g*).

The various forms of the parasite seen in the human blood may now be described more in detail.

1. *The Merozoites (Enhæmospores, Lankester)* are the youngest and smallest forms resulting from the segmentation of the adult amœbula or schizont. They are of round or oval shape and of small size, usually not exceeding  $2\ \mu$  in diameter; the size, however, varies somewhat in the different types of fever. A nucleus and peripheral protoplasm can be distinguished (Fig. 162). The former appears as a small rounded body which usually remains unstained, but contains a minute mass of chromatin which stains a deep red with the Romanowsky method, the peripheral protoplasm being coloured fairly deeply with methylene-blue. The merozoites show little or no amœboid movement; at first free in the plasma, they soon attack the red corpuscles, where they become the intra-corpuscular amœbulæ. If the blood, say in a mild tertian case, be examined in the early stages of pyrexia, one often finds at the same time schizonts, free merozoites, and the young amœbulæ within the red corpuscles.

2. *Intra-corpuscular Amœbulæ or Trophozoites.*—These include the parasites which have attacked the red corpuscles; they are at first situated on the surface of the latter but afterwards penetrate their substance. They usually occur singly in the red corpuscles, but sometimes two or more may be present together. As seen in fresh blood, the youngest or smallest forms are minute colourless specks, of about the same size as the spores; they exhibit more or less active amœboid movement, showing marked



variations in shape. The amount and character of the amœboid movement varies somewhat in different types of fever. As they increase in size, pigment appears in their interior as minute dark brown or black specks, and gradually becomes more abundant (Figs. 158, 159, Plate V., Fig. 21 *c, d, e*, Fig. 22 *e*). This pigment is elaborated from the hæmoglobin of the red corpuscles, the parasite growing at the expense of the latter. The red corpuscles thus invaded may remain unaltered in appearance (quartan fever), may become swollen and pale (tertian fever), or somewhat shrivelled and of darker tint (malignant fever). In stained specimens a nucleus may be seen in the parasite as a pale spot containing chromatin which may be arranged as a single concentrated mass or as several separated granules, the chromatin being coloured a deep red by the Romanowsky method. The protoplasm of the parasite, which is coloured of varying depth of tint with methylene-blue, shows great variation in configuration (Fig. 159). The young parasites not infrequently present a "ring-form," a portion of the red corpuscle being often enclosed by the parasite. These ring-forms are met with in all the varieties of the parasite, but they are especially common in the case of the malignant parasite, where they are of smaller size and of more symmetrical form than in the others (Fig. 163).

Within the red corpuscles the parasites gradually increase in size till the full adult form is reached (Fig. 160). In this stage the parasite loses its amœboid movement more or less completely, has a somewhat rounded form, and contains a considerable amount of pigment. In the malignant form it only occupies a fraction of the red corpuscle. The adult parasites may then undergo schizogony, but not all of them do so; some become degenerated and ultimately break down.

3. *Schizonts*.—In the process of schizogony the nuclear outline becomes lost, and the chromatin becomes divided into a number of small granules which are scattered through the protoplasm; the latter then undergoes corresponding segmentation and the small merozoites or enhæmospores result. The pigment during the process becomes aggregated in the centre and is surrounded by a small quantity of residuary protoplasm. (Schaudinn has found in the case of the tertian parasite that schizogony begins by a sort of primitive mitosis, which is then followed by simple multiple fission.) The merozoites are of rounded or oval shape, as above described, and are set free by the rupture of the envelope of the red corpuscles. The pigment also becomes free and may be taken up by leucocytes.





FIG. 157.

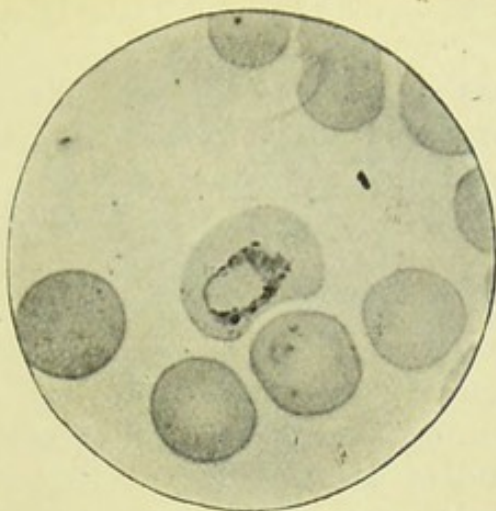


FIG. 158.

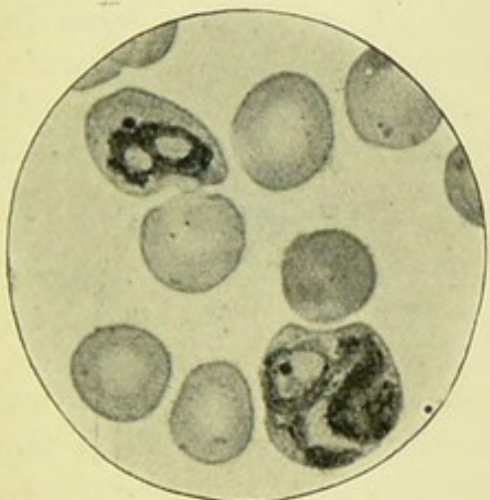


FIG. 159.



FIG. 160.

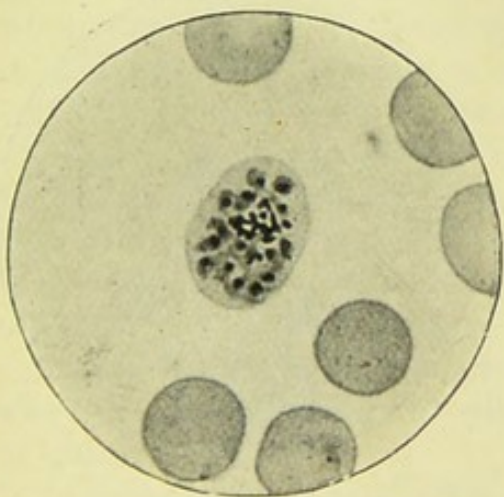


FIG. 161.



FIG. 162.

FIGS. 157-162.—Various phases of the benign tertian parasite.

FIG. 157. Several young ring-shaped amœbulæ within the red corpuscles, one of the latter enlarged and showing a dotted appearance. FIG. 158. A larger amœbula containing pigment granules. FIG. 159. Two large amœbulæ, exemplifying the great variation in form. FIG. 160. Large amœbula assuming the spherical form and showing isolated fragments of chromatin—preparatory to schizogony. FIG. 161. Schizont, which has produced eighteen merozoites, each of which contains a small collection of chromatin. FIG. 162. A number of merozoites which have just been set free in the plasma.  $\times 1000$ .



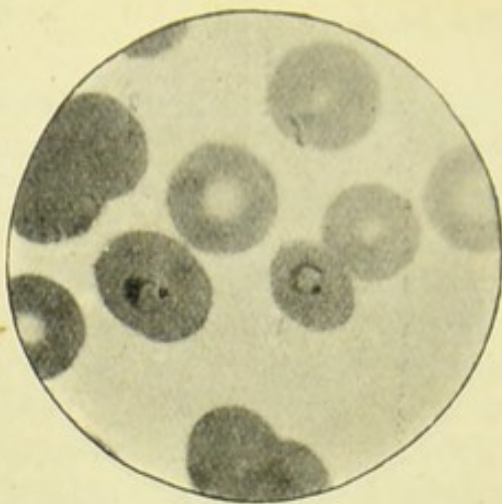


FIG. 163.



FIG. 164.



FIG. 165.



FIG. 166.



FIG. 167.

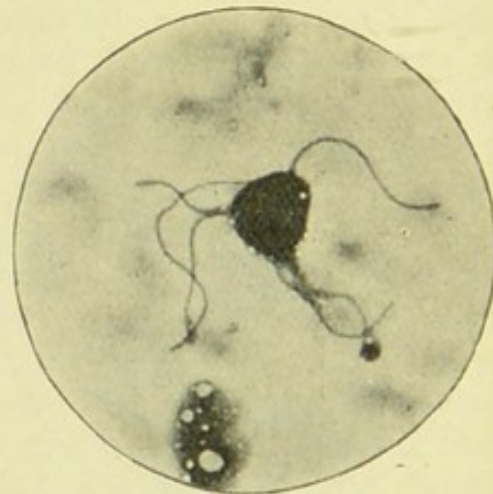


FIG. 168.

FIGS. 163-168.—Exemplifying phases of the malignant parasite.

FIG. 163. Two small ring-shaped amebulae within the red corpuscles. FIG. 164. A "crescent" or gamete showing the envelope of the red corpuscles; also an amebula. FIGS. 165-168 illustrate the changes in form undergone by the crescents outside the body. In the interior of the spherical form in FIG. 167 evidence of the flagella can be seen. FIG. 168. A male gametocyte which has undergone exflagellation, showing the thread-like microgametes or spermatozoa attached at the periphery.  $\times 1000$ . (The figures in this plate are from preparations kindly lent by Sir Patrick Manson.)



The number and arrangement of the merozoites within the schizont vary in the different types. In the quartan there are 6-12, and the segmentation is in a radiate manner, giving rise to the characteristic daisy-head appearance; in the tertian they number 15-20 or more, and have a somewhat rosette-like arrangement (Fig. 161); in the malignant there are usually 6-20 merozoites of small size and somewhat irregularly arranged.

*Gametocytes.*—As stated above, these are sexual cells which are formed from certain of the amœbulæ, and which undergo no further development in the human subject. In the mild tertian and quartan fevers they are rounded and resemble somewhat the largest amœbulæ. The female cells, *macrogametocytes*, are of large size, measuring up to  $16\ \mu$  in diameter; they contain coarse grains of pigment, and the protoplasm stains somewhat deeply with methylene-blue. The male cells, *microgametocytes*, are smaller, and the protoplasm stains faintly; the nucleus, generally in the centre, is rich in chromatin. In the malignant fevers the gametocytes have the special crescentic or sausage-shaped form mentioned above. They measure 8 to  $9\ \mu$  in length, and occasionally a fine curved line is seen joining the extremities on the concave aspect, which represents the envelope of the red corpuscle (Fig. 164). They are colourless and transparent, and are enclosed by a distinct membrane; in the central part there is a collection of pigment and granules of chromatin. The male crescents can be distinguished from the female by their appearance; in the former the pigment is less dark and more scattered through the cell, and there are several granules of chromatin; in the latter the pigment is dark and concentrated, often in a small ring, and there are one or two masses of chromatin in the centre of the crescent (Plate V., Fig. 22 *f, g*). According to the Italian observers, the early forms of the crescents are somewhat fusiform in shape and are produced in the bone-marrow. The fully developed crescents do not appear in the blood till several days after the onset of the fever, and they may be found a considerable time after the disappearance of the pyrexial attacks. They are also little, if at all, influenced by the administration of quinine.

It is well known that after a patient has apparently recovered from malarial fever a relapse may take place without fresh infection occurring, sometimes several years afterward, and Schaudinn has published interesting observations bearing on this point. He has found, and his observations on this point have been confirmed, that the macrogametocyte of tertian fever may by a process of parthenogenesis give rise to merozoites,



which in their turn infect the red corpuscles and start the cycle again. As described and figured by him, the chromatin of the macrogametocyte divides first into two portions, one of which is smaller and stains more deeply than the other. This more deeply staining portion then divides, and the protoplasm becomes segmented as in ordinary schizogony, and a young brood of parasites results. The more faintly-staining chromatin along with part of the protoplasm breaks up and disappears.

**The Cycle in the Mosquito.**—As already explained, this starts from the gametocytes. After the blood is shed, or after it is swallowed by the mosquito, two important phenomena occur, namely, (a) the full development of the sexual cells or gametocytes, and (b) the impregnation of the female (Plate V., Fig. 21 *m-y*). If the blood from a case of malignant infection be examined in a moist chamber, preferably on a warm stage, under the microscope, both male and female gametocytes may be seen to become oval and afterwards rounded in shape (Figs. 165–167). Thereafter, in the case of the male cell, a vibratile or dancing movement of the pigment granules can be seen in the interior, and soon several flagella-like structures shoot out from the periphery (Fig. 168). They are of considerable length but of great fineness, and often show a somewhat bulbous extremity. By the Romanowsky method they have been found to contain a delicate core of chromatin, which is covered by protoplasm. They represent the male cells proper, that is, they are sperm-cells or spermatozoa; they are also known as microgametes. They become detached from the sphere and move away in the surrounding fluid. In the female cell, which has also assumed the rounded form, maturation takes place by the giving off of part of the nuclear chromatin, this process corresponding to the formation of a polar body. Impregnation occurs by the entrance of a microgamete, the chromatin of the two cells afterwards becoming fused. Impregnation was first observed by McCallum in the case of halteridium, and he found that the female cell afterwards acquired the power of independent movement or became a “travelling vermicule.” He also observed the impregnation of the malignant parasite. The fertilised female cell is now generally spoken of as a *zygote* or *oökinete*.

It has been established that the phenomena just described occur within the stomach of the mosquito, and that the fertilised cell or zygote penetrates the stomach wall and settles between the muscle fibres; on the second day after the mosquito has ingested the infected blood small rounded cells about 6 to 8  $\mu$  in diameter, and containing clumps of pigment, may be found in



this position. (It was in fact the character of the pigment which led Ross to believe that he had before him a stage in the development of the malarial parasite.) A distinct membrane called a *sporocyst* forms around the zygote, and on subsequent days a great increase in size takes place, the cysts coming to project from the surface of the stomach into the body cavity. The zygote divides into a number of cells called *blastophores* or *sporoblasts*, and these again divide and form a large number of filiform cells which have a radiate arrangement; these were called by Ross "germinal rods," but are now usually known as *sporozoites* or *exotospores* (in contradistinction to the *enhæmospores* of the human cycle). The full development (*sporogony*) within the sporocyst occupies, in the case of *proteosoma*, about seven days, in the case of the malarial parasites a little longer. When fully developed the cyst measures about  $60\ \mu$  in diameter, and appears packed with sporozoites. It then bursts, and the latter are set free in the body cavity. A large number settle within the large veneno-salivary gland of the insect, and are thus in a position to be injected along with its secretion into the human subject. The sporozoites enter red corpuscles and become amœbulæ as above described. Daniels found that in the case of the malignant parasite an interval of twelve days at least intervened between the time of feeding the mosquito and the appearance of the sporozoites in the gland.

It will thus be seen that in the human subject the parasite passes through an indefinite number of regularly recurring asexual cycles, with the giving off of collateral sexual cells, and that in the mosquito there is one cycle which may be said to start with the impregnation of the female gamete.

**Varieties of the Malarial Parasite.**—The view propounded by Laveran was that there is only one species of malarial parasite, which is polymorphous, and presents slight differences in structural character in the different types of fever. It may, however, now be accepted that there are at least three distinct species which infect the human subject. Practically all are agreed as to a division into two groups, one of which embraces the parasites of the milder fevers—"winter-spring" fevers of Italian writers—there being in this group two distinct species, for the quartan and tertian types respectively; whilst the other includes the parasites of the severer forms—"æstivo-autumnal" fevers, malignant or pernicious fevers of the tropics, or irregularly remittent fevers. There is still doubt as to whether there are more than one species in this latter group. Formerly Italian writers distinguished (1) a quotidian; (2) a non-pig-



mented quotidian; and (3) a malignant tertian parasite, though the morphological differences described were slight. Further observations have, however, thrown doubt on this distinction, and the evidence rather goes to show that there is a single species. Opinion also varies as to the cycle of this parasite; according to some observers it is twenty-four hours, according to others forty-eight hours, though there is more evidence in support of the latter view; and the term "malignant tertian" is frequently used. The fever is often of an irregular type and multiple infection is probably common. Although the question cannot be considered as finally settled, we shall speak of three species of human parasites. The zoological position may be shown by the following scheme, generally followed by English writers, the terminology being chiefly that of Grassi and Feletti:—

Family: HÆMAMÆBIDÆ (Wasielewski).

Genus I. *Hæmamæba*. The mature gametes resemble in form the schizonts before segmentation has occurred.

Species 1. *Hæmamæba Danilewski* or *halteridium*.

Parasite of pigeons, crows, etc.

Species 2. *Hæmamæba relictæ* or *proteosoma*.

Parasite of sparrows, larks, etc.

Species 3. *Hæmamæba malaria*.

Parasite of quartan fever of man.

Species 4. *Hæmamæba vivax*.

Parasite of tertian fever of man.

Genus II. *Hæmomenas*. The gametocytes have a special crescentic form.

Species: *Hæmomenas præcox*.

Parasite of malignant or æstivo-autumnal fever of man.

In addition there are other species belonging to the same family of blood parasites, which infect monkeys, bats, frogs, lizards, etc., especially in malarial regions.

We shall now give the chief distinctive characters of the three human parasites:—

1. *Parasite of Quartan Fever*.—The cycle of development in man is seventy-two hours, and produces pyrexia every third day; double or triple infection may, however, occur. In fresh specimens of blood the outline is more distinct than that of the tertian parasite, and amœboid movement is less marked. Only the smaller forms show movement, and this is not of active character. The infected red corpuscles do not become altered



in size or appearance, and the pigment within the parasite is in the form of coarse granules, of dark brown or almost black colour. The fully developed schizont has a "daisy-head" appearance, dividing by regular radial segmentation into from six to twelve merozoites, which, on becoming free, are rounded in form.

2. *The Parasite of Mild Tertian Fever.*—The cycle of development is completed in forty-eight hours, though a quotidian type of fever may be produced by double infection. The amœbulæ have a less refractile margin than in the quartan type, and are thus less easily distinguished in the fresh blood; the amœboid movements are, however, much more active, while longer and more slender processes are given off. The infected corpuscles become swollen and pale, and may show deeply stained points by the Romanowsky method—"Schüffner's dots." The pigment within the parasite is fine and of yellowish-brown tint. The mature schizont is rather larger than in the quartan, has a rosette appearance, and gives rise to from fifteen to twenty merozoites, though sometimes even more occur; these have a somewhat oval shape.

In both the quartan and tertian fevers all the stages of development can be readily observed in the peripheral blood. The gametocytes have a rounded form as described above.

3. *The Parasite of Malignant or Æstivo-autumnal Fever or Tropical Malaria.*—The cycle in the human subject probably occupies forty-eight hours, though this cannot be definitely stated to be always the case (*vide supra*). The amœbulæ in the red corpuscles are of small size, and their amœboid movements are very active; they often, however, pass into the quiescent ring form (Fig. 163). The pigment granules, even in the larger forms, are few in number and very fine; the infected red corpuscles have a tendency to shrivel and assume a deeper or coppery tint, sometimes they are swollen and decolorised. The fully developed schizont usually occupies less than half the red corpuscle, and gives rise to from six to twenty merozoites, somewhat irregularly arranged and of minute size. Schizogony takes place almost exclusively in the internal organs, spleen, etc., so that, as a rule, no sporocytes can be found in the blood taken in the usual way. The proportion of red corpuscles infected by the amœbulæ is also much larger in the internal organs. The gametocytes have the crescentic form, as already described.

Cases of infection with the malignant parasite sometimes assume a pernicious character, and then the number of organisms in the interior of the body may be enormous. In certain fatal



cases with coma the cerebral capillaries appear to be almost filled with them, many parasites being in process of sporulation; and in so-called algid cases, characterised by great collapse, a similar condition has been found in the capillaries of the omentum and intestines. The process of blood destruction, present in all malarial fevers, reaches its maximum in the malignant class, and the brown or black pigment elaborated by the parasites—in part after being taken up by leucocytes, chiefly of the mononuclear class—becomes deposited in various organs, spleen, liver, brain, etc., especially in the endothelium of vessels and the perivascular lymphatics. In the severer forms also brownish yellow pigment is apparently derived from liberated hæmoglobin, and accumulates in various parts, especially in the liver cells; most of this latter gives the reaction of hæmosiderin.

**General Considerations.**—The development of the malarial parasites in the mosquito and infection of the human subject through the bites of this insect, have, by the work of Ross and others, as detailed above, become established scientific facts. These facts, moreover, point to certain definite methods of prevention of infection, which have to a certain extent already been practically tested. The extensive observations recently carried out go to show that all the mosquitoes which act as hosts of the parasite belong to the genus *anopheles*; of these there are a large number of species, and in at least eight or nine the parasite has been found. Some of these anopheles occur in England, especially in regions where malaria formerly prevailed. The opportunity for infection from cases of malaria returning from the tropics to this country thus exists, and such infection has occurred. The breeding places of the insects are chiefly, though not exclusively, in stagnant pools and other collections of standing water, and accordingly the removal, where practicable, by drainage of such collections in the vicinity of centres of population, the covering in of wells, etc., and the killing of the larvæ by petroleum sprinkled on the water, have constituted the most important measures in localised areas. This procedure has been carried out in various places, for example, in Freetown and Ismailia, with marked success. On the other hand, where there are large populous areas, as in India, it has been found almost impracticable to carry out these measures with any success. Another measure is the protection against mosquito bites by netting, it being fortunately the habit of the anopheles to rarely become active before sundown. The experiments of Sambon and Low in the Campagna proved that individuals using these means of protection may live in a highly malarial district with-



out becoming infected. The administration of quinine to persons living in highly malarial regions, in order to *prevent* as well as to treat infection, has also been recommended and carried out, and the general agreement appears to be that in India the properly controlled administration of quinine must, in the meantime at least, be the chief means of combating the disease. In the tropics the natives in large proportion suffer from malarial infection, and one would accordingly expect that infection of the mosquitoes in the neighbourhood of native settlements would be common. This has been found to be actually the case, and it has accordingly been suggested that the dwellings of whites should as far as possible be at some distance from the native centres of population.

So far as is known, none of the lower animals have been found to take the place of man as intermediate host to the parasites of malaria, but the possibility of such being the case cannot be as yet definitely excluded. On the death of infected mosquitoes the exotospores or sporozoites will become set free, and therefore theoretically there is a possibility that they may enter the human subject by inhalation or by some other means. We have no facts, however, to show that this really occurs, and the evidence already obtained establishes the bites of mosquitoes as the most important if not the only mode of infection.

It may also be mentioned as a scientific fact of some interest, though not bearing on the natural modes of infection, that the disease can also be communicated from one person to another by injecting the blood containing the parasites. Several experiments of this kind have been performed (usually about  $\frac{1}{2}$  to 1 c.c. of blood has been used), and the result is more certain in intravenous than in subcutaneous injection. In such cases there is an incubation period, usually of from seven to fourteen days, after which the fever occurs; the same type of fever is reproduced as was present in the patient from whom the blood was taken.

**The Pathology of Malaria.**—While much work has been done on the malarial parasite, relatively less attention has been directed to the processes by which it produces its pathogenic effects. It may be said that the organisms are not always equally prevalent in the circulating blood, and probably at certain stages tend to be confined in the solid organs; thus they may be scanty at the height of the paroxysm. Some of the pathogenic effects are probably associated with particular stages in the life cycle. Thus the pyrexia occurs when the stage of schizogony is actively in progress. No opinion can be stated,



however, as to the cause of the fever,—whether it is due to a toxic process or to general disturbance of metabolism. We can better explain the anaemia which is so pronounced in cases where the disease is of long standing, and which is due to the actual destruction of red blood corpuscles. The parasite in its sojourn in these cells absorbs their pigment and thus destroys their function; this is further evidenced by the activity displayed by the red marrow in its attempts to make good the loss sustained by the blood. One of the most interesting events in malaria, and one that links it with bacterial infections, is the reaction of the colourless cells of the blood. It has been shown that during the apyrexial stages the total number of leucocytes may be diminished, but that there is always an increase of the mononuclear cells, these frequently numbering 20 per cent. of the whole, and sometimes even outnumbering the polymorphs. This is such an important feature that in cases where the parasites themselves cannot be demonstrated in the blood, the mononuclear reaction along with the presence of pigment in the mononuclear cells (due to phagocytosis of pigmented parasites), has been taken as evidence that the case is really one of malaria. The mononuclear reaction is specially interesting from the fact that in other protozoal diseases an activity of the same elements has been observed.

The question of the possibility of immunity to malaria being developed naturally arises, and this is especially interesting in the light of the leucocytic reaction which we have seen must be looked on as an element in immunity against bacterial infection. With regard to Europeans developing immunity, it is difficult to speak. In such a malaria-stricken region as the West Coast of Africa, the death-rate in residents of more than four years' standing is less than in the previous years, but this may be due to the survival of the more resistant immigrants. But there can be little doubt that malaria in the negro is a much less serious condition than in the European. Koch from his observations in New Guinea attributes this to the infection of the native children leading to the development of immunity in the adult community. He found, what has been independently noted by Stephens and Christophers in West Africa, that the greater number of the children harboured malarial parasites in their blood. The widespread presence of parasites in children might appear to preclude the immunity of the adult being due to survival of the most resistant, but the infant mortality in these regions may be very high, and such a survival may be the real explanation. On the other hand, Koch states that while an immunity appears to exist



in native adults in malarial districts, this is only true of those born in the locality,—natives coming from neighbouring non-malarial districts into the malarial region being liable to contract the disease. At present it must be held that the facts available do not enable us to determine the relative parts played by the development of artificial immunity on the one hand, and the existence of a natural immunity on the other, in apparent insusceptibility to malaria.

Our knowledge on the relationship of blackwater fever to malaria is also in an unsatisfactory condition. Blackwater fever is a condition often occurring, especially in Europeans, in tropical countries. It is characterised by pyrexia, darkly-coloured urine,—the colour being due to altered hæmoglobin pigment,—delirium and collapse, frequently ending in coma and death. By some the condition has been looked on as a separate disease, by others as the terminal stage of a severe malaria. With regard to the former view no special parasite has yet been demonstrated. Stephens sums up the evidence for the second view by saying that malaria, apart from the occurrence of blackwater fever, is a relatively non-fatal disease; that in the great majority of cases there is direct or indirect evidence of the subject of the condition having suffered from repeated attacks of malaria; that while in all cases there must be an agent at work causing hæmolysis, there is evidence that in many cases there is the possibility of that agent being quinine. In a recent important work, Christophers and Bentley come to the conclusion that the essential feature in blackwater fever is an extra-cellular destruction of red corpuscles in the blood plasma, a lysæmia as they call it, but that this is not directly due to parasitic, osmotic, or chemical actions, but to a specific hæmolysin arising in the body as the result of the repeated blood destruction. They have shown, for example, that the addition of fresh serum (complement) to the red corpuscles of blackwater fever, as well as of malarial, patients may produce lysis, this apparently being due to a substance corresponding to immune-body united to the corpuscles in question. The development of this hæmolysin (autolysin) results from the extensive and repeated destruction of red corpuscles by the malarial parasite. Thus though the latter is not the immediate cause of the lysæmia, which is the essential feature of blackwater fever, it is the means of inducing the development of the hæmolysin. If this view of the process is found to be correct, it would of course explain the relationship of malaria to the condition. They also consider that in the conditions mentioned, *i.e.* where there has been repeated destruction of an



individual's corpuscles by the malarial parasite, the occurrence of lysæmia may be precipitated by an acute attack of malaria especially when under certain circumstances this is associated with the administration of quinine. On this view, however, it still remains to be determined whether the lysis at the onset of an attack of blackwater fever is due to a sudden liberation of complement or to some other cause.

**Methods of Examination.**—The parasites may be studied by examining the blood in the fresh condition, or by permanent preparations. In the former case, a slide and cover-glass having been thoroughly cleaned, a small drop of blood from the finger or lobe of the ear is caught by the cover-glass, and allowed to spread out between it and the slide. It ought to be of such a size that only a thin layer is formed. A ring of vaseline is placed round the edge of the cover-glass to prevent evaporation. For satisfactory examination an immersion lens is to be preferred. The amœboid movements are visible at the ordinary room temperature, though they are more active on a warm stage. With an Abbé condenser a small aperture of the diaphragm should be used.

Permanent preparations are best made by means of dried films, which films are fixed by one of the methods already given (p. 94), or by placing in absolute alcohol for five minutes (Manson). The films thus prepared and fixed may be stained for two or three minutes in a saturated watery solution of methylene blue or in carbol-thionin-blue (p. 105); the solutions must be carefully filtered (especially the latter), and the films must be washed well after staining. They are then dried and mounted in balsam. In the case of thionin-blue, sharper results are obtained by dehydrating in alcohol and clearing in xylol before mounting. The best results are, however, obtained by one of the Romanowsky methods as described on p. 113.

The fact that in many cases the parasites may be few in number led Ross to devise a method for making their recognition more easy by using blood films of unusual thickness. Here about as much blood as is used in a hæmoglobin determination (20 c.mm.) is taken on a slide, and, being spread out only so much as to occupy the area of an ordinary cover-glass, is allowed to dry. There is then dropped on it by means of a glass rod a little of the watery eosin used in making up the Romanowsky dye (*vide* p. 113). This is allowed to act for about a quarter of an hour, and then very gently washed off with distilled water. The Romanowsky methylene-blue solution is then applied for a few seconds and also carefully washed off, and the preparation



dried and mounted. The hæmoglobin of the red corpuscles is washed out by the eosin solution, and the smaller forms of the malarial parasite stand out as round circles containing the characteristic chromatin dots; and in consequence of the greater number present in a given area as compared with an ordinary preparation their recognition is very easy. For the large forms of the parasite Ross has found it useful to make such a film and, hæmolysing the red cells with distilled water, to examine it unstained. The presence of pigment in the parasites enables them to be readily seen.



## APPENDIX D.

### AMŒBIC DYSENTERY.

IN a previous chapter it has been pointed out that the term "dysentery" has been applied to a number of conditions of different etiology, and the relations of bacteria as causal agents have been there discussed (*vide* p. 384). We shall here consider that variety of tropical dysentery which is believed to be due to an amœba, and hence often known as *amœbic dysentery*.

Amongst the early researches on the relation of organisms to dysentery probably the most important are those of Lösch, who noted the presence and described the characters of amœba in the stools of a person suffering from the disease, and considered that they were probably the causal agents. Further observations on a more extended scale were made by Kartulis with confirmatory results, this observer finding the same organisms also in liver abscesses associated with dysentery. Councilman and Lafleur, working in Baltimore, showed that this variety of dysentery can be distinguished from other forms, not only by the presence of amœbæ, but also by its pathological anatomy. The intestinal lesions, to which reference is made below, are of a grave character, mortality is relatively high, and recovery, when it occurs, is protracted on account of the extensive tissue changes. The subject was, however, complicated by the fact that a similar organism—the *amœba coli*—had been previously found in the intestine in normal conditions and in other diseases than dysentery (by Cunningham and Lewis and others), and additional research confirmed these results. The matter is still far from being satisfactorily cleared up. While we may say that the pathogenic rôle of amœbæ has been established, much remains to be done in determining what species have pathogenic properties and how these species may be identified. The characters of the common amœba of the colon and an amœba of dysentery were carefully worked out by Schaudinn, who recognised them to be quite distinct species, and gave to them



the names of *entamœba coli* and *entamœba histolytica* respectively. We shall give the chief points in his description, but it must be kept in view that amœbæ of dysentery studied by others present differences in character. To these also reference will be made below.

*Entamœba histolytica*, as seen in dysenteric stools, occurs in the form of rounded, oval, or pear-shaped cells, measuring 12 to 50  $\mu$  in diameter (Fig. 169, and Plate VI., Fig. 23). Considerable variations in size are met with in different cases of dysentery; in some acute cases few amœbæ may exceed 20  $\mu$  in diameter. When at rest, a somewhat clear, highly

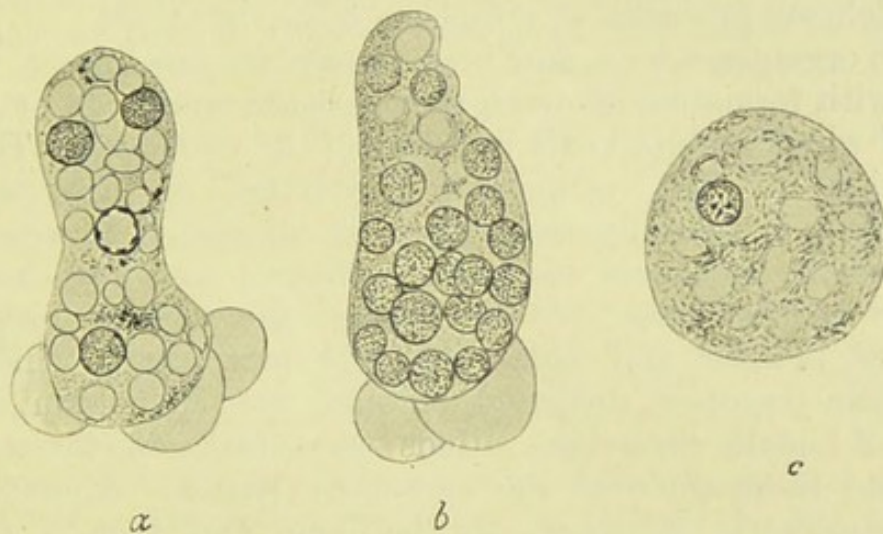


FIG. 169.—Amœbæ of dysentery.

*a* and *b*, amœbæ as seen in the fresh stools, showing blunt amœboid processes of ectoplasm. The endoplasm of *a* shows a nucleus, three red corpuscles, and numerous vacuoles; that of *b*, numerous red corpuscles and a few vacuoles.

*c*, an amœba as seen in a fixed film preparation, showing a small rounded nucleus (Kruse and Pasquale).  $\times 600$ .

refractile ectoplasm and a granular endoplasm can be distinguished, a feature which differentiates the organism from the *entamœba coli*. The nucleus is rounded or oval, and is seen with difficulty; its position is usually excentric, and is sometimes quite at the margin of the ectoplasm. In the fresh condition, and especially when examined on a warm stage, the organism shows very active amœboid movements. The pseudopodia, which are quickly protruded and retracted, are blunt and appear to be of tough consistence, a property which Schaudinn considers of importance, as enabling the organism to penetrate the mucous membrane, etc. The amœbic movements are often of an active kind, and locomotion may be



fairly rapid; and red corpuscles, bacteria, cells, etc. may often be seen in the interior, though the ingestion of red corpuscles is by no means a constant feature. The organism usually dies and undergoes disintegration in a comparatively short time after being removed from the body; the stools ought therefore to be examined in as fresh a state as possible. Multiplication takes place by simple amitotic division and also by budding. The *entamoeba coli* is an organism of about the same size. When at rest it shows no differentiation into ectoplasm and endoplasm, and the nucleus, usually situated in the centre, shows a highly refractile membrane with chromatin masses scattered in the interior. During amœboid movement some delicate processes of ectoplasm come into view.

Both organisms have now been shown to pass into a resting stage with formation of cysts, the character and mode of formation of which are markedly different in the two cases. The cyst formation of the *entamoeba histolytica*, as described by Schaudinn, is specially seen when the disease is in process of cure and the stools are beginning to have a less fluid character. In the earliest stage of the change the nuclear membrane becomes broader and fades into the protoplasm, whilst the chromatin becomes dispersed through the endoplasm in the form of small chromidia. Buds then form on the surface, and into these some of the chromatin passes. Around these buds concentric striation can be seen, and then a hyaline cyst wall is formed, which is highly refractile in character. The cyst then becomes separated from the rest of the cell. Several cysts which measure 2 to 7  $\mu$  in diameter may be formed from the same amœba, and the remnant of the cell undergoes disintegration. These cysts, as will be shown below, represent a resting-stage with high powers of resistance to external agencies, and are concerned in producing infection of another subject. The cellular changes in the encysting of the *entamoeba coli* have also been worked out by Schaudinn. They are of a somewhat complicated character, involving the formation of reduction bodies and copulation of nuclei, but the ultimate result is the formation of a fairly large cyst, which contains eight small cells. The process of cyst formation accordingly in the two organisms is of a widely different character.

The description of the encystment of amœbæ from cases of dysentery as given by some other observers differs considerably from that of Schaudinn. In fact, in the majority of the investigations published no process of encystment of buds on the surface of the amœba has been observed; on the contrary, the



whole cell becomes enclosed in a cyst, which is of considerable size. The facts already ascertained point strongly to there being more than one pathogenic species which have not yet been satisfactorily distinguished.

The whole subject of the classification and means of distinguishing the species of pathogenic and non-pathogenic amœbæ is still in a very unsatisfactory state, and much further work is necessary. We may, however, refer to some of the facts recorded. Musgrave and Clegg, working in Manila, cultivated amœbæ from drinking water and from various other external sources as well as from cases of dysentery, and found that they possessed similar characters. The cysts as shown in their photographs are of fairly large size, and do not correspond to Schaudinn's description. By means of amœbæ, cultivated from sources apart from dysentery, they were able to produce dysenteric symptoms and lesions in monkeys, Lesage cultivated amœbæ from cases of dysentery in Saigon and Toulon, and found that the process of encystment as studied in agar plates agreed with the account given by Schaudinn. Craig, as the result of studies on amœbæ in San Francisco, confirms the work of Schaudinn with regard to *E. coli* and *E. histolytica*. Viereck found an amœba in two cases in Hamburg which resembled *E. coli*, from which, however, it differed in its cysts containing only four cells. He gave to it the name *E. tetragena*. Hartmann found the same organism in African dysentery, and was able by means of it to produce dysentery in cats, though the disease was milder than with *E. histolytica*. Noc, working in Cochin-China, cultivated amœbæ from the intestines in dysentery, from liver abscesses and from drinking water, and found that they all had the same characters. The process of encystment was different from that described by Schaudinn, the whole cell becoming enclosed by the cyst. In addition to the ordinary method of fission, it formed numerous small cells or merozoites by a process of budding within the protoplasm; these afterwards becoming free.

*Cultivation.*—Various attempts have been made to cultivate the amœba of dysentery, and Kartulis considered that he obtained growth in straw infusions. Within recent years cultures of amœbæ in association with various bacteria have been obtained on agar media by various workers, *e.g.* Lesage, Musgrave and Clegg, Noc, and others. For this purpose a plain agar without peptone is used, and its reaction is made distinctly alkaline to phenolphthaleine. The presence of bacteria seems to be essential for the growth of the amœbæ, and it is found that some species favour growth whilst others act prejudiciously; amongst the former may be mentioned the *sp. cholerae*, *b. subtilis*, and various members of the *coli* group, though organisms from a great variety of sources have been found to be equally efficient.

In such cultures, which are most conveniently made in Petri dishes, the stages of growth and encystment of the amœbæ can be readily studied; the organisms seem to flourish best at a



temperature of about  $25^{\circ}$  C. Although cultures without bacterial growth have not been obtained, means have been devised to ensure that only one species of amœba is present. For this purpose Musgrave and Clegg select, by means of a low-power objective, an amœba well separated on the agar plate, place it in the middle of the field, then swing into position a high-power objective, and, having ascertained by means of it that the amœba is still there, lower the point of the lens on to the agar. By this means the amœba may have been picked up, and it may then be transferred to a fresh plate. These observers consider suitable bacterial symbiosis to be of great importance in increasing the virulence of the amœbæ, and probably to play an important part in the pathology of the disease.

**Distribution of the Amœbæ.**—As already stated, they are usually found in large numbers in the contents of the large intestine in tropical amœbic dysentery. They also, however, penetrate into the tissues, where they appear to exert a well-marked action. In this disease the lesions are chiefly in the large intestine, especially in the rectum and at the flexures, though they may also be present in the lower part of the ileum. At first there are seen local swellings on the mucous surface, chiefly due to a sort of inflammatory gelatinous œdema with little leucocytic infiltration; soon, however, the mucous membrane becomes partially ulcerated, more or less extensive necrosis of the subjacent tissues occurs, and gangrenous sloughs result. The ulcers thus come to have irregular and overhanging margins, and the excavation below is often of wider extent than the aperture in the mucous membrane. The amœbæ are found in the mucous membrane when ulcers are being formed, but their most characteristic site is beyond the ulcerated area, where they may be seen penetrating deeply into the submucous and even into the muscular coats. In these positions they may be unattended by any other organisms, and the tissues around them show œdematous swelling and more or less necrotic change without much accompanying cellular reaction beyond a certain amount of swelling and proliferation of the connective tissue cells. This action of the amœbæ on the tissues explains the character of the ulcers as just described. These lesions are considered to be characteristic of amœbic dysentery.

As a complication of this form of dysentery, liver abscesses are of comparatively common occurrence. They are usually single and of large size; sometimes there are more than one, and occasionally numerous small ones may be present. The contents are usually a thick pinkish fluid of somewhat slimy consistence,



and are largely constituted by necrosed and liquefied tissue with admixture of blood in varying amount. Microscopic examination shows chiefly necrosed and granular cells and débris resulting from their disintegration, whereas ordinary pus corpuscles are scanty or may be practically absent. In such abscesses associated with dysentery the amœbæ are usually to be found, and not infrequently are the only organisms present, no cultures of bacteria being obtainable by the ordinary methods (Fig. 170). They are most numerous at the spreading margin, and this probably explains a fact pointed out by Manson, that examination of the contents first removed may give a negative result, while they may be detected in the discharge a day or two later. The action here on the tissues is of an analogous nature, namely, a necrosis with softening and partial liquefaction, attended by little or no suppurative change. The amœbæ have also been found in the sputum when a liver abscess has ruptured into the lung, as not very infrequently happens. Kartulis records two cases of brain abscess occurring secondarily to dysentery in which numerous amœbæ were present.

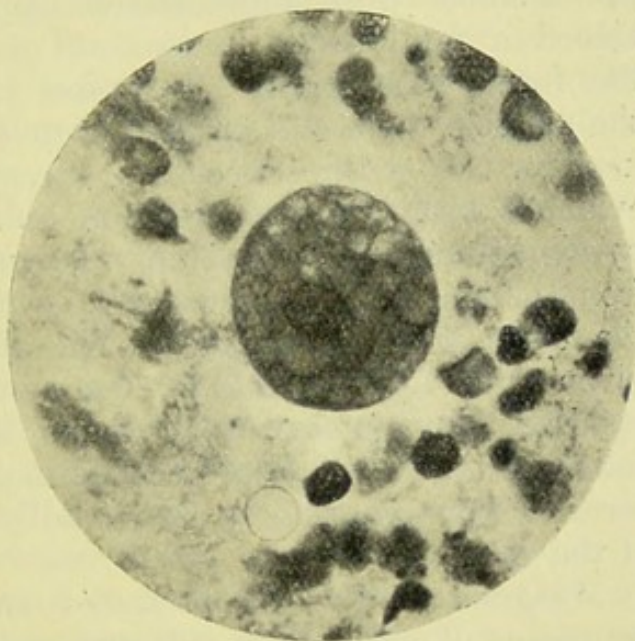


FIG. 170.—Section of wall of liver abscess, showing an amœba of spherical form with vacuolated protoplasm. From a case published by Major D. G. Marshall.  $\times 1000$ .

**Experimental Inoculation.**—The anatomical changes in dysentery, as above described, give strong presumptive evidence as to the causal relationship of the amœbæ, and practically conclusive evidence is afforded by animal experiments. Dysentery occurs occasionally in animals, *e.g.* in monkeys, but it is of comparatively rare occurrence. The disease sometimes results in the dog by experimental inoculation with dysenteric material. Kartulis, for example, records two cases, in one of which liver abscess was present. Cats are, however, found to be more susceptible, especially young animals. Dysenteric changes have been produced in this animal by Kartulis, Kruse and Pasquale, and others. The method generally adopted is the introduction



of a small quantity of mucus from a dysenteric case into the rectum. The resulting disease is of an acute character, and sometimes leads to a fatal result. The changes in the large intestine resemble those found in the human disease, and microscopic examination shows the amœbæ penetrating the wall of the bowel in the characteristic manner. Kruse and Pasquale obtained corresponding results when the material from a liver abscess, containing amœbæ without any other organisms, was injected. Quincke and Roos obtained no effects when the amœbæ were administered by the mouth, but they obtained a fatal result in two out of four cases when the cyst-like forms were given. They also found that the cysts, unlike the amœbæ, were still present even after the material had been kept for two or three weeks. Extremely important confirmatory evidence with regard to infection by the cysts has been supplied by experiments of Schaudinn. Dysenteric material was obtained from China, and portions of it which were found to contain the cysts were thoroughly dried. Some of this material was given with food to cats by the mouth, and typical dysentery resulted, the amœbæ being found in the stools. No results follow when the material ingested merely contains the vegetative form of the organism, as it is readily destroyed in the contents of the stomach.

Musgrave and Clegg produced amœbic colitis in monkeys by means of cysts from cultures, and such results were obtained whatever was the source of the amœbæ,—that is, with those obtained from water, vegetables, etc., as well as with those from dysenteric material. They also produced liver abscess by direct injection into the liver, and in some instances only amœbæ were present in the abscesses.

Investigations with regard to *entamœba coli* seem to show that it is a harmless organism and that it is frequently present in the intestines of healthy individuals. Schaudinn found that in East Prussia as many as 50 per cent. of the population were infected with it. The administration of the amœbæ, or of the cysts by the methods mentioned above, produced no result in animals. It has, however, been shown that when the eight-celled cysts are swallowed by persons who are free from the parasite the *entamœba coli* appears in the large intestine in a comparatively short period of time. It accordingly appears that in the case of both organisms it is the cysts alone which give rise to infection. Confirmatory results with regard to the common occurrence of *E. coli* were obtained by Craig in San Francisco.



From the above facts, all of which have received ample confirmation, there can be no doubt that the amœbæ described are the cause of the form of dysentery with which they are associated. As already stated, much information is still required as to the different species of pathogenic amœbæ and as to the means of distinguishing them, if this is possible, from harmless forms. In this connection it is interesting to note that Musgrave and Clegg obtained pathogenic effects with amœbæ resembling the *E. coli*. But the causal relationship of amœbæ to dysentery has been completely established by the anatomical and experimental evidence. It is also of importance to note that the serum of patients suffering from amœbic dysentery gives no agglutinating reaction with Shiga's bacillus of dysentery (*vide* p. 384).

It is important to note that cases of amœbic dysentery have been recorded both in France and England in patients who have never resided outside these countries.

**Methods of Examination.**—The fæces in a case of suspected dysentery ought to be examined microscopically as soon as possible after being passed, as the amœbæ disappear rapidly, especially when the reaction becomes acid. A drop is placed on a slide without the addition of any reagent, a cover-glass is placed over it but not pressed down, and the preparation is examined in the ordinary way or on a hot stage, preferably by the latter method, as the movements of the amœbæ become more active, and it is difficult to recognise them when they are at rest. Hanging-drop preparations may also be made by the methods described. Dried films are not suitable, as in the preparation of these the amœbæ become broken down; but wet films may be fixed with corrosive sublimate or other fixative (*vide* p. 96). In sections of tissue the amœbæ may be stained by methylene-blue, by safranin, by hæmatoxylin and eosin, and iron hæmatoxylin, etc. Benda's method of staining with safranin and light-green is also a very suitable one. Sections are stained for several hours in a saturated solution of safranin in aniline oil water (p. 105), they are then washed in water and decolorised in a  $\frac{1}{2}$  per cent. solution of light-green in alcohol till most of the safranin is discharged, the nuclei, however, remaining deeply stained. In this method the nuclei of the amœbæ are coloured red (like those of the tissue cells), the protoplasm being of a purplish tint.



## APPENDIX E.

### TRYPANOSOMIASIS—LEISHMANIOSIS—PIRO- PLASMOSIS.

#### THE PATHOGENIC TRYPANOSOMES.

THE trypanosomata are protozoal organisms belonging to the sub-class Flagellata, and many members of the genus have come to be recognised as living in the blood and tissues in various animals and as causing important disease conditions. As long ago as 1878 the *Trypanosoma Lewisi* was observed infesting the blood of rats, and it has been found to be sometimes capable of causing death. Other diseases in which similar organisms have been found are Surra, which occurs in cattle, horses, and camels in India, and which is associated with the *Tr. Evansi*; Dourine, a condition affecting horses in especially the Mediterranean littoral (*Tr. equiperdum* or *Rougeti*); Mal de Caderas, a disease of South American horses (*Tr. equinum* or *Elmassiani*); Tse-tse Fly Disease or Nagana, affecting horses and herbivora in South Africa (*Tr. Brucei*); trypanosomiasis of African cattle (*Tr. Theileri*); and—most important from the human standpoint—the trypanosomiasis and sleeping sickness of West and Central Africa associated with the *Tr. gambiense* and *Tr. ugandense*, which are now believed to be the same organism. These diseases present many general resemblances to one another. They tend to be characterised by wasting, cachexia, anæmia, fever often of an intermittent type and irregular œdemas, and frequently have a fatal result. In many cases the infective agent is conveyed from a diseased to a healthy animal by the agency of blood-sucking insects.

**Morphology and Biology of the Trypanosomata.**—If a drop of blood containing trypanosomes be examined, the organism will be seen to be a fusiform mass of protoplasm which at one end passes into a pointed flagellum. In the living condition the trypanosome is usually actively motile by an undulatory move-



ment of its protoplasm and a lashing of the flagellum. The size varies, but those mentioned above are about  $30\ \mu$  long and about  $1.5$  to  $3\ \mu$  broad. Much smaller forms exist, however, and one, *Tr. ingens*, which is  $7$  to  $10\ \mu$  broad and  $72$  to  $123\ \mu$  long, has been described by Bruce. From the fact that in progression the flagellum is in front, the flagellated end is denominated the anterior end of the organism. It is stated that the method of examining the fresh blood by merely allowing it to spread itself out in a fairly large drop beneath a cover-glass is more likely to reveal the presence of trypanosomes, if these are present in small numbers, than is the examination of stained specimens; but the minuter structure of the organisms can best be studied in dried preparations stained by Romanowsky dyes such as those of Leishman or Giemsa.

For staining trypanosomata (or the Leishman-Donovan bodies) *in sections* so as to bring out the chromatin structures, Leishman recommends the following method:—Sections of  $5\ \mu$  thickness are made and carefully fixed on slides. The paraffin is very thoroughly removed by melting it before applying the first xylol, and then washing with alternate baths of alcohol and xylol three or four times. The last alcohol is thoroughly washed off by distilled water, and the excess of water is removed with cigarette paper. A drop of fresh blood serum is then placed on the preparation and allowed to soak in for five minutes. The excess is removed by blotting, and the remainder is allowed to dry on the section, which is now treated with a mixture of two parts of Leishman's stain and three of distilled water, and placed in a Petri dish for  $1$  to  $1\frac{1}{2}$  hours. The preparation is very deeply stained, the nuclei being almost black, and decolorisation and differentiation are effected by alternately applying the acetic acid and caustic soda solutions (commencing with the acid) used in the application of the stain to ordinary histological sections (*v. p.* 114), the effects being carefully watched with a low power. The essential part of the method is the application of the blood serum, though what effect this has is not known; Leishman suggests that it restores the normal alkalinity of the tissue.

In preparations stained by the above methods the protoplasm of trypanosomata stains blue, and in some species some parts are more intensely coloured than others. Sometimes it contains violet-coloured granules (chromatin granules), and occasionally there appears in it slight longitudinal striation. Two bodies are always present in the protoplasm. Usually near the middle there is an oval granular body staining purple,—the trophonucleus or macronucleus,—and towards the posterior end is a minute intensely stained purple granule known as the kinetonucleus, blepharoplast, micronucleus, or centrosome (that this body represents the centrosome is strongly held by Laveran from the analogy of appearances in certain spermatozoa which



closely resemble trypanosomes in structure). This micronucleus is often surrounded by an unstained halo, and in its neighbourhood, in certain species, a vacuole has been described as existing; this has been considered by some to be analogous to the contractile vacuole present in many protozoa, and its shape and position have been made the basis of specific distinctions; Laveran, however, thinks it is an artefact. From the micronucleus or from its neighbourhood there arises an important structure in the trypanosome,—the undulatory membrane. This is of varying breadth, has a sharp undulating free margin, and surmounts the protoplasm of the organism like a cock's comb; it narrows towards the anterior end where it passes into the flagellum. Motion is chiefly effected by the undulations of this membrane and of the flagellum. The latter is continuous with the protoplasm of the body of the organism; it stains uniformly like it, except the free edge which has the reddish hue of the chromatin. In different species of trypanosomes variations occur in shape, in length, in breadth, in the position of the micronucleus (and therefore in the length of the undulating membrane), in the breadth of the membrane, in the length of the free part of the flagellum, in the shape of the posterior end, which is sometimes blunt, sometimes sharp, and in the presence or absence of free chromatin granules in the protoplasm.

Multiplication in the body fluids ordinarily occurs by longitudinal, amitotic division (see Fig. 171). First of all the micronucleus divides, sometimes transversely, sometimes longitudinally, then the macronucleus and undulating membrane, and lastly the protoplasm. In some species the root of the flagellum only divides, so that in the young trypanosomes the flagellum is short and subsequently increases in length (*Tr. Lewisi*); usually the whole flagellum takes part in the general splitting of the organism.

In the cases of several of the trypanosomata it has been found possible to cultivate them outside the body, the first work here having been done by Novy and MacNeal, who succeeded with the *Tr. Lewisi*, *Tr. Evansi*, and *Tr. Brucei*. They used a special medium (see p. 45), on which it was found that multiplication went on readily, the organisms dividing longitudinally as in the tissues. Sometimes very small forms result, and often these are found in rosettes which are formed by a number of individuals arranging themselves in a circle with the flagella directed towards the centre of the agglomeration. These results have been confirmed by other observers, and by repeated subcultures several of the trypanosomata named have been kept



alive for more than a year, and when re-introduced into appropriate hosts have been found not to have lost their infective properties.

The main fact in the biology of those trypanosomata with which the pathologist is concerned, is that in the higher animals infection takes place by the parasite being transferred from one host to another by the agency of biting or blood-sucking insects, or by other similar agencies such as leeches. It may be said that the mere mechanical transference of the parasite by, say, a blood-sucking insect, while it may sometimes occur, probably plays a subsidiary part in infection. Several instances will be given in which it is known that an insect does not become actively infective until some days have elapsed after it has sucked the blood of an infected animal. The analogy of the malarial organisms suggests the occurrence of a sexual conjugation within the insect, but definite proof of this is still wanting, and, as Minchin points out, while we must admit the existence of a cyclic development, it by no means follows that this includes a definitely sexual stage, although many are of opinion that such a stage does take place.

The starting-point of the sexual theory lies in the slight differences in form which have been observed in the organisms in the body fluids of the vertebrate hosts. Such differences have been described in *Tr. Lewisi* and *Tr. Brucei* by Prowazek, and in *Tr. ugandense* by Minchin, and have been made the basis of a classification into three types, which are looked on as representing male, female, and indifferent individuals. The male type is rather slender both in body and in nucleus, the free part of the flagellum is longer than the body, and the protoplasm is free from granules; the female is broader, its nucleus is larger and rounder, the undulating membrane narrower, the free part of the flagellum is shorter than the body, and the protoplasm contains many chromatin granules, which are looked upon as reserve food material. The indifferent individuals present intermediate characters. All multiply by fission as described, and, according to the supporters of the sexual theory, the indifferent individuals can on occasion become differentiated into male or female forms. The females are the most hardy, and next come the indifferent individuals; if all but the females die out, these can undergo parthenogenesis, and representatives of all three types can be again reproduced. The sexual cycle is represented as occurring in the invertebrate host. In *Tr. Lewisi*, according to Prowazek, this is found in the rat louse, *haematopinus spinulosus*. When this insect sucks the blood of an infected rat, copulation occurs by the male trypanosome entering the female near the micronucleus and the various parts of the two individuals becoming fused. A non-flagellated oökinete results, which, passing through a spindle-shaped gregarine-like stage (*crithidium*), can develop into a trypanosome in the stomach of the louse. A resting-stage in an immature trypanosome-like form is described as occurring in or on the intestinal epithelium, and the parasite is supposed to reach the body cavity, and ultimately the pharynx of the insect, and



thus to find the opportunity for passing into the body of a fresh host. Minchin, however, has been unable to find evidence of a sexual stage in this trypanosome.

A still further development of the views held as to the life-history of the trypanosomes is found in the work of Schaudinn, who investigated the *trypanosoma noctuae* found in the owl (*athene noctua*), and which is carried from bird to bird by the common mosquito (*culex pipiens*). In the blood of the owl is a halteridium hæmameba showing pigmented male and female forms, closely corresponding to those observed by Macallum in the crow. These, according to Schaudinn, on reaching the mosquito's stomach undergo ordinary changes—the microgametocyte develops microgametes, one of which fertilises the macrogamete. An oval motile ookinete results, and in the formation of its nucleus from the male and female elements a reduction of chromosomes takes place, while the superfluous nuclear structures along with the pigment are cast out of the cell. In these ookinetes a differentiation into male, female, and indifferent forms can be recognised, but the important new departure is that each can go on to develop into a trypanosome. In the indifferent ookinete a portion of the new nucleus breaks off by a sort of heteropolar division, and the broken-off part becomes the micronucleus or blepharoplast of the trypanosome and gives origin to the undulatory membrane. Longitudinal division of these forms in the mosquito's intestine may occur, and here it may be said that Schaudinn differed from other observers in holding that on division the membrane does not split, but that one of the individuals gets its membrane by this being laid down along the root of that already in existence. Further, a resting-stage of the trypanosome may occur, in which it becomes attached to the intestinal epithelium, and, losing more or less its flagellate form, may resemble a gregarine. The female ookinete is plumper and contains more chromatin granules in its protoplasm than the male and indifferent forms, and is in virtue of the reserve material in its protoplasm much more resistant than the male or indifferent forms; when these die out, as they do when, for instance, the insect is starved, it reproduces all three forms by a process of parthenogenesis. In the female ookinete the smaller nucleus which goes to form the blepharoplast of the indifferent trypanosome divides into eight small nuclei, all of which perish, and the blepharoplast and membrane are formed by a fresh division in the large nucleus remaining. In the male ookinete, which differs from the female in the clearness of its protoplasm, a similar heteropolar mitosis takes place, and again eight small nuclei are produced. These evidently represent the essentially male element, for they persist, and each, appropriating to itself a portion of the cellular protoplasm, detaches itself so that eight small trypanosomes are budded off from the ookinete. This male ookinete Schaudinn holds to be homologous with the microgametocyte occurring in the blood of the owl, and the small trypanosomes are similarly homologous with the microgametes formed when the blood of the host reaches the stomach of the mosquito. These small trypanosomes and the male trypanosomes readily die, probably because, by a reduction process in their genesis, the assimilative powers of the larger nucleus have been diminished. In degenerating they often are found in the intestinal tract of the mosquito arranged in rosettes, with sometimes the anterior ends, sometimes the posterior ends, directed towards the centre of the rosette. The next stage of development takes place when the parasites by the mosquito's bite reach the blood of the vertebrate host, and it is



in connection with this stage that Schaudinn's observations are very far-reaching. The indifferent forms by this time, by repeated longitudinal division, have become small. In the owl they attach themselves to red blood corpuscles, which they penetrate, and, assuming a halteridium form, grow in size for twenty-four hours; they then leave the cells, elongate, again assume the flagellate form, move freely in the blood till the next night, when they again enter fresh cells. This cycle is repeated for six nights, the organism gaining in size with each sojourn in a corpuscle. When the full size is thus attained, repeated longitudinal division occurs, and when the smallest forms are again reached the intra-cellular cycle recommences. The largest female forms probably cannot pass through the mosquito's proboscis, but when small forms reach the owl they enter the red corpuscles. They assimilate food material, and appear not to migrate so frequently as the indifferent forms. They lose their capacity of assuming the free trypanosoma form, and ultimately their capacity of migration, so that they are found lying surrounded by the remains of the last cell they entered. The male forms when they reach the blood of the host rapidly die, and the microgametocytes are always recruited from the indifferent forms, or from parthenogenesis of the female forms.

It may be said that, according to Schaudinn, the trypanosomes gain access to the tissues of the mosquito by perforating the intestinal wall, and, passing through the body till they reach the wall of the pharynx, they burst through this, and are in a position to be ejected when next the insect bites.

Such are the views put forward by this observer on the cycle of life-history of the *Tr. noctuæ*. It will be recognised that the essential point is the occurrence, both in the vertebrate and invertebrate host, of halteridium stages alternating with those in which the trypanosome form is assumed. It is evident that, if this were substantiated, important effects would follow in our views as to the morphology not only of the trypanosome group, but also of that to which the malarial parasites belong.

Certain criticisms of these results have been made, especially by Novy and McNeal, who sought confirmatory evidence by means of their culture method. These observers are of opinion that the appearances described by Schaudinn were due to his dealing with a mixed infection of the owls by trypanosomes on the one hand and hæmamebæ of the halteridium type on the other. They examined a very large number of different species of birds, and established the fact that infection with halteridium parasites on the one hand and with trypanosomes on the other is extremely common, and further, that in the blood of the same bird both halteridium and trypanosome infection could be observed. The bird trypanosomes could be readily cultivated, and it was observed that no cultures were obtainable from birds in which halteridia were alone found, and further, that when a trypanosome isolated from a case where both forms of parasite had been seen was injected into a fresh bird, only trypanosome forms were found to develop in its body. These results are, of course, not quite conclusive, as the halteridium stage might only follow on the sexual portion of the cycle.



Reference may here be made to the views put forward by Schaudinn regarding the relationship of certain spirilla to the trypanosomes. In the *athene noctua*, besides the *Tr. noctuæ* already referred to, there is a protozoal parasite infesting the leucocytes known as *spirillum Ziemanni* or *leucocytozoon Ziemanni*, whose invertebrate host is also the *culex pipiens*. Ziemann had described the male and female forms in the owl, and microgametes had been observed forming from the microgametocytes. Schaudinn observed the formation of an oökinete in the mosquito; in certain cases this oökinete elongates, and the vermicule rolls itself up into a ball with great proliferation of the nucleus. Each little nucleus attaches to itself a portion of the protoplasm, and, becoming a miniature trypanosome, swarms off and becomes free. These minute trypanosomes elongate and develop into typical spirilla by rolling their ribbon-shaped bodies spirally along their longitudinal axes, the individuals possessing male, female, or indifferent characters, just as in *Tr. noctuæ*. These spirilla multiply by longitudinal division, and often after fission the two individuals remain attached to each other by their posterior ends, and in this way there is made possible what is often seen in spirilla, namely, a capacity to move in either direction. The spirilla often divide so frequently that ultimately the individuals become invisible by means of the microscope, and can only be seen when lying in clumps. In this stage Schaudinn thinks the organism would be able to pass through a Chamberland filter, and this may be a very important observation, as throwing light on the etiology of certain diseases, such as yellow fever, in which no visible parasites have been found.

Schaudinn's views on the trypanosomal characteristics of *Sp. Ziemanni* raised important questions regarding the morphology of other similar forms which have been long familiar, such as *Sp. Obermeieri*, and also of the *Spirochaete pallida* which Schaudinn himself discovered. It is as yet too soon to express any opinion on the ultimate effect of these views. According to Schaudinn, a trypanosomal spirillum consists of a central thread which represents the posterior nucleus of the trypanosome; round this thread the undulating membrane is spirally wound, and the principal nucleus is represented by minute chromatin dots sometimes seen in the course of the spiral. Whether all spirilla have this structure must be left for future investigation to determine. Difficulties arise with regard to the significance of an undulatory membrane as a spirillary characteristic, and also with regard to the terminal flagellum which Schaudinn himself found in *Spirochaete pallida*, and which he had previously thought did not occur in protozoal spirilla. It is possible that two groups of organisms have hitherto been classed together under the name spirillum, and that one of these may still have to be placed with the bacteria.



We now pass to consider in detail some of the more important trypanosomes.

**Trypanosoma Lewisi.**—In 1878 Lewis described in rats in India the occurrence of the parasite which now goes by his name, and since that time this trypanosome has been found to be very common in the blood of rats all over the world, though the percentage of animals affected varies in different localities. Though the organism has no importance from the standpoint of human pathology, several significant points arise in connection with it. The condition in the rat is of great interest, as, though the infection runs a very definite course, it is very rarely fatal; in fact, many observers have been unable to produce death by infecting even large series of animals. There is, however, little doubt that a fatal issue does occur sometimes in young individuals, especially when these are infected with strains of the organism imported from other localities. The trypanosome, which is actively motile, is of the usual length but is somewhat narrow, and its protoplasm does not contain any granules. It multiplies by fission, of which Laveran describes two varieties. In one, the organism splits longitudinally and gives rise to smaller individuals than the parent. In the other, the trypanosome loses its ordinary shape and becomes more oval; nuclear division, which is often multiple, then takes place, and on subsequent division of the protoplasm a number of small flagellate organisms result; these last may attain the full form and size before dividing again, or they may divide when still small. When a rat is infected by injection into the peritoneum, active multiplication goes on in the cavity for a few days and then comes to an end. Very soon after infection the organisms begin to appear in the blood and there rapid multiplication occurs, the extent of which is sometimes so great that the trypanosomes may seem to equal the red blood corpuscles in number. The animal usually shows no symptoms of illness. The infection goes on for about two months, and then the organisms gradually disappear from the blood. In the great majority of cases the rat is now immune against fresh infection. If trypanosomes be introduced into its peritoneum they are, according to Laveran, taken up by mononucleate phagocytes and destroyed. The serum of a rat which has been infected shows agglutinating capacities towards the trypanosomes, causing them to agglomerate in rosettes in which the flagella are directed outwards, and the serum of immune rats has a certain degree of protective action if injected along with the organism into a susceptible animal. As has already been noted, this trypanosome has been cultivated on artificial media, on which it multiplies freely, large numbers of small forms being often produced. These when injected into rats give rise to the usual infection, but not so rapidly as when blood from an infected animal is used. The organism multiplies at the body temperature, but a lower temperature is preferable, and at 20° C. Novy and McNeal succeeded in carrying a growth through many sub-cultures. The trypanosome is very resistant to cooling, and has been exposed for fifteen minutes to the temperature of liquid air (−191° C.) without being killed. With regard to this infection Minchin and Thomson have shown that the rat flea, *ceratophyllus fasciatus* transmits the parasite by the cyclical method (mechanical infection not having been proved). The flea becomes infective about a week after biting, and remains infective for a long period,—possibly for the rest of its life. Infection may also take place through another species of flea and through a louse.



**Nagana or Tse-tse Fly Disease.**—This is a disease affecting under natural conditions chiefly horses, cattle, and dogs; it is prevalent especially in certain regions of South Africa, though it probably may occur elsewhere. In the horse the chief symptoms are the following:—The animal is observed to be out of condition, its coat stares, it has a watery discharge from the eyes and nose, and the temperature is elevated; swellings appear on the under surface of the abdomen and in the legs; it gradually becomes extremely emaciated and anæmic, and dies after an illness of from two or three weeks to two or three months. In other animals the symptoms are of the same order, though the duration of the disease varies much; thus in the dog the illness does not last more than one or two weeks, while in cattle it may continue for six months. It is doubtful whether a domestic animal attacked by the disease ever recovers. The popular idea regarding the etiology of the disease was that it was contracted by animals passing through certain rather restricted and sharply defined areas or belts characterised by heat and damp, usually lying beside rivers, and always infested by the tse-tse fly (*glossina morsitans*), to the bite of which the disease was attributed; in this connection it is important to note that though man is frequently bitten by the tse-tse fly he does not contract nagana. Modern knowledge on the subject dates from the discovery made by Bruce in 1894 that the blood of animals suffering from nagana swarmed with a trypanosome now known as the *Tr. Brucei*, and in 1895 he was instructed by the Governor of Natal to undertake the investigation which led him to work out the true etiology of the disease. It may be said that this research forms the starting-point of the important work done during the last decade with regard to infections by trypanosomes. In his earlier work Bruce found that the parasite was present in the blood of every animal suffering from nagana and absent from the blood of healthy animals in the affected districts; further, that the fever which marks the onset of the disease was accompanied by the appearance of the trypanosome in the blood; and finally, that the transference of the smallest quantity of blood from an affected to a healthy animal originated the disease. He then proceeded to investigate the part played by the tse-tse fly in the condition. He found that if flies taken from the fly belt were transported to a place where nagana did not occur, kept for a few days, and then allowed to bite susceptible animals, the latter did not contract the disease—this result showing that it was not, as had been supposed by



some, a poison natural to the insect which was the pathogenic agent. But if such a fly was allowed to bite a dog suffering from the disease and then to bite a healthy dog, the latter contracted the malady and abundant trypanosomes were found in its blood. Again, threads dipped in the blood of an infected animal and allowed to dry caused the disease in healthy animals up to, but rarely beyond, twenty-four hours after being dried; if, however, the blood were kept moist, then it retained its infectiveness up to between four and seven days; up to forty-six hours living trypanosomes could be seen in the tube of the fly's proboscis. This corresponds roughly with what was found regarding the limits of the infectiveness of the fly, in that twenty-four hours after it has been fed on an infected animal its bite is usually innocuous.<sup>1</sup> Further, Bruce showed that infection did not occur by any food or water partaken of by an animal while going through a fly belt, for he took horses through such a region without allowing them to eat or drink, and found that they still contracted the infection, if during their few hours' journey through the belt they had been bitten by the tse-tse fly. Finally, he showed that if flies were taken from an infected area to a healthy one a few miles off and allowed at once to bite infected animals, the latter contracted nagana.

By those experiments it was thus determined that nagana could be transmitted by the blood of the infected animal, that is, without the agency of the fly; that the latter had no inherent power to produce the disease; that it could, however, by successively biting infected and healthy animals transmit the disease to the latter; and that specimens of the insect caught in infected areas harboured the parasite and were thus infective. The question remained as to how the flies might become infected in nature. It had been observed that in districts where the tse-tse fly lived the prevalence of the disease in imported animals was related to the presence in the locality of wild herbivora. Bruce now found that, if considerable amounts of the blood of the latter were taken to another locality and injected into dogs, these in a proportion of cases contracted nagana, and from this he deduced that the wild animals harboured the parasites in small numbers in their blood and thus kept up the possibility of infection. A further fact was that other blood-sucking flies besides the tse-tse appeared incapable of acting as carriers of infection. Bruce's work as a whole pointed to the

<sup>1</sup> This observation probably only applies to infection so far as this may be merely mechanical. There is evidence that a cyclic development occurs in glossina, and that thus after an interval its bite is again infective.



trypanosome as the cause of nagana, and this has since been finally established by the origination of the disease by artificial cultures of the organism.

The *Tr. Brucei* (Fig. 171), according to Laveran, measures in the horse from 28 to 33  $\mu$  long and from 1.5 to 2.5  $\mu$  broad; in the rat and dog it is somewhat shorter. It is motile, but its activity is less than that of *Tr. Lewisi*. When stained it presents the usual appearances; its posterior end is usually blunt, and



FIG. 171.—*Trypanosoma Brucei* from blood of infected rat. Note in two of the organisms commencing division of micronucleus and undulating membrane.  $\times 1000$ .

the body often contains granules in the anterior portion of its protoplasm. It divides longitudinally, and, according to Bradford and Plimmer, a form of longitudinal conjugation occurs in the blood. According to the same observers, it can be kept alive for five to six days in blood outside the body. It is less resistant to the action of cold than *Tr. Lewisi*, perishing in a few days at 5 to 7° C., but, like the other organism, it can withstand short exposures to temperatures down to -191° C.; it is quickly



killed at 44 to 45° C. Novy and McNeal succeeded in cultivating this trypanosome also, though here it was very difficult to obtain a first growth from the blood on their blood-agar medium; once started, however, it was kept alive through many sub-cultures, the optimum temperature of growth being 25° C., and it was from these sub-cultures that the infection was obtained which definitely proved the organism to be the cause of the disease. In cultures, as with *Tr. Lewisi*, short forms occur, and there is sometimes a rosette formation with the flagella directed outwards; agglutination phenomena are also observable in defibrinated blood. Under unfavourable conditions involution forms occur, the organism dividing frequently to form round flagellated individuals.

Nearly all laboratory animals are susceptible to infection, and the duration of the illness corresponds to what has been observed in the natural infection of these animals. The rat has been largely used for experiment and usually succumbs in about ten days, there being very few symptoms up till a few hours before death. A very important fact has been observed with regard to this animal, namely, that individuals which have gone through infection with *Tr. Lewisi* and which are immune are still susceptible to the *Tr. Brucei*; from this it has been deduced that the two organisms are to be looked on as distinct species.

**Trypanosoma of Sleeping Sickness.**—Since the year 1800 the disease called sleeping sickness, sleeping dropsy, or negro lethargy has been recognised as prevailing on the West Coast of Africa from the Senegal to Lagos, and in the parts lying behind the coast between these regions. It has also been found to be rife from Cameroon to Angola and in the Congo valley, and to a less extent up the Niger and its tributaries. In 1901 it began to appear in the Uganda Protectorate, and it is in that region that the investigations have been carried on which have led to a knowledge of its cause; here it has wrought very serious havoc amongst the native population. It is characterised in the early stages by a change in disposition leading to moroseness, apathy, disinclination for work or exertion, and slowness of speech and gait. There may be headache, indefinite pains about the body, the evening temperature may be elevated several degrees, the pulse tends to be soft and rapid, and in a very large number of cases the superficial glands of the body are enlarged. In a rapid case the lethargy becomes more pronounced; fine tremors, especially of the tongue and arms, develop; progressive emaciation occurs; blood changes appear, consisting of a progressive diminution of the red cells and of the hæmoglobin, and of a



lymphocytosis in which the percentage of both the large and small mononuclear cells is increased, so that the former may constitute from 20 to 30 and the latter from 30 to 40 per cent. of all the white cells present. As the disease progresses the drowsiness increases till it deepens into a coma from which the individual cannot be roused. Often during the disease there occur irregular œdematous patches on the skin, and sometimes erythematous eruptions, and effusions into the serous cavities. Not every case runs a progressively advancing course. Sometimes along with enlargement of glands the chief early feature is the occurrence from time to time of attacks of fever which may be mistaken for malaria, and from these apparently complete recovery may take place; recurrence, however, follows as a rule, and ultimately the typical terminal phenomena may commence. Such cases may go on for years, and it is probable that many patients die of pneumonia without exhibiting typical manifestations of the malady from which they really suffer. The disease is an extremely fatal condition, and probably no case where the actual lethargy is developed ever recovers.

On considering the disease from the standpoint of pathological anatomy there is little to be said. As Mott described, the most striking feature is the presence of a chronic meningo-encephalitis and meningo-myelitis. The pia-arachnoid is sometimes opaque and slightly thickened and may be adherent to the brain, and its vessels usually show some congestion. The sub-arachnoid fluid is sometimes in excess and occasionally may even be purulent. The membranes of the spinal cord show similar changes. The chief other feature is the presence of enlarged lymphatic glands in the body, but otherwise there is nothing special to note. With regard to the microscopic changes, the chief feature, according to Mott, is a proliferation and overgrowth of the neuroglia cells, especially of those which are related to the sub-arachnoid space and the perivascular lymph spaces, with accumulation and probably proliferation of lymphocytes in the meshwork. He further points out that the changes in the lymph glands are of similar nature and resemble the infiltration of the perivascular lymphatics of the central nervous system. These changes are specially significant in view of the lymphocytosis present in the blood, which has already been noted, and which so often occurs in protozoal infections. In the nervous structures there is comparatively little change, there being merely, according to Mott, some atrophy of the dendrons of the nerve cells, a diminution of Nissl's granules, and an excentricity of the nucleus.



*Trypanosoma gambiense*.—Before going further we must refer to the observation of a trypanosome in the blood of persons not evidently suffering from sleeping sickness. The first case of this was recorded by Dutton in 1901, the patient being a European then living at Bathurst on the Gambia. The progress of the disease was here very slow, and was characterised by general wasting and weakness, irregular rises of temperature, local œdemas, congested areas of the skin, enlargement of spleen,



FIG. 172.—*Trypanosoma gambiense* from blood of guinea-pig.  $\times 1000$ .  
See also Plate VI., Fig. 25.

and increased frequency of pulse and respiration; death occurred a year after the case came under observation after an access of fever, and a striking fact was the absence of any gross causal lesion. During the time the patient was under observation trypanosomes were repeatedly demonstrated in the peripheral blood, and they also developed in the bodies of monkeys and white rats inoculated with the blood. Pursuing further inquiries, Dutton and Todd demonstrated similar parasites in other Europeans and in several natives in the Gambia region,



whilst about the same time Manson reported a case of the same kind in the wife of a missionary on the Congo. It thus came to be recognised that in man there occurred a disease having characters somewhat resembling nagana and in which trypanosomes could be demonstrated in the blood, and this was usually referred to as human trypanosomiasis, or trypanosoma fever,—the trypanosome being named the *Tr. gambiense*.

*Relation of Trypanosomes to Sleeping Sickness.*—Several views as to the etiology of this disease had been advanced. A Portuguese Commission in 1902 described a diplococcus, tending to grow in chains, which they isolated from the cerebro-spinal fluid taken from cases during life, and to which they were inclined from the constancy of its occurrence to attribute a causal rôle. The seriousness of the epidemic in Uganda had led the Royal Society of London in 1902, at the instigation of the Foreign Office, to despatch a Commission to investigate the condition on the spot. Soon after commencing work, Dr. Castellani found in some cases in the cerebro-spinal fluid, especially when this was centrifugalised, living trypanosomes resembling the *Tr. gambiense*; he also found in 80 per cent. of the cases post mortem a coccus resembling if not identical with that observed by the Portuguese Commissioners. At first Castellani was inclined to look on the presence of the protozoon as accidental, but Colonel Bruce, on going out with Nabarro and Greig in 1903 to pursue the work of the Commission, realised the significance of the observation, urged Castellani to further inquiries, which he himself continued after the departure of the latter, with the result that in a series of examinations carried out in several infected localities, the trypanosome was demonstrated in every case of the disease. This work formed the starting-point for inquiries, the results of which make it practically certain that the parasite is the causal agent of the condition. The organisms were not demonstrable in the cerebro-spinal fluid of patients dying of other diseases in the sleeping sickness area. On the other hand, it was found that if cerebro-spinal fluid withdrawn from cases of the disease was injected into monkeys (especially *macacus rhesus*), trypanosomes appeared in the blood, and in many cases in three or four months the animals died of an illness indistinguishable from sleeping sickness, and with the parasites in the central nervous system. It was further found that in the parts round the north end of Victoria Nyanza where sleeping sickness was rife, the distribution of the disease exactly corresponded with the distribution of a blood-sucking insect, the *glossina palpalis*, a species closely allied to the *glossina morsitans*.



of nagana. It was found that, when one of these flies was fed on a sleeping sickness patient and then allowed to bite a monkey, frequently trypanosomes appeared in the animal's blood, and that when fresh flies caught in the sleeping sickness area were placed on a monkey a similar occurrence took place.

The trypanosome of sleeping sickness is 17 to 28  $\mu$  long and 1.4 to 2  $\mu$  broad (Fig. 172); when about to divide it is both longer and broader. According to Laveran, the free part of the flagellum often equals a fourth of the whole length, but occasionally the body protoplasm extends quite to the end of the organism. The undulating membrane is narrow, and the posterior end may be either sharp or blunt. The trypanosome contains the macro- and micronucleus characteristic of the group, and the protoplasm often shows chromatin granules. Castellani attached great importance to a vacuole often seen in the neighbourhood of the micronucleus, but, as stated above, Laveran holds this to be an artefact. The organism divides longitudinally in the usual manner, and often two can be seen to approach each other and lie more or less side by side, but whether this indicates conjugation or not is not known. The organism does not usually long survive removal from the body, but it has been found to be motile for nineteen days when kept on rabbit-blood agar at 22° C. As we have said, when *Tr. ugandense* is inoculated into monkeys they often contract an illness which ultimately presents the features of typical sleeping sickness. Inoculation of other species of animals is not usually so successful, though in nearly every case, *e.g.* in the guinea-pig, a proliferation of the parasite, as indicated by its appearing in the blood, takes place; but often either no disease occurs or this runs a very chronic course. The relative insusceptibility of animals, especially of the dog, to the *Tr. ugandense* is taken as evidence that this organism is essentially different from *Tr. Brucei*.

By means of microscopic examination the organisms may be found in the cerebro-spinal fluid, the blood, or the juice of glands. In the case of the first about 10 c.c. of the fluid is to be centrifugalised for fifteen minutes and the deposit placed under a cover-glass for examination; it is better to make a little cell on a slide by painting a ring of ordinary embedding paraffin, to place the droplet of fluid in its centre, and to support the cover-glass on the paraffin; in this way injury to the delicate structure of the organism is avoided. In fresh cerebro-spinal fluid the trypanosomes can be seen to be actively motile; the number in which they occur varies very much, and the same is



true to a greater degree of the blood, in which they are, however, usually very scanty. With regard to the examination of the blood, Bruce and Nabarro state that it is difficult by ordinary centrifugalisation to concentrate the organisms, as these are not readily precipitated. They accordingly recommend that the blood be mixed with citrate of sodium solution (equal parts of blood and of a one per cent. citrate solution) and centrifuged for ten minutes, that the plasma be removed and centrifuged afresh for the same time, and that this be repeated three times, the deposit from each centrifugalisation after the first being carefully examined. Greig and Gray have insisted that the examination of the glands in a suspected case forms the most ready means of arriving at a diagnosis, and this opinion has found strong support from the work of Dutton and Todd. The method is to push a hypodermic needle into the gland, suck up a little of the juice, and blow it out on to a slide. In all cases where films of any kind are to be prepared the staining methods of Leishman or Giemsa are to be recommended. Often in cerebro-spinal fluid and gland juice the staining of the chromatin is difficult, but good preparations are obtained by the procedure recommended by Leishman for studying the parasite in sections (p. 114).

Greig and Gray have studied the trypanosome in the body of the glossina. They found evidence of its multiplying in the stomach of the insect, and it also was seen to undergo changes not elsewhere observed. These consisted in alterations in the position of the micronucleus, which often became anterior to the macronucleus; there also occurred rosettes, consisting of from four to twenty individuals attached by their posterior extremities. Oval forms were also observed. It was at first supposed that monkeys could not be inoculated with the trypanosomes from the stomach of the fly, but recently Bruce has succeeded in originating an infection in this way; probably, however, the organism remains alive in only a small proportion of flies biting an infective case. Minchin in this connection has described in the gut of the fly different types of the parasite corresponding with the male, female, and indifferent forms found in other trypanosomes. This was confirmed by Koch and by Klein, who also found in the intestine agglomerations of immature forms which they ascribed to the results of sexual conjugation. The most important fact established by the last observer was, however, that when *Gl. palpalis* was allowed to bite an animal suffering from nagana it did not become infective for some days. This has been confirmed for *Gl. palpalis* in the case of monkeys suffering



from *Tr. gambiense* by Bruce and those associated with him in 1908-9. Here it was found that infectivity did not appear till about thirty-two days after the fly had fed, and continued until at least seventy-five days. In this connection certain facts having a serious bearing on the continued infectivity of a locality have emerged. It was found that a certain island on Lake Victoria Nyanza, which had been cleared of infective natives two years previously, still harboured infective flies. To account for this it must be supposed either that the glossina has an extended duration of life or that the trypanosome exists among the wild animals. As it has been found that cattle may be infected with the parasite, and may through the medium of the fly infect monkeys, it is possible that wild herbivora, while not suffering in any serious way themselves, are the means of maintaining infectivity. There is no definite evidence that, as Koch supposed, the crocodile harbours the trypanosome.

Early in the Uganda investigations the question arose as to whether the trypanosoma of sleeping sickness was different from *Tr. gambiense*. This was forced on the inquirers by the fact that a very large proportion of the natives in the sleeping sickness area were found to harbour trypanosomes in their blood, although not apparently suffering from the disease. Several cases were carefully examined in which trypanosomes were constantly present in the blood, but in which the patients from time to time suffered from fever, and during these pyrexial periods trypanosomes were found in the cerebro-spinal fluid. It was suggested that these cases were on the way to develop sleeping sickness. A very important observation was that while in sleeping sickness areas a large proportion of the native population harboured trypanosomes, this was not the case where sleeping sickness did not occur. Further, it was found that trypanosomes from the cerebro-spinal fluid of sleeping sickness cases and from the blood of persons harbouring trypanosomes, but not suffering from disease symptoms, gave rise in monkeys to the same group of chronic effects which resembled the last stages of the disease in man. These facts led the Commissioners to incline to the idea that trypanosoma fever and sleeping sickness are due to the same cause, and represent different stages of the same disease. It has already been pointed out that a fatal termination can occur in trypanosoma fever by an acute febrile attack or from intercurrent disease, and thus the terminal lethargic stage may only develop in a certain proportion of cases. Continued observation of prolonged cases of trypanosoma fever, both in Uganda by Greig and Gray, and in this country by



Manson, has shown that sometimes the termination of a case is by the onset of typical sleeping sickness. There is now practically no doubt that the two conditions are etiologically identical. The best authorities are agreed that morphologically no difference between the *Tr. gambiense* and the *Tr. ugandense* can be recognised, and from considerations of priority the former term is now alone employed.

The prevalence of trypanosomes in the blood of apparently healthy natives has raised the question of the possibility of tolerance existing and of immunity being established. It is possible that both phenomena occur, that not every infection results in multiplication of the parasite in the body of the victim, and that in certain cases where multiplication does occur a resistance is developed which enables the body to kill the parasites. The occurrence of the mononuclear reaction is here significant; it has been suggested that, when this resistance is weak, the organism gains entrance to the spinal canal, and that then sleeping sickness results.

The whole of the recent work on the disease is of the highest interest and importance. The strongest evidence may be said to exist that the *Tr. gambiense* is the cause of sleeping sickness, and action taken on this supposition has had a very important effect in checking the ravages of the disease in Uganda, where the natives have been deported from the fly areas, and the brushwood in which the insects lodge has been cut down in the neighbourhood of ferries.

Not much success has attended remedial efforts in those suffering from infection. Here attention has been chiefly concentrated on the action of organic arsenical compounds, the application of which in the shape of atoxyl was first recommended by Thomas. A great range of such substances and also of aniline derivatives has been investigated by Ehrlich and his co-workers, and under certain conditions of artificial infection in animals a complete or partial destruction of the parasites has followed administration of these bodies, but their application to natural infections has not as yet met with decided success. Sufficient, however, is known to justify further investigations of a similar kind. It has been observed that a tolerance of such reagents can be developed by the trypanosomes, and this fact may complicate the problem at issue.

*Other Pathogenic Trypanosomes.*—Apart from sleeping sickness no other important disease of man has been found to be associated with trypanosomal infection, but some observations on a condition observed in Brazil may be alluded to.



**Trypanosoma Cruzi.**—Chagas, working in Brazil, observed this trypanosome in a monkey, the intermediate host being a hemipterous insect of the genus *Conorhinus*. As this insect also feeds on man, the possible relationship of the trypanosome to a human disease occurring in that region was considered. This disease affects children, and is characterised by pronounced anæmia, the occurrence of œdema, and enlargement of lymphatic glands, the spleen and liver; it is associated with a mental condition of infantilism, and ends in death with convulsions. The parasite was only in one case found in the blood of infected individuals, but when the blood was injected into guinea-pigs, or into callithrix monkeys, a definite disease occurred, leading to death. In the peripheral blood in such animals, besides free forms, an infection of the red blood corpuscles with a body resembling a malarial merozoite was seen, this body apparently developing into a trypanosoma-like organism. A special development of the parasite seemed to occur in the lungs, the result of which was the formation of cells containing eight bodies resembling the merozoite forms seen in the circulation, and analogous to what Schaudinn described in the mosquito's stomach in connection with trypanosoma noctuæ. A cycle of development was also observed in the intestinal tube of the conorhinus, and cultures were obtained on Novy and MacNeal's medium.

It is beyond the scope of this work to deal at length with the other diseases of animals caused by trypanosomes. The chief of these have been mentioned in the opening paragraph, but it may be said that many others have been described in various species of mammals, birds, and fishes, and that these are spread either by flies or by leeches. The most interesting of those mentioned is Dourine, a condition resembling in many ways nagana. It, however, presents this peculiarity, that infection does not take place by an intermediate host, but apparently directly through coitus, as it occurs only in stallions and in mares covered by these.

In several of the trypanosomal infections of animals it appears as if, as in the case of *Tr. Lewisi*, the animal suffers little inconvenience from the presence of the parasite in its blood, and the view has even been put forward that with all pathogenic trypanosomes there exists a host which acts as a "reservoir" and carries the organism without being affected by its presence more, for example, than is the rat by *Tr. Lewisi*. Though no opinion can be expressed on this point, it is necessary to bear the fact in mind that either natural or acquired immunity can exist against such protozoa. Not only is this important from the point of view of the investigation of the conditions under which such tolerance arises, but also from the bearing which the existence of this tolerance may have on the spread in nature of the parasites to a susceptible species from immune animals which still harbour trypanosomes in their blood. We are, however,



as yet quite ignorant of many of the processes at work in the body during a trypanosomal infection, and of the causes of the symptoms and other morbid effects.

### LEISHMANIOSIS.

Under this term there are grouped three human diseases, but the exact zoological place of the parasites among the protozoa cannot be said to be at present definitely settled. These organisms are the *Leishmania donovani*, associated with the human disease, *kála-azar*; *Leishmania infantum*, derived from a similar disease occurring in children; and *Leishmania tropica*, which has been found in a skin ulceration of widespread geographical distribution. Microscopically the organisms are practically identical, but at present it is convenient to look upon the three species as being distinct.

**Leishmania Donovan.**—Leishman noticed in several soldiers invalided from India for remittent fever and cachexia that the most careful examination of the blood failed to reveal the presence of the malarial parasite. From the fact that such patients had almost invariably been quartered during their service at Dum-Dum, an unhealthy cantonment near Calcutta, he suspected he had to deal with an undescribed disease. In 1900 he noticed in the spleen of such a case peculiar bodies which, from comparison with certain appearances found in degenerating forms of *Tr. Brucei*, he suggested might be trypanosomes, and on publishing his observations in 1903 he put forward the view that trypanosomiasis might prevail in India and account for the aberrant cases of cachexial fever met with there. Soon after Leishman's paper appeared, his observations were confirmed in India by Donovan, and the bodies associated with the disease are usually called the "Leishman" or the "Leishman-Donovan" bodies. They were found by Bentley, and later by Rogers, in the disease known in Assam as *kála-azar*, the pathology of which had long puzzled those who had worked at it, from the fact that, while it resembled malaria in many ways, no parasite could be demonstrated to occur in those suffering from it. This disease has gone under various synonyms, *e.g.* cachetic fever, Dum-Dum fever, non-malarial remittent fever, but is now recognised as a single entity.

*Kála-azar* (or "black disease,"—so called from the hue assumed by chocolate-coloured patients suffering from it) has been known since 1869 as a serious epidemic disease in Assam, where it has spread from village to village up the Brahmaputra



valley. The disease is now known to occur in various sub-tropical centres between the forty-ninth parallels—cases where the Leishman bodies have been found having been met with in many parts of India, China, the Malay Archipelago, North Africa, the Soudan, and Arabia. The disease is characterised by fever of a very irregular type, by progressive cachexia, and by anæmia associated with enlargement of the spleen and liver, and often with ulcers of the skin and dropsical swellings. Rogers has pointed out that there occurs a leucopenia which differs from that of malaria in that it is almost always more marked,—the leucocytes usually numbering less than 2000,—and further, in that the white cells are always reduced in greater ratio than the red corpuscles, which condition, again, does not occur in malaria. The disease is chronic, often going on for several years, and, at any rate in the great majority of cases, has a fatal issue. Post mortem, there is little to note beyond the enlargement of the liver and spleen, but in the intestine, especially in the colon, there are often large or small ulcers, and there is evidence of proliferation in the bone marrow, the red marrow encroaching on the yellow.

In a film made from the spleen and stained by Leishman's stain, the characteristic bodies can be readily demonstrated (Fig. 173). They are round, oval, or, as Christophers has pointed out, cockle-shell shaped, and usually  $2.5$  to  $3.5 \mu$  in diameter, though smaller forms occur. The protoplasm stains pink, or sometimes slightly bluish, and contains two bodies taking on the bright red colour of nuclear matter when stained by the Romanowsky combination. The larger stains less intensely than the smaller, is round, oval, heart-shaped, or bilobed, and lies rather towards the periphery of the body—in the region of the "hinge" in the cockle-shaped individuals. The other chromatin body is usually rod-shaped, and is set perpendicularly or at a tangent to the larger mass, with which only exceptionally it appears to be connected. Usually the protoplasm contains one or two vacuoles. Though in spleen smears many free bodies are seen, the study of sections shows that ordinarily their position is intra-cellular,—the cells containing them being of a large mononuclear type (Fig. 174). The view held is that on their entering the circulation they are taken up by the mononuclear leucocytes and by such cells as the endothelial lining of the splenic sinuses or by those lining capillaries or lymphatics, that in these cells multiplication takes place—it may be to such an extent as to rupture the cell,—and that if thus the bodies become free they are taken up by other



cells and the process is repeated. The clusters of bodies sometimes seen in smears are probably held together by the remains of ruptured phagocytes. In capillaries the endothelial cells after phagocytizing the bodies probably become detached from the capillary wall, as they are often observed free in the lumen of the vessel,—this being well seen in the hepatic capillaries. In the body generally the parasites are found in greatest abundance in the spleen, liver, and bone marrow, and also in

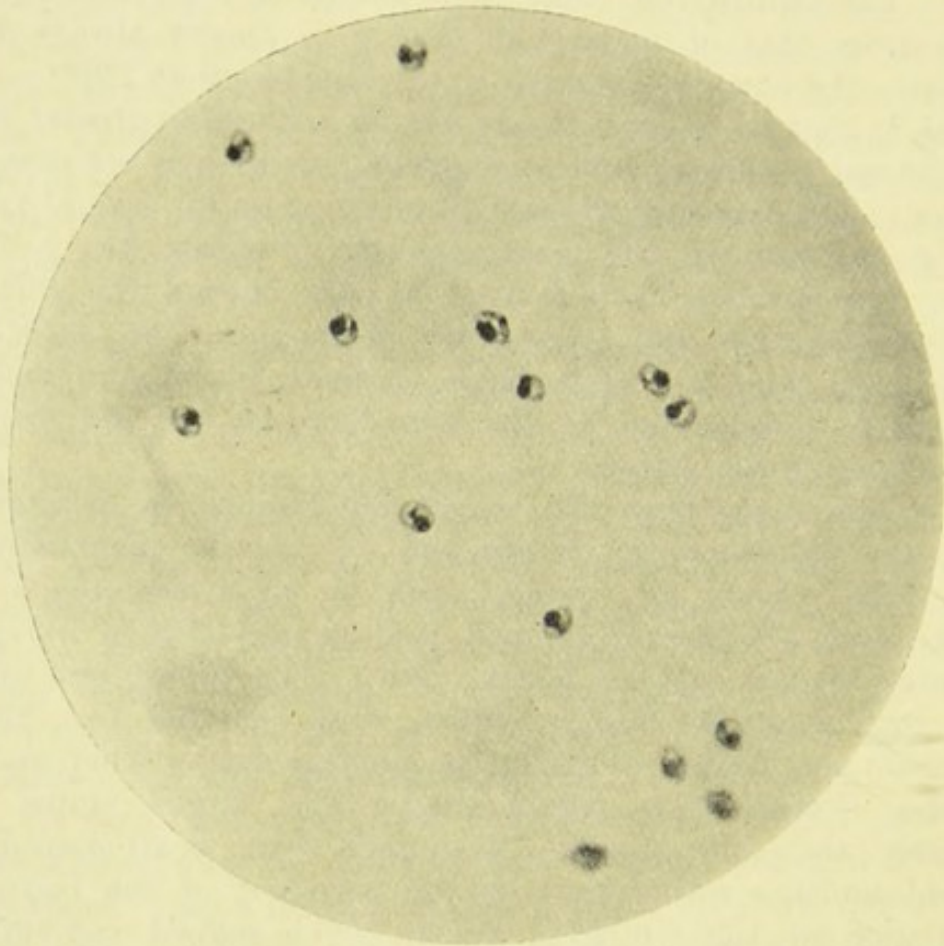


FIG. 173.—Leishman-Donovan bodies from spleen smear.  $\times 1000$ .

mesenteric glands, especially in those draining one of the intestinal ulcers; less frequently they occur in the skin ulcers, and in other parts of the body. Donovan described them as occurring in the peripheral blood, especially within the leucocytes, and this has been confirmed by other observers, though sometimes prolonged search is necessary.

In the body the parasite multiplies by simple fission, both nuclei dividing amitotically, and two new individuals being formed; but sometimes a multiple division takes place, each nucleus dividing several times within the protoplasm and a corresponding number of new parasites resulting.



In view of Leishman's original opinion an extremely important discovery was made by Rogers and later confirmed by Leishman himself, to the effect that in cultures a flagellate organism developed from the Leishman-Donovan body. Cultivation was effected by taking spleen juice containing the parasite, placing it in 10 per cent. sodium citrate solution and keeping it at 17 to 24° C. Under such conditions there occurs an enlargement of the organism, but especially of the larger nucleus. This is followed by the appearance of a pink-staining vacuole in the neighbourhood of the smaller nucleus. Along with these changes, in from twenty-four to forty-eight hours the parasite becomes elongated and the smaller nucleus and its vacuole move to one end; from the vacuole there then appears to develop a red-staining flagellum, which when fully formed seems to take its origin from the neighbourhood of the small nucleus. The body of the parasite is now from 20 to 22  $\mu$  long and 3 to 4  $\mu$  broad, with the flagellum about 22  $\mu$  long. The whole development occupies about ninety-six hours. The formation of an undulating membrane was not observed, and, although the flagellated organism moved flagellum first, like a trypanosome, it is evident that here the relationship of the micronucleus is different, as this structure lies anterior to the macronucleus. In his cultures, which kept alive for four weeks, Leishman made a still further important observation. In certain of the flagellate forms he saw chromatin granules develop in the protoplasm often in couples, a larger and a smaller. There then occurred a very unequal longitudinal division of the protoplasm, and a hair-like undulating individual containing one of the pairs of chromatin granules would be split off. At first these would be non-flagellate, but later a red-staining flagellum would appear at one end. The analogies between these observations and those of Schaudinn (*v. p. 616*) on the relations of spirochaetes

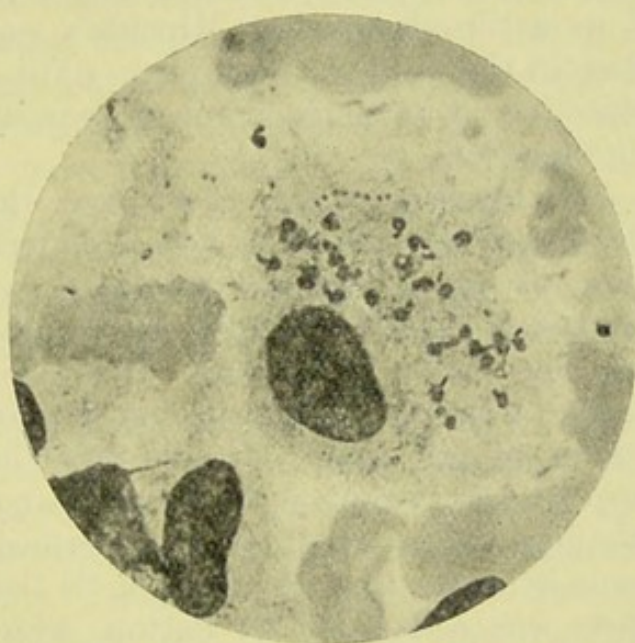


FIG. 174.—Leishman-Donovan bodies within endothelial cell in spleen. See also Plate VI., Fig. 24.  $\times 1000$ .



to trypanosomes will be at once apparent; the further development of these spirillary forms in Leishman's organism could not, however, be traced.

The facts just detailed have been the basis for discussion of the classification of the organism, which now usually goes by the name *Leishmania donovani*, originally given to it by Ross. According to one view, it is to be looked on as a trypanosome, and although, as we have noted, its flagellated form differs from the typical trypanosoma form, it bears considerable resemblance to the members of this group, and, as Leishman has pointed out, his cultures may not represent the full development of the organism in the trypanosoma direction. Others have looked on it as a piroplasma, but Minchin's suggestion has been accepted that in the present incomplete state of knowledge it is well to place it and its congeners in a provisional genus, *Leishmania*, of the flagellata.

The question arises, given that the *Leishmania donovani* is the cause of *kála-azar*, how is infection spread? On this we have as yet no certain information. The fact that in some centres of the disease natives who are supplied with good water are less liable than those who rely on the ordinary polluted native cisterns, has led to the opinion that water may be the carrier of infection. On the other hand, the possible relationship of the organism to the trypanosomata naturally suggests the idea of an insect as an intermediary, and Rogers adduced some evidence that the bed-bug is the extra-human host. This view was elaborated by Patton, who brought forward facts to show that multiple cases might occur in a house while neighbouring houses were free from the disease. This observer also fed the common insect parasites of man in Madras on patients whose peripheral blood contained the *Leishmania*, and found that the parasite could be observed only in the *pediculus capitis*, and in the bug, *cimex macrocephalus*. In the midgut of the latter, forms similar to those seen in the earlier stages of cultures could be found. Patton compares the organism to an allied protozoon occurring in the intestine of the common fly. The rarity of the *Leishmania* in the peripheral blood has been advanced as an argument against infection taking place by means of a blood-sucking insect, but it has been pointed out that invisible spirillary forms may be instruments of infection. It may be said here that all attempts to communicate the disease to animals have been hitherto unsuccessful.

With regard to *kála-azar* as a whole, we may say that we are dealing with a distinct disease fairly widespread in various sub-



tropical regions. All attempts to include it among the malarial cachexias, which clinically it so much resembles, have failed. In this atypical cachexial fever there is always present a parasite of very special characters belonging or closely allied to a group which contains many varieties capable of giving rise to similar diseases. Beyond this we cannot go, but at present we must admit that there is strong presumptive evidence of the parasite described being the cause of the disease.

*Methods of Examination.*—The *Leishmania donovani* can be readily seen in films or sections of the organs in which we have mentioned its occurrence. These should be stained by the Romanowsky stains. Fluid taken from the enlarged spleen with a perfectly dry needle during life may be examined, but it is probable that in this disease puncture of the spleen may not be a very safe operation, as death from hæmorrhage from this organ is a not uncommon natural terminal event. During life the main points on which a pathological diagnosis may be based are the demonstration of the parasite in the circulating blood which should always be attempted, the absence of the malarial parasites from the blood, and the features of the leucopenia which have been alluded to.

**Leishmania Infantum.**—Nicolle, working in Tunis, observed a disease clinically identical with *kála-azar*, but presenting the peculiarity of only affecting children of about two years of age. He found in the spleen in such cases an organism, microscopically indistinguishable from the *Leishmania donovani*. It was cultivated on a modified Novy and MacNeal's medium, the cultures presenting characters similar to those observed by Rogers and by Leishman in the other *Leishmania*. It was found that dogs could be infected with the parasite, and, taking into account the fact that this animal is not susceptible to infection with the *Leishmania donovani*, and the further fact that the disease is apparently confined to infants, Nicolle considered the organism to be a separate species and gave it the name, *Leishmania infantum*. In his view, the infection of the dog possesses a further significance in that this animal may be the reservoir from which, by means at present unknown, children become infected. In support of this, he observed the fact that a certain proportion of dogs destroyed in Tunis contained the parasite in the spleen. This disease occurs in other parts of the Mediterranean littoral; in 1905 Pianese described it in children in Italy, and the parasites have been found in cases in that country and also in Sicily and Malta.

**Leishmania Tropica.**—In various tropical and sub-tropical



regions (India and the East, Northern Africa, Southern Russia, South America) there is widely prevalent a variety of very intractable chronic ulceration which goes by various names in different parts of the world—Delhi sore, tropical ulcer, Aleppo boil, etc. Various views were formerly held as to the pathology of the condition, but the work of J. H. Wright makes it practically certain that a protozoal parasite is concerned in its etiology. In the discharge from the ulcer and in sections of a portion of tissue excised from a case coming from Armenia, Wright observed great numbers of round or oval, sharply defined bodies, 2 to 4  $\mu$  in diameter. When stained by a Romanowsky combination there was found to be a peripheral portion coloured a pale blue and a central portion tending to be unstained; there were also two chromatin bodies, one larger, occupying a fourth or a third of the whole and situated in the periphery, another smaller, round or rod-shaped, and of a deeper colour than the larger mass. It was found that the bodies were usually intracellular in position in the lesion, as many as twenty being in one cell, and that the type of cell containing them was, as in *kála-azar*, that derivable from endothelial tissues.

Wright's observations have been fully confirmed by workers in various parts of the world, and it is now recognised that in these tropical ulcers we have a third example of the activity of a *Leishmania*. This is corroborated by the work of Row, who has obtained cultures in citrated blood, corresponding to those of the other two species. Nicolle and Manceaux have also cultivated the organism on Novy and MacNeal's medium, and have reproduced the condition in man, the monkey, and the dog, both by virus obtained from the natural infection and from cultures. The lesions were identical with those naturally occurring, but the incubation period was often many months. It may be said that Thompson and Balfour have described in the Soudan a condition in which subcutaneous nodules without ulceration occurred in man and these contained *Leishmania* bodies.

At present the tendency is to look upon the three *Leishmaniae* as representing different species, but further investigation is here necessary. It has been pointed out that in *kála-azar*, skin ulcerations occur which might link this condition with tropical ulcer, but it is to be noted that, while in the latter enormous numbers of the parasite are found, in the ulcers of *kála-azar*, on the other hand, parasites are difficult to find. Again, Nicolle has found that dogs infected with *Leishmania tropica* appeared to be not so susceptible to subsequent infection with *Leishmania*



infantum. These facts, however, might be consistent with the existence of three species.

**Histoplasma Capsulatum.**—Under this name, Darling has described a parasite observed by him in Panama, in certain cases characterised during life by continued irregular fever, spleno-megaly, emaciation, and anæmia, and post mortem showing minute granulomata in the lungs, irregular necrosis and cirrhosis of the liver,—the spleen, naked-eye, resembling that of spleno-myelogenous leukemia. In smears from the lung nodules, the liver, and spleen, stained by Leishman's method, there were observed enormous numbers of small bodies sometimes crowding endothelial cells, often free. These bodies were round or oval and from 1 to 4  $\mu$  in diameter. Each contained an irregularly placed chromatin mass, the shape of which was globular, oval or kidney-shaped, the remainder of the parasite consisting of blue-staining basophilic substance. The parasite is surrounded by a non-staining refractile capsule, one-sixth of the diameter of the parasite in width and sometimes containing a single minute chromatoid dot, and similar granules are sometimes seen in the non-chromatoid part of the body of the parasite. Darling considers this organism to be different from the *Leishmania donovani* in the form and arrangement of its chromatin and in not possessing a blepharoplast.

#### PIROPLASMOSIS.

Up to the present no human disease has been proved to be associated with the presence of piroplasmata. The observations of Donovan, which seemed to indicate that the parasite of kála-azar might be found within the red blood corpuscles, and which led Laveran to denominate the *Leishmania donovani* the *piroplasma donovani*, have, as already indicated, not been confirmed; the same is true of the association of piroplasms with the occurrence of the Rocky Mountain spotted fever sometimes prevalent in Montana. But several important diseases of the lower animals are almost certainly caused by protozoan parasites of this group, and a short account of the organisms may be given.

The piroplasmata are pear-shaped unicellular organisms about 1 to 1.5  $\mu$  long and varying in breadth. The peripheral part is denser than the central, which often appears as if vacuolated, and at the broad end there is a well-staining chromatin mass. Sometimes irregular and ring-, rod-, or oval-shaped individuals occur. The organisms are found within the red blood corpuscles of the infected animal and also free in the blood. In the former situation there is sometimes only one within a cell, but the numbers vary under different circumstances and in different species. Multiplication takes place by fission, and the new individuals, remaining for longer or shorter times in apposition, account for some of the appearances seen in cells. Especially in the forms free in the blood pseudopodial prolongations of the protoplasm, usually from the pointed end, are developed, and it may be by means of such pseudopodia that entrance to the red cells is obtained. Infection is usually carried from infected animals by means of ticks. In one case Koch has described the development in the organism, in the stomach of the tick, of spiked protoplasmic processes sprouting out from the broad end of the piroplasm, and the occurrence of conjugation of two such individuals by their narrow ends to form a zygote. Further observations, however, here are necessary,



and nothing is known of the further history of the parasite within the insect except that the eggs in the ovary may become infected, so that insects developed from these can carry infection to animals. Frequently when an animal has passed through an attack of a piroplasmosis it is immune to the disease, and with regard to this immunity in certain cases very interesting facts have been observed. For instance, the condition may not be associated with the disappearance of the parasite from the blood of the immune animal, and the latter may thus be a source of danger to other non-immune animals with which ticks harboured by it may come in contact.

The following are the chief piroplasmata causing disease in animals :—

(1) *Piroplasma bigeminum*. This was first described by Theobald Smith and is the cause of Texas or red-water fever, a febrile condition associated with hæmoglobinuria, which occurs in the Southern States of America, the Argentine, South and Central Africa, Algeria, various parts of Northern Europe, and in Australia. The organism gets its name of bigeminum from the fact that it is often present in the red cells in pairs, which may be attached to one another by a fine thread of protoplasm; this probably results from the complete separation of two individuals being delayed after division has occurred. Infection is here spread by the tick *boophilus bovis*, and some of the characteristics of the disease epidemiologically are explained by the fact that this insect goes through all its moultings on the same individual host. (2) *Piroplasma parvum*. This organism was discovered by Theiler in the blood of cattle suffering from African East Coast fever, a disease closely resembling Texas fever, which prevails endemically in a narrow strip along a long extent of the east coast, and which occurs epidemically inland. As its designation implies, the organism is small, and it is also attenuated. Its insect host is the tick *rhhipicephalus appendiculatus*, and it may be noted that this tick drops off the animal on which it may be feeding when it is about to go through one of its several moultings. It can thus carry an infection much more quickly and widely through a herd than can the carrier of ordinary red-water fever. It may be said that in England there occurs a red-water fever also associated with the presence of a piroplasm in the blood, but the relationship of this organism to the other varieties has not yet been fully worked out. (3) *Piroplasma equi*. This organism gives rise to biliary fever in horses, another South African disease, and it is carried by the tick *rhhipicephalus evertsii*. In this disease Theiler made the interesting observation that when the blood of a donkey which had recovered from the disease was injected into a horse, the latter suffered a slight illness only, although the organisms were present in the blood injected. Such a fact is of importance, as attenuation of virulence in pathogenic protozoa seems, so far as our present knowledge goes, a not very common event. (4) *Piroplasma canis*. This causes a piroplasmosis occurring in dogs.

With regard to the pathology of infection by piroplasmata we know nothing. The diseases are often extremely fatal, carrying off nearly every individual attacked, but we do not know the nature of the changes originated.



## APPENDIX F.

### YELLOW FEVER.

YELLOW fever is an infectious disease which is endemic in the West Indies, in Brazil, in Sierra Leone and the adjacent parts of West Africa, though it is probable that it was from the first-named region that the others were originally infected. From time to time serious outbreaks occur, during which neighbouring countries also suffer, and the disease may be carried to other parts of the world. In this way epidemics have occurred in the United States and elsewhere, infection usually being carried by cases occurring among the crews of ships. In the parts where it is endemic, though usually a few cases may occur from time to time, there is some evidence that occasionally the disease may remain in abeyance for many years and then originate *de novo*. There is, therefore, reason to suspect that the infective agent can exist for considerable periods outside the human body. It is possible, however, that continuity may be maintained by the occurrence of a mild type of the disease, which may be grouped with the "bilious fevers" prevalent in yellow fever regions. This would explain the degree of immunity which is shown during a serious epidemic by the older immigrants.

Great variations are observed in the clinical types under which the disease presents itself. Usually after from two to six days' incubation a sudden onset in the form of a rigor occurs. The temperature rises to 104–105° F. The person is livid, with outstanding bloodshot eyes. There are present great prostration, pain in the back, and vomiting, at first of mucus, later of bile. The urine is diminished and contains albumin. About the fifth day an apparent improvement takes place, and this may lead on to recovery. Frequently, however, the remission, which may last from a few hours to two days, is followed by an aggravation of all the symptoms. The temperature rises, jaundice is observed, hæmorrhages occur from all the mucous



surfaces, causing, in the case of the stomach, the "black vomit"—one of the clinical signs of the disease in its worst form. Anuria, coma, and cardiac collapse usher in a fatal issue. The mortality varies in different epidemics from about 35 to 99 per cent. of those attacked. Both white and black races are susceptible, but those who have resided long in a country are less susceptible than new immigrants. An attack of the disease usually confers complete immunity against subsequent infection.

Post-mortem the stomach is found in a state of acute gastritis, and contains much altered blood derived from hæmorrhages which have occurred in the mucous and submucous coats. The intestine may be normal, but is often congested and may be ulcerated; the mesenteric glands are enlarged. The liver is in a state of fatty degeneration of greater or less degree, but often resembling the condition found in phosphorus poisoning. The kidneys are in a state of intense glomerulo-nephritis, with fatty degeneration of the epithelium. There is congestion of the meninges, especially in the lumbar region, and hæmorrhages may occur. The other organs do not show much change, though small hæmorrhages under the skin and into all the tissues of the body are not infrequent. In the blood a feature is the excess of urea present, amounting, it may be, to nearly 4 per cent.

**Etiology of Yellow Fever.**—Although a large amount of bacteriological work has been done on yellow fever, this has merely a historical interest, as it is now known that the causal agent is not one of the ordinary bacteria, but belongs to the group of ultra-microscopic organisms.<sup>1</sup> A mosquito acts as the intermediate host, and the facts detailed below point to the organism passing through some cycle of development in the body of the insect. The analogy of malaria makes it extremely probable that the organism is a protozoon, but this has not yet been completely proved. As bacteriological work led up to the establishment of our knowledge regarding the nature of the disease, some reference must be made to it.

A very full research into the bacteriology of yellow fever was that of Sternberg, and one of the organisms isolated, which he called the bacillus *x*, appeared possibly to have some relationship to the disease. Sanarelli in 1897 isolated an organism which he called bacillus *icteroides*, and which he considered to be the cause of yellow fever; it was probably identical with the bacillus

<sup>1</sup> In several diseases the existence of such causal factors is probable. Examples in animals are foot and mouth disease, South African horse sickness, and the contagious pleuro-pneumonia of cattle.



x of Sternberg, but subsequent observations made by others gave conflicting results. The bacillus icteroides, as described by Sanarelli, belongs to the paratyphoid group, possessing lateral flagella, growing on gelatin without liquefaction, and fermenting glucose but not lactose. Reed and Carroll found that it was practically identical with the bacillus of swine cholera. It must now be considered merely as an organism which may occur in the organs and tissues in yellow fever as a secondary infection, but without any etiological significance.

The facts of importance which have been established regarding the etiology of the disease are due to the labours of the United States Army Commission, which began its work in 1900. The members of the Commission first directed their inquiries towards determining whether the bacillus icteroides was present in the blood during life, and a series of cases was investigated bacteriologically, with entirely negative results in each instance. They then resolved to test the hypothesis of Dr. Carlos Finlay of Havana, promulgated several years previously, that the disease was carried by mosquitoes. Selecting mosquitoes which they reared from eggs, they allowed them to bite yellow fever patients and then to bite healthy men. Of several experiments of this nature two were successful in the first instance, the first individual to be infected in this way being Dr. James Carroll, a member of the Commission, who passed through a severe attack of typical yellow fever. Experiments were then performed on a larger scale, with completely confirmatory results, as to the conveyance of the disease by mosquitoes. Of twelve non-immunes living under circumstances which excluded natural means of infection, ten contracted yellow fever after having been bitten by mosquitoes which had previously bitten yellow fever patients; happily all of these recovered. Two of the men who were thus infected had been previously exposed to contact with fomites from yellow fever patients without results. These results were confirmed by Guitéras, whose investigations were carried out along similar lines; of seventeen individuals bitten by infected mosquitoes, eight took yellow fever, and three of these died.

The species of mosquito used by the American Commission was the *Stegomyia fasciata*, and up to the present time no other species has been found capable of carrying the infection. It has also been determined that a certain period must elapse after the insect has bitten a yellow fever patient before it becomes infective to another subject. In summer weather this period is about twelve days; at a lower temperature somewhat longer. This



probably means that, as in the case of malaria, the parasite must pass through certain stages of development before it reaches the salivary gland and is thus in a position to be transferred to a fresh subject. Infected mosquitoes, however, retain the power of infection for a considerable time afterwards, probably as long as sixty days. It has also been shown that mosquitoes may become infective after biting a patient on the first, second, or third day of the disease, but at a later period the results are usually negative, apparently because the virus is no longer present in the blood.

Interesting results were also obtained with regard to the communication of the disease directly from patient to patient, the conclusion arrived at, after careful experiments, being that the disease cannot be transferred in this way, even when the contact is of a close character. In a specially constructed house seven men were exposed to the most intimate contact with the fomites of yellow fever patients for a period of twenty days each, the soiled garments worn by the patients being in some cases actually slept in by these men; the result was that not one of those thus exposed contracted the disease. The conclusions on this point have been subsequently confirmed by other workers.

The American Commission also found it possible to transmit yellow fever to a healthy man by injecting small quantities of blood or of serum taken from a yellow fever patient at any period up till the third day of the disease. The period of incubation in this case is somewhat shorter than when the disease is conveyed by the bite of mosquitoes, the average duration in the former case being about three days, and in the latter about four days, though these times may be considerably exceeded. It is also interesting to know that in these experimental injections the blood or serum used was found to be free from bacteria. Up till the present time, we know of only these two methods of infection, namely, indirectly by the bite of a mosquito infected with the yellow fever germ; or directly by the injection of some of the blood from a yellow fever patient. In these respects there is a striking similarity to what has been established in the case of malarial fever.

Experiments with regard to the nature of the yellow fever organism were carried out by Reed and Carroll, and interesting results were obtained. They found that the organism of the disease was very easily killed by heat, as blood from a yellow fever patient lost its infective power on being heated to 55° C. for ten minutes. On the other hand, blood or serum was found to be still infective after having been passed through a Berkefeld



filter. This has been confirmed by the French Commission, with the additional result that the virus passes through a Chamberland F filter, but not through a Chamberland B. These facts would show that the parasite is of extremely minute size, and apparently belongs to the group of ultra-microscopic organisms. Up till the present time all attempts to find by microscopic examination the yellow fever parasite, either in the blood of patients suffering from the disease or in the tissues of infective mosquitoes, have been attended with negative results. It has been recently stated that it is possible to produce yellow fever in the chimpanzee by the injection of blood from a patient.

Though nothing has been determined regarding the actual nature of the virus, yet the results already obtained have supplied the basis for preventive measures against the disease, these being directed towards the destruction of mosquitoes and the protection of those suffering from yellow fever, and also the healthy, against the bites of these insects. Already a striking degree of success has been obtained in Havana. Such measures came into force in February 1901, and in ninety days the town was free of yellow fever, and for fifty-four days later no new cases occurred; and although subsequently the disease was reintroduced into the town, no difficulty was experienced in stamping it out by the same measures. In recent years the results have also been highly gratifying, and the disease may be said to be practically eradicated from Havana. In other places also successful results have been obtained, and epidemics would appear to be now under control if the proper measures are taken.



## APPENDIX G.

### EPIDEMIC POLIOMYELITIS.

DURING the past twenty years, the occurrence at one time and place of groups of cases of acute anterior poliomyelitis has favoured the idea that the condition might be of an infective nature. This view was supported by the work of Landsteiner and Popper, who, in 1909 in Vienna, succeeded in producing the disease in a monkey by the intraperitoneal injection of an emulsion of the spinal cord of a child who had succumbed on the fourth day of illness. The occurrence in New York in the summer of 1907 of an epidemic in which probably over 2000 cases occurred, 762 of which were carefully investigated by a Commission, concentrated attention in America upon the condition, and a recrudescence in the summer of 1909 furnished Flexner with material for investigation. In earlier experiments it had been observed that the cerebro-spinal fluid was non-infective, and that, while it was possible to infect monkeys with the disease, the transference of the condition from monkey to monkey was not successful. Flexner found that if for intraperitoneal injection intracerebral inoculation was substituted, the brain and cord of the infected animals was infective for other monkeys, the incubation period being from four to thirty-three days. In this way the disease was kept up by subdural and intravenous injection, and also by injection into the sheath of nerves such as the sciatic,—the intraperitoneal and subcutaneous methods being found also to give results. The disease produced resembled in every way the disease naturally occurring in children, and frequently resulted fatally, as in the natural illness. When the virus was injected into the sheath of a nerve, the paralytic symptoms first appeared in relation to that part of the cord from which the nerve emerges. Infective material preserved in glycerin retained its virulence,—a fact which rather militates against there being a bacterial virus,—and the virus could also withstand prolonged freezing and drying for a considerable period.



In animals other than monkeys no result followed infection. A careful microscopic examination of the nervous system in natural and experimental cases did not disclose the presence of bacteria or protozoa, and it was found that the virus could pass through a Berkefeld filter without losing its infectivity, and that the cords of animals thus infected were still infective for further animals,—mere toxic action being thus excluded. These results have been confirmed by Levaditi working with the chimpanzee, and there is thus little doubt that here again we are dealing with an ultra-microscopic virus. Flexner found that when the virus was mixed with bouillon a turbidity developed, but no formed organic body could be detected. He further found that monkeys which had passed through the illness following inoculation were insusceptible to re-inoculation, and both he and Levaditi noted that the serum of such insusceptible monkeys had the capacity of neutralising the virus, a similar result being obtained with the serum of human cases which had recovered.

With regard to the distribution of the virus in an infected animal, the chief concentration is found in the nervous system, but it was also found in lymphatic glands; as has been stated, the cerebro-spinal fluid is apparently inert. No facts are known bearing on the channel of natural infection or on the path by which the poison reaches the cord,—whether by the general lymph stream or by the sheaths of nerves, but the virus can be eliminated by the nose, and infection can also be effected through the scarified nasal mucosa. In its pathology the condition bears many resemblances to rabies.

These recent observations are of the greatest importance in relation to the etiology of the condition and also possibly in relation to treatment.



## APPENDIX H.

### PHLEBOTOMUS FEVER.

IN Dalmatia, Herzegovina, and neighbouring parts of the Adriatic littoral there occurs a disease known as "pappataci," characterised by fever lasting for about three days, followed by somewhat prolonged prostration, but very rarely having a fatal issue. Doerr, after failing to isolate any organism from the blood, found that the subcutaneous injection of from .5 to 1 c.c. of the serum from a case into a healthy individual was followed about eight days later by an attack of the disease. A similar effect was produced with the serum after it had been passed through a Berkefeld filter,—all the inoculation experiments being performed at a distance from the original location of the disease. The view is therefore put forward that here we have to deal with another example of an ultra-microscopic virus. The disease has been only observed in the summer season, and Doerr considered there was justification for the popular view that it was associated with the bite of the dipterous fly, *phlebotomus pappatasi*. This was borne out by the fact that on feeding such flies on a sick person, transporting them to a locality free from the disease and allowing them to bite healthy individuals, the affection was reproduced. An apparently identical disease occurs in Malta and has been investigated by Birt under the name of "Phlebotomus Fever." This observer fully confirmed Doerr's results, the condition again being reproduced by infected flies which, however, were found not to manifest infectivity earlier than seven days after biting.

These results are of importance of themselves as throwing light on the etiology of a troublesome disease of the Mediterranean littoral, but they are also interesting as having a possible bearing on the pathology of a group of similar affections occurring in various parts of the world,—chiefly in coastal areas,—and going under a variety of names. Examples are dengue, the three-day fever of various regions, Canary fever, Shanghai



fever, Chitral fever, and the seven-day fever or simple continued fever of India. Of these, that presenting the most definite clinical picture is dengue,—a condition for long well known and having an extensive distribution, and it may be said that Ashburn and Craig in the Philippines found the blood in dengue as in pappataci to be infective even after filtration. Whether all these disease conditions are identical further research must decide; at present Birt believes that at any rate pappataci and dengue are distinct, and certainly Doerr does not in his description allude to the terminal skin eruption which Manson believes to be of very constant occurrence in the latter. The rarity of a fatal result in these diseases makes their investigation by inoculation of the human subject relatively safe.



## APPENDIX J.

### TYPHUS FEVER.

UP till recently all attempts to elucidate the etiology of this disease by ordinary bacteriological methods have given equivocal results. Certain experiments, however, performed by Nicolle in 1909, during an outbreak in Tunis, are of importance. This observer found that the inoculation of the monkeys, *macacus cynomolgus* and *macacus sinicus*, with typhus blood gave a negative result. On injecting 1 c.c. of such blood into a chimpanzee, however, an illness presenting the features of the disease in man, including the eruption, resulted three days later, and death occurred. It was then found that the blood of this animal was capable of originating a similar disease after an incubation period of ten to fourteen days in the lower apes referred to, and this was kept up through several passages. The virulence of the material, however, gradually died out. The dog and the white rat were insusceptible. It was found that *macacus sinicus* could be infected by means of the body louse, the incubation period, however, being lengthened to forty days. These experiments probably throw important light on the etiology of the condition, and on the means by which the disease is spread in man.



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