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BACTERIOLOGY

A MANUAL
OF
BACTERIOLOGY
CLINICAL AND APPLIED

By

R. TANNER HEWLETT

M.D., F.R.C.P., D.P.H.(Lond)., F.R.M.S.

Professor of Bacteriology in the University of London; Director of the Bacteriological Department, King's College, London; Director of Pathology, Seamen's Hospital, Greenwich; Lecturer on Bacteriology, London School of Tropical Medicine; Examiner in Bacteriology, University of London

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PREFACE TO THE SIXTH EDITION

In the present edition the text has been revised throughout, many sections have been re-written and much new matter has been introduced. The following is a summary of the principal alterations and additions :—

Under methods, media, etc., Donald's method of drop measurements, throttled pipettes, Douglas's trypsin agar, Fildes' method of sterilising fluid serum and McIntosh and Fildes' method for anaërobic cultivation have been added. Considerable additions have been made to the sections dealing with anaphylaxis, agglutination and immunity. Chapter VI, dealing with suppuration and septic conditions, has been materially altered, and the account of cerebro-spinal fever re-written.

Chapter X, dealing with the Typhoid-Colon Group of organisms, has been extended, the accounts of the paratyphoid and dysentery bacilli have been re-written, and a fuller table of fermentation reactions and the Oxford Standard Method of Agglutination have been introduced.

Chapter XIII, dealing with anaërobic organisms, has been much added to and an account given of the relation of this group of organisms to war-injuries. The section on cholera has been extended, and in Chapter XV Chalmers' and co-workers' classification and nomenclature of the so-called streptothrix infections have been adopted.

In Chapter XVIII, dealing with the Protozoa, the sections on the intestinal forms have been re-written, and Spirochaetosis has received many additions, while in view of the prominence now accorded to the control of venereal disease the section on syphilis has been modified and added to, and a full account of the technique of the Wassermann reaction is given.

Under Disinfection reference is made to new methods of testing and to new antiseptics.

Prominence is given throughout to those microbic conditions having a bearing on war medicine and surgery.

Several new figures and plates have been introduced, and for the photo-micrographs I am indebted to Mr. J. E. Barnard, F.R.M.S., Lecturer in Microscopy, King's College, London.

My best thanks are again due to Dr. F. E. Taylor, Lecturer in Bacteriology, King's College, London, for many suggestions and for much assistance in the revision of the proofs.

R. T. H.

University of London,
King's College.

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A MANUAL OF BACTERIOLOGY

INTRODUCTION

BACTERIOLOGY is a branch of Biology which deals with the study of Micro-organisms, particularly the minute vegetable ones known as *Bacteria*. The scope of bacteriology is difficult to define exactly, for the term is often used in a comprehensive sense equivalent to micro-pathology, or even micro-biology, and all investigations connected with micro-organisms, animal and vegetable, may be included under it. So extensive, however, has the subject become that the animal micro-organisms are now being studied as a separate branch, PROTOZOOLOGY. Bacteriology deals with micro-organisms particularly in their relation to processes—disease, fermentation, putrefaction, and the like—while the study of their structure, functions, and life-history belongs to the botanist and zoologist. There is no space in this work to enter into the history of the science, but the names of Leeuwenhoek (1675), Müller (1786), Schwann (1837), Cohn, Pasteur, Lister, Koch and Ehrlich will ever hold an honourable place in its annals.

The study of micro-organisms must always be of importance in general biology, for their vital phenomena are comparatively simple, and shed light on the more complex processes occurring in the higher orders of living

beings. Weismann based his theory of heredity on the fundamental conception of the immortality of these unicellular organisms. Excluding accidents, they are immortal—they reproduce themselves by a process of simple division, an individual dividing, and two daughter forms taking the place of the original parent one, and although the parent has disappeared yet there has been no death, no dissolution; its protoplasm or living material is still existent in its progeny and is immortal, since this process of reproduction apparently may go on indefinitely. Moreover, the study of the mutability and possible transformation of species of micro-organisms is likely to throw light on the theory of evolution. Organisms such as bacteria multiply so rapidly that fifty or sixty generations may develop in thirty hours, a number which would take years to attain if even the most rapid breeder among mammals were the subject of experiment, and as they occur in vast numbers the opportunity for variation is extensive. These are some of the relations which micro-organisms have with general biology.

In what may be termed the economy of nature micro-organisms are all-important; without them there would be no putrefaction, no decay, and the dead remains of animal and vegetable life would accumulate and encumber the earth, which would become barren for the want of the organic matter originally derived from it, but of which there was no return. In fact the higher plants, and indirectly, therefore, animals also, are largely dependent for their existence upon the presence of bacteria in the soil, which fix atmospheric nitrogen and break up and render assimilable complex substances used as manures.

The question of life, animal and vegetable, without bacterial activity is an important and interesting one. It would seem from the experiments of Duclaux [1] that

[1] *Comp. Rend.*, t. 100, p. 66.

the higher plants in ordinary circumstances are unable to obtain nutriment unless the complex compounds, proteins, urea, and other nitrogenous bodies, which form the important constituents of many manures, are broken down into simpler ones through the agency of bacteria. He sowed seeds in sterile soil free from nitrates, nitrites, and ammonia, which was plentifully watered with sterile milk and solutions of sugar and starch. No changes occurred in these substances, the seeds lost weight, and the seedlings dwindled and died. As regards the higher animals various views have been expressed. Pasteur considered that their life also would probably be impossible without the presence of bacteria in the intestinal tract. Nencki expressed the opinion that this idea of Pasteur's was an erroneous one, and his experiments in conjunction with Macfadyen and Sieber [1] showed that any considerable decomposition of the food by bacteria first takes place in the large intestine, and that the digestive juices alone, without the co-operation of bacteria, are able to prepare the constituents of the food for absorption. Nuttall and Thierfelder obtained unborn guinea-pigs by Cæsarian section with antiseptic precautions, and afterwards kept them in a sterile environment and fed them on sterilised food. Not only did the animals live, but they were even in a more thriving condition than those naturally brought up. The intestinal tract was found to be sterile on the eighth day. Schottelius, however, found that chickens reared on sterile food were retarded in development, and experiments by Moro on turtle larvæ point to the same conclusion, viz. that intestinal bacteria are necessary for normal nutrition. On the other hand, Cohendy [2] was able to rear chickens satisfactorily without the presence of bacteria. Levin found

[1] *Journ. of Anat. and Physiol.*, xxv, p. 390.
[2] *Ann. de l'Inst. Pasteur*, xxvi, 1912, p. 106.

that the intestinal tract in many Arctic animals—the polar bear, reindeer, seal, eider duck, etc.—is generally sterile, and in these instances, therefore, bacteria are not required for normal nutrition.

Commercially, micro-organisms are of the utmost importance. Without them there would be no fermentation, and the wine, beer, and indigo industries, the ripening of cheese and tobacco, and many like processes would be non-existent. From a financial aspect also micro-organisms cannot be ignored; thus, many of the so-called "diseases" of beer and wine, which often occasion serious loss, are due to the entrance of adventitious forms, and the silk industry and sheep farming in France were once threatened with extinction owing to the ravages of pébrine and of anthrax respectively, but through the genius of Pasteur were restored to their former prosperity. There is no need to emphasise the importance of micro-organisms from a medical and hygienic point of view, but the fact may be recalled that sixty years ago the mortality after operations was very high, and that 40 per cent. of these deaths were caused by pyæmia, septicæmia, and hospital gangrene, conditions which are due to the entrance of micro-organisms, and which are now almost preventable, thanks to the antiseptic system introduced by Lord Lister and its further development, the aseptic system.

The theory of spontaneous generation or abiogenesis is intimately connected with the study of bacteria. The putrefaction of animal and vegetable fluids even after boiling, and the growth in them of minute living forms, were held by many to be a sure proof of the development of life from inanimate matter, of the spontaneous generation of the living from the non-living. A succession of investigators, however, showed (1) that if the fluids be boiled sufficiently long, and be then sealed up so as to

prevent the access of air, they do not undergo putrefaction; (2) that the sealing up may be dispensed with, provided the air be first filtered through cotton-wool before being admitted to the flasks; (3) that even the cotton-wool is not needed if the air be passed slowly through a long and tortuous channel, so as to deposit its solid particles. Tyndall showed that putrescible fluids may be exposed in open vessels in a closed chamber in which the air has been undisturbed for some time and its solid particles thereby deposited on the walls of the chamber, which had been smeared with glycerin; he also proved that vegetable infusions and the like, which putrefy after having been boiled for ten minutes, do not do so if the boiling be repeated on two or three successive days, and explained this by the supposition that while the fully developed bacteria are destroyed by the first boiling, their more resistant spores remain alive, but these on being left for twenty-four hours germinate into the less resistant bacterial forms, which are destroyed by the second boiling, and by the repetition of the process complete sterilization may ultimately be obtained. It is this process of "discontinuous sterilization," as it is termed, which is employed by the bacteriologist for the preparation of sterile culture media.[1]

The occurrence of abiogenesis (or as he preferred to term it, "archebiosis") was maintained by Bastian up to his death in 1915. He claimed that certain saline solutions which had been boiled or even heated above the point-boiling in sealed tubes after a time show the development of various living organisms, including bacteria and yeasts.[2]

[1] The writer believes that this explanation is only partially true, and would ascribe some of the sterilising effect of repeated heatings simply to the injurious action of alternate heating and cooling.

[2] See various papers in the *Proc. Roy. Soc. Lond.*; *The Evolution of Life*, Methuen, 1907; and *Proc. Roy. Soc. Med.* 1913.

Dunbar,[1] as the result of a series of experiments conducted over a long period and with every care to prevent contamination, has come to the conclusion that the bacteria are not an independent group of organisms, but that the bacteria, yeasts, and moulds are stages in the life-history of green algæ. The observations were carried out both by culture methods and by microscopical examination. A culture of a single-celled alga belonging to the *Palmellacea* was obtained, but by modifying the culture medium in which a pure culture of the alga was growing, by the addition of acid, of alkali, or of traces of copper salts, other organisms, generally bacteria, occasionally moulds and yeasts, and even spirochaetes, made their appearance. Granting that there is no flaw in the experimental methods, and every care seems to have been taken to exclude contamination, etc., the results are susceptible of another explanation, viz. that the secondary growths were derived by transformation of the algal cells, in fact by the phenomenon of hetero-genesis which has been claimed by Bastian to occur.

Undoubtedly bacteria exhibit variations and mutations, not only in morphology (see p. 16) but also in function. Thus pathogenic organisms may become non-pathogenic, and Twort has succeeded in training *B. typhosus* to ferment lactose, which ordinarily it does not. Some experiments by Horrocks suggest that the *B. typhosus* may, by symbiosis with *B. coli*, be converted into *B. alcaligenes*, and Revis has found many variations occur with coliform organisms as a result of cultivating in malachite green media, etc.[2]

As a result of exposure of sporing anthrax to ultra-

[1] See *Journ. Roy. Inst. Pub. Health*, vol. xv, No. 11, 1907, p. 679.

[2] *Proc. Roy. Soc. Lond.*, B, vol. 85, p. 192, and vol. 86, p. 373; *Centr. f. Bakt.*, Abt. II, 1912 and 1913.

violet rays, Mme. Henri[1] states that stable coccoid and Gram-negative thin filamentous forms are obtained.

Minchin in a presidential address to the Quekett Microscopical Club pointed out that syngamy (sexual reproduction, *e. g.* conjugation) is of the greatest importance in preserving differentiation of species, and that without it a species will tend to break up into races. It therefore follows that there are no true species among organisms of the bacterial grade, if it be true, as is usually held, that syngamy does not occur amongst them, and the so-called species of bacteria are to be regarded as mere races or strains capable of modification in any direction.

While much progress has been made during the last two or three decades, a vast amount still remains to be done. We have only touched the fringe of the explanation of the perplexing problems of susceptibility and immunity, and of the important question of cure in, and prevention of, infective diseases, while the chemistry of the products of bacterial activity is still in its infancy.

The literature of Bacteriology has now become somewhat extensive. In the following pages references to original papers have been freely introduced, many of which contain a more or less full bibliography on the subject referred to, so that further information may be obtained if required. Kolle and Wassermann's *Handbuch der Pathogenen Mikroorganismen*, ed. ii, is the most encyclopædic work on pathological bacteriology yet published.

[1] *Comp. Rend. Acad. Sc.*, vol. 158, No. 14, 1914, p. 1032.

CHAPTER I

THE NATURE, STRUCTURE, AND FUNCTIONS OF THE BACTERIA: THEIR CLASSIFICATION, GENERAL BIOLOGY, AND CHEMISTRY—BACTERIA AND DISEASE

THE Bacteria or Schizomycetes ("fission fungi") are minute vegetable organisms for the most part unicellular and devoid of chlorophyll, which multiply by simple transverse division or fission; this distinguishes them from the yeasts, in which multiplication takes place by budding or gemmation. A certain number of filamentous forms are also included, serving to connect the unicellular ones with the multicellular true fungi. The "fission plants" may be placed in a sub-kingdom, the Schizophyta, which may be divided into two classes: Class I, Schizophyceæ, the blue-green algæ, and Class II, Schizomycetes, the bacteria.

The unicellular plants are sometimes termed the "Protophyta." It must be understood that there are connecting links between the different groups, and that there is no sharp line of demarcation between them.

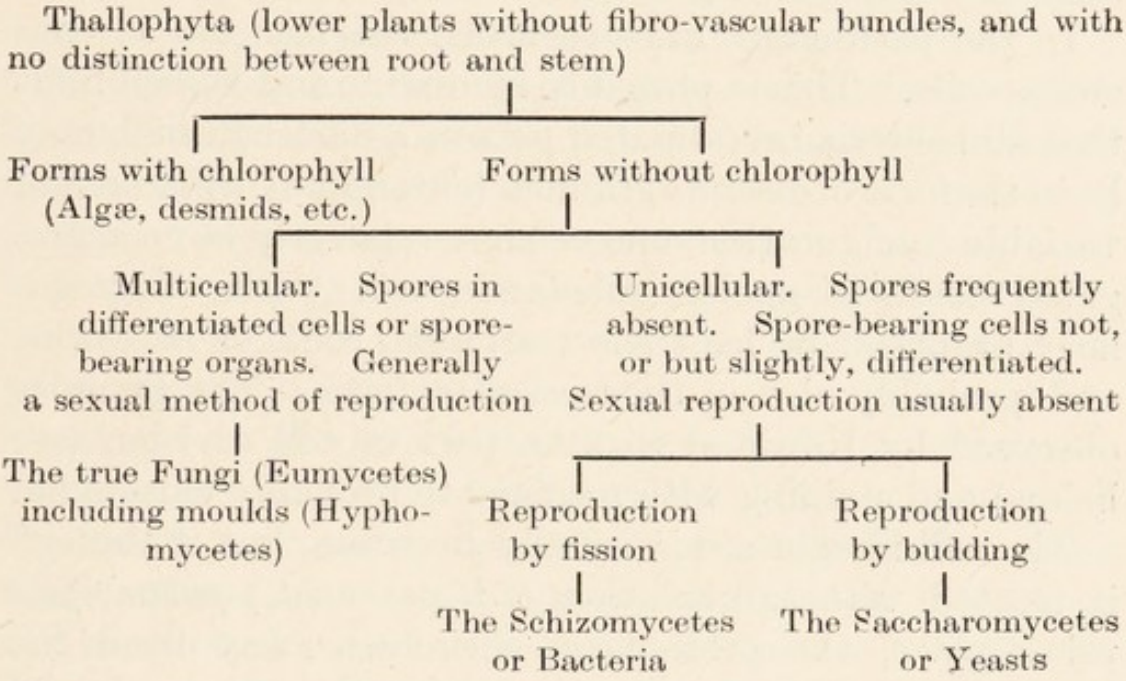
The relation of the bacteria to other lower plants is shown in the following scheme (p. 9):

The Bacteria have been supposed to have affinities with the Fungi, with the Protozoa, or with the Cyanophyceæ. There is little or no evidence to connect them with the first two groups and not much with the last one, though the resemblances here are greater. Though usually re-

garded as simple forms, the Bacteria display considerable morphological and structural differentiation and physiological complexity and are by no means primitive forms.

The size of the bacteria is variable, but they are all microscopic, measuring 0·3μ to 30–40μ in diameter or in length.[1] Their shape likewise is very different in the different species ; some are spherical, others ovoid, others

Relation of Bacteria to Lower Plants



rod-shaped or filamentous, while in some the rod or filament is twisted into a spiral. The end of the cell is occasionally almost rectangular, but is usually more or less rounded ; it is probably never pointed except in the Spirochaetæ, if these be true Bacteria (see p. 19). The bacterial cell consists of a cell-membrane enclosing the transparent, more or less structureless living matter or protoplasm, the cell-plasma or cytoplasm. Bütschli has described the bacterial plasma as having a reticular structure, but in the young cell this is probably either an artifact or a " false image " due to faulty illumination ;

[1] μ = micron = 0·001 mm.

the most that can be seen is a fine granulation. The protoplasm frequently contains granules composed of fatty or protein matter, pigment, and in some species of sulphur; occasionally certain granules stain blue with iodine. In some species "metachromatic" granules occur, chiefly at the poles; these stain red or pink with many blue dyes, *e. g.* methylene blue, are composed of nucleic acid combined with an organic base and are to be regarded as non-living reserve material (Dobell).

In the past many have regarded the bacteria as enucleate cells. This is probably incorrect, and Dobell finds that all bacteria investigated possess a nucleus which may be in the form of discrete granules (chromidia), a filament of variable configuration, one or more relatively large aggregated masses of nuclear substance, or a system of irregularly branched or bent short strands, rods, or networks, and probably also in the vesicular form. The granules observed by Rowland to take part in cell division (see below) and staining with roseine are probably chromidia.

The cell-membrane is usually invisible, but if the cell is treated with salt-solution (2·5 per cent.) *plasmolysis* takes place, the protoplasm shrinking away from the membrane, which then becomes visible. It can also be stained *in vivo* with very dilute solutions of roseine. The cell-membrane sometimes becomes thickened, swollen, and gelatinous on its outer surface, forming a layer or so-called "capsule" around the organism. The clear spaces frequently seen around bacteria in dried and stained preparations, especially in those from blood and lymph, are generally artifacts and not true capsules. In *Cladothrix* and some other forms the cell-membrane becomes hardened, leading to the production of a firm sheath. When bacteria assume the resting stage groups of them adhere together in a jelly-like matrix, forming what is known as a "zooglœa."

The chemical composition of bacteria varies much, not only in different species, but even in the same species when grown on different nutrient media. All bacteria contain proteins, lipoid substances, and salts. Nencki termed bacterial protein "mykoprotein," and asserted that it differs from ordinary protein matter in not being precipitated by alcohol and in not containing sulphur. This does not appear to be the case with the proteins obtained by grinding bacterial cells, which seem to agree with other proteins in heat-coagulation, etc.

The proteins are mainly globulins and nucleo-proteins. The cell-wall is relatively insoluble, and generally consists chiefly of a material like *chitin*, and not of cellulose; in this respect bacteria resemble animal rather than vegetable cells. Carbohydrates are generally scanty. Spores differ from the parent cells in containing a larger proportion of solids and less water.

All species of bacteria, but especially the smaller ones, when suspended in a fluid exhibit what is known as Brownian movement, consisting of an oscillation with some amount of rotation about a fixed point, but there is little actual movement of translation, unless due to flotation. This Brownian movement is physical and not vital in origin, and occurs with all fine particles suspended in a fluid, and must be clearly distinguished from a true vital motility.[1] Some bacteria are always motionless, others are more or less motile, but these, too, have a resting stage. For motility to occur the cells must be young, and the conditions favourable to growth and development. Motility is due to delicate protoplasmic threads termed "flagella" connected with the outer layer of the cell protoplasm; these vibrate to and fro and

[1] Brownian movement is due to "the incessant movements of the molecules of the liquid which, striking incessantly the observed particles, drive them about irregularly through the fluid" (Perrin).

propel the organism through the medium. A cell will, however, move indifferently in either direction; if a motile organism be watched it will often be seen to proceed rapidly in one direction, stop, and then return without turning round. The flagella are not visible in the living state, unless dark ground illumination be used, nor by the ordinary methods of staining, unless a mordant is previously used and are extremely liable to be broken off. They vary considerably in number and in length; some organisms have but a single flagellum at one pole (*monotrichic*), *e. g. Bacillus pyocyaneus*, others have two or more flagella forming a brush or tuft (*lophotrichic*), *e. g. Spirillum rubrum*, while others may be almost entirely covered with them (*peritrichic*), *e. g. B. typhosus*; in some the flagella are short and straight, and in others long and sinuous. The motility of organisms does not necessarily depend directly upon the number of flagella they possess, an organism with a few flagella often being more active than another possessing many, and some are apparently non-motile, though well-marked flagella can be demonstrated. Generally speaking, however, an organism with several flagella will be more motile than a similar form with a few.

Darwin says: "In looking at Nature it is most necessary never to forget that every single organic being may be said to be striving to the utmost to increase in numbers," and in no group perhaps of the animal and vegetable kingdoms is this more marked than among the Bacteria. Reproduction is generally considered to be always non-sexual, and takes place in two ways—by simple division or fission and by spore formation. Dobell considers that all the evidence is definitely against the view that a sexual process occurs at any stage in the life-history of Bacteria. Schaudinn, however, described an apparent conjugation in one species (*B. flexilis*) and Nadson states

that in a few species sister cells conjugate and from this conjugation a spore arises. Hort[1] also claims that the life-cycle of Bacteria is by no means a simple one, and Löhnis and Smith[2] state that bacteria live alternately in an organized and in an amorphous stage. In the latter, the living matter of several cells unites and undergoes a thorough mixing, a large mass or "symplasm" being formed, from which small bodies, "regenerative units and bodies," develop and ultimately become cells of normal shape. Direct conjugation between two or more cells was also observed. Small bodies or "gonidia" were also found to be liberated from the bacterial cells, the gonidia in some cases being so small as to be filterable through a porcelain filter. The gonidia form either regenerative bodies or occasionally exospores.

Reproduction by transverse fission is common to all bacteria; the bacterial cell becomes constricted at its middle and finally separates into two parts, and thus two young cells take the place of the parent one; reproduction by fission is therefore also an increase in numbers. The fission is always transverse, never longitudinal,[3] the rule being in cell-division that the new membrane is formed in the most economical manner. Longitudinal division, on the other hand, is comparatively common among the Protozoa. Previous to division the rod-forms become elongated and the spherical ones ellipsoidal, and there is an increase in the number of the roseine-staining granules, partly by division of pre-existing ones and partly by new formation. The constriction in the majority of cases involves and passes through one of the granules.

[1] *Brit. Med. Journ.*, 1917, vol. i, p. 571.

[2] *Journ. Agricult. Research* (Washington), vol. vi, 1916, No. 18.

[3] Longitudinal division has been described in a few species, but its occurrence is so rare that it seems doubtful if these forms be true bacteria.

In the monotrichous and lophotrichous bacteria it is always the non-flagellated end of the dividing cell which bears the flagella of the new cell. Under favourable conditions reproduction may be very rapid, fission occurring every twenty or thirty minutes (Klein), so that, the increase being in a geometrical ratio, the number of individuals which might arise from a single bacterium in three or four days is almost inconceivable, and would *en masse* weigh thousands of tons ; fortunately there are many checks to such a rapid multiplication. Frequently, although the protoplasm divides, the division of the cell-membrane is incomplete, resulting in a loose union of the cells with the formation of a pseudo-filament. These filaments often become much curved and twisted, forming tangled masses, owing to fission taking place in the cells in the middle of the filament as well as at the ends, so that the filaments have to become curved to make room for the new cells.

Reproduction by spore formation is met with in some species, and is generally described as being of two varieties. In one, endogenous or endospore formation, a refractile round or ovoid body forms within the bacterial cell, the development of which can be watched under the microscope. Rowland described the process of spore formation as follows : Refractile, oily-looking droplets, which do not stain with roseine, appear and ultimately coalesce, forming the spore. The cell-plasma at the same time diminishes and retracts from the cell-membrane. The roseine-staining granules increase in number and aggregate into two spherical masses, which dispose themselves one at each end of the cell. The cell-membrane collapses somewhat, and, when the spore is fully formed, ruptures transversely, leaving two cup-shaped receptacles, in which the granules and remains of the plasma are still recognisable. Only one spore develops in each cell, and

the spores serve to perpetuate the race when it is threatened with extinction from adverse circumstances. Each spore consists of a little mass of protoplasm enclosed within a very tough and resisting membrane, which tends to preserve its vitality even under unfavourable conditions; for spores resist the action of desiccation and germicidal agents to a much greater degree than the fully developed organisms. Endospores vary much in size and in the position they occupy within the bacterial cell in the different species; their diameter is usually about the same as that of the cell in which they are developed, but may be much greater, and in position they may be central or terminal, and sometimes the spore-bearing cells are swollen or club-shaped; these are termed "clostridia." Endospores are still unknown in a large number of species. The other variety of sporulation, arthrospore formation, is of doubtful occurrence, but is stated to take place as follows: Some of the elements formed by fission are slightly larger, more refractile, and more resisting than their fellows, and are stated to have the properties of spores.

Placed in favourable circumstances, the spore in either case germinates, it becomes swollen and granular, and loses its refractile appearance; a slight protuberance forms, this increases in size, and an organism similar to the parent one is finally reproduced; the empty spore membrane at first frequently encloses one extremity, and is afterwards cast off. In certain instances the spore germinates without casting its membrane, the spore membrane becoming the cell-wall of the young organism. The ellipsoidal spores of the *B. anthracis* sprout from the end, those of *B. subtilis* from the side ("polar" and "equatorial" germination respectively).

On the Morphology, etc., of the Bacteria see Dobell, *Quart. Journ. Micr. Sci.*, vol. 56, 1911, p. 395 (Bibliog.); Pénau, *Comp.*

Rend., clii, 1911, p. 53 ; Prazmowski, *Bull. Internat. de l'Acad. des Sci. de Cracovie*, No. 4, *B*, April 1913, p. 105 (Bibliog.).

Classification of the Bacteria

Many classifications of the Bacteria have been proposed, but none can be said to be strictly scientific, or even satisfactory from the standpoint of convenience. A somewhat heterogeneous group of organisms has undoubtedly been described under the term Bacteria, and many forms exist intermediate between those unicellular organisms with and those without chlorophyll, so that a hard and fast line cannot be drawn. Moreover, bacterial cells are so minute that only a few broad differences can be observed in the morphology and reproductive processes of different species, and therefore ordinary criteria are not available for the classification of the Bacteria.

One of the most prominent of the older classifications was that of Cohn. He divided the Bacteria into four principal groups :

I. The Sphærobacteria or spherical forms.
II. The Microbacteria or short rod-forms.
III. The Desmobacteria or long rod-forms.
IV. The Spirobacteria or spiral forms.

Zopf's classification (1885) has many points to commend it, but is largely based on the occurrence of pleomorphism. By pleomorphism is meant a variation in the form of an organism during its life-cycle, a coccus, for example, growing into a rod, or a straight rod becoming a spiral. In a peach-coloured bacterium examined by Lankester, coccoid, rod, filamentous, and spiral forms occurred, and the doctrine of pleomorphism received considerable support from his work, though it may be questioned whether he was working with pure cultures.

Be that as it may, a certain amount of pleomorphism undoubtedly occurs in some organisms. In the colon, typhoid, and plague bacilli, for example, the rods may sometimes be so short as to be almost cocci, while at others they are well-marked rods and even filaments (see also p. 6).[1]

The abnormal forms of cells which are commonly seen in old cultures are termed "involution forms," and are generally regarded as altered, degenerate and decrepid cells. Some of these "involution" forms are, however, considered by Löhnis and by Hort to be developmental forms (see p. 13).

The following is an outline of Zopf's classification, the Bacteria being divided into four principal groups or families, which again are subdivided into smaller groups or genera :

Family I. Coccaceæ.—Spherical forms only ; division occurs in one or more directions.

Genus 1. Micrococcus (Staphylococcus).—Division in one direction only, but irregular, so that the cocci after division form irregular clusters.

Genus 2. Streptococcus.—Division in one plane, but regular, so that the cocci form chains.

Genus 3. Merismopedia (Tetracoccus).—Division in two directions at right angles to each other, but in the same plane, so that lamellæ or plates are formed.

Genus 4. Sarcina.—Division in three directions at right angles to one another and in two planes, so that cubical masses are formed.

Genus 5.—Ascococcus.—Cocci which develop in a gelatinous matrix.

[1] See Dobell, *Journ. of Genetics*, ii, pp. 201, 325.

Family II. BACTERIACEÆ.—Rods, straight or curved, at some period of the life-history, though coccoid and other forms may occur.

Genus 1. BACTERIUM.—Straight rods; endospore formation does not occur.

Genus 2. BACILLUS.—Straight rods; endospore formation occurs.

Genus 3. LEUCONOSTOC.—Cocci and rods; arthrospore formation occurs in the coccoid forms.

Genus 4. CLOSTRIDIUM.—The same as bacillus, but the spore-bearing rods are enlarged and club-shaped.

Genus 5. SPIRILLUM.—Spiral rods; spore formation does not occur.

Genus 6. VIBRIO.—Spiral rods; spore formation occurs.

Family III.—LEPTOTRICHEÆ.—These are unbranching thread forms.

Family IV. CLADOTRICHÆ.—These are thread forms showing true but not dichotomous branching.

There are many features in this classification which are of practical value. The distinction made between a bacterium and a bacillus, for example, is convenient. Formerly a short rod was termed a bacterium, and a long rod a bacillus, but such a division is an arbitrary one, and at one stage of its life-history an organism might be a bacterium and at another a bacillus. The term "bacterium" is now but little used in this sense, and any straight rod is termed a bacillus. The term "staphylococcus" is one frequently met with; it is practically synonymous with micrococcus, and refers to cocci which are aggregated into groups or clusters. Of the twisted rods, a simple curved rod is now known as a vibrio, a definitely cork-screw form of three or a few turns is a

spirillum, a long and flexible twisted filament is a spirochaeta. The systematic position of the Spirochaetæ has given rise to controversy. The parasitic ones (*e. g.* that of relapsing fever) are commonly regarded as Protozoa, but Dobell [1] dissents from this view and considers them all to be much more closely allied to the Bacteria, which he classifies as follows :

SCHIZOPHYTA { Cyanophyceæ ; Bacteria { Trichobacteria ; Haplobacteria { Coccoidea ; Bacilloidea ; Spirilloidea ; Spirochaetoidea { *Spirochaeta* ; *Treponema* ; *Cristispira* ; *Saprospira*

Another classification is that proposed by Migula.[2] The Bacteria are divided into two orders : the Eubacteria —bacteria proper—the cells of which contain neither sulphur granules nor a colouring matter, bacteriopurpurin ; and the Thiobacteria, the cells of which contain sulphur granules and may be coloured with bacterio-purpurin. The Eubacteria are divided into five families : (1) Coccaceæ, (2) Bacteriaceæ, (3) Spirillaceæ, (4) Chlamydo-bacteriaceæ, and (5) Beggiatoaceæ. These, again, are subdivided into many genera, based partly on the mode of division and partly on the number and on the arrangement of the flagella upon the organisms. The Coccaceæ—globular cells—contain the genera Streptococcus, Micrococcus, Sarcina (non-motile), and Planococcus and Planosarcina (motile) ; the Bacteriaceæ are defined as long or short cylindrical rods, straight and never spiral ; division in one direction only after elongation of the rods ; and this family has three genera : (*a*) Bacterium—non-flagellated cells, often with endospore formation ; (*b*) Bacillus—cells possessing both lateral and polar flagella, often with endospore formation ; (*c*)

[1] *Proc. Roy. Soc. Lond.*, B, vol. 85, 1912, p. 186.
[2] *System der Bakterien*, 1897.

Pseudomonas—cells with polar flagella only, rarely endospore formation. The Spirillaceæ are curved or spiral rods, and include (*a*) Spirosoma, non-motile forms, (*b*) Microspira, motile forms with one polar flagellum, (*c*) Spirillum, motile forms with two or more polar flagella.

Various pointed organisms have been described as "fusiform Bacteria," *e. g.* in Vincent's angina (see Chap. VIII), but Dobell expresses the opinion that these more probably belong to the Fungi.

The nomenclature of bacterial species is at present in a chaotic condition. In botanical and zoological nomenclature every species has a binomial name, the first being the generic, the second the specific name. Many bacterial species have received trinomial or multinomial names, which should be inadmissible. The specific name first given to an organism must stand unless it has been used for some other species.

Conditions of Life of Bacteria

Bacteria, being living organisms, must be supplied with suitable nutritive substances in order that their life-processes—nutrition, reproduction, and the like—may be carried on in a normal manner. Being devoid of chlorophyll they are mainly dependent upon complex organic compounds for the carbon, hydrogen, and nitrogen which enter into their composition, these elements being derived for the most part from proteins and carbohydrates. Some bacteria, however, are able to obtain the requisite nitrogen from such comparatively simple compounds as ammonia, ammonium carbonate, or nitrates, and one group can make direct use of the atmospheric nitrogen. Certain inorganic salts, sulphates, phosphates, and sodium chloride, also seem to be necessary for normal development. These nutrient substances must be presented to the bacteria in association with water, for without water bacterial activity ceases, though in the dry state

many forms, and especially their spores, may retain their vitality for a considerable time; absolute desiccation, however, is rapidly fatal to many.

Temperature is also an important factor. Though the growth of many species occurs through a wide range, there is for almost all an optimum at which growth is best, and of a range not exceeding 5° or 10°. Growth usually ceases below 10° C., but cold does not destroy bacterial life; after exposure to the intense cold produced by the evaporation of liquid oxygen (— 170° C.) for weeks, or of liquid hydrogen (— 252° C.) for ten hours, bacteria and their spores will grow and germinate, and their chromogenic and pathogenic properties seem to be unaltered.[1] On the other hand, bacterial growth usually ceases when the temperature exceeds 40° C. or thereabouts, and most bacteria without spores are destroyed within half an hour by a temperature of 65° C. The spores are far more resistant; some may even be boiled for a short time without losing their vitality, but prolonged boiling is fatal to both bacteria and their spores. There is, however, a group of so-called thermophilic bacteria, which thrive best at a temperature of 60° to 70° C. They occur in the soil and in water, and are probably of considerable importance in the natural fermentations accompanied by the evolution of heat, such as are met with in manure heaps, the heating of hay, and firing of moist cotton.[2]

Free oxygen is essential to the growth of some organisms; these are termed strictly aërobic. Others will not develop in its presence, strictly anaërobic; others, again, while preferably aërobic or anaërobic, will grow in the absence, or in the presence, of oxygen, and are respectively termed facultative anaërobic or facultative

[1] Macfadyen and Rowland, *Proc. Roy. Soc. Lond.*, 1900.

[2] Macfadyen and Blaxall, *Journ. of Path. and Bact.*, November 1894, and *Trans. Jenner Inst. of Prev. Med.*, vol. ii, 1899, p. 162.

aërobic. The distinction between aërobic and anaërobic organisms though in the main true has limitations, for it is possible to "educate" strictly anaërobic forms to grow aërobically. Some organisms are strictly parasitic on animals or plants; others live in water, soil, decaying matter, etc.—these are termed saprophytes; and many are able to exist either as parasites or as saprophytes.

Bacterial development is much influenced by the presence of foreign substances in the nutrient medium. A number of metallic and other salts, chlorine, bromine, and iodine, carbolic acid, salicylic acid, etc., have an injurious effect upon bacterial life, inhibiting or stopping growth, or killing the organisms outright; these are of considerable practical importance and are known as germicides, antiseptics, and disinfectants. The products produced in the nutrient medium by the bacteria themselves also sooner or later inhibit or stop further growth; a familiar instance of this is seen in the alcoholic fermentation of sugar by yeast, which ceases when the amount of alcohol reaches 12 to 14 per cent. The same reason probably accounts for the fact that growths of bacteria in culture tubes frequently do not spread all over the surface of the nutrient medium, and why our cultures sometimes die out more rapidly than might be expected.

Another point affecting bacterial life is the presence of a mixture of organisms in the same nutrient medium. If there be a very vigorous form, it may ultimately grow and multiply to such an extent as to crowd out and finally kill the other forms with which it is associated, and if the nutrient medium equally favour two species, that one which is in an excess at the beginning may outgrow the other. The occurrence of what has been termed symbiosis is of considerable interest in the life of micro-organisms, and too little attention has hitherto been paid

to it. This is the co-existence of two or more species which together bring about certain changes. For example, in the well-known ginger-beer plant, Marshall Ward [1] isolated several yeasts, bacteria, and moulds; of these, one of the yeasts and one of the bacteria together induce the particular changes in a saccharine fluid to which ginger has been added, which render the mixture like ginger-beer, and these changes do not occur unless both species develop together.

Another extraordinary feature exhibited by bacteria is the selective action exerted on certain substances which contain isomerides or right- and left-handed modifications of a substance. The *Bacillus ethaceticus* attacks mannitol but not dulcitol, two alcohols which are very similar in taste and properties and possess the same simple chemical formula.

By a series of most brilliant researches Emil Fischer succeeded in determining the constitution of the various sugars, and, what is more, has produced them artificially in the laboratory. The natural sugars are all compounds with dissymmetric molecules, rotating a beam of polarised light, but when prepared artificially they are without action on polarised light, because the artificial product consists of equal numbers of left-handed and right-handed molecules, and the molecules of the one neutralise the molecules of the other, thus giving rise to a mixture which does not affect the polarised beam.

By the action of micro-organisms, however, on such an inactive mixture the one set of molecules is sought out by the microbes and decomposed, leaving the other set of molecules untouched, and the latter now exhibit their specific action on polarised light, an active sugar being thus obtained.

Fructose was one of the principal artificial sugars pre-

[1] *Phil. Trans. Roy. Soc. Lond.*, vol. clxxxiii, 1892, p. 125.

pared by Fischer ; it is inactive, but consists of an equal number of molecules of oppositely active sugars termed "lævulose." One set of these lævulose molecules turns the plane of polarisation to the right, another set to the left—right- and left-handed lævulose. The left-handed lævulose occurs in nature, while the right-handed lævulose, as far as is known, does not. Now, on putting brewer's yeast into a solution of fructose, the inactive artificial product, the yeast organisms attack the left-handed lævulose molecules and convert them into alcohol and CO_2, while the right-handed lævulose is left untouched.

Pressure, unless very great, has little effect on bacteria. Roger investigated the effects of high pressure on certain organisms in bouillon cultures. Pressures of 200 to 250 kilos. per square centimetre had no effect ; by raising the pressure to 3000 kilos. per square centimetre one-third of streptococci were killed, and of anthrax without spores a good many ; while sporing anthrax, *Micrococcus pyogenes*, var. *aureus*, and the colon bacillus were unaffected.[1]

Our countrymen Downes and Blunt first called attention to the injurious effect of light upon bacteria. If plate cultures be prepared and exposed to sunlight, a portion of the plate being protected from its action, as by sticking on a letter cut out of black paper, and the preparation afterwards incubated, it will be found that the colonies develop at the protected portion only, those parts which have been exposed to sunlight remaining sterile. Although this action of sunlight may sometimes be due to chemical changes in the medium, resulting in the production of ozone or other germicidal bodies, the experiments of

[1] Bacteria being so minute, the actual pressure on a bacterial cell, even with these high pressures, is small. If, for example, a bacterium measures 1 μ by 5 μ, a pressure of 1000 kgrm. per square centimetre would be but 0·05 grm. ($\frac{3}{4}$ grain) on the cell.

Marshall Ward and others have conclusively shown that germicidal action may be caused by the direct action of the light, the violet and ultra-violet rays being those concerned, and the red end of the spectrum having no effect. Exposure to ultra-violet rays for short periods may produce mutations of the anthrax bacillus (see p. 6). The Röntgen rays seem to have little or no influence upon bacteria, but the results that have been obtained are somewhat contradictory.

The radium emanations with prolonged exposure and near contact are germicidal to non-sporing organisms.[1]

Electricity, *per se*, has also usually little effect. When the current is passed directly through the cultures electrolysis takes place, and the products formed may destroy the bacteria ; currents of high potential, however, may inhibit growth.

Living motile bacilli are very sensible to induced currents of electricity, immediately orientating themselves in the direction of the current, while dead or paralysed bacilli are unaffected.

Bacterial Products

The chemical changes produced by micro-organisms are chiefly analytic or destructive—the formation of simpler from more complex bodies. This analytic faculty is present to a marked degree in the process known as putrefaction. *Putrefaction* is a term applied to the decomposition of organic, especially protein, matter after the death of the animal or plant. It is usually accompanied by the evolution of foul-smelling gases and by solution of the solid material. A large number of organisms are concerned in this process, particularly a group to which Hauser gave the name of *Proteus*. The first changes which occur are the formation of proteoses

[1] See Green, *Proc. Roy. Soc. Lond.*, vol. lxxiii, 1904, p. 375.

and peptone, then leucin, tyrosin, and glycocol, and basic compounds to which the name of ptomine has been given; next indole, skatole, and phenol, and volatile fatty acids; and lastly, mercaptans, sulphuretted hydrogen, marsh gas, ammonia, carbonic acid, and hydrogen.

In view of its practical importance in bacteriological analysis and the identification of species, indole may here be referred to at some length.

Indole.—Indole (C_8H_7N) is a product of the putrefactive decomposition of proteins containing a tryptophane nucleus and is formed during the growth of many organisms, and, since one species may produce it and another allied one may not, the determination of its presence or absence in the culture may be of value in the identification of organisms. The detection of indole is based on the reaction with nitrous acid, with which it gives a fine purplish-red coloration. In order to test for it, the organism is grown in a fluid medium for twenty-four to forty-eight hours or longer, 1 c.c. of a 0·1 per cent. solution of sodium nitrite is added to every 10 c.c. of the culture and a few drops of pure concentrated sulphuric acid or of hydrochloric acid are allowed to trickle slowly down the side of the test-tube, which is inclined with its mouth away from the operator. As the acid runs down, it is mixed with the fluid; a colour varying from pale pink to pale purple indicates the presence of indole. A control tube, uninoculated, should also be similarly tested to make sure that the reaction is due to the products of the growth of the organism. The culture fluid usually employed is peptone water, preferably 2 per cent., but some samples of "peptone" occasionally fail to give the indole reaction when organisms are grown in media prepared from them; the right kind of peptone must, therefore, be used. The formation of indole is probably dependent upon the presence of tryptophane in the protein. As the dilute

solution of sodium nitrite is unstable, a stock 5 per cent. solution may be kept ; 2 c.c. of this solution are diluted to 100 c.c. with distilled water at the time of making the test, and 1 c.c. of this dilution is added to every 10 c.c. of the culture. The addition of the acid liberates free nitrous acid, which reacts with any indole present and yields a pink colour. Sometimes when the reaction is apparently absent or feeble, it may be obtained or intensified by placing the tube in the blood-heat incubator for half an hour. The sulphuric acid should be pure and free from oxides of nitrogen, hence hydrochloric acid is often preferable.

A more delicate method of testing is to run a little hydrochloric acid down the side of the tube, so that a layer forms at the bottom, the nitrite having been previously added to the culture if required. A pink ring at the juncture of the hydrochloric acid and culture indicates the presence of indole. The pink pigment, the product of the reaction, may be extracted by shaking with a little amylic alcohol.

Other delicate and more certain reagents for the detection of indole are para-dimethylamidobenzaldehyde (4 grm., dissolved in absolute alcohol 380 c.c., hydrochloric acid 8 c.c.). To about 10 c.c. of culture 5 c.c. of this solution are added, and then 5 c.c. of a saturated aqueous solution of potassium persulphate ; indole gives a pink or red colour. Another test is β-naphthaquinone-sodium-mono-sulphonate (2 per cent. aqueous solution), which gives, when the mixture is rendered alkaline with caustic potash, a blue or blue-green colour or precipitate. The coloured compound may be extracted with chloroform, in which it yields a red solution.

Peptone water is by no means a good culture medium, and broth may therefore be employed, but it should be free from dextrose. Peptone water with the addition of a little rabbit's serum is perhaps the best culture medium for the production of indole.

The presence of dextrose, saccharose, glycerin, or lactose in quantity exceeding about 0·25 per cent. prevents the formation of indole in broth by bacteria. Broth prepared in the ordinary way usually contains a little dextrose derived from the glycogen in the meat, and this probably explains why the indole reaction is generally much more marked in a peptone water than in a broth culture, although the latter is a better nutrient soil. In order to prepare a soil free from dextrose, the acid beef-broth used in the preparation of nutrient broth should be inoculated with the colon bacillus and incubated for twenty-four hours, and the nutrient beef-broth prepared from it. The dextrose is consumed and no indole is formed.[1]

Homer [2] suggests that in a medium containing glucose there is lessened indole production because of the formation of a glucose-tryptophane complex which is not so readily attacked as tryptophane alone.

Some bacteria not only form indole but also produce nitrites in the culture medium by the reduction of the nitrates present in the peptone, etc., used in making the nutrient medium, in which case the addition of pure sulphuric or hydrochloric acid alone suffices to bring out the pink indole reaction. This forms, therefore, an additional means of distinguishing organisms, and is employed especially for the recognition of the cholera spirillum, which, if grown in peptone water, gives the indole reaction (or, as it has been termed, "the cholera red reaction") on the addition of acid alone. The reaction can be obtained as early as twelve hours after inoculation, and becomes very marked in twenty-four to forty-eight hours.

If indole is formed only in small quantities, 100 c.c.

[1] T. Smith, *Journ. of Exper. Med.*, vol. ii, 1897, p. 543.
[2] *Journ. of Hygiene*, vol. xv, 1916, p. 401.

of the culture may be distilled; the first 20 c.c. of the distillate will contain the bulk of the indole.

This "indole-reaction" is not necessarily always due to indole; the writer has shown [1] that the indole-like reaction obtained with cultures of the diphtheria and pseudo-diphtheria bacilli is owing to the presence of skatole-carboxylic acid. This substance is distinguished from indole by being non-volatile. To make sure of the presence of indole, the culture should therefore be made alkaline with caustic soda and distilled.

Skatole (methyl indole) seems also to be formed by some organisms. It is volatile like indole, but if a solution containing it be boiled with an acid solution of paradimethylamidobenzaldehyde (5 per cent. in 10 per cent. sulphuric acid) it yields a blue colour, which gives a blue solution in chloroform.

Nitrification.—Another important series of changes is that included under the term "nitrification." As mentioned before, protein, albuminoid, and other complex nitrogenous matters and urea, all of which are valuable manures for plant life, cease to be so unless bacteria are present.

It is necessary, in fact, for the nitrogenous matter to be converted into nitrates, in which form alone is it available for the nutrition of plants.

Although so important, extremely small quantities of nitrates are present in the soil; in fertile soils, for example, under some conditions there may be as little as one part of nitrogen in 1,000,000, and there is often less than ten parts. The bodies yielding nitric acid in the soil are: (1) free nitrogen; (2) small quantities of nitrates in rain-water; (3) ammonium salts, applied intentionally or carried to the soil by rain or derived from the decay of organic matter; (4) various nitrogenous organic sub-

[1] *Trans. Path. Soc. Lond.*, vol. lii, pt. ii, 1901, p. 113.

stances arising from the decay of animal and vegetable matters.

With regard to the production of nitric acid from nitrogenous organic matters very little was formerly known. In 1877 Schloesing and Müntz by an ingenious experiment showed that nitrification (as the production of nitric acid is termed) of nitrogenous organic matter is brought about by living organisms in the soil. Sewage was passed continuously through a tube containing a mixture of ignited quartz sand and limestone. After three weeks nitrates began to appear in the effluent and increased to such an extent that finally the filtered sewage contained no ammonia. After this had continued for some weeks chloroform vapour was passed at the same time through the tube, with the result that in ten days after the introduction of the chloroform all nitrates disappeared from the effluent.

Subsequently the passage of chloroform vapour was discontinued, but nitrification did not resume until the washings from 10 grm. of garden soil were added. Eight days after this addition nitrates again appeared in the effluent (this was confirmed by Warington). Evidently the chloroform vapour acted as an antiseptic and killed the nitrifying organisms, while the addition of soil washings re-inoculated the material.

Shortly after this Schloesing and Müntz found that exposure of soil to 100° C. for an hour destroyed the power of inducing nitrification. Soils thus treated were exposed to a current of air, purified by ignition, without nitrification taking place; the addition of a little unheated mould was, however, sufficient to cause nitrification to recommence. They also tried seeding the sterilised soils with various Hyphomycetes, etc., without result.

In 1884 Warington concluded that the factor determining the formation sometimes of nitric acid and some

times of nitrous acid was a difference in the character of the organisms; for it is possible to have two similar solutions under identical conditions, and for nitrites to be produced in the one, and nitrates in the other.

In 1886 Munro showed that the process of nitrification could take place in solutions practically destitute of organic matter.

Nitrification in the soil takes place in three stages:

I. *Ammonisation.*—When complex organic compounds such as albuminoids are applied to the land they are broken up; first they become liquefied, peptone-like bodies being produced; these are then further acted upon and we get alkaloidal substances in small quantity, indole, skatole, leucin, and tyrosin and amino-acids, valerianic acid, volatile fatty acids, lactic acid, etc.

These changes are brought about by numbers of organisms, among which the varieties of *Proteus* (formerly known as *Bacterium termo*), *B. mesentericus*, *B. mycoides*, *B. fluorescens liquefaciens*, and *B. putrificus* are the more important.

The nitrogenous compounds are then further acted upon and ammonium salts are formed. According to Emile Marchal, ammonisation takes place essentially under the influence of microbes living in the upper layers of the soil. The *Bacillus mycoides* is one of the most energetic of these, and seems to play a double *rôle*, being ammonising in the presence both of nitrogenous organic substances and of nitrates. Urea is ammonised especially by the *Micrococcus ureæ.*

II. *Nitrosation.*—The ammoniacal salts are next converted into nitrites. The nitrous organisms can probably attack nitrogenous organic substances such as asparagine and milk, but only feebly, milk being much more rapidly nitrified when the nitrous organisms are mixed with other species. The organisms bringing about this change are

short, stumpy, motile bacilli with single polar flagella which are grouped under the generic name of *Pseudomonas*.

III. *Nitratation*.—These nitrites are then converted into nitrates. The "nitric" organisms are minute non-motile bacilli known as *Nitrobacter*.

Stages II and III are brought about by different species, the nitric organisms having no effect whatever on ammonia, but acting only after this has been oxidised into nitrous acid by the nitrous forms.

The discovery of Dr. Munro that organisms will grow in purely inorganic solutions has been made use of for the isolation of the different species. Solutions such as the following have been used:

For the Nitrous Organisms.	For the Nitric Organisms.
Ammonium chloride, 0·5 grm.	Potassium nitrite, 0·3 grm.
Potassium phosphate, 0·1 grm.	Potassium phosphate, 0·1 grm.
Magnesium sulphate, 0·02 grm.	Magnesium sulphate, 0·05 grm.
Calcium chloride, 0·01 grm.	Calcium carbonate, 5 grm.
Calcium carbonate, 5 grm.	Distilled water, 1000 c.c.
Distilled water, 1000 c.c.	

These are seeded with traces of earth, and by carrying on the cultivation for many generations a large number of organisms are eliminated. This method does not lead to a pure cultivation, for several forms besides the nitrifying organisms persistently maintain themselves in these mineral solutions.

So recourse was had to gelatin plate cultivations. Although several organisms were isolated in this manner, none of them possessed the slightest nitrifying power.

Frankland, and later Warington (1890), succeeded in isolating nitrous organisms by the dilution method. Nitrifying solutions were diluted, and traces inoculated into ammoniacal solutions; in some of these nitrification occurred, although no growth could be obtained on gelatin, and they were found to contain the nitrous organism only. A little later Winogradsky isolated nitrous organisms, first by modified gelatin plates, and afterwards by the silica jelly method.

This is carried out as follows: Sodium carbonate is fused in the blowpipe, and fine white sand is added so long as effervescence is produced. The mass is allowed to cool, and is then dissolved in

water. The solution is poured into an excess of very dilute hydrochloric acid (silicic acid and sodium chloride being formed). The solution is dialysed and sterilized. For use, some of this is placed in a sterile dish and is mixed with the following solution and inoculated :

Ammonium sulphate . . .	0·4 grm.
Magnesium sulphate . . .	0·5 ,,
Di-potassium hydrogen phosphate .	0·1 ,,
Calcium chloride	trace
Sodium carbonate. . . .	0·6–0·9 grm.
Water	100 c.c.

This mixture sets to a jelly in five to fifteen minutes.

Winogradsky has also made use of agar for plates, but this medium is not so suitable as the silica jelly. A 2 per cent. aqueous agar is prepared and poured into Petri dishes ; the film is then sown with *Proteus*, and allowed to grow for seven to ten days. It is then thoroughly washed, collected, melted, and mixed with the salts mentioned above. The object of growing the *Proteus* upon it as a preliminary is to eliminate the organic matter admixed with the agar.

Nitrification in the soil is thus brought about by two groups of organisms. The first oxidises ammonia into nitrous acid, and is isolated by successive cultivation in solutions of ammonium carbonate. The second group oxidises nitrous acid into nitric acid, and may be separated by successive cultivations in a solution of potassium nitrite containing a little sodium bicarbonate. In the soil the nitric and nitrous organisms are equally active.

Besides the derivation of nitrogen from nitrogenous compounds, the free atmospheric nitrogen is also " fixed " through the agency of certain micro-organisms and rendered available for plant life.

Thus, the Leguminosæ are able to obtain their nitrogen directly from the nitrogen of the air. If the roots of a pea, bean, or vetch be examined, numerous little nodules will be found upon them ; these contain minute irregular and Y-shaped bodies, which have been termed " bac-

teroids," and seem to be of the nature of involution forms. On inoculation into suitable culture media [1] the bacteroids give rise to a growth of a motile bacillus known as *Pseudomonas radicicola*; this "fixes" the atmospheric nitrogen. The organisms penetrate the young roots through the root-hairs, multiply and form a filamentous zooglœa, which grows into the tissue of the root and pentrates the cells. Large amounts of nitrogen are taken up by the bacteroids, and are converted into nitrogenous compounds which can be assimilated by the plant. Leguminous plants grown from sterile seeds in a sterile soil dwindle and die, but if inoculated with the organisms derived from another plant of the same species growth becomes vigorous; if inoculated with those derived from another species growth still takes place, but not nearly to the same extent. The Leguminosæ thus store up one of the most important elements of plant food, and hence their value in the rotation of crops. There is apparently no increase of nitrogen compounds in the soil, the excess found being due to the root residues remaining. A substance termed "nitragin," consisting of a culture of these root organisms, has been prepared as a fertiliser. Nobbe's "nitragin" did not prove a success, apparently because the organisms soon lose their vitality. A better preparation, "nitro-bacterine," was devised by Moore of the United States Department of Agriculture. Besides the leguminous organisms, other bacteria are present in the surface layers of the soil which fix atmospheric nitrogen. The principal of these are ovoid organisms known as *Azotobacter*. This group can be cultivated in a mannite medium, *e. g.* di-potassium

[1] Such as wood-ashes, maltose, agar. Boil 8 grm. of wood-ashes with 500 c.c. of water for one minute; filter. To 400 c.c. of this extract add 4 grm maltose and 4 grm. agar. Boil until dissolved; filter, tube, and sterilise.

phosphate 0·2 grm., mannite 20 grm., water 1 litre. This may be used for isolation by converting into an agar medium by the addition of 2 per cent. agar. Prof. Bottomley has succeeded in obtaining a powder preparation of *Azotobacter*, which retains its vitality for months, and the preparation properly applied to *poor* soils produces astonishing results.

It has been found that partial sterilisation of the soil, *e. g.* by heat, *increases* its fertility, whereas it might have been supposed that such a procedure would *decrease* the fertility by destruction of nitrogen-fixers. Russell and Hutchinson suggest that in ordinary soil amœbæ and other protozoa devour and keep down the bacteria; by the sterilisation the protozoa are destroyed and the more resistant bacteria are then free to develop. Greig-Smith,[1] however, denies that phagocytic protozoa possess any power of limiting the number of bacteria in the soil, and ascribes the effect of soil sterilisation to an action on the bacterio-toxins and nutrients of the soil.

Besides nitrifying bacteria many de-nitrifying organisms occur in the soil. They may (1) reduce nitrates to nitrites; (2) remove oxygen from nitrates and nitrites and form ammonia; (3) form nitrous and nitric oxides or nitrogen from nitrates and nitrites.

Fermentation.—Another important group of changes produced by micro-organisms is that comprised under the comprehensive title of "fermentation," of which it is difficult to give an accurate definition, for the distinction between it and other chemical changes due to the activity of micro-organisms is conventional rather than scientific. The original conception of the term involved the occurrence of frothing of the fermenting liquid, owing to the escape of gaseous products. Fermentation is brought about by the action of ferments, two classes of which are

[1] *Proc. Linn. Soc.* N.S.W., xxxvii, 1913, p. 655.

recognised, viz. the living or organised ferments, which, in other words, are micro-organisms ; and the unorganised or chemical ferments, bodies such as pepsin, which in minute amount produce changes in a considerable quantity of the substance acted upon, without themselves undergoing alteration.

It is better to reserve the term "fermentation" for the changes brought about by the organised ferments or living organisms, and to call the unorganised ferments enzymes, and the changes which they produce zymolysis. As fermentations are investigated more critically, the tendency is to find that they are brought about by enzymes, extra-cellular or intra-cellular, so that in course of time this distinction may no longer hold good.

The following are the chief varieties of fermentation :

The alcoholic fermentation.—This is mainly brought about by the decomposition of sugars of the hexose group ($C_6H_{12}O_6$), principally dextrose and lævulose, by yeasts into alcohol and carbonic acid, but some of the bacteria and moulds also produce appreciable quantities of alcohol. Other carbohydrates by the action of enzymes secreted by the organisms may be converted into hexoses, which are then fermented. The general reaction is as follows :

$$C_6H_{12}O_6 = 2C_2H_6O + 2CO_2.$$

As a matter of fact small amounts of by-products appear in addition to the alcohol and carbonic acid, viz. glycerin, succinic acid, and higher alcohols. Until 1897 no enzyme had been obtained which would carry out this change ; it only occurred when the living yeast-cells were present, but in that year Buchner, by grinding up the living yeast-cells, obtained a juice which decomposed dextrose with the formation of alcohol and carbonic acid. This "zymase" Buchner claimed to be the alcoholic enzyme of yeast.

The lactic acid fermentation.—This is brought about chiefly by bacteria. Hexoses are converted into lactic acid, the reaction being

$$C_6H_{12}O_6 = 2(HC_3H_5O_3),$$

but it is probably not actually so simple as this, for carbonic acid is given off at the same time. A familiar example of this form of fermentation is the souring of milk. Lactose, which is a di-saccharide ($C_{12}H_{22}O_{11}$) is first split up into two molecules of dextrose :

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6,$$

and the dextrose so formed is then acted upon.

The butyric acid fermentation.—Butyric acid is formed from carbohydrates by the action of bacteria, mainly the *Bacillus butyricus* and *Clostridium butyricum*, the latter an anaërobic organism, some by-products being formed in addition. Milk which has been just boiled usually undergoes the butyric rather than the lactic fermentation, the spores of the butyric organisms surviving. Lactic acid is first formed, and this is then converted into butyric acid :

$$2C_3H_6O_3 = C_4H_8O_2 + 2CO_2 + 2H_2.$$

The acetic acid fermentation.—The conversion of alcohol into acetic acid is also due to bacteria, familiar examples of which are the souring of beer and wine.

The reaction is :

$$C_2H_6O + O_2 = CH_3COOH + H_2O$$

Citric, oxalic and other acid fermentations are also known.

Bacterial enzymes.—Many changes brought about by bacteria and other micro-organisms are due to enzymes, which may be not only intra-cellular but may escape from the cells into the medium in which they are growing.

The most familiar examples are the peptonising enzymes produced by bacteria which liquefy gelatin and digest coagulated protein, fibrin, etc. The enzymes differ : an organism which liquefies gelatin does not necessarily digest blood-serum. The proteolytic enzyme is tryptic in nature and escapes from the cells into the surrounding medium, so that some of the liquefied gelatin free from cells or in which their action is inhibited by an antiseptic, liquefies other gelatin if added to it. Amylolytic enzymes are also produced, such as amylase (digesting starch), maltase, lactase, inulase, and invertase. Lipases and rennet-like enzymes also occur. "Fermentation" of urea takes place by means of an enzyme secreted by the *Micrococcus ureæ*, etc., with the formation of ammonium carbonate. These enzymes do not seem to possess any poisonous action.

Formation of pigment.—Numerous organisms, especially those of air and water, during their growth produce various coloured pigments. They are termed "chromogenic bacteria," examples of which are the *Sarcina lutea* and *Micrococcus cereus*, var. *flavus*, which form citron-yellow pigments ; the *Bacillus prodigiosus* and *Spirillum rubrum*, red pigments ; the *Bacillus violaceus* forms a rich violet one ; and the *Bacillus pyocyaneus*, a blue. A large number of chromogenic organisms require oxygen for the production of the pigment, and potato is often the most favourable culture medium. In some cases the medium may become coloured, and the property of fluorescence be conferred upon it, as is the case with the *Bacillus fluorescens liquefaciens*. Usually the pigment is extra-cellular, occasionally, as in *B. violaceus*, it is intra-cellular.

A group of organisms producing purplish pigments has been described under the name of "purple bacteria." It is doubtful if these organisms are true bacteria, and the pigment may exercise a respiratory function analogous to chlorophyll.

Phosphorescence, or light-production, is developed by some bacteria, notably by many marine forms, and is well seen in decomposing fish. Some spirilla are also known occasionally to produce phosphorescence.

A necrotic action on the tissues is produced by many pathogenic organisms. For example, the tubercle and glanders bacilli cause necrosis and caseation of the surrounding tissues.

Gas production.—This is common to many organisms. The gas may consist of carbonic acid, hydrogen, or marsh gas, and in some cases of foul-smelling sulphur compounds, sulphuretted hydrogen, mercaptans, etc.

Sulphuretted hydrogen may be detected by the blackening of lead acetate paper. Methyl mercaptan may be detected by aspirating a current of air through the culture, through a calcium chloride drying-tube, and then through a test-tube or small flask containing isatin dissolved in concentrated sulphuric acid. The red colour of the isatin solution is changed to olive or grass-green by the mercaptan.

Toxic bacterial products.—Almost without exception the pathogenic action of bacteria is brought about by means of the chemical substances produced in one way or another by their metabolic processes. The toxic bacterial products may be classified as follows :

(1) *Decomposition products.*—These are substances produced by the decomposition of the medium upon which the bacteria are growing. Thus proteoses appear to be formed by the anthrax bacillus and by the pyogenic cocci.

The *ptomines* form another group of these substances. These are a very important group of nitrogenous bodies, analogous to the vegetable alkaloids and mostly solid and crystalline in nature, which are formed by the action of bacteria on protein and albuminoid matter. They often occur naturally in decomposing and putrefying food,

meat, fish, etc., and as many of them are virulent poisons they are of considerable practical import. Poisoning by tainted food may be due to the absorption of such toxic ptomines, and this form of food-poisoning is known as *ptomine poisoning*. A number of toxic ptomines were isolated by Brieger from cultivations of pathogenic microbes, and great importance was once attached to them. They are referred to in the descriptions of the various pathogenic organisms.

Brieger's work, however, needs revision, for his methods were not such as to exclude alteration by the reagents employed.

Stevenson obtained traces of a highly poisonous crystalline ptomine from some sardines that had caused death. Vaughan isolated a body, tyrotoxicon, apparently identical with diazobenzene, from poisonous cheese and milk. It seems to be developed by the action of organisms belonging to the *B. coli* or *B. lactis aerogenes* types. Mytilotoxin ($C_6H_{15}NO_2$) is the specific poison of toxic mussels. Such mussels have invariably been subjected to sewage pollution and the poison is probably produced by the action of bacteria derived from sewage. Neurin and muscarin are extremely poisonous and may occur in decomposing flesh. Some of the ptomines produced by putrefaction are very similar to certain vegetable alkaloids and are thus of considerable medico-legal importance. The ptomines are not specific like the true toxins, and toxic ones may be produced by non-pathogenic bacteria.

(2) *Toxins.*—These are the soluble poisons elaborated by the bacteria and excreted by the cells into the surrounding medium. They are regarded by Martin and others as being allied to the proteoses. Roux and Yersin suggested that the diphtheria poison might be an enzyme, while Brieger and Fränkel regard it as albuminous. The toxins are non-basic substances closely related to the

proteins and hence have been named tox-albumins, and are considered to be the specific toxic poisons of the pathogenic bacteria. It is difficult or impossible to prepare them in a state of purity and their chemical constitution is therefore unknown, and they are characterised by extreme specificity. Such are the poisons of the diphtheria and tetanus bacilli.

(3) *Endotoxins.*—These are toxic substances elaborated by the bacteria which do not to any extent escape from the cells. They are as specific as the toxins and possess analogous properties (see below).

(4) *Bacterial proteins.*—These are toxic constituents of the bacterial cells which do not diffuse from the cells, are not specific, and which probably usually play little part in the production of the disease symptoms.

LITERATURE

On Nitrification, see Warington, *Journ. Chem. Soc.*, 1886, *et seq.*; Frankland, *Cantor Lectures*, 1892; *Nature*, 1890, *et seq.*; Löhnis, *Handbuch der landwirtschaftlichen Bakteriologie* (Borntraeger, Berlin, 1910, full bibliography). *On Bacterial Products*, see *Cellular Toxins*, by Vaughan and Novy, 1902 (*Bibliog.*), *Ueber Ptomaine*, by Brieger, 1885; Macfadyen, *The Cell as the Unit of Life* (Churchill, 1908); Wells, *Chemical Pathology*, 1913; Hewlett, Art. "Toxins and Antitoxins," Thorpe's *Dict. of Chemistry*, 1913. For General Bibliography, see Kolle and Wassermann, *Pathogenen Mikroorganismen.*

Endotoxins

The majority of pathogenic micro-organisms do not excrete any appreciable amount of toxin; the toxin remains within the cells. To such an intra-cellular toxin the name of "endotoxin" has been given. The toxins of the staphylococci and streptococci, the typhoid-colon group, plague, cholera, etc., are endotoxins. Various methods have been employed to prepare these endotoxins, such as extraction of the cells by the action of weak alkalies and enzymes, and by autolysis or self-digestion.

The late Dr. Allan Macfadyen conceived that if the intra-

cellular toxins (endotoxins) of such organisms as the typhoid bacillus, cholera vibrio, etc., could be obtained free from the bacterial cells, it might be possible to prepare sera (anti-endotoxic sera) of much more therapeutic potency than the ordinary anti-microbic sera.

The disintegration of the bacterial cells in the presence of intense cold, to prevent chemical change in the bacterial juice obtained, was the method devised by Macfadyen to attain this end. With the aid of his colleagues, Mr. Rowland and Mr. Barnard, and of his laboratory assistants, Messrs. Burgess and Thompson, apparatus and methods were evolved to effect this.

By growing on the surface of agar or other suitable medium in plate bottles (Fig. 19), scraping off the growth and suspending this in salt solution, centrifuging at high speed, and collecting the bacterial cell-mass on the walls of the centrifuge vessels, sufficient material is readily obtained to grind or triturate, and thus disintegrate the bacterial cells so as to liberate their contents. This is accomplished by means of a special machine, the essential part of which consists of a metal cone revolving at a high speed in a metal pot, the bottom of which is shaped so as to fit the cone. The pot with its contents is immersed in a vessel of liquid air or other freezing mixture and the bacterial mass is ground.

After grinding, the ground material is made up with distilled water or with 0·1 per cent. sodium hydrate so as to form a 10 per cent. solution (calculated on the original weight of the moist bacterial paste); this is centrifuged, and the fluid is filtered through a sterile Berkefeld filter.

The filtrate thus obtained is the endotoxin, and is used to immunise horses and other animals in the same manner as with any other toxin; it should be used as fresh as possible. The amounts of a typhoid or cholera endotoxin employed for immunising must at first be small, 0·2–0·5 c.c., as it produces considerable disturbance on injection, and the amount is gradually increased. After some weeks' treatment a dose of 20–30 c.c. may be injected. When tests show that the serum has attained the necessary potency, the horse is bled and the serum obtained and bottled.

The endotoxins also possess immunising properties to a high degree, and may be used as prophylactic or as curative vaccines; they markedly raise the opsonic index.

Another machine has been devised by Barnard for disintegrating bacterial and other cells. It is supplied by Messrs. Baker, of High Holborn, and is depicted in Fig. 1, p. 43.

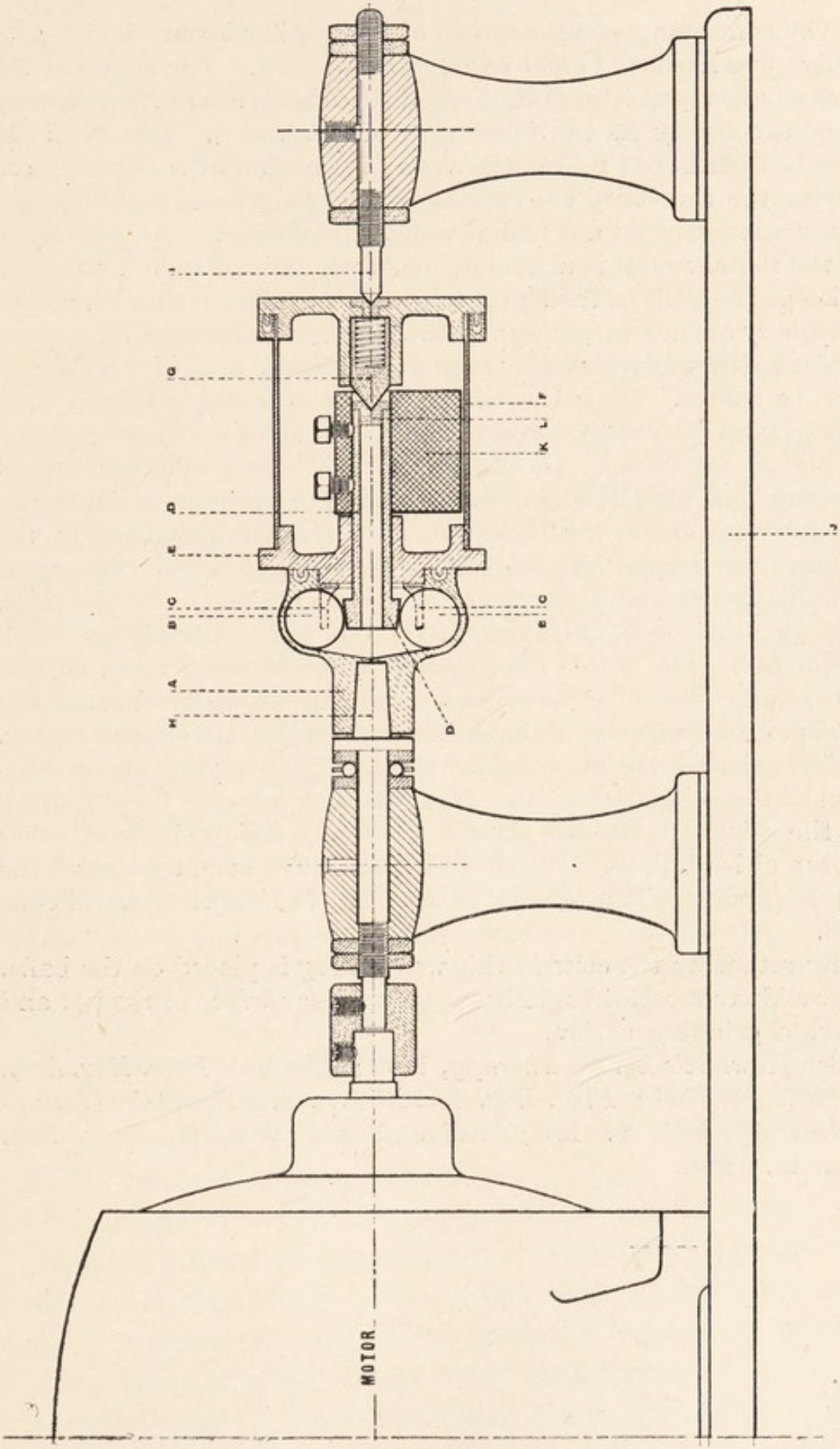


FIG. 1.—Barnard machine for disintegrating bacterial and other cells.

The containing vessel consists of a phosphor-bronze body, A, in which five hardened steel balls, B, are placed. The shape of the containing vessel is such that when these balls are at its periphery they accurately fit the inner side of the vessel. The balls are evenly distributed round the vessel by means of a cage, C, and during the time they are running this cage ensures that they are equidistant and do not collide one with another. At the centre of the metal vessel is a steel cone, D, which is of such a size that it keeps the balls in their proper position in close contact with the periphery of the containing vessel. The vessel is closed by a screw cap, E, through which the steel cone passes, and in which it is free to rotate. Over the whole of this a metal cylinder, F, is placed, and is screwed down, completely sealing the upper opening in the metal vessel. In the top of this metal cylinder a steel bearing, G, is placed, which has freedom of movement in a horizontal direction, but is kept down on the top of the steel cone by the action of a spring. It therefore follows that when this metal cylinder is screwed down the steel cone is pressed on to the balls, and the balls are in their turn forced out to the periphery of the metal pot. The whole appliance is mounted on a cone, H, and a centre, I, which are carried by two uprights attached to the base plate, J ; one end of the shaft is attached to the electric motor.

The grinding action is brought about by retarding the revolution of the central cone, D. This has been effected by mounting on the spindle of the central steel cone, D, a semi-cylindrical mass of iron or lead, K, the weight of which must be such that when the whole apparatus is rotated it is sufficient to hold the central cone still.

By retarding the cone in this way a drag is placed on the balls, they slide to a certain extent over the inner surface of the pot and exert a grinding action.

See Hewlett's *Serum Therapy*, 1910 ; Hewlett, *Proc. Roy. Soc.*, B, 1909 and 1911 ; *Proc. Roy. Soc. Med.*, vol. iii, 1909–10 (Pathological Section), p. 165 ; Barnard and Hewlett, *Proc. Roy. Soc.*, B., 1911.

CHAPTER II

METHODS OF CULTIVATING AND ISOLATING ORGANISMS

It is necessary for the satisfactory study of micro-organisms in their relation to the various processes of infection and disease, of fermentation, putrefaction, and the like, to separate and isolate the different species occurring in a mixture, and, having done so, to cultivate, grow, or propagate each species on suitable soils through successive generations. A slight consideration will show that unless we work with pure cultures—that is, cultures consisting of a single species—we can never be sure that a particular result is due to a given organism ; in a mixture several or all of the forms present may conduce to the effect produced. With regard to the pathogenic organisms, or disease germs, Koch laid down certain conditions which have been termed "Koch's Postulates" (p.160), which must be complied with before the relation of an organism to a disease process can be said to be completely demonstrated, one of which is that "the organism must be isolated and cultivated outside the animal body on suitable media for successive generations."

In order to isolate organisms in a state of purity it is absolutely necessary to employ vessels, instruments, and culture media which are sterile, that is, free from any living organisms, and to possess the means of manipulating them in such a way that the entrance of organisms from without is prevented and contamination avoided.

Various methods of destroying and of getting rid of organisms are known, such as the use of chemical "germicides," heat, and filtration through porous porcelain. The addition of chemical germicides, such as carbolic acid or corrosive sublimate, is out of the question ; for although the vessels and media might be rendered sterile thereby,

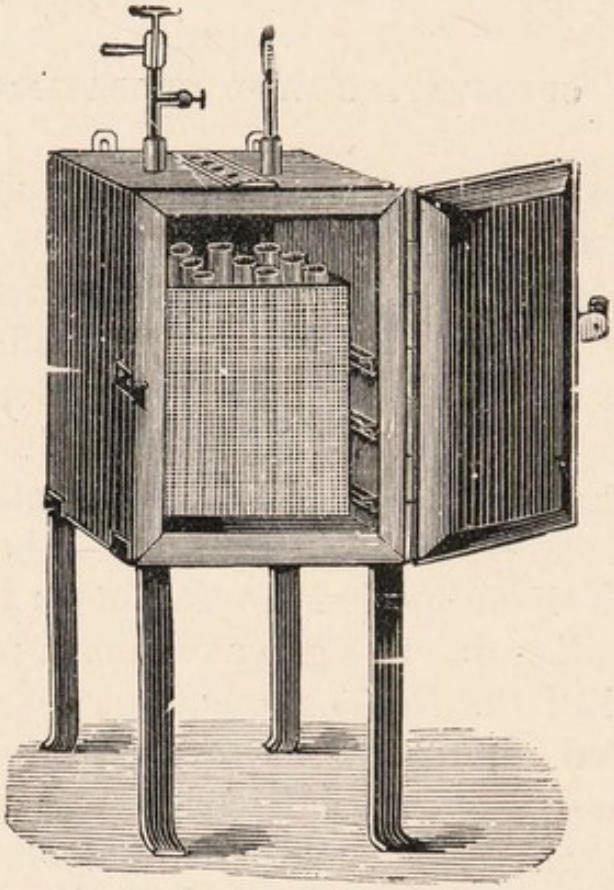


FIG. 2.—Hot-air steriliser.

the growth of the organisms which are being investigated would equally be prevented, so that the two last, viz. heat and filtration, are those which are employed, the former being used for vessels, instruments, and culture media, solid and fluid, the latter for fluid culture media only.

Various apparatus are needed for sterilisation and the preparation of culture media. These will now be described.

Hot-air steriliser (Fig. 2).—This is a rectangular box of

sheet iron or copper with double walls, having an air-space of nearly an inch between them, and furnished with a door. The joints should be brazed, riveted, or folded, not soldered. The outer skin at the bottom should have a large hole cut in it in which a loose piece of sheet iron or copper should be inserted to protect the inner skin from oxidation and may be renewed as it "burns" away. The top is perforated with a couple of holes, through one of which a chemical thermometer, registering to 200° C., is inserted in a cork, while through the other some form of mercurial regulator can be introduced if required, but is not usually needed. In the hot-air steriliser all thin-glass vessels and cotton-wool are sterilised by heating to a temperature of about 150° C. by means of a Bunsen or a small ring burner under the steriliser, which is supported on a suitable iron stand. If the steriliser is placed on a table or other wooden support, a piece of sheet iron, asbestos cardboard or uralite should be laid over the wood to protect it from the heat. An inexpensive substitute for the hot-air steriliser may aredily be devised, any iron box or even a biscuit-tin being used for the purpose.

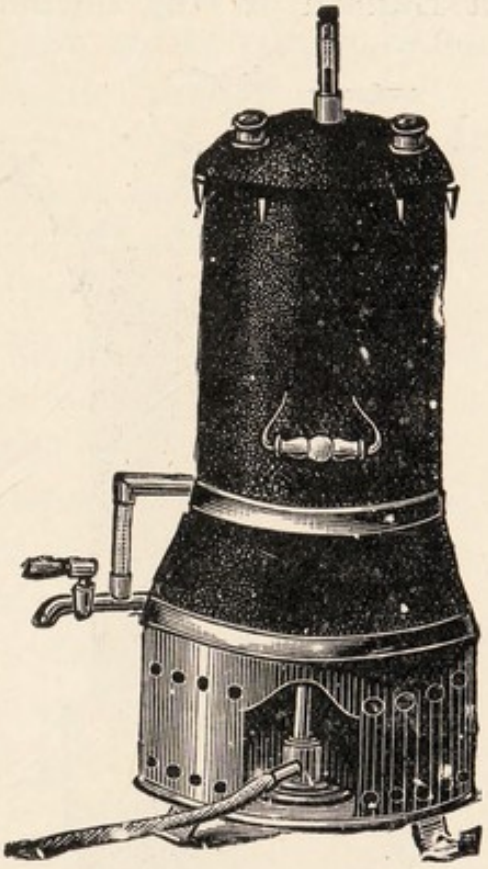


FIG. 3.—Steam steriliser.

Steam steriliser (Fig. 3).—This consists of a cylindrical or rectangular vessel of tinpate, galvanised iron, or copper, covered on the outside with a layer of felt or asbestos, having a false perforated bottom supported a

few inches above the true bottom, and provided with a movable lid. In the steam steriliser or "steamer" the culture media, and thick glass vessels and other apparatus which would crack or be damaged by the high temperature of the hot-air steriliser, are sterilised by steam. The lower chamber of the steamer, below the false bottom, is partly filled with water, which is boiled by means of a Bunsen or ring burner. Above the false bottom the culture media or apparatus are placed, and are sterilised by the steam at 100° C. which fills this space.

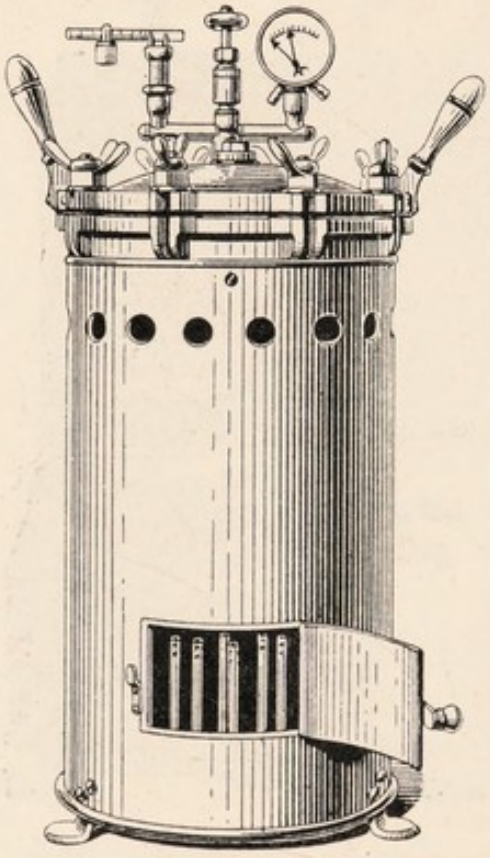


FIG. 4.—Autoclave.

Here again an inexpensive substitute may be devised; the ordinary kitchen saucepan with steamer will do well for many purposes, while a "warren pot" answers admirably.

Autoclave (Fig. 4).—This is most useful for many purposes, but it is expensive and not a necessity, as the steam steriliser will serve almost every purpose for which the autoclave is employed with the expenditure of a little more time and trouble. It consists of a strong boiler of brass or gun-metal with a removable lid, which is attached to the boiler by means of screw-bolts. The lid is provided with a safety valve, a gauge for indicating the pressure and temperature, and a stopcock to relieve the pressure if required. A small quantity of water is placed in the bottom, and the media or apparatus to be sterilised having been introduced, the lid is screwed down. It is

heated by means of one or more Bunsen burners, which are turned down when the required temperature has been reached. The temperature usually employed is about 115° to 125° C. When sterilising media care should be taken that the vessels are not too full, and that the autoclave is allowed to cool down below 100° C. before opening the stopcock, or some of the contents may be lost by violent ebullition. While the temperature is rising, the stopcock should always be left open until steam is being freely generated so that the air may be expelled.

Air-pump.—An exhaust pump is useful for many purposes, such as evaporating to dryness *in vacuo*, filtration through porous porcelain filters, etc. Any form will serve, but of the more elaborate ones the Fleuss pump (Fig. 5, p. 51) made by the Pulsometer Engineering Company is one of the best. In using it care must be taken that no fluid or moisture gains access to the barrel ; to avoid this the connecting pipe may be intercepted with a vessel containing strong sulphuric acid (D, Fig. 5), over the *surface* of which the exhausted air has to pass. A double-necked Woulfe's bottle is suitable for this, the inlet and outlet tubes extending nearly down to, but not dipping below, the surface of the sulphuric acid.

For greasing the vessels, etc., to make air-tight joints, beeswax dissolved in the Fleuss pump oil with the aid of heat to a stiff paste is a good composition, or the resin ointment of the Pharmacopœia may be used. Another good grease is made by melting together one part of black rubber, one part of vaseline, and one-third part of paraffin wax.

Centrifuge.—A small centrifuge holding two or four 10 c.c. tubes is a necessity in the laboratory. A form driven by hand may be used, but one driven by water or electricity is almost essential. If milk is examined, a centrifuge driven by power and containing two or more

tubes having a capacity of not less than 70 c.c. each is required. Many forms of centrifuges are supplied by Messrs. Hearson.

Bell-jars with ground rims and one or two tubules are useful for evaporation *in vacuo*. They should stand on a square of thick ground glass. To make an air-tight joint the surface of the rim of the bell-jar, which must be quite clean, should be well greased and pressed thoroughly home on the ground-glass plate. A thick ridge of grease should then be plastered all round the angle formed by the rim of the bell-jar and the glass plate. Thick rubber pressure tubing must be used for connections, and all joints should be well greased. For evaporating large quantities of fluid the writer devised a copper stand with shelves, the shelves supporting glass dishes containing alternately strong sulphuric acid and the fluid to be evaporated, the whole being placed under a suitable bell-jar. A mercurial gauge is a useful addition to show the amount of exhaust and the occurrence of leakage. The ordinary glass filter pumps used in chemical work and actuated by a stream of water are also useful for many purposes.

Porous filter candles.—The forms which are generally employed are the Pasteur-Chamberland, the Doulton, and the Berkefeld. These consist of "candles" composed in the first two of unglazed porous porcelain, in the last of a specially prepared diatomaceous earth. The filtration through the Pasteur-Chamberland is much slower than through the Berkefeld. All give a germ-free filtrate, but the last should be employed if the fluid is thick or contains many particles; a preliminary filtration through paper is an advantage. A useful method of conducting filtration is the following: The filter "candle" (B, Fig. 5, p. 51) is connected by a short length of pressure tubing with a piece of glass tubing passing through a rubber cork

in the neck of an ordinary filtering flask C. The "candle" is placed in a jar A, such as a glass measure or urine-jar, which is filled up with the solution to be filtered. The lateral branch of the filter flask is then connected with the air-pump. On exhausting, the fluid passes through the filter "candle" over into the filtering flask, in which it is collected. Before use the "candle" should be well scrubbed and some water or $\frac{1}{2}$ per cent. carbolic run through to clean it, and the whole may be sterilised in the

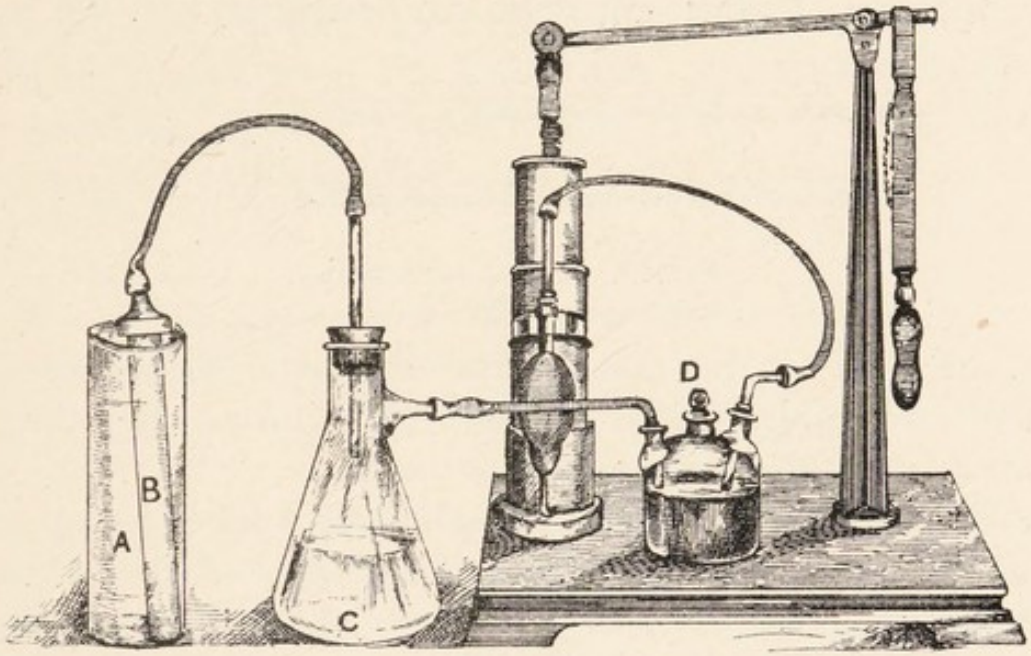


FIG 5.—Fleuss exhaust pump, arranged for filtration.

steamer for an hour or two. After use the same process should be repeated to cleanse it.

Flasks, beakers, and test-tubes.—A good supply of these of various sizes is required: Erlenmeyer and ordinary shapes, tall and short forms of beakers, etc. A few "yeast flasks" are also useful (see Fig. 17, p. 86). Beakers and flasks of "Jena" glass are to be preferred. Enamelled iron ware, jugs, saucepans, mugs, etc., may replace glass for many purposes.

The most useful size of test-tube is $5'' \times \frac{5}{8}''$; a few larger sizes and "boiling tubes" should also be kept.

Platinum needles. (Fig. 6).—Two or three platinum needles are required. They consist of about two inches of platinum wire in a handle of glass rod. One end of a glass rod is softened in the Bunsen or blowpipe flame, and about an eighth of an inch of the platinum wire is embedded in it with a forceps, the wire having been first heated to a red heat. The glass-wire joint is then well annealed in the flame and allowed to cool slowly. Two thicknesses of platinum wire are desirable, viz. 0·4 mm.

FIG. 6.—Platinum needles.

(27–28 B.W.G.) for most purposes, but a thicker wire of about 0·7 m.m. where stiffness is required, and one or two 3 in. or more in length are useful.

When platinum is unobtainable (as under the present war conditions), iron wire may be employed instead, and needles formed of it are quite serviceable. The wire cannot, however, be sealed into glass *rod*, but may be sealed into glass *tubing*. Metal holders with screw sockets for the wires are also obtainable and aluminium knitting needles make excellent holders if drilled or split to receive the wire and the end then fused in the blowpipe flame.

Forceps, needles, etc.—Several forceps are necessary, the ordinary dissecting form in two or three sizes, one or two pairs of fine pointed, two or three small brass ones, and two or three pairs of the "Cornet" pattern. A few ordinary sewing needles of various sizes mounted in wooden handles serve all purposes.

Glass pipettes and capillary tubes.—These are useful for preserving or storing blood or pus, etc., for examination, for sterile water in making film specimens, and for many other purposes. A blowpipe worked by a foot bellows is required for making pipettes, etc. A piece of glass tubing is heated in the blowpipe flame until quite soft; it is then *taken out of the flame* and the two ends are pulled steadily apart; this forms a capillary tube of greater or lesser length and smaller or larger diameter, and it can be sealed off in convenient lengths. To make a pipette proceed in the same way: seal off the capillary tube two or three inches from the wide tube, then heat this close up to where it was heated before, and draw out again and seal off two or three inches from the bulb. In this

Fig. 7.—Glass pipette.

way a capillary tube with a bulb at its middle is formed (Fig. 7). "Vaccine tubes," pipettes made of glass tubing drawn out at one end, and Wright's capsules (see Fig. 37, *a* and *d*, p. 237) are also useful.

Graduated Measures, etc.—A few graduated cylinders of 10 c.c., 25 c.c., 100 c.c., 500 c.c., and 1000 c.c. capacity are necessary. For making standard solutions and the solutions of disinfectants for determinations of the carbolic coefficient, some graduated stoppered flasks are necessary: the usual sizes are 100 c.c., 500 c.c., and 1000 c.c. Graduated pipettes of various forms are also required, *e. g.* 1 c.c., 2 c.c., 2·5 c.c., 3 c.c. and 5 c.c., bulbous for carbolic coefficient determinations, 1 c.c. straight pipettes divided into hundredths for water examination, and 5 c.c., 10 c.c., and 50 c.c. straight with central bulb for

ordinary measurements. One or two 25 c.c., and 50 c.c. burettes are required for standardisation of culture media (p. 60).

The ordinary 1 c.c. pipettes graduated in hundredths will not measure with any accuracy quantities smaller than 0·05 c.c. Finer pipettes—0·1 c.c. divided into tenths—can be obtained by which approximately 0·01 c.c. may be delivered.

For the measurement of quantities less than 0·1 c.c. however the most practicable and accurate method is the drop method elaborated by Donald.[1] While the method is more suitable for arbitrary volumes it may also be used for definite measured volumes. The volume of a drop depends upon three principal factors : the *external* diameter of the dropping point, the surface tension of the liquid, and the temperature of the liquid : if measurements are done at room temperature, the last named may be neglected. Pipettes drawn out of glass tubing are employed, similar to those used for opsonic determinations (Fig. 37 *a*, p. 237). A pipette is calibrated by gently pushing it into the suitable hole of a wire gauge until arrested : it is then cut above and flush with the steel plate with a glass-cutting knife. The end of the pipette will now have an external diameter corresponding with that of the hole of the wire gauge. A wire gauge is a steel plate pierced with holes which are numbered. Various wire-gauges are on the market, *e. g.* the Stubbs Lancashire, the Morse, and the Birmingham. The table (see p. 55) from Donald's papers gives some of the data for the Stubbs and Morse gauges (temp. = about 20° C.) : The dropping should be done with the pipette held nearly vertical and the drops should fall at a rate of about one per second. The point of the pipette should be untouched with the finger : it must be absolutely clean

[1] *Lancet*, 1915, vol. ii, p. 1243, and 1916, vol. ii, p. 423.

Diameter in mm.	Gauge Number.		Drops per c.c.	Cb. mm. per drop.
	Stubbs.	Morse.		
0·406	77	78	112·9	8·86
0·457	76	77	101·0	9·9
0·508	75	76	90·0	11·1
0·610	72	73	80·0	12·5
0·660	71	71	73·0	13·7
0·787	67	68	59·6	16·78
0·813	66	67	57·7	17·33
0·838	65	66	56·6	17·67
0·889	64	65	54·6	18·32
0·914	63	64	53·7	18·62
0·940	62	63	52·9	18·94
0·965	61	62	52·0	19·23
0·991	60	61	51·2	19·53
1·016	59	60	50·4	19·84
1·041	58	59	49·7	20·12
1·067	57	58	49·0	20·41
1·397	54	54	40·0	25·0

and free from grease. For different liquids Donald has worked out the following factors :

Water	1·0	Peptone broth . .	1·2
Saline	1·0	Standard agglutinable typhoid culture .	1·0
Serum, human. .	1·1		
Serum, guinea-pig.	1·06	Alcoholic antigens .	2·5
Cerebro-spinal fluid	1·02		

This means, for example, that 6 drops of peptone broth measure the same volume as 5 drops of water or saline, from the same diameter pipette. For steady dropping the pipette should be furnished with a rubber teat and may be "throttled" as described below. For the measurement of any number of equal volumes, Wright's method may be employed. A similar pipette is used as in the drop method (Fig. 37, *a*, p. 237). Clean mercury is sucked into the pipette so as to fill any convenient volume. A mark is then made on the pipette with

grease pencil at the upper limit of the mercury column ; this forms the " unit volume." By carefully tilting the pipette and regulating the flow with the finger placed on the point, the mercury is brought so that the lower end of the column corresponds with the first mark. The limit of the upper end of the mercury column is again marked, giving a second volume, and by repeating the process the steam of the pipette may be graduated into any number of equal volumes. It is not easy with such a pipette without considerable practice to regulate the flow of the contained fluid with a rubber-teat. By adopting the method of " throttling," however, any degree of control may be obtained. In order to throttle, a short length of glass tubing is taken of a diameter small enough to slip into the end of the undrawn extremity of the pipette. This piece of tubing is then drawn out in the flame and cut off. The fine extremity is then again drawn out in a by-pass flame and cut off so as to get a short length of very fine bore. This piece is then sealed into the pipette by means of sealing-wax. Fig. 8 shows such a throttled pipette and explains its construction better than words.

FIG. 8.—" Throttled " pipette. The upper figure shows the fine point of the " throttle."

A little practice is necessary in order to obtain just the right throttle. It is used with a rubber teat as shown in the figure. In using, care must be taken to avoid wetting the fine point of the throttle, for this prevents it from acting.

By using a measured volume of mercury, the graduation may be made to correspond to a definite volume. For instance, for Wassermann work the writer uses as unit volume 20 cub. mm., which is obtained by measuring out mercury with a hæmoglobinometer pipette, the volume of which is 20 cub. mm. One c.c. of mercury weighs 13·5 grams, any volume may therefore be obtained by *weighing*.

For marking these pipettes grease-pencil or ink may be employed. To render the mark more stable it should be passed through the Bunsen flame. Blue-black ink heated nearly to the softening point of the glass gives the most stable marking the writer knows, as stated by Donald.

India-rubber caps.—A few indiarubber caps for capping test-tube or flask cultures are required. They retard evaporation and the desiccation of the medium, and prevent the entrance of moulds. For use they should be soaked in 1–500 corrosive sublimate solution; they should not be *kept* in the solution, as vulcanised rubber absorbs mercuric chloride (Glenny and Walpole). Tinfoil, gutta-percha tissue (sealed down by warming), paraffin wax, sealing wax, or plasticine may also be used to cover the tops of tubes and flasks.

Preparation of Sterile Test-tubes, Flasks, etc., for the Reception or Manipulation of Culture Media

To sterilise cotton-wool.—Non-absorbent cotton-wool, best or No. 2 quality, is used for plugging purposes. The wool should be pulled apart so as to assist the penetration of heat; in the compressed condition the interior is difficult to sterilise. The separated wool is placed in the hot-air steriliser and the temperature is slowly raised to 145° C. and maintained at this for at least an hour. Above 150° C. cotton-wool becomes brown and brittle.

It is a common practice now to use various coloured wools for the different culture media, especially the carbohydrate ones, so that they are readily distinguishable by the eye. The coloured wools may be purchased, or the ordinary white wool may be dyed with the " Dolly " dyes.

Glass vessels.—The vessels (usually test-tubes, flasks, and dishes) are thoroughly washed and rinsed in water, then rinsed with 25 per cent. hydrochloric acid, and afterwards washed well with tap-water and drained. A final rinse with distilled water or alcohol is an advantage, as no deposit then occurs on drying. The cleansed vessels should be dried before sterilising, either in the air or by placing in the hot-air steriliser for half an hour. When dry, the vessels are plugged with a firm plug of the sterilised cotton-wool, and are placed in the hot-air steriliser, the temperature of which is then raised to about 150° C. They should remain at this temperature for not less than half an hour, after which the steriliser and its contents are allowed to cool slowly.

Petri dishes for plate cultures, graduated pipettes, etc., are cleaned as described for tubes and flasks. They may be sterilised and kept in sheet-iron or copper boxes of appropriate size and shape.

If tubes, flasks, pipettes, etc., are required in a hurry they may be rapidly sterilised as follows : After washing in water they are rinsed with 5 per cent. carbolic, then with absolute alcohol, and finally with ether, and are then well flamed over a Bunsen flame, holding in a suitable forceps or holder. The ether evaporates and burns at the mouth, and when dry, a pledget of cotton-wool is held in the forceps and singed in the flame, and, while burning, the tube or flask is plugged with it.

When thick glass vessels, such as measures, etc., have to be sterilised, it is not safe to do this in the hot-air steriliser unless the heating and cooling are carried out

very slowly, as they are very liable to crack. It is preferable, after cleaning and plugging with sterile wool, to steam in the steam steriliser or the autoclave, the heating and cooling being conducted slowly.

Culture Media

Culture media are employed (*a*) for the artificial cultivation of micro-organisms in the laboratory, (*b*) for the isolation of micro-organisms from the medium in which they are naturally present, and (*c*) for the identification of the organisms so isolated and cultivated.

The methods of preparing the culture media commonly used are here given, but certain special media will be described as required. The requisite procedures comprise (*a*) preparation of sterilised vessels, (*b*) preparation of the media, (*c*) neutralisation or standardisation of the media, (*d*) filling into the vessels, and (*e*) sterilisation. The preparation of sterilised vessels is described above (p. 58). For ordinary laboratory cultures test-tubes are generally used. Media which are solid at ordinary temperatures, *e. g.* agar, gelatin, and serum, are prepared either as deep, upright tubes (Fig. 9 A), for which 8–15 c.c. of the medium are required for a tube, or as sloping tubes (Fig. 9 C), for which 4–5 c.c. are required for a tube. Of fluid media 5–15 c.c. are used for a tube. The prepared media are introduced into the test-tubes, etc., through a funnel, care being taken to avoid soiling the mouths or the wool plugs may stick. Fluid media may be run in from a large burette or from a funnel with a piece of rubber tubing, with a spring clip, attached to the stem. The filled tubes are then sterilised in the steam steriliser (p. 47) by steaming for twenty to thirty minutes on two or three successive days, or in the autoclave (p. 48) by heating to 115°–120° C. for half an hour on one occasion. Culture media may also be kept in bulk in flasks, bottles or milk-

bottles; these need somewhat longer sterilisation than tubes. Some forms of culture media can also be purchased ready for use. Certain media can be obtained in powder form (Chopping's) from Messrs. Baird and Tatlock, and in tabloid form (Thompson's) from Messrs. Burroughs and Wellcome. These are convenient when small quantities are required for occasional use.

Neutralisation.—For the cultivation of the pathogenic micro-organisms a slightly alkaline medium is generally to be preferred, and as many of the media are somewhat acid when prepared, the addition of a certain amount of alkali is required: this is termed "neutralisation." For this purpose a 10–20 per cent. solution of caustic soda (preferably) or of sodium carbonate is employed, and *glazed* litmus paper is used as an indicator. A few drops of the alkaline solution are added at a time and well mixed with the medium, and between each addition a drop of the medium is taken with a glass rod and smeared on to both red and blue litmus papers. At first the blue paper will be reddened, while the red paper will be unchanged. Subsequently, both papers will frequently be changed, the "amphoteric reaction." Neutralisation should be continued beyond this stage by the addition of more alkali, until the red paper is well blued and the blue paper is unaltered; the reaction will now be about right.

Standardisation.—Slight variations in the composition of the nutrient media have a marked influence upon the characters of the growths of micro-organisms developing upon them. In order to obtain more uniformity for descriptive purposes, etc., a committee of the American Public Health Association drew up a scheme for the preparation of nutrient media of approximately constant composition and reaction; the latter is obtained by a chemical titration with standard alkali or acid.

Media so "neutralised" are termed "standard nutrient media." [1]

Standardisation may be most simply described in the case of nutrient broth. A 100 c.c. Erlenmeyer flask is rinsed out with boiling distilled water, 25 c.c. of the nutrient broth are introduced into it, and 0·5 c.c. of phenolphthalein solution is added (0·5 per cent. phenolphthalein in 50 per cent. alcohol). This is kept boiling, and decinormal caustic soda solution [2] is run in from a 25 c.c. burette until a faint pink tinge appears in the boiling fluid. From the amount of soda solution used the amount of normal or deka-normal soda solution required to neutralise a given volume of the broth (*e.g.* a litre) can be calculated, and this amount is then added. Although neutral to phenolphthalein, the medium is now strongly alkaline to litmus—too alkaline for the optimum growth of most organisms. The reason for this is that the di-sodium hydrogen phosphate (Na_2HPO_4) present in the medium is alkaline to phenolphthalein. To reduce the alkalinity (to litmus) normal hydrochloric acid is then added. The American Committee recommended an acidity of + 1·5—that is, to every 100 c.c. of the medium neutral to phenolphthalein 1·5 c.c. of the normal hydrochloric acid are added. Eyre advises a reaction of + 1·0 (*i.e.* 1 c.c. of normal hydrochloric to every 100 c.c.), while Chester considers that the acidity should not exceed + 0·5. Whatever the reaction adopted, it should be stated. Similarly, if a

[1] Eyre, *Brit. Med. Journ.*, 1900, vol. ii. p. 921; 1901, vol. ii, p. 788.

[2] By a "normal" solution is meant the equivalent weight in grammes of a substance dissolved in (*i.e.* made up to) a litre of water; a "decinormal" solution contains one tenth of, a deka-normal ten times, this amount. A normal solution of caustic soda contains 40 grm. of pure NaOH (NaOH = 40), of sulphuric acid 49 grm. of pure H_2SO_4 $\left(\frac{H_2SO_4}{2} = 49\right)$, per litre.

medium is used which is alkaline to phenolphthalein, this is expressed by the minus sign ; *e.g.* a reaction of — 1·5 indicates that to every 100 c.c. 1·5 c.c. of normal hydrochloric acid must be added to render it neutral to phenolphthalein, or, what is almost (but not quite) the same thing, that to the *neutral* medium 1·5 c.c. of normal caustic soda solution have been added to every 100 c.c. Various methods are adopted to obtain the final reaction ; the American Committee recommend first neutralising and then adding sufficient acid (or alkali) ; Eyre, having calculated the acidity, adds only sufficient alkali to *reduce* the reaction to the required point. Eyre describes the reaction as that represented by the number of c.c.'s of normal alkali or acid per litre, *e.g.* + 10 on Eyre's scale is equivalent to the American + 1·0. Nutrient gelatin and agar are standardised in the same manner after preparation, being kept fluid by heating during the process.

Preparation of Culture Media

The more important culture media for the cultivation of the pathogenic micro-organisms, which may also be employed for the cultivation of a large number of saprophytic organisms, are nutrient broth, nutrient gelatin, and nutrient agar-agar. These may be prepared either from an infusion of meat, usually beef, termed *acid beef-broth*, or with one of the commercial meat extracts such as " Lemco."

The constituents composing the medium are weighed out and measured and are introduced into a glass flask or a beaker or into an enamelled iron jug or saucepan.

Acid-Beef Broth.—Constituents : 1000 c.c. of water, preferably distilled ; 500 grm. of finely minced fresh gravy beef, free from fat. Heat over a water-bath at 40°–45° C. for twenty minutes with frequent agitation,

then boil for ten minutes, strain through cotton-wool, and filter through paper. Add sufficient water to the filtrate to make up to 1000 c.c.

Nutrient Beef-Broth.—Constituents : 1000 c.c. of acid-beef broth ; 10 grm. of peptone [1] ; 5 grm. of sodium chloride.

Nutrient "Lemco" Broth.—Constituents : Lemco, 10–20 grm. ; peptone, 10–20 grm. ; sodium chloride, 5–10 grm. ; water, preferably distilled, 1000 c.c.

In either case, boil over a water-bath or steam in the steam-steriliser until the constituents are completely dissolved. Then neutralise or standardise (p. 60). Again heat over the water-bath or in the steamer for half an hour, then filter through two thicknesses of coarse filter-paper. The broth should now be quite clear and bright, but if it should filter at all cloudy, cool to 50° C., add the white of an egg beaten up with the shell, and heat for half an hour, filter, tube, or keep in bulk, and sterilise. (For the preparation of dextrose-free broth see p. 28).

FIG 9.—Tubes of culture media. A. Upright agar. B. Potato. C. Sloping agar.

Trypsin-broth.—To avoid the use of prepared peptones, Douglas [2] has devised a medium made from meat and trypsin. A fresh average-sized bullock's heart is obtained and freed from fat and the large vessels. The meat is

[1] Witte's peptone was formerly almost exclusively used but is now unprocurable. Fairchild's make an efficient substitute, or Douglas's trypsin broth may be employed. The French "Peptone Chapoteaut" is another form.

[2] *Lancet,* 1914, vol. ii, p. 891.

then finely minced and four litres of water are added, the mixture is thoroughly stirred, rendered faintly alkaline to litmus, and heated to 70°–80° C. The mixture is cooled to 45° C., 1 per cent. of trypsin solution (*i.e.* 40 c.c. to the 4 litres) is added (Allen & Hanbury's Liquor Trypsinæ Co.), and the whole is kept for two or three hours at 37 °C. At the end of that time, the mixture is rendered slightly acid with acetic acid and then brought to the boil, whereby unaltered albumin, etc., is precipitated : the mixture is then strained through cotton wool or fine muslin. The fluid is rendered alkaline, 0·5 grm. of calcium chloride and 10 grm. of sodium chloride are then added and the mixture is autoclaved for an hour at 115° C. This procedure precipitates phosphates and the broth should now filter clear through paper.

This broth may also be used for the preparation of agar and gelatin. For agar, the agar powder should be added before autoclaving ; for gelatin, the gelatin should be added after autoclaving. These media are quite suitable for most purposes, but in the writer's experience are not so suitable for the growth of some organisms, particularly some of the saprophytes, as the media made with peptone. Some of the pathogenic organisms seem also to die out more readily than on peptone media.

Veal-broth.—For some purposes veal presents advantages over beef, *e.g.* for growing the tubercle bacillus. When obtained from the butcher's the veal is frequently powdered with flour ; this should be brushed and washed off as completely as possible, as it renders the broth turbid and difficult to clarify. The veal-broth is made in precisely the same way as nutrient beef-broth. It is, however, often slightly alkaline, so that less alkali is required for neutralisation. For the cultivation of the tubercle bacillus about 4 to 6 per cent. of glycerin should be added.

Glycerin beef-broth is prepared in the same manner, 4 to 6 per cent. of the best glycerin being added to the fluid after filtration.

Glucose broth.—For the cultivation of anaërobic organisms the addition of 0·5 to 2 per cent. of grape sugar is an advantage. It should be added after filtration.

Egg broth.—Besredka and Jupille [1] describe the composition of this as follows :

White of egg (10 per cent. solution) .	4 parts
Yolk of egg (10 per cent. solution) .	1 part
Nutrient broth	5 parts

The egg-white is beaten up with ten times its volume of distilled water, filtered through cotton-wool, heated to 100° C., and filtered through " papier Chardin." The liquid is tubed and sterilised at 115° C. for twenty minutes. The yolk is beaten up with ten times its volume of distilled water and a sufficiency of normal caustic soda solution is added to clarify it (about 1 c.c. per 100 c.c.). It is then treated as the egg-white. The authors recommend the use of L. Martin's broth for this medium.

Peptone water.—Add to distilled or tap water 1 to 2 per cent. of peptone and $\frac{1}{2}$ per cent. of common salt, dissolve by heat, make faintly alkaline, steam for one hour and filter.

For the cholera vibrio it is an advantage to add 1 per cent. instead of $\frac{1}{2}$ per cent. of common salt (Dunham's solution).

Beer-wort.—Procure beer-wort (preferably unhopped) from the brewery. Allow it to stand in a cool place for twelve hours, filter, and then steam for an hour and filter again. Fill into sterile test-tubes and sterilise. This medium is *not* neutralised.

Nutrient gelatin.—This is prepared in precisely the same manner as nutrient beef or Lemco broth with the addition

[1] *Ann. de l'Inst. Pasteur*, xxvii, 1913, p. 1009

of 100 grm. of the best "gold label" gelatin (Coignet's) per litre. After the addition of the egg, steam for an hour and then filter through two thicknesses of filter-paper in a hot-water funnel (this is best, but it may be done in the steamer at a low temperature, *e.g.* 35° C.). Fill into test-tubes and sterilise. After the third steaming the tubes are allowed to solidify, either in the upright or oblique position, according as they are required for stab or surface cultivation. It may also be kept in bulk for plate cultures, etc.

In hot summer weather 15 or even 20 per cent. of gelatin (150 grm. or 200 grm. to the litre) are necessary for the product to remain solid, as nutrient gelatin melts at 24° C. or a little under. Prolonged boiling diminishes and ultimately destroys the gelatinising power of gelatin, so the less it is heated the better. It must not be autoclaved.

Glucose gelatin.—Ordinary gelatin with the addition of 1 to 2 per cent. of grape sugar.

Beer-wort gelatin.—This is one of the best culture media for yeasts and some of the fungi (*e.g.* ringworm). Procure from the brewery some beer-wort, preferably unhopped, and add to every litre 100 grm. of gelatin. Dissolve, clarify, and filter, as in the case of ordinary gelatin. It is not neutralised.

Nutrient agar-agar.—This is one of our most valuable culture media, and has the advantage over nutrient gelatin that it remains solid at blood-heat.

Agar is a carbohydrate substance of high melting-point and considerable gelatinising power, obtained from Eastern seaweeds. The powdered form is now generally used. It is prepared in the same manner as nutrient beef- or Lemco broth with the addition of 15 grm. (*i.e.* 1½ per cent.) of powdered agar to the litre. Heat over the water-bath or in the steamer until dissolved (an hour or more), then neutralise or standardise. Cool to

50° C., add the white of an egg and return to the steamer for an hour and a half, then filter through an *agar filter-paper* ("papier Chardin") in a hot-water funnel or in the steamer. By this treatment a litre of agar should pass through the filter in two to three hours. If it does not come through clear, add another white of egg and repeat the process. Agar requires *well cooking*, otherwise a soft watery jelly results.

If an autoclave is available, a quicker and better method is, after neutralising and adding the white of an egg, to place in the autoclave with a small beaker inverted over the mouth of the flask, and heated to 134° C. (two atmospheres pressure) for half an hour. Turn the gas out, and allow to cool without opening the stopcock. When cool, open, and filter through the *special agar filter-paper* in a hot-water funnel; the agar will pass through in about ten minutes or a quarter of an hour. Fill into test-tubes and sterilise. Solidify in the upright or oblique position as required. It may also be kept in bulk.

In the case of bar or stick agar, first steep the agar in 1 per cent. acetic acid for a quarter of an hour, then drain and wash it so as to thoroughly remove the acid. The further procedure is the same as detailed above. This yields a very clear, pale product, and is perhaps preferable when an autoclave is not available.

Glycerin agar.—Add 4 to 6 per cent. of glycerin to the nutrient agar after filtration and proceed as before.

Glucose agar.—One or two per cent. of grape sugar is added to the nutrient agar after filtration.

Litmus media.—The addition of neutral litmus to the various culture media is a useful method of demonstrating the production of acid or of alkali by organisms. To prepare the litmus solution take the lump litmus, powder finely, and boil with distilled water so that a saturated solution is obtained. Filter, and preserve in a flask

stoppered with cotton-wool, after sterilising by boiling for half an hour on two successive days. For some purposes a special solution of litmus, the Kubel-Tiemann solution, which can be procured ready for use, is employed. It must not have any antiseptic added to it (as is sometimes done to preserve it for use in the chemical laboratory).

Sufficient of this litmus infusion is added to the nutrient media, after filtration, to tinge them a distinct purplish colour. After steaming the colour has usually disappeared, but returns as the tubes cool.

Milk.—Use separated milk, but failing this, centrifugalise ordinary new milk, or place it in a tall cylinder and allow it to stand overnight in a cool place, preferably in an ice safe. Then pipette off the milk from the bottom, rejecting the cream. Introduce the separated milk into test-tubes to the depth of about an inch to an inch and a half and steam for an hour on two successive days. The milk is usually tinged with litmus before tubing, forming *litmus milk.*

Potatoes.—Choose sound potatoes, and scrub them well with water to remove dirt. Cut off the ends, and with a cork-borer, slightly smaller than the test-tubes which are used, bore through the potato so that a cylindrical piece is removed. Push this out of the borer, and divide it into two portions by a very oblique transverse cut, so that two wedge-shaped pieces are obtained, and in this manner prepare as many pieces as there are tubes to be filled. Place them in a basin under the tap, and allow the water to flow over them for about two hours. This prevents the darkening of the potato in the subsequent steaming, as does also the use of a *silver* borer. The test-tubes for the potato-wedges are prepared as follows : After plugging and sterilising in the ordinary way, introduce a small pledget of sterilised wool into each, push

to the bottom, and moisten with a little sterilised distilled water. Drop the potato-wedges into the tubes, plug, and sterilise by steaming for three-quarters of an hour on two successive days (Fig. 9 B). The object of the moist wool is to prevent drying, and for the same purpose Roux's tubes (Fig. 10) may be used, the lower bulb being filled with water.

FIG. 10.—Roux's tube for potato.

Blood-serum.—Clean some glass jars of about 1 to 3 litres capacity, plug with wool, and sterilise in the steamer for an hour on three successive days. Bleed a horse, with aseptic precautions, and catch the blood in these sterilised jars. Allow the jars to stand in a cool place for twelve hours. Then pipette off the clear serum with a sterile pipette, and fill the sterilised test-tubes to the depth of 2–4 cm. The tubes are then arranged in a sloping position on the shelves of the serum inspissator, or failing this in a hot-water oven, the temperature of which should be about 50° C. At this temperature they remain for thirty hours ; it is then raised to 65° C., at which temperature the serum coagulates in from four to six hours and the tubes are now ready for use. It is well, however, to place them in the bloodheat incubator for a night, so that any contaminating bacteria may form colonies, and the contaminated tubes may then be rejected. Ox or sheep blood may also be used and may be obtained from the slaughterhouse ; the corpuscles do not separate so well.

Löffler's blood-serum is prepared by adding one part of glucose broth to three parts of the serum before inspissation.

The serum inspissator is practically an incubator with slightly inclined (10–15°) shelves, on which the tubes rest, and thus the serum is coagulated in a sloping position.

Fluid serum, etc.—Fluid blood-serum, ascitic and hydrocele fluids, etc., are sometimes useful, and may be used alone or mixed with peptone beef-broth in various proportions.

Ascitic or hydrocele fluid may be obtained by using sterile trocars, etc., and carrying out the tapping with aseptic precautions, collecting the fluid in sterilised flasks. It is better to collect in several small flasks than in one large one.

Fluid blood-serum may be obtained by collecting blood with aseptic precautions in sterilised flasks. When the blood has coagulated and the serum separated, the serum is pipetted off with a sterile pipette into sterile flasks.

The flasks of serum, etc., should be kept in a warm place for two or three days to make sure that they are sterile, those in which a growth appears being rejected.

Serum, ascitic fluid, etc., may also be obtained sterile by filtering through a sterilised Berkefeld filter into sterile flasks.

Serum, ascitic and hydrocele fluids, etc., may be preserved in bulk and used as required. The material is collected as aseptically as possible, 0·5 per cent. of chloroform is added, and the whole is well mixed. The mixture is then placed in a well stoppered bottle and heated for an hour in a water-bath at 45° C., with occasional shaking. It may then be stored. For use pipette off 50 c.c. of the serum aseptically and place in a sterile 200 c.c. bottle. Heat rapidly to 55° C. and shake thoroughly, or, better, place under the receiver of an

air-pump, partially exhaust, and shake. This procedure removes the chloroform.[1]

Serum agar.—This may be prepared by adding sterile serum or hydrocele or ascitic fluid, warmed to 45° C., to sterile nutrient agar (2 to 3 per cent. agar) melted and cooled to 45° C. Equal parts of the serum and agar may be mixed, or 1 part of serum to 2 or 3 parts of agar is usually sufficient. It must not be sterilised.

Blood agar.—This may be prepared by smearing the surface of the agar in sloping agar-tubes with blood obtained aseptically from the finger or from a rabbit. Or blood obtained aseptically may be defibrinated by shaking with glass beads or with a coil of fine wire, and the defibrinated blood, warmed to 45° C., is added to sterile agar liquefied by boiling and cooled to 45° C. *Hæmoglobin agar* may be prepared by laking defibrinated blood by the addition of sterile distilled water and adding to the liquid agar as before. Blood agar cannot be sterilised after preparation, and the blood therefore must be sterile.

Noguchi's medium.—For the cultivation of certain organisms which refuse to grow on all the ordinary media, Noguchi makes use of serum, serum broth, simple or glucose agar or gelatin, to which is added a piece of rabbit kidney, removed with careful aseptic precautions. Anaërobic cultivation is generally required.

Alkali albumen (Lorrain-Smith).—To 100 c.c. of fresh serum add 1 to 1·5 c.c. of a 10 per cent. caustic soda solution ; mix and introduce into test-tubes in the ordinary way. Place the test-tubes in the slanting position in the autoclave at 115° C. for twenty minutes, or in the steamer on three successive days.

Egg cultures (Hueppe).—These are very useful for some purposes. A hen's egg is taken and one end sterilised

[1] For exact details see Fildes, *Lancet*, 1917, vol. i, p. 492.

by washing with carbonate of soda solution, rinsing in sterile water, soaking in 1–500 corrosive sublimate solution, and washing in alcohol and in ether. A small hole is then chipped in the shell with a sterile needle and the inoculation made through this. The hole is afterwards closed with a little sterilised wool and collodion.

Uschinsky's Fluid.	Parts
Sodium chloride . .	5–7
Calcium chloride . .	0·1
Magnesium sulphate .	0·2–0·4
Di-potassium phosphate	2–2·5
Ammonium lactate .	6–7
Sodium asparaginate .	3–4
Glycerin . . .	30–40
Water . . .	1000

Pasteur's Fluid.	Parts.
Cane sugar . . .	10
Tartrate of ammonia .	1
The ash of 1 grm. of yeast	—
Water	100

Uschinsky's fluid is a solution of known composition without protein which can be used for investigating the chemical products of bacteria. Pathogenic organisms grow well in it and produce their toxins.

Pasteur's fluid is a good culture medium for yeasts, etc.[1]

Fermentation Media.—Fermentation reactions obtained with various fermentable substances, sugars, alcohols and glucosides, are of considerable value in the differentiation of organisms. The changes brought about by growth are acid reaction, alkaline reaction, or the former changing into the latter, or acid formation with gas production. The substances chiefly employed for this purpose are glucose, lactose, saccharose, maltose and galactose, dulcitol, mannitol, sorbitol and adonitol, starch, inulin and dextrin, and amygdalin and salicin.

For the detection of fermentation and gas production, stab cultures in agar or shake cultures in gelatin may be employed, a sufficiency (1 per cent. or so) of the particular fermentable substance having been added to the medium. For a shake culture a tube of gelatin is melted

[1] Several formulæ for synthesised media will be found in the *Journal of Experimental Medicine*, vol. iii, p. 666.

at a low temperature, inoculated with the organism, and allowed to solidify in the upright position; the organism is thereby distributed throughout the medium. Fermentation with gas production is indicated by the presence of gas bubbles (see Fig. 44 p. 436) or even by the disruption of the medium. To demonstrate glucose

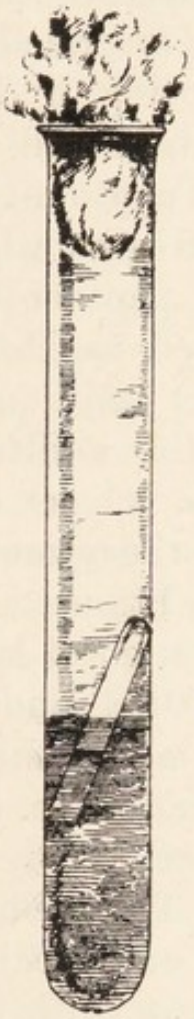


Fig. 11.—Durham's fermentation tube.

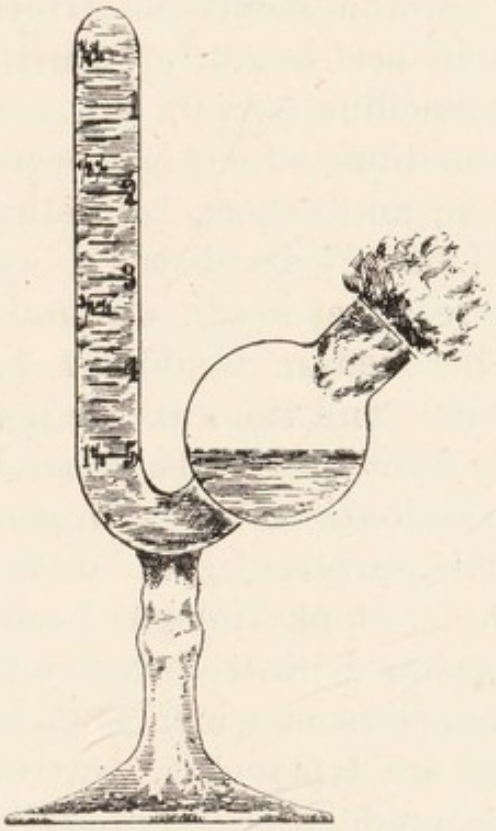


Fig. 12.—Einhorn's saccharimeter.

fermentation, ordinary nutrient gelatin is frequently employed for the "shake," but it must be meat, and not Lemco, gelatin. Durham's fermentation tubes are very convenient for showing fermentation. These are test-tubes containing suitable fluid media into which small glass tubes closed at the upper end are placed; the

latter become filled during the sterilisation. The tubes are inoculated and incubated, and if fermentation occurs the little tube becomes filled with gas (Fig. 11). Einhorn's saccharimeter may also be used (Fig. 12). The tube is filled with the medium, sterilised, inoculated, and incubated. Any gas produced collects in the closed limb of the tube. When the amount of gas ceases to increase, a little strong caustic potash solution may be added ; this absorbs the CO_2, the residue probably being hydrogen, and thus the H : CO_2 ratio may be determined. The most suitable media for fermentation are peptone broth, the acid beef-broth for which has been treated with the colon bacillus (see p. 28), 1–2 per cent. peptone water, or a medium which has been largely used by Houston, Gordon, and others, consisting of a 1 per cent. solution of "Lemco" in distilled water with the addition of peptone 1 per cent., sodium bicarbonate 0·1 per cent. ; to either medium is added 1–2 per cent. of the fermentable substance and the mixture is tinged with litmus.

The fermentation tube has been much used of late for the examination of fæces in abnormal intestinal conditions. For this purpose 1 grm. of fæces is thoroughly emulsified in 10 c.c. of physiological salt solution and 1 c.c. of the suspension is introduced into the fermentation tube, the long arm of which is 95 mm. long. The media employed are 1 per cent. dextrose, lactose, and saccharose broths made with "Lemco" (as above) or with sugar-free meat broth (see p. 28). With such tubes normal stools yield the following amounts of gas : [1]

Dextrose.	Lactose.	Saccharose.
26·75	29·9	19·5 mm.

[1] See Herter and Kendall, *Studies from the Rockefeller Institute* (Reprints), x, 1910.

The Cultivation and Isolation of Micro-organisms

It should be clearly understood that micro-organisms cannot usually be identified by their microscopical characters alone. We can state from a microscopical examination the form of an organism, that it is a bacillus or a micrococcus, or a sarcina, its size, that it is motile or non-motile, sporing or non-sporing, but we cannot as a rule go beyond this. It is necessary in most cases to ascertain the characters of the growths of organisms on the various culture media before species can be identified, and this is the principal reason for having a varied assortment of nutrient soils. It is likewise necessary for the successful cultivation of pathogenic organisms, *i.e.* those connected with disease processes and developing in or upon the bodies of man and of animals, to maintain the cultures at a temperature approximating to that of the host. For this purpose some form of incubator is required. This consists of a box or chamber of copper or iron with double walls (Fig. 13), the space between which is filled with water, the outside being covered with wood or felt, or some other non-conductor. The water between the walls is heated by means of a small burner, the gas supply for which passes through some form of regulator inserted in the water, so that the temperature, indicated by a thermometer inserted through a hole in the top, can be kept constant. The regulator is usually a mercurial one, such as Page's or Reichert's, the principle of its action being that as the temperature rises the mercury expands and at a certain point cuts off the greater part of the gas supply, only sufficient gas then passing to keep the flame of the burner alight. This point can be varied either by a sliding tube, in Page's, or by a screw, in Reichert's, so that the temperature may be set at any desired point. In this country Hearson's incubators are

now generally employed (Fig. 13). In these the regulator consists of a capsule containing a fluid of a certain boiling-point, which when ebullition takes place raises a lever and so partially cuts off the gas supply. While the Hearson regulator is a very constant one, it has the

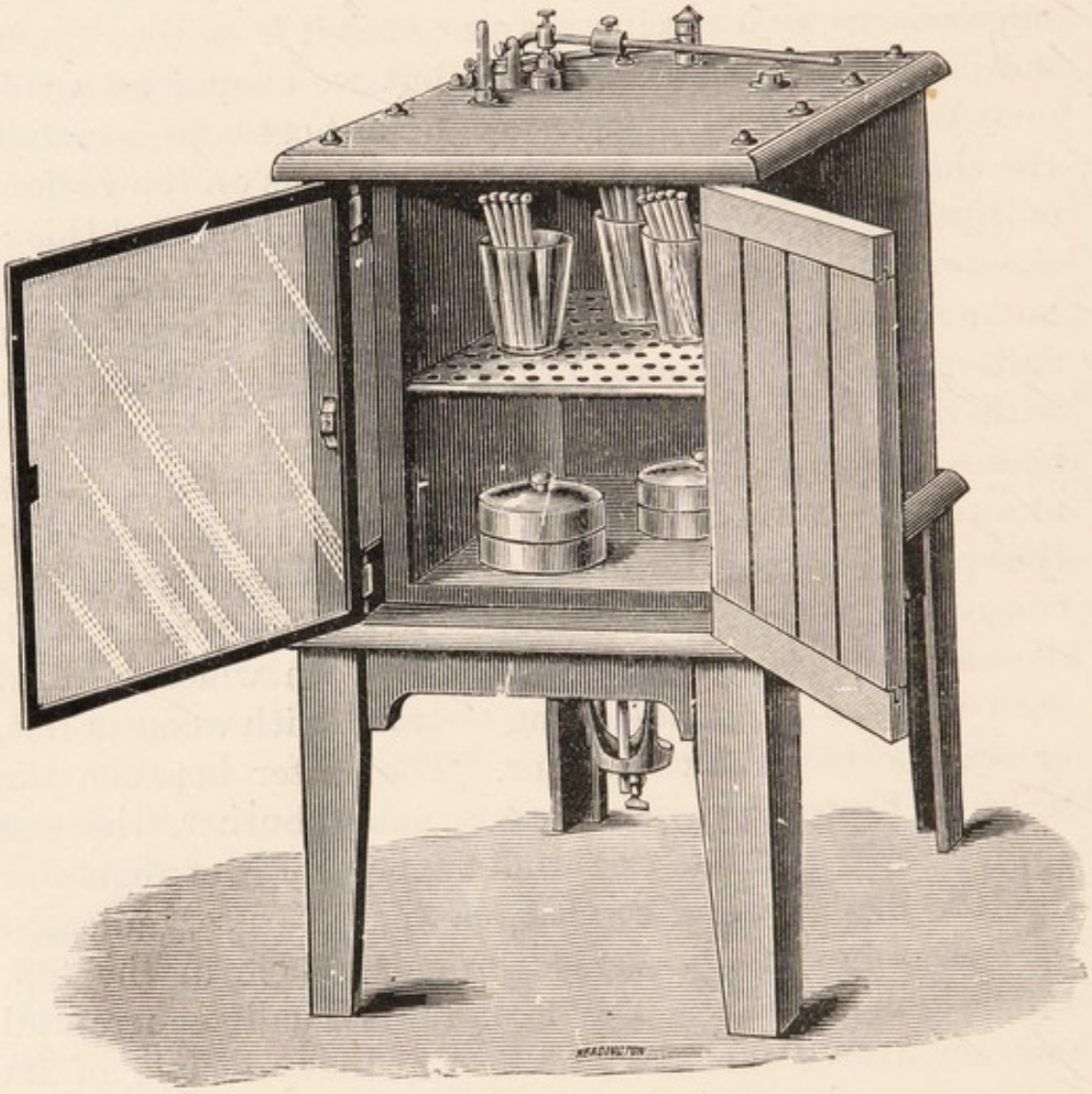


Fig. 13.— Hearson's incubator.

disadvantage that it can only be used for a range of temperature of a few degrees unless the capsule be changed. The Hearson incubators are heated with gas or oil, or in the latest forms by electricity. At least one incubator is required, and it is convenient to have two or

three. If there be only one the regulator should be set for a temperature of 37° C.; if more, another should be kept at about 20° C. The incubator at 37° C. is termed the warm or blood-heat, and that at 20° C. the cool or room temperature one. A warm room or cupboard will serve most of the purposes of the cool incubator. A third incubator set for 42° C. is useful for water examination, and a fourth at 25° C. for fermentation work. For maintaining the cool incubator at 20° C. in summer or in a hot climate a form of Hearson incubator has been devised in which a stream of cold or iced water runs through the jacket.

A substitute for the large and expensive incubator can readily be devised. An ordinary chemical hot-water oven may be employed, or simply a smaller tin set in a somewhat larger one, the interspace being filled with water; and, with a little scheming, regulators can be dispensed with by making use of a small gas or lamp flame, varying its size and distance from the bottom until the right temperature has been attained. A Thermos flask, or a Dewar's vacuum flask, or the writer's Milk Pasteuriser (made by Messrs. Allen and Hanbury) filled with water at the proper temperature may be utilised as small incubators.

Gelatin will remain solid only at temperatures below 24° C., and cannot therefore be placed in the blood-heat incubator without becoming for practical purposes a fluid medium. Agar, however—and this is one of its most valuable properties—does not liquefy below a temperature of 97°–99° C., though when once liquefied it does not set again until the temperature has fallen to about 45° C. Gelatin is therefore usually reserved for use at low temperatures, while agar, blood-serum, potato, and the fluid media can be used indifferently either at low or at high temperatures. Agar is often a

better cultivating medium than gelatin, even at low temperatures, probably because it is so much moister. The growths in fluid media are usually of the nature of a general turbidity and are not particularly characteristic, but sometimes an organism produces a film on the surface which another similar organism does not, or the medium remains clear, the growth forming a flocculent deposit, thus affording a distinction. Not only do the characters of the growths of organisms on media differ more or less, but in some instances chemical changes occur in the media which afford valuable information in the differentiation of species. Thus many organisms exert a peptonising effect on gelatin, and render it fluid sooner or later, while others have no such action. Milk is coagulated by some organisms, the coagulation being brought about in one of two ways, either by the production of acids and precipitation of the caseinogen, or by the action of a rennet-like ferment with the formation of a clot of casein. Most organisms which liquefy gelatin coagulate milk, but the converse is not the case. Agar is carbohydrate, not albuminoid, in nature, and only two or three organisms are known which liquefy it. In fluid media, such as broth and peptone water, chemical tests can be applied especially for indole, which is formed by some organisms but not by others.

Method of inoculating tubes.—The following is the procedure by which sub-cultures are prepared from an original test-tube or other culture : Tubes of the culture media selected are placed in a test-tube rack. Their mouths are then singed by holding in the Bunsen flame for a few seconds, and with a forceps, also sterilised by heating in the flame, the wool plugs are loosened by a rotatory motion, and then partially withdrawn. The mouth of the original culture-tube is similarly singed and its plug partially withdrawn. A platinum needle is

selected and carefully straightened. The original tube is then taken in the left hand between the thumb and index finger with the palm upwards, and is held obliquely, the mouth of the tube pointing to the right, a tube of sterile medium being held side by side with the original culture in an exactly similar manner. The wire of the platinum needle is then heated to redness by holding nearly vertically in the flame, and the lower part of the handle is also carefully heated. Holding the sterilised needle between the finger and thumb of the right hand, the plug of the original culture is now withdrawn by grasping between the ring and little fingers of the right hand, and is held there while the platinum needle is carefully introduced into the tube without touching the mouth or sides, and a trace of the growth is picked up with it, preferably from the margin. To ensure that the needle is cool, it may first be touched on the medium where there is no growth. The needle is quickly withdrawn without touching the sides of the tube and the plug at once replaced. The plug of the sterile tube is now withdrawn in the same manner, and the inoculated needle introduced. If a typical surface culture is desired, a single light streak is made with the needle from the bottom to the top of the medium without penetrating the surface ; if an abundant growth be required for any purpose the whole surface of the medium may be rubbed with the needle ; if a stab culture, the needle is plunged steadily into the centre of the medium and withdrawn ; if a fluid one, the growth removed is rubbed upon the side of the tube at the margin of the fluid, and the emulsion washed down by tilting the tube. The inoculation having been completed, the plug is quickly replaced, and the needle is again heated in the flame to destroy the remains of the growth upon it. If the original culture is in a deep stab, or a fluid medium, a looped platinum needle may sometimes be used with

advantage. The inoculations completed, the mouths of the tubes are singed and the wool plugs pushed in level with the lip. Before replacing the plugs each may, if desired, for greater safety, be taken with the forceps, held in the flame for a second or two, and pushed while burning into the tube, and this procedure must always be adopted if the plug be dropped or brush against anything. If the tubes have to be kept for any length of time, especially in the bloodheat incubator, each should be capped with a rubber cap, tinfoil, or gutta-percha tissue which has been soaked in 1–500 corrosive sublimate solution.

Anaërobic cultures.—Many organisms refuse to grow in the presence of free oxygen, and various expedients have to be adopted to exclude or remove it. The simplest of all is to make the cultivation in a deep stab in glucose-agar or gelatin. Narrow test-tubes filled three parts full with the medium are best, and immediately before the inoculation they should be placed upright in a beaker of water, boiled for five minutes, and then cooled and solidified in cold water. The object of this is to soften the medium so that it does not split, as a dry medium will, when the needle is plunged into it ; moreover, the needle track closes up more readily, and the dissolved oxygen is expelled. The tubes being cool, the inoculation is made with a long thin wire, either straight or with a closed loop at the end. It is inoculated and plunged steadily into the centre of the medium, nearly to the bottom, rotated, and then withdrawn, and the wool plug is replaced and singed. The tube is then carefully heated at the upper border of the medium so as to melt this slightly and seal the puncture, and a well-fitting rubber cap is applied while the tube is hot. The heating expels a portion of the air, and, with a well-fitting cap, creates a negative pressure within the tube, so that the residual oxygen is not so readily absorbed, or the tubes may be

placed in a Buchner apparatus (see below). The tubes are placed in the incubator at a suitable temperature, and it will be found that the most strictly anaërobic organisms can be cultivated in this way.

When, however, an organism is required to grow anaërobically on the surface of the medium, or in a fluid medium, some other method may be adopted. The tubes may be placed under the receiver of an air-pump and exhausted as completely as possible. This is not very convenient, for it is difficult without great care to maintain a vacuum, and special receivers must be used when the cultures have to be kept in the incubator, while with fluid media ebullition may cause considerable difficulty.

FIG. 14.—Buchner's tube arranged for anaërobic cultivation.

For fluid cultures Hamilton's method is the simplest of all. The fluid in the tubes is covered with a layer of olive oil or, better, liquid paraffin 1–2 cm. thick, and the tubes are then sterilised. The layer of oil prevents the access and entrance of oxygen. The only disadvantage is that the inoculation, or the withdrawal of culture, must usually be performed with a sterile glass pipette; if a wire needle be used the material is very liable to be detached in the oil.

Another method (Buchner's) is that usually adopted, and consists in absorbing the oxygen by means of alkali and pyrogallic acid, and so cultivating in an atmosphere of nitrogen. This can be carried out in two ways—either in a wide-mouthed bottle with well-fitting glass stopper, sufficiently large to contain the test-tubes, or in a Buchner's tube. For the first the inoculated culture tubes are

placed in the bottle, into which a few cubic centimetres of a strong aqueous solution of pyrogallic acid have previously been poured. By means of a thistle funnel, an equal volume of 20 per cent. caustic potash [1] or soda solution is then added. As quickly as possible the thistle funnel is withdrawn without mixing the solutions, and the stopper, well vaselined, inserted and twisted well home, and some melted paraffin may be poured all round the joint and melted in with a hot iron. The solutions in the bottle are now well mixed, and the whole is placed in a suitable incubator. The glass-jars used for bottling fruit may be employed instead of a stoppered bottle. The Buchner's tube (Fig. 14) is convenient for single test-tube cultures. It consists of a strong glass test-tube, large enough to take an ordinary test-tube, and having a constriction about an inch and a half from the bottom. The constriction supports the test-tube culture, while the mixture of pyrogallic acid and caustic potash fills the portion below the constriction. A well-fitting rubber cork closes the mouth of the tube, and the joint may be paraffined for additional security. If a Buchner's tube is not available, the cotton-wool plug of the culture tube may be pushed into the tube for an inch, some solid pyrogallol is placed on the wool plug, this is just *moistened* with caustic potash solution and the tube is stoppered with a rubber cork.

McIntosh and Fildes' [2] recommend the use of palladium black. Asbestos wool, 0·25 grm., is soaked in 1·5 c.c. of a 10 per cent. aqueous solution of palladium chloride, rendered soluble by the addition of a little strong hydro-

[1] Thirty-two grm. of pyrogallic acid and 64 grm. of caustic potash dissolved in 100 c.c. of water will absorb 9200 c.c. of oxygen. At the same time some carbon monoxide is evolved (122·5 c.c.). The evolution of CO is a minimum when the potash is in excess and only one-fifth of the theoretical absorbable amount of O is absorbed.

[2] *Lancet*, April 8, 1916.

chloric acid to the water. The wool is moulded with a glass rod into a flat pad 1 inch square and dried. The wool pad is then well smoked in a smoky gas flame and heated in a blowpipe flame to reduce the palladium. The prepared wool pad is enclosed in fine brass wire gauze which is clipped in a bracket made of thin sheet brass so

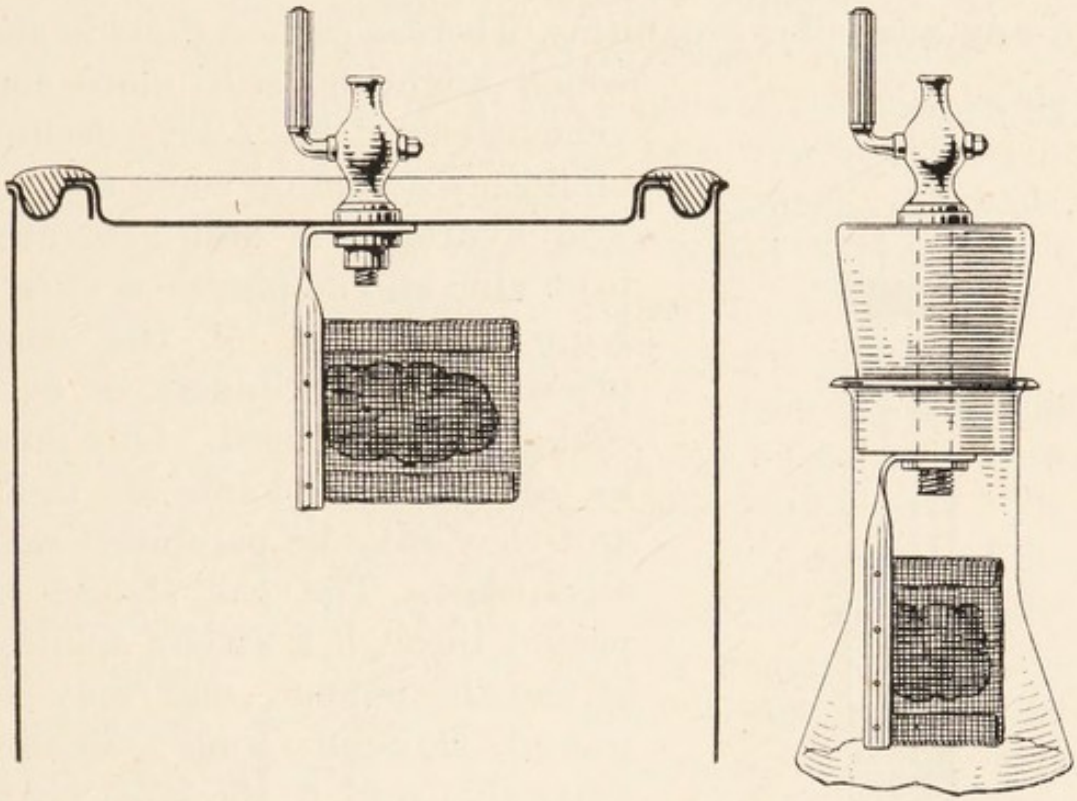


FIG. 15.—McIntosh aud Fildes' apparatus for anaërobic cultivation.

that it can be suspended from the cork or lid of the apparatus. A glass bottle or a tin with lever top may be used, and the cork or lid is provided with a tap through which hydrogen is passed into the apparatus. Before use the asbestos is heated in the Bunsen flame and immediately placed in position in the tin or bottle, into which the cultures have already been introduced. Hydrogen, which need not be purified, is then passed into the apparatus, until increasing pressure stops the generation of the gas or the tin is absolutely cold. The palladium

black causes the combination of the hydrogen with any oxygen that may be present forming water, so that an absolutely anaërobic condition is obtained. The figure (Fig. 15) shows the details of the apparatus.

The displacement of the atmosphere by means of hydrogen may be adopted, and is to be preferred for fluid cultures. Hydrogen does not seem to inhibit the growth of any anaërobic organisms, whereas carbon dioxide gas, which might be still more conveniently used, has a very decided inhibitory action on some species. The hydrogen is best generated from zinc and sulphuric acid in a Kipp apparatus, or the compressed gas in cylinders, or even coal-gas, may be used. Care must be taken that all joints are tight, and they may be paraffined with advantage. The gas should be passed through a strong solution of caustic potash, and may be passed through some alkaline pyrogallic acid if the most rigorous condition of anaërobiosis is desired, but for ordinary purposes this is not essential; it should also pass through two or three fairly firm plugs of cotton-wool to remove organisms; these must be dry, for if moist the passage of the gas may be stopped.

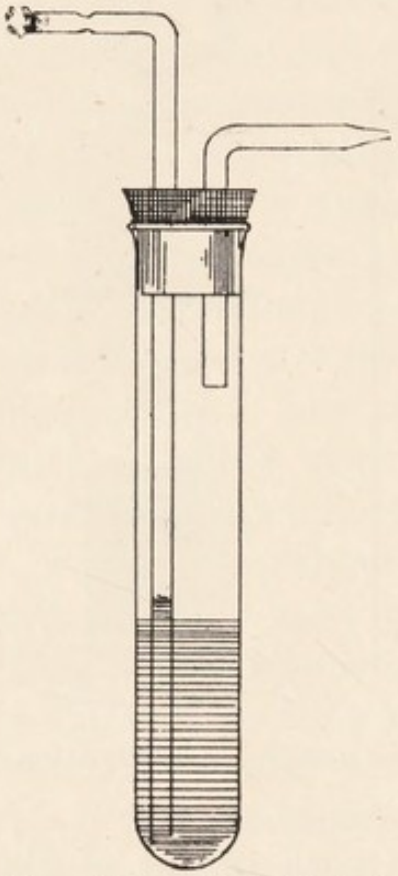


FIG. 16.—Fränkel's tube for anaërobic cultivation.

For tube cultures Fränkel's method may be adopted (Fig. 16). The broth or gelatin is introduced into a large strong test-tube which is plugged with a rubber cork, through which two pieces of glass tubing pass, one to the

bottom of the tube, the other just through the cork. Outside the cork these tubes are bent over at right angles, and each is drawn slightly out so as to contract its lumen at about the middle. The long tube is connected with the hydrogen supply, and a current of the gas is passed through and escapes by the shorter tube. After the gas has been passing for twenty minutes to half an hour, and *all oxygen has been expelled*, the distal, *i.e.* shorter, tube is sealed off at the contracted portion in the Bunsen or blowpipe flame, and then the proximal or longer one in the same manner. The rubber cork must, of course, fit well, and the joints should be paraffined. If gelatin be the medium, it should be kept fluid in a bath of warm water while the hydrogen is passing.

For broth or other fluid cultures, which are essential for obtaining toxic products, flasks are used which are fitted up like the Fränkel tube described above. The ends of the tubes are plugged with cotton-wool, and the whole—flask, cork, tubes and medium—is sterilised. The medium is inoculated from a recent culture by momentarily removing the cork. Hydrogen is then passed through from a Kipp apparatus, the long tube being connected with the hydrogen supply. After passing for about half an hour, the tubes are sealed off and the flask is incubated. For convenience of sealing the tubes should be drawn out slightly.

As many organisms produce gas during their growth, it may be necessary to provide for its escape, or the flasks may burst owing to the pressure. This can be done by adjusting a mercury valve, and may be carried out in a simple manner by a method devised by the writer. "Yeast flasks," which can be obtained in various sizes, are made use of, and are filled three parts full with a 2 per cent. grape-sugar bouillon. The neck is corked with a perforated rubber cork (A, Fig. 17), through which a

glass tube, B, passes to the bottom of the flask, projecting two inches above the rubber cork and here plugged with cotton-wool. The lateral tube of the yeast flask is also plugged with cotton-wool, care being taken that the plugs are loose enough to allow air to pass freely. The whole is sterilised and inoculated. The glass tube, B, which passes through the rubber cork, is then connected with a Kipp or other hydrogen-generating apparatus by means of a rubber tube, and a current of hydrogen is passed through the flask. The hydrogen bubbles through the bouillon and escapes by the lateral tube. After the gas has been passing for half an hour a small tube containing mercury, C, is applied to the end of the lateral branch, so that the open end just dips below the surface of the mercury, and the tube, B, which passes through the rubber cork, is sealed off in the blowpipe flame, care being taken that all the air has been expelled from the flask by a free current of hydrogen. The flask, with the capsule of mercury applied to the end of the lateral branch, can then be placed in the incubator. The mercury thus forms a valve through which air cannot enter, while gases formed by the growth of the organism have free exit.

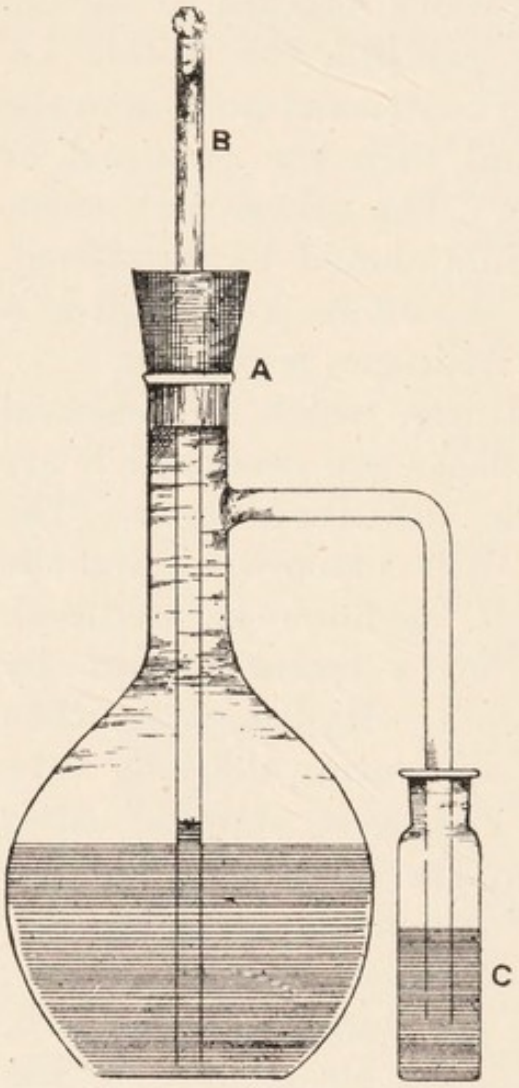


FIG 17.—Yeast flask arranged for anaërobic cultivation.

For large flasks, the lateral tube may be just bent down and a little capsule of mercury attached.

The addition of ½ to 1 per cent. of sodium formate to the culture media much simplifies anaërobic cultivation; the tetanus bacillus, for example, can be grown in formate broth in a stoppered bottle without any elaborate precaution for excluding the last traces of air. The sodium formate should be added immediately before the last sterilisation, not previously, or decomposition may occur. Sodium sulphindigotate (0·3 per cent.) may be similarly used.

With such a broth, Dean's bottle may be used for anaërobic cultivation. This consists of a bottle around the neck of which a gutter for mercury is formed. A glass cap loosely fits over the mouth of the bottle, and its edge dips into the mercury in the gutter, thus sealing the bottle but allowing the escape of gas.

Plate cultivations.—The method of plate culture is one of the most important in bacteriology. It is used for three purposes: (1) for obtaining pure cultivations, *i.e.* cultures containing a single species, from a mixture of organisms; (2) for the enumeration of organisms; and (3) for ascertaining the characters of the colonies of organisms as an aid in the identification of species.

Before the introduction of plate cultivations pure cultures of many organisms could only be obtained by chance, or by the dilution method, which was also by no means certain. The dilution method consisted in estimating approximately the number of organisms in a given volume of fluid by means of an instrument on the same principle as the hæmatocytometer. The fluid is then diluted by the addition of some sterile fluid so that a given volume of the dilution contains a single organism only, assuming the organisms to be evenly distributed throughout the fluid. By transferring this volume to tubes of

sterile media pure cultivations can in some cases be obtained, a single organism having been sown in a tube.

It is obvious, however, that this method is at best an uncertain one, but the plate-culture method to a large extent obviates this uncertainty. It depends upon the following principles: Gelatin and agar media, when melted, remain fluid down to 25° and 45° C. respectively, temperatures which will not affect the vitality even of delicate organisms. By inoculating the fluid gelatin or agar, thoroughly mixing, and then pouring on to a level sterilised surface, so that the medium solidifies in a thin film ("plating"), the organisms, wherever they may be situated, are fixed and are unable to wander, and, being in a good nutrient soil, grow and multiply and ultimately form visible growths or colonies. Many of these colonies will have arisen from a single organism; the growth, therefore, is "pure," *i. e.* consists of a single species, and pure cultures can be obtained by inoculating tubes of sterile media from them.

When suitable, sterile nutrient gelatin is usually employed for the preparation of plate cultivations, as it is more easily manipulated than agar. Three tubes of sterile nutrient gelatin are melted at a low temperature in a beaker of water (gelatin melts at 24° C.; the temperature should not exceed about 45° C.). The tubes may be termed respectively 1, 2, and 3. Tube No. 1 is inoculated, by means of a platinum needle, with a trace of the growth from which pure cultivations are desired. The trace of growth is thoroughly mixed up and distributed throughout the melted gelatin. If this mixture be "plated," so many organisms may be present in the film that the colonies which develop will not be separate, but will form a confluent growth. To obviate this difficulty a second and a third dilution are prepared. The second dilution is made by inoculating the tube of melted

gelatin No. 2 with one platinum loopful from tube No. 1, and thoroughly mixing up ; and to be quite sure that the resulting colonies will be isolated from one another, a third dilution is prepared in the same manner by inoculating the tube of melted gelatin No. 3 with two to four platinum loopfuls from tube No. 2. The organisms having been distributed throughout the gelatin by rolling and gentle shaking, the wool plug is in each case withdrawn from the mouth of the tube, the mouth of the tube is sterilised in the Bunsen burner to prevent contamination, then cooled for a few seconds, and finally the melted gelatin is poured on to a level sterile glass surface. Formerly plates of glass were used (hence the name) ; but now shallow glass dishes with lids, about three or four inches in diameter, known as Petri dishes (Fig. 18), are almost always employed ; they are previously sterilised in the hot-air steriliser in suitable iron or copper boxes holding a dozen or so, or each dish with cover may be wrapped in paper ; the melted gelatin having been poured in, the dish is tilted to diffuse the gelatin over the bottom of the dish, placed on a level surface for the gelatin to set, and then stored in the cool incubator. The plates are examined daily, with a hand lens if necessary, or with a low power of the microscope, the dish being turned bottom upwards on the stage of the microscope for this purpose. When the colonies have developed, inoculations can be made from them by means of a platinum needle on to tubes of sterile media. The colonies, having arisen from single organisms, are pure, and the resulting sub-cultures are therefore also pure (it sometimes happens

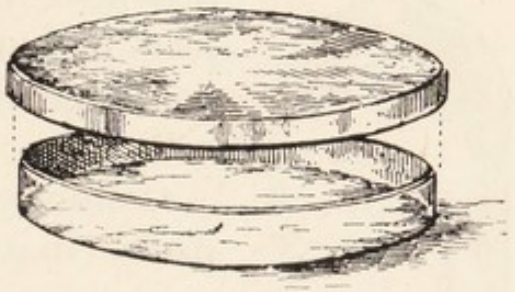


FIG 18.—Petri dish for plate cultivation.

that the colonies are mixed owing to two or more organisms being close together). Different species of organisms usually form colonies having different appearances, so that the colonies are an aid in diagnosis and enable the various species to be picked out from a mixture. The colonies in gelatin are as a rule much more distinctive than those in agar. Whereas the plate cultivation prepared from tube No. 1 is generally too crowded, plates 2 or 3, or both, can be made use of, and it is apparent that, to make certain of isolating all the organisms from a mixture, several sets of plates should be prepared. Flat bottles (Fig. 19) may likewise be used for plate culturing, and are also very useful for growing organisms in bulk for the examination of the constituents and actions of the bacterial cells.

FIG 19.—"Plate" bottle.

Golding has devised flat wedge-shaped flasks (having sides at an appropriate angle) for plate-culturing, and these are very useful, as the culture medium may be kept in them ready for use.

Agar plate cultures may be prepared in a similar way. The agar must, however, be brought to a temperature of nearly boiling before it melts; it is then allowed to cool to nearly 45° C. and the tubes are inoculated in the same manner as for a gelatin plate culture described above. Unless the manipulations be carried out expeditiously the agar will solidify, or the agar film in the Petri dish be lumpy.

Agar plates should usually be inverted during incubation, or the growth may become confluent owing to the condensation water carrying the organisms all over the film.

The plate-culture method can be modified to suit

particular circumstances : for example, the melted gelatin or agar, uninoculated, may be poured into the dishes and allowed to solidify, and the film then inoculated by streaking or painting with the material, or by pouring a few drops of broth containing the organisms upon it. This is practically the only way in which blood-serum can be used, the sterile blood-serum being placed in the Petri dish, solidified in the inspissator in the same manner as for blood-serum tubes, and the coagulated film inoculated.

For many purposes plates are unnecessary, the same result being obtained by rubbing over the surface of two or three or more tubes of sloping agar or gelatin successively the *once* charged needle, straight or looped. In the last tube or two isolated colonies generally develop.

The plate-culture method may fail if the organism to be isolated forms but a small minority of the total organisms present in the mixture ; the only alternative then is to multiply the number of plates, which, however, may entail great labour in their examination, or to adopt some special method (see p. 95).

Esmarch's roll cultures.—Another modification of the plate-culture method is known as Esmarch's roll culture. For this purpose large test-tubes (" boiling tubes "), at least an inch in diameter and six inches long, are sterilised and plugged with cotton-wool. The sterile melted gelatin, about 10 c.c., is poured in and inoculated, the wool plug replaced, and the tube held in the horizontal position and rotated under a stream of cold water, or in warm weather on a block of ice, until the gelatin has set. In this way the gelatin forms a thin film over the inside of the tube, but a little practice is required to get it evenly distributed. The colonies then develop in the film of gelatin, which is quite analogous to a film in a Petri dish.

Anaërobic plate cultivations are sometimes required.

The plate culture after preparation as described above, using a *deep* Petri or other dish, is inverted, and some alkaline pyrogallol is placed in the lid; this absorbs the oxygen within the dish. The preparation must be kept under observation for the next hour or so, and more alkaline pyrogallol is added from time to time to compensate for the rise of fluid within the dish until absorption of the oxygen from the contained air is complete.

McLeod has devised a useful porcelain dish for containing the alkaline pyrogallol over which the Petri dish is inverted, the joint being made air-tight with plasticine.

In Botkin's method a bell-jar standing in a glass dish is made use of. The Petri dishes are placed on a support within the bell-jar, and mercury or oil is poured into the glass dish. By means of a piece of bent glass tubing a steam of hydrogen is passed into the bell-jar under its rim so as to displace the air, which bubbles out through the oil or mercury. When the air has been entirely displaced the glass tube is removed, the bell-jar weighted, and the whole placed in the incubator. Bulloch's apparatus is somewhat similar to this. Wide-mouthed jars with well-ground glass lids, which are luted down, are very convenient, the oxygen being absorbed with alkaline pyrogallol placed at the bottom, and the Petri dishes stacked on a glass capsule or other support to raise them above the fluid.

The Esmarch roll cultures can be adapted for anaërobic cultures. The wool plug is replaced by a rubber cork with two holes, through which inlet and outlet glass tubes pass, as in Fränkel's anaërobic tubes (Fig. 16). The gelatin (or agar) having been melted and inoculated, the medium is kept melted in a water-bath at appropriate temperature, the hydrogen is passed through for a quarter of an hour, the tubes are sealed off, and the roll-culture is prepared.

Golding's flask (p. 90) or a "plate" bottle (Fig. 19) may be similarly used, or a Golding flask may be inverted over a beaker of alkaline pyrogallol.

Single-cell cultures.—With large cells, such as yeasts, it is not difficult to obtain growths from single cells by making miniature plate cultures on ruled cover-glasses and ascertaining where single cells are located in the film by examining the preparation with a $\frac{1}{4}$ or $\frac{1}{6}$ in. objective (see Chapter XVI). But with the minute bacterial cells this method is inapplicable. By the use of Burri's Indian ink method,[1] however, single-celled cultures of bacteria can be obtained. Fluid Indian ink is diluted with 6–10 volumes of distilled water and the mixture is sterilised in the autoclave. Several loopfuls of this are deposited in series on a sterile slide. The first drop is inoculated with the culture which is being investigated, the second drop is inoculated from the first, the third from the second, and so on. A fine mapping-pen, sterilised in the flame, is then dipped into the third, fourth, or fifth drops, and the trace of Indian ink mixture so picked up is deposited on a gelatin or agar plate. The droplet is covered with a sterilised cover-glass and is examined with a $\frac{1}{6}$ in. or $\frac{1}{8}$ in. objective, with a high eyepiece. An organism shows up white on a black background. Many drops are deposited on the plate and examined, and those in which only a single organism can be found are noted and the plate is then incubated so that colonies may form, from which sub-cultures may be prepared.

Barker [2] has devised a method by which a single organism or cell can be picked up with a capillary pipette. The fluid containing the organisms and suitably diluted is mounted on the under surface of a cover-glass forming the roof of a special cell. The capillary pipette is mounted

[1] *Das Tuschverfahren* (G. Fischer, 1909).

[2] See *Philippine Journ. of Science*, vol. ix, Sec. B, 1914, p. 307.

on a stand provided with three screws by means of which motion in the three directions of space can be obtained. The film is examined microscopically and a suitable organism having been found the pipette is brought into position and the organism taken up into it.

Isolation of Organisms.—If the organism be present unmixed with other organisms, and provided it will grow on culture media, there is no difficulty in obtaining pure cultures by transferring a little of the material to tubes of the appropriate medium. This is frequently the case in many diseases and by culturing from the blood or tissues pure cultures may be obtained.

As regards appropriate media, these must be adjusted to the food requirements of the particular class of organisms to be cultivated. Thus for pathogenic organisms of animals, media rich in protein are generally required, *e.g.* blood serum, nutrient agar, gelatin and broth, etc. For the organisms of plant diseases, vegetable infusions of the plant itself or from other sources, with or without the addition of vegetable proteins and carbohydrates may be used. For organisms of fermentations beer-wort, grape or fruit juice, and carbohydrate solutions will probably prove most serviceable, while for the nitrifying organisms saline solutions, etc., must be employed (p. 32). Some of the organisms of milk will grow only in milk or media containing milk products. A considerable number of the common saprophytic bacteria of air, soil and water, etc., will develop in the ordinary nutrient agar, gelatin and broth.

If a mixture of organisms be present, isolation of the individual species may be difficult and complicated. Plate cultures will then usually give the best hope of success, either in Petri dishes, etc. (p. 89) or in tubes (p. 91). Aërobic and anaërobic cultivation should in all cases be employed.

In some instances single-cell cultures must be made use of.

Various devices will sometimes aid isolation. Thus aërobic and anaërobic cultivation will separate the strict aërobes from the strict anaërobes. If sporing and non-sporing forms be present, the former may be separated from the latter by heating the material to 80° C. for fifteen to thirty minutes; the heating kills most non-sporing forms but does not affect the vitality of the spores.

To separate a pathogenic organism from non-pathogenic forms, inoculation into a susceptible animal may succeed.

The use of "selective" culture media may be of service in some instances. This method is largely employed, for instance, for the isolation of the typhoid-coli group from the fæces, such media as the Conradi, malachite green, and bile-salt being utilised.

Germicidal agents are occasionally employed. Thus antiformin will destroy all or most of the organisms in tuberculous sputum, except the tubercle bacillus.

"Enrichment" may sometimes be practised. This means placing the material under such conditions that a particular species grows and multiplies considerably in the material. This is sometimes used for the isolation of the meningococcus from cerebro-spinal fluid; the fluid is incubated at 37° C. for twenty-four hours and then cultured, and for the isolation of the typhoid bacillus from water, some peptone is added to the water, which is then incubated at 37° C. for twenty-four hours.

"Concentration" may also be utilised. This may be done by sedimentation, by centrifuging at high speed, by the formation of an inert precipitate and subsequent sedimentation or centrifuging, or by filtering through a porcelain filter. The organisms will tend to be concentrated in the deposit.

CHAPTER III

THE PREPARATION OF TISSUES AND ORGANISMS FOR STAINING AND MOUNTING—STAINING AND STAINING METHODS

A SELECTED few of the numerous methods devised for the preparation and staining of tissues, bacteria, etc., are here given. Special methods occasionally employed will be described when required.

Preparation of Tissues

In bacteriological work the demonstration of the bacteria in the tissues is the primary object, and, therefore, the elaborate methods which have been devised for fixing the tissue elements are not usually required, unless it be that the minuter changes in the latter are being studied. The tissues should always be obtained as fresh as possible, because within a few hours of death they are invaded by numerous bacteria, derived from the air and from the intestine, which may mask the original bacterial infection and lead to serious mistakes if this source of error be not carefully borne in mind. In all cases the tissue should be cut into pieces of convenient size, not more than about 1 cm. in thickness, and organs if kept *en masse* should be sliced. Having been thus prepared, the material may be treated by one of the following methods :

(*a*) Place directly in alcohol [1] for a week or a fortnight.

(*b*) Place in alcohol 1 part, water 2 parts, for twenty-four to forty-eight hours, transfer to alcohol and water, equal parts, and finally to absolute alcohol, for like periods.

(*c*) Place in rectified spirit (86 per cent. alcohol) containing 1 per cent. of corrosive sublimate for twelve to forty-eight hours, and pass through increasing strengths of alcohol as in (*b*).

(*d*) Place for six to twenty hours in a saturated aqueous solution of corrosive sublimate. This is prepared by saturating boiling distilled water with the corrosive sublimate, cooling, and filtering. Keep in the dark. When removed from the corrosive sublimate solution the tissues must be washed in a stream of running water for an hour, or, better, placed for a day in 70 per cent. alcohol deeply coloured with iodine, to remove the excess of corrosive sublimate and prevent precipitation. The tissues are then passed through increasing strengths of alcohol, as in (*b*).

(*e*) Formalin, a 40 per cent. aqueous solution of formic aldehyde, is an excellent fixing agent. A solution of 1 part of formalin and 9 parts of water, or better, physiological salt solution, may be used, the pieces of tissue remaining in this for twelve to twenty-four hours. They are then washed in running water for an hour or two

[1] Methylated spirit may usually be employed for all purposes when an alcohol of not more than 90 per cent. strength suffices. *It must, however, be free from mineral naphtha*, which is present in all "shop" methylated spirit. Methylated spirit free from mineral naphtha can be obtained in quantities of five bulk gallons, "for scientific purposes only," by special order from the Inland Revenue Authorities, Somerset House, W.C. If it cannot be procured, absolute alcohol must be employed. Duty-free absolute alcohol can also be obtained at a low price under somewhat similar conditions. In the following pages when the unqualified term "alcohol" is used, the naphtha-free methylated spirit may be generally employed.

and passed through increasing strengths of alcohol, as in (*b*).

All tissues after fixing and hardening should be preserved in alcohol—70–80 per cent.

The methods (*c*), (*d*), and (*e*) are to be recommended, especially the two last, as the tissue elements are well fixed thereby. In all cases the fixing fluid should be used in considerable excess. Fixing fluids containing potassium bichromate (as in Müller's fluid) and chromic acid tend to prevent the bacteria from staining with any certainty, and should be avoided.

Section Cutting

In order satisfactorily to demonstrate bacteria in tissues, and their relation to the tissue elements, it is usually necessary to prepare sections. For this purpose either the freezing or the paraffin method should be employed.

(*a*) *Freezing method.*—The tissue, in suitable pieces, must first be soaked in water to remove the alcohol. A convenient way of doing this is to place the material in a wide-mouthed bottle, into the mouth of which an ordinary glass funnel is introduced, and the bottle with the funnel is placed under a stream of running water; the funnel, while allowing the water to flow out, retains the pieces of tissue in the bottle. With running water the alcohol will be completely removed in from one to two hours; in still water, which should be changed two or three times, this result may not be attained for several hours, during which time there is an ever-increasing risk of bacterial contamination from without. *It is essential to remove all the alcohol, or the tissue will not freeze.*

When the alcohol has been removed, which is known by the tissue *sinking* in the water (lung is an exception —it always floats unless solid from any cause), the

pieces are transferred to a strong mucilage of gum acacia :

Gum acacia	5 grm.
Cane sugar	0·5 grm.
Water	100 c.c.

Add a piece of thymol or a little carbolic acid to prevent decomposition. Hamilton saturates the solution with boric acid.

In this gum solution the pieces remain for twelve to forty-eight hours, according to their size and the time

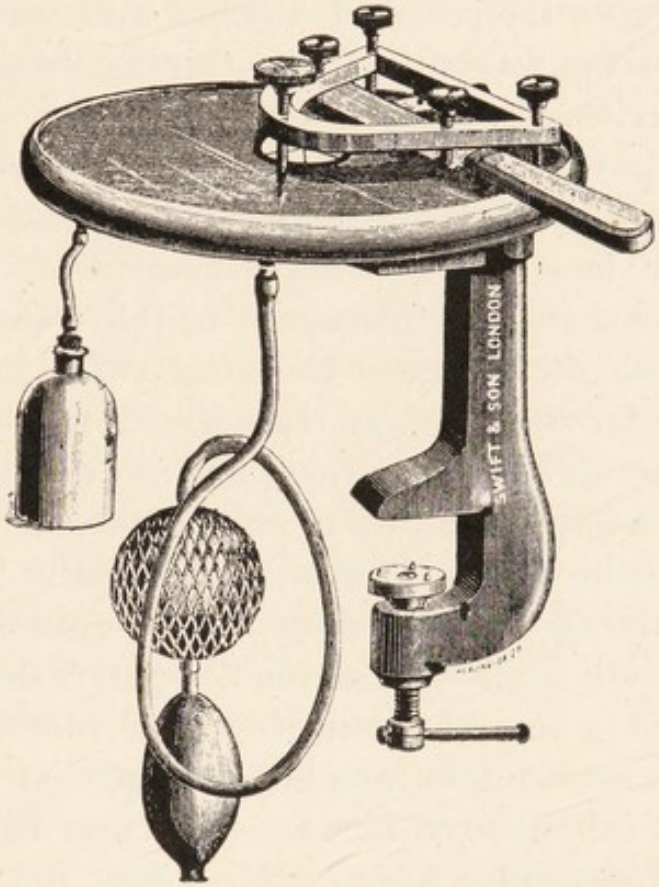


Fig 20.—Swift's ether-freezing microtome.

at the disposal of the investigator, and are then cut on one of the numerous ether-freezing microtomes now to be obtained, such as Swift's (Fig. 20) or Cathcart's. A microtome in which the freezing is effected by carbonic acid is now frequently employed and acts well. Liquid carbonic acid, contained in a cylinder, sprays by its own pressure on to the under surface of the plate on which the

block of tissue rests; the tissue quickly freezes and is then cut. This form of microtome works satisfactorily in the hottest weather. The material must not be frozen so hard that the sections roll up and fall off the knife; the sugar in the above solution should prevent this. The sections are transferred successively to two or three lots of distilled water, preferably slightly warmed, to remove the gum, and can then be stained at once, or may be preserved in equal parts of alcohol and water.

Bacteria seem to retain their staining properties better in the tissue in bulk than in sections preserved in alcohol. This objection does not hold with *paraffin* sections.

(*b*) *Paraffin method.*—Nothing can surpass the paraffin method for the thinness and beauty of the sections obtainable by it, and for some friable tissues, such as actinomycosis, it is almost essential. The tissue, in suitable pieces for cutting, is transferred from the diluted spirit preservative solution to pure methylated spirit for two or three hours, and then to absolute alcohol—which may have to be changed once unless a fairly large volume is employed—for from four to twenty-four hours. It is then removed from the alcohol, lightly dried between the folds of a *dry* cloth or piece of blotting-paper to remove the superfluous alcohol, and placed in an excess of xylol, in which it remains for from four to twenty-four hours until cleared. This is recognised by the material assuming a more or less semi-transparent condition, and the process may be much accelerated by warming the xylol to from 37° to 50° C. in the blood-heat incubator or paraffin oven, the bottle containing the xylol being well stoppered. When cleared it is ready to go into the bath of melted paraffin. A paraffin of a fairly high melting-point is perhaps the best, viz. 45° to 55° C., and is placed in glass capsules in an oven which can be kept uniformly heated to the required temperature. An ordinary chemi-

cal hot-water oven answers the purpose quite well, and is heated by a special form of small Bunsen burner with mica chimney, the temperature being regulated by some form of mercurial regulator, which is set a degree or two above the melting-point of the paraffin employed. The tissue is taken out of the xylol, blotted to remove the excess, and placed in the melted paraffin for from six to twenty hours. It is then embedded by pouring a little of the melted paraffin into a watch-glass, or into a small box formed of folded paper or lead-foil, or by bringing together two L-shaped pieces of brass on a glass plate so that a rectangular cavity is produced. The pieces of tissue are then taken out with a small warmed forceps or needle, adjusted to the position they are required to occupy, and more melted paraffin is poured in, so as to cover them. When a film of solid paraffin has formed, the whole is immersed in cold water so as to cool it rapidly.

A new paraffin is frequently crystalline in structure, and acts much better after it has been kept melted for some weeks, or is much improved by heating nearly to its boiling-point for five or six days (P. T. Beale). The xylol for clearing may be used several times and the paraffin repeatedly, the remains of old tissues being removed. The time which the tissues require to remain in the alcohol, xylol, and paraffin depends upon their size; *very small* pieces may be treated in an hour or two, large ones may require 12–24 hours.

Other clearing agents, such as chloroform, turpentine, and cedar oil, may be used instead of xylol. The paraffin method is usually straightforward, but *small* pieces of tissue must not be left too long either in absolute alcohol or in the paraffin bath, for they are liable to become too hard to cut. Thyroid tissue and skin are also rather troublesome; they become very hard unless the whole process is carried out as rapidly as possible. If the

pieces of tissue be large, the capsule of melted paraffin containing the tissue may be placed under the receiver of an air-pump, which is then exhausted. This causes the paraffin to penetrate better, and the process may be repeated two or three times during the period of infiltration. A special form of paraffin oven has been devised by Cheatle for infiltrating under diminished pressure, and is made by Messrs. Hearson, of Regent Street, London.

In order to prepare sections from material embedded in

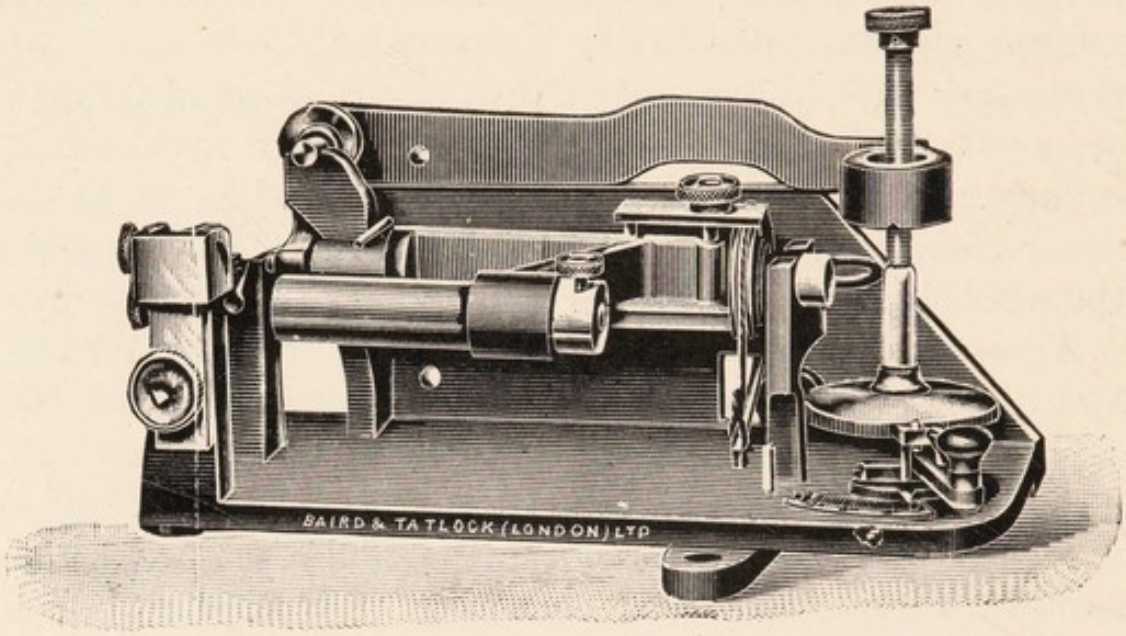


Fig 21.—Cambridge rocking microtome.

paraffin some form of microtome must be employed. An ether-freezing microtome *can* be made use of with some manipulation, the paraffin block being placed in a little melted paraffin on the freezing plate so that it is cemented there, and sections are cut with the razor or plane iron, as though it had been frozen (it is *not* to be frozen). It is better, however, to use some special form of microtome, the Cambridge "Rocker" (Fig. 21), or a modification of it, or the "Minot," being perhaps the best. The block of paraffin containing the tissue is trimmed with a knife to

remove the excess, and is cemented to the carrier of the microtome with a little melted paraffin, or by melting the paraffin on it with a hot iron (end of a file, etc.) or a match. The union may be made more secure by melting the paraffin around the base of the block with a hot iron.

Having fixed the paraffin block to the carrier, sections may then be cut of any degree of thinness. In order to do this it is essential for the knife or razor to have a keen edge and one of the right nature, for a knife may be perfectly sharp and yet the sections as they are cut may roll up in such a manner that it is difficult to flatten them. Though this may be due to a wrong consistence of the paraffin, owing to cold weather or some other factor, in the majority of instances it is the edge of the knife which is at fault. Provided the knife be sharp, stropping on the palm of the hand will usually remedy this difficulty. The paraffin being of the right consistence, and the knife in good order, the sections as they are cut should be flat and should adhere together at adjacent margins so that a ribbon of greater or shorter length is formed.

Satisfactory sections having been obtained, they are transferred with a needle or camel's-hair brush to a tin pan containing a little water, or spirit and water warmed to about 40° C. The sections float and the paraffin *softens* so that they spread out perfectly flat (the water must not be hot enough to *melt* the paraffin). A clean slide is then introduced underneath the section, raised so that the section is lifted up on it, and by fixing the section with a needle and tilting the slide the section is deposited in the required position on the slide and allowed to dry. If preferred, the section may be transferred to a slide flooded with water, which is warmed over the Bunsen. The slides can be manipulated in an hour or two if dried at 37° C., but it is best to allow them to dry in the incubator all night. It will be found after this treatment

that *thin* sections generally adhere sufficiently firmly to the slides for all the ordinary methods of staining to be carried out without detaching them ; thick sections, however, do not adhere nearly so well.

To prevent the risk of detachment, it is generally better to cement the sections to the slides by the following method. Equal parts of egg-white and glycerin are mixed and filtered through muslin, and to every 100 c.c. of the mixture 1 grm. of sodium salicylate is added. The slide is smeared thinly with this, the section is transferred to it and afterwards dried in the manner above described.

Supposing that the sections, in spite of all precautions, curl up as they are cut, it is still often possible to obtain a few that can be mounted. They may sometimes be unrolled by cautious manipulation with a couple of needles after having been softened by warming, or a needle or knife-blade may be held close to the edge of the microtome knife during cutting, so that curling is prevented.

Tissues embedded in paraffin may be kept indefinitely in labelled pill-boxes and cut all at once or from time to time as required, or the ribbons of sections may be preserved in a box in a cool place until wanted. The slides also, with the sections attached, can be kept until it is convenient to stain, if preserved free from dust in a slide box.

Cover-glass and Film Specimens

The satisfactory preparation of cover-class and film specimens is one of the most important in bacteriology, for they are used for the examination of cultivations of bacteria, and of blood or other fluids or secretions, organs, etc., for the presence of micro-organisms.

Films and smears are now usually made on the slide, but may be made on the cover-glass (" cover-glass specimens "). In either case the glass must be clean and free

from grease. Cover-glasses must be thin, otherwise the higher powers cannot be employed to examine the preparations, and those described as "No. 1" should be purchased, "$\frac{3}{4}$-in. squares" being a convenient size. These serve both for cover-glass specimens and for covering sections; it is well also to have a few of the same thickness but larger, viz. $\frac{7}{8}$-in. or 1-in. squares, for large sections. Slides and cover-glasses may be cleaned by boiling them in a porcelain dish with 10 per cent. carbonate of soda solution for a few minutes, well washing, and then treating with strong sulphuric acid, warmed carefully in a porcelain dish, for a few minutes. The acid having been poured off, they are well rinsed in several changes of water, and should be kept in a stoppered glass pot or capsule in absolute alcohol.

A clean slide (or cover-glass) is taken, dried with a clean soft linen or silk rag or handkerchief, or with Japanese paper, or it may be momentarily introduced into the Bunsen flame and the spirit burnt off, and placed flat on a convenient support on the work-table—a white glazed tile is excellent—with the end or corner projecting so that it can be conveniently picked up.[1] A droplet (*i.e. small* drop) of tap-water or of physiological salt solution (*not* distilled water) is then placed on it, in the middle, by means of a looped platinum needle, or with a small glass pipette (Fig. 7). Theoretically, physiological salt solution [2] sterilised by boiling should be used, but ordinary tap-water may generally be employed. A thin film of organisms has now to be formed on the glass, and the following is the method of procedure with a culture

[1] The writer has devised a useful support for staining. It consists of a square of plate glass, painted half white and half black at the back, and having a narrow strip of thick glass cemented across it on which the glass rests. It is made by Messrs. Baird and Tatlock.

[2] 0·75–0·95 per cent. of sodium chloride dissolved in distilled water

on a solid medium such as agar or gelatin. The culture tube and platinum needle are held and manipulated in precisely the same manner as that described for the inoculation of tubes (p. 78).

A mere trace of the growth from a culture should be taken, just sufficient to soil the tip of the straight platinum needle, or the preparation will be too crowded, and this is well rubbed up with the droplet of water on the glass, so as to form an emulsion, which is then spread over the surface. As a general rule the material should be well emulsified, but in some instances this is inadvisable, as a particular formation or characteristic grouping may be disturbed thereby, in which case, after a slight admixture with the water, the emulsion is gently spread. The thin moist film is allowed to dry, or may be dried by gentle warming over the Bunsen flame, preferably holding the preparation in the fingers and moving backwards and forwards over the flame. The film, when dry, must next be fixed, which is accomplished by passing the slide, film side up, six times through the Bunsen flame (a cover-glass is held in the forceps and passed three times through the flame). Films may also be fixed in alcohol and ether (p. 107). The object of this "fixing" is to thoroughly dry the film and coagulate albuminous material, whereby the film adheres better to the glass, and is not so likely to be detached in the subsequent processes of staining and washing, etc. Fixing may also tend to diminish the staining capacity of the extraneous matter mixed with the organisms. The preparations are now ready for staining.

When the culture is in a fluid medium, such as broth, the tube is manipulated in the same way, the deposit at the bottom having been shaken up if necessary, and a loopful or two of the fluid removed with a looped platinum needle, transferred to the glass, spread, dried, and

fixed as before, but as the medium is fluid there is usually no need to add any water.

If a specimen of blood, pus, or sputum is required, the procedure is much the same. A little of the material is taken up with a looped platinum needle and spread in a thin film over the slide or cover-glass, which is then dried and fixed, as described above. If necessary, a droplet of tap water or physiological salt solution may be used to dilute the material so as to obtain a thinner film. If a specimen is to be made from an organ, a particle of the pulp is picked up and an emulsion made as before, or a small piece of the organ may be held in sterile forceps and the cut surface gently smeared over the slide or cover-glass, which is then similarly dried and fixed ; these are termed " smear preparations."

To obtain the rest results it is preferable before staining to submit films of blood [1] or pus or smear preparations to the action of some chemical fixing agent, unless the film is stained with Leishman's solution, which both fixes and stains. The simplest method of doing this is to immerse the films, after *air*-drying, in a mixture of equal parts of absolute alcohol and ether for ten to thirty minutes. In hot countries a saturated aqueous solution of corrosive sublimate (five to fifteen minutes) is perhaps as satisfactory as anything. Another method, combining both fixing and staining, is to immerse the films as soon as they are prepared and without drying for a few minutes in the following solution :

Absolute alcohol, saturated with eosin .	25 c.c.
Pure ether	25 c.c.
Alcoholic solution of corrosive sublimate (2 grm. in 10 c.c.)	5 drops

The specimens are then removed with a forceps and

[1] For the method of preparing blood-films see the section on " Malaria," Chapter XVIII

well rinsed in water, stained for not more than a minute in a saturated aqueous solution of methylene blue, washed quickly, dehydrated in absolute alcohol, cleared in xylol, and mounted in xylol balsam. This solution may be used for fixing blood, pus, sputum, etc., if the eosin be omitted, and the preparations may then be stained or otherwise treated in any desired manner.[1]

Scott [2] recommends the following as giving the most perfect results with blood films, etc. :

(1) Hold the freshly prepared and still wet film in the mouth of a wide-mouthed bottle half filled with the ordinary formalin solution, film side downwards, for five seconds.

(2) Drop, *while still wet*, film downwards, into absolute alcohol. Leave for fifteen minutes, or, for convenience, for any time up to forty-eight hours.

The preparations may then be stained with methylene blue, hæmatoxylin and eosin, or with the Leishman or Giemsa stain. (See also under "Malaria," Chapter XVIII.)

Impression specimens.—These are employed to examine and preserve permanently the colonies or growths of organisms so that their characteristic formation may be observed. With plate cultivations this is very simple. A clean cover-glass is sterilised in the flame, and, having cooled, is cautiously lowered on to a selected surface colony with a sterile needle, avoiding all lateral movement. It is then gently pressed on to the colony and then carefully raised by means of a couple of needles ; the colony should adhere to the glass, and may be dried and fixed. The colonies in gelatin tube cultures may also be used if the gelatin is removed from the tube. This can be done by dipping the tube for a few seconds into hot

[1] Gulland, *Brit. Med. Journ.*, 1897, vol. i, p. 65.

[2] *Journ. Path. and Bact.*, vol. vii, No. 1, p. 131.

water, the gelatin round the walls of the tube will be melted, and the gelatin mass can then be tilted out of the tube on to a glass dish or tile.

Stains and Staining Methods

Micro-organisms being so minute and transparent, it is usual to stain or dye them, so that they can be more readily examined. In some instances organisms may have a peculiar staining reaction which may serve as an aid to their identification. But when an organism is being investigated, examination in the fresh and living condition must never be omitted, for it is only thus that its motility and life-history can be studied. Only general methods are detailed here; special ones will be given when they are required.

(1) Löffler's alkaline methylene blue:

Saturated alcoholic solution of methylene blue	30 c.c.
Solution of caustic potash, 0·01 per cent. .	100 c.c.

A very useful, though somewhat feeble, staining solution. Cultures should be quite fresh, or the organisms do not stain well. When the organisms are mixed with extraneous material, as in smears, or there is much *débris*, this is one of the best staining solutions to employ. Methylene blue preparations are, however, not very permanent, and in hot countries rapidly fade. Thionine blue is then preferable. (See also p. 110.)

Film specimens are stained for three to ten minutes, and sections half to twenty-four hours.

(2) Carbol-methylene blue (Kühne):

Methylene blue	1·5 grm.
Absolute alcohol	10 c.c.
Five per cent. aqueous solution of carbolic acid	100 c.c.

A more intense staining solution than the former, and very useful for sections, which are stained for from half to six hours.

(3) Anilin gentian violet:

Saturated alcoholic solution of gentian violet	30 c.c.
Anilin water	100 c.c.

The anilin water is prepared by shaking 3 c.c. of anilin with 90 c.c. of distilled water, allowing the mixture to stand for a few minutes, and filtering.

This solution is a useful general stain for films, which are stained for two or three minutes, and is employed in Gram's method of staining. It does not keep well.

Instead of anilin gentian violet, a carbol-gentian violet may be used, and keeps much better than the foregoing (saturated alcoholic solution of gentian violet, 1 part; 5 per cent. aqueous solution of carbolic acid, 10 parts).

For anilin gentian violet two stock solutions may be employed, and these seem to keep indefinitely, viz. :

No. 1

Gentian violet	2 grm.
Anilin	9 c.c.
Alcohol (95 per cent.)	33 c.c.

No. 2

Gentian violet	2 grm.
Distilled water	100 c.c.

For use, mix 1 c.c. of No. 1 with 9 c.c. of No. 2, and filter; this mixture will keep for about a fortnight.

(4) Carbol-fuchsin (Ziehl-Neelsen solution) :

Fuchsin	1 part
Absolute alcohol	10 parts
Five per cent. aqueous solution of carbolic acid	100 parts

The fuchsin is dissolved in the absolute alcohol and then mixed with the carbolic acid solution. It must always be filtered before use.

An intense staining solution. For films it is best diluted with five to ten parts of water; stain for two to five minutes.

(5) Carbol-thionine blue (Nicolle) :

Saturated solution of thionine blue in alcohol (90 per cent.)	10 c.c.
One per cent. aqueous solution of carbolic acid	100 c.c.

Sections can be stained in from a few minutes to half an hour. This solution may be used for a modified Gram's method (see p. 116). Can be substituted for methylene blue for all purposes, and is more permanent than the latter.

(6) Eosin (alcohol-soluble and water-soluble) :

A somewhat diffuse stain. Is used for counter-staining the tissues in Gram's method, and for staining red blood-corpuscles and acidophile granules in leucocytes.

A ½ to 1 per cent. aqueous or alcoholic solution may be used, and the staining should not, as a rule, be prolonged for more than about half a minute.

(7) Bismarck brown (Vesuvin) :

A saturated aqueous solution should be prepared and diluted somewhat for use. A good counter-stain for the tissues in Gram's method. Stain for two to five minutes.

(8) Orange-rubin :

Prepare saturated aqueous solutions of orange G. and rubin S. Mix equal volumes and dilute with water until of a light port-wine colour. Stain tissues for five to fifteen minutes. A good contrast stain for tuberculosis and actinomycosis.

(9) Picro-carmine :

This is best bought ready prepared. Sections are stained in the solution for half to one hour, washed, then placed in a watch-glass of spirit, to which three or four drops of hydrochloric acid have been added, for two or three minutes, then well washed in water. The section can now be counter-stained with Löffler's blue or by Gram's method.

(10) Hæmatoxylin :

Ehrlich's formula is one of the best and simplest to use, and can be obtained ready for use. It must be "ripe." It is a histological and not a bacterial stain. Sections are treated as follows :

(1) Distilled water, one to two minutes.

(2) Stain with the hæmatoxylin solution for five to thirty minutes. In some cases the solution is preferably diluted somewhat with distilled water.

(3) Rinse in distilled water.

(4) Rinse in distilled water containing a *trace* of acetic acid.

(5) Treat with distilled water containing a *trace* of ammonia. The sections remain in this until they assume a deep blue colour. (Tap-water, five to ten minutes, may also be used.)

(6) They can be dehydrated, cleared and mounted, or counter-

stained with eosin, orange-rubin, or Van-Gieson, and then mounted.

Hæmatoxylin makes a good contrast stain for the tubercle and the leprosy bacillus and for *Actinomyces*.

Mayer's hæmalum (see section on the "*Entamœba coli*") and Delafield's hæmatoxylin are also good hæmatoxylin stains.

(11) Ehrlich-Biondi triple stain :

This is best bought ready for use. It is a good histological stain for tissues and blood films, and actinomycosis stains well by it.

Stain for ten to sixty minutes, then treat with methylated spirit until the section becomes greenish. Pass through absolute alcohol, clear, and mount.

(12) Leishman's stain :

Like the Jenner, Wright, and other similar ones, a modification of the Romanowsky stain, a double compound of eosin and methylene blue. The solution will keep for some time, but is best freshly prepared. Grübler's powder or Burroughs Wellcome's soloid may be used, and is dissolved in *pure* methyl alcohol. Failure frequently proceeds from the use of a so-called pure methyl alcohol, which is not really so. (For method of using, see "Malaria," Chapter XVIII.)

(13) Giemsa stain :

An eosin-azur mixture dissolved in pure glycerin and methyl alcohol. Useful for blood-films, smears, etc., and has been much used to demonstrate the spirochaetes in syphilitic material. (For method of using, see "Syphilis" and "Malaria.")

Safranin and acid fuchsin are also used as counter-stains. Malachite green, neutral red, and rosein may be used for *intra-vitam* staining of protozoa, etc.

Eosin, orange-rubin, hæmatoxylin, and picro-carmine keep well in solution ; the remainder may or may not, and are best used fairly fresh. All stains should be filtered before use, and may be conveniently kept in bottles having a funnel fitted with a filter-paper, so that they are always ready. Or smaller bottles may be used, fitted with pipettes, and several arranged in a stand.

Methylene-blue, Leishman and Giemsa preparations are more permanent if kept unmounted. After examination with the oil-

immersion, the oil may be removed from the film with xylol. Coles mounts these preparations in parolein.

The best stains were Grübler's, but are now almost unobtainable. Messrs. Burroughs, Wellcome and Co. supply most of the anilin dyes and some other reagents, iodine, etc., in "soloids," which are very convenient and good.

Gram's method.—This is a most useful method, especially for sections, specimens of blood, or films or impression preparations, as the tissue or ground substance can be counter-stained so that the organisms show up in marked contrast. Ordinary films of cultures do not usually require this method, unless *débris* or ground substance is present and the best result is desired. Unfortunately Gram's method is not applicable for all organisms, as many do not retain their colour by the process. This disadvantage, however, is counterbalanced by the fact that it forms a valuable means of distinguishing organisms, and is always one of the characters to be ascertained in bacteriological diagnosis. Most of the moulds, yeast, and streptothrix forms, and cocci stain by it, though there are exceptions; the spirilla and protozoa do not stain by it, but as regards the bacilli no rule can be laid down (see p. 115). Old and degenerate organisms stain indifferently, and some organisms in tissue or exudate may be Gram-positive, while in culture they are mostly Gram-negative. Films are stained for five to ten minutes, and sections for ten minutes to half an hour, in anilin- or carbol-gentian violet solution. The superfluous stain is then drained or blotted off, *not* washed away, the specimen is rinsed with Gram's iodine solution and is treated with fresh iodine solution for from one-half to two minutes.

GRAM'S IODINE SOLUTION

Iodine	1 part
Potassium iodide	2 parts
Distilled water	300 parts

The purple colour of the gentian violet changes to a dirty yellowish brown, and sections become much like a used tea-leaf. The specimens must not be passed on to the next solution until they have assumed the brown colour. Cover-glass specimens are best *immersed* in the solution in a watch-glass, *film side up*.

The specimens are removed from the iodine solution, drained, and then immersed in alcohol, preferably methylated spirit. In this the purple colour of the gentian violet returns and is dissolved out, so that they ultimately become colourless ; this is aided by moving them gently about, and for sections two or more baths of alcohol may be an advantage, a fresh one being substituted when the first has become deeply coloured. Films decolorise much more readily than sections, and they should be removed from the alcohol when no more colour dissolves out, or the stain may be entirely removed ; usually twenty to forty seconds in the alcohol suffices, thick preparations taking longer than thin ones. After decolorising, films are washed in water, dried, and mounted, or, after washing, the ground substance may be counter-stained, if required, with eosin for a few seconds, or Bismarck brown for two or three minutes, washed again in water, dried, and mounted. With films it is important to remember on which side of the glass the film is, for it may be very difficult to ascertain this after decolorisation. Sections after decolorising are passed through absolute alcohol and xylol before mounting, or, if required to be counter-stained, are immersed in eosin for fifteen to thirty seconds, or Bismarck brown for three to five minutes, and then passed through alcohol, absolute alcohol, and xylol.

Sections frequently are somewhat difficult to decolorise with alcohol alone, in which case it is well to treat them with a slightly acid alcohol (3 per cent. of hydrochloric

acid) for a few seconds, and then return to the alcohol (Günther's method).

The iodine in Gram's method seems to act as a mordant, precipitating the stain in a relatively insoluble form in certain species of bacteria. The staining of organisms by Gram is relative ; some forms do not stain at all, are *Gram-negative—i.e.* the colour is removed by the alcohol with the greatest facility ; others stain intensely, are *Gram-positive*, but even these may become decolorised by prolonged treatment with alcohol. In order to ascertain whether an organism is or is not stained by Gram's method, it is sometimes useful to mix with it in making the preparation some undoubted Gram-staining organism—*e.g.* if a bacillus, the *Micrococcus pyogenes* ; if a coccus, *B. anthracis* or *B. subtilis*. The admixed organism then serves as an index.

The following organisms are Gram-positive : *B. anthracis*, *B. diphtheriæ*, *B. tetani*, *B. botulinus*, *B. tuberculosis*, *B. smegmatis*, *B. lepræ*, *B. murisepticus*, *Actinomyces*, *B. subtilis*, *B. mesentericus*, *B. megaterium*, *B. mycoides*, the pyogenic cocci, the streptococci, including the pneumococcus, most cocci, yeasts, moulds and streptothrices. *B. perfringens*, *B. œdematis maligni*, and *B. Chauvœi*, are usually Gram-positive in the tissues, but tend to become Gram-negative under cultivation.

The following organisms are Gram-negative : *B. typhosus*, *B. enteritidis*, *B. dysenteriæ*, *B. coli*, *B. pestis*, *B. influenzæ*, *B. mallei*, *B . pseudo-tuberculosis*, *B. pyocyaneus*, *B. prodigiosus*, *B. proteus*, the septicæmic bacilli, such as chicken cholera, the spirilla and vibrios, spirochaetes and protozoa, *M. gonorrhœæ*, *M. meningitidis*, *M. melitensis*, and *M. catarrhalis*.

Gram's method of staining depends upon the formation of an iodine-pararosanilin-protein compound which is not readily dissociable in the case of the Gram-positive organisms. Pararosanilin dyes, such as gentian violet, methyl violet and victoria and thionine blues, are alone suitable for the method.

In Claudius's modification of Gram's method,[1] staining

[1] *Ann. de l'Inst. Pasteur*, xi, 1897, p. 332.

is done in a 1 per cent. aqueous solution of methyl violet (films for one minute, sections for two minutes). The preparations are washed, treated with a half-saturated aqueous solution of picric acid for one to two minutes, washed again, and dried with filter-paper. Decolorisation is then carried out in the case of films with chloroform, in that of sections with clove oil. After decolorising, the preparations are treated with xylol and mounted. By this method the ordinary Gram-positive organisms are stained. Counter-staining may be carried out with lithium carmine.

Weigert's modification of Gram's method.—In this process the sections, whether frozen or paraffin ones, should be manipulated *on the slide.* They are stained with the anilin gentian violet and treated with Weigert's iodine solution (iodine 4–5 per cent., potassium iodide 6 per cent.) as in the simple Gram's method. The iodine is then removed with filter-paper and the sections are flooded with anilin oil two or three times. This removes the colour and dehydrates. The anilin oil is removed by flooding two or three times with xylol.

Thionine blue may be used for Gram's method, the carbol solution being employed (No. 5, p. 110). Sections are stained for two or three minutes, then treated with an iodine solution somewhat stronger than Gram's (200 parts of water instead of 300 parts). The sections, after remaining in this for one to two minutes, are decolorised in alcohol containing 1 per cent. of acetone (methylated spirit does very well), and subsequently treated as in Gram's method.

The Staining of Film Specimens

To stain films, smear, and impression preparations, the film is flooded after fixing with a drop or two of the solution, or the preparation, if a cover-glass, may be

floated, film side down, on the solution contained in a watch-glass ; if it should sink it makes little difference. Various baths or pots can be obtained for staining slides. Warming intensifies the staining properties of all staining solutions, and may be necessary if deep staining is required or if the temperature of the laboratory be low (see also p. 121). When stained sufficiently, the preparation is rinsed in a beaker or tumbler of water, or in a fine stream of water, preferably distilled, to remove the superfluous colour, after which it is dried and mounted in a drop of solution of Canada balsam in xylol. The preparation may be dried either by *gentle* warming over the Bunsen flame after the film has been blotted with filter paper, or the film may be allowed to dry spontaneously in the air, in which case it should always be set up on edge to drain. *The preparations must be completely dried before being mounted in balsam.*

To prevent the stain flowing all over a slide, two thick lines may be made across the slide with a grease pencil or with a stiff grease made by melting together vaseline and paraffin wax, one on either side of the area to be stained.

If there be much *débris* or other material which, when stained, would interfere with a clear view of the organisms, various expedients may be adopted. One is to stain for a short time with a solution which does not give a very dense colour, the best for this purpose being Löffler's methylene blue, or Gram's method may be made use of if the organism stains by it, and will give the best result of any. Another plan is to treat the specimen with acetic acid before staining ; it may be just dipped in glacial acetic acid and immediately washed in distilled water, or immersed in 20 per cent. acetic acid for five to ten minutes, washed in distilled water, and then stained. A third is, after staining and washing, to rinse the pre-

paration in dilute alcohol (alcohol 1 part, water 1 or 2 parts), and immediately to wash again in water to stop the further action of the alcohol. If the film be thick, two or three rinses in the dilute alcohol may be necessary. This process gives excellent results with the sarcinæ, but the staining agent should be anilin gentian violet or dilute carbol-fuchsin and not Löffler's blue, unless it is allowed to act for fifteen to twenty minutes. The treatment with acetic acid before staining may be combined with decolorisation with alcohol after.

Preparations can always be examined in water with the $\frac{1}{6}$-in. objective, after washing and before permanently mounting, in order to see whether they are satisfactory. If the film is on a slide, a drop of water is put on and covered with a cover-glass, if on a cover-glass, this is mounted in a drop of water on a slide. If satisfactory, the preparation can be dried, and mounted in balsam; or if not stained sufficiently, or if stained too deeply, it can be stained again, or further decolorised, as the case may be.

Another process for demonstrating the presence of organisms in films is by the method of "relief staining" in which the organisms are left unstained on a coloured background. This may be done by the Indian-ink method (see "Syphilis") or by Benian's Congo method.[1] For this a small drop of a 2 per cent. solution of Congo red in distilled water is placed on a slide and a very small quantity of the bacterial culture, or of exudate, is rubbed up with it with a platinum wire; the drop is then spread out into a tolerably thick film, either with the wire or with another slide. The film is allowed to dry in the air and is then washed with a 1 per cent. solution of hydrochloric acid in absolute alcohol and dried in the air; preferably *not* blotted. The background is an opaque blood red and the organisms appear white. Broth and

[1] *Brit. Med. Journ.*, 1916, vol. ii, p. 722.

saline solutions do not form a satisfactory mixture with the Congo red and the organisms should be centrifuged out of them. Serous exudates mix readily and evenly. Care must be taken that the distilled water used in making the Congo red solution contains no organisms.

Treatment of Sections for Staining and Mounting

(*a*) *Frozen sections.*—If preserved in spirit they should be rinsed in distilled water or in fresh alcohol before staining, according as the staining solution is an aqueous or an alcoholic one. After staining they are well rinsed in water or alcohol to remove the excess of stain, and are then dehydrated and cleared before being mounted. For dehydrating, if they have been washed in water, they should be well rinsed in methylated spirit [1] to remove the excess of water, and then transferred to absolute alcohol for a few seconds to two minutes, the time varying with the size and thickness of the section. In many cases—for instance, when the anilin dyes have been used for staining—the sections must be passed as rapidly as possible, *consistent with thorough dehydration*, through the absolute alcohol to avoid removing too much of the colour. If it is important to avoid any decolorisation, anilin oil may be used for dehydration, as in Weigert's method (pp. 116 and 122). For clearing, xylol or cedar oil is the best agent, for neither dissolves the anilin dyes; they will only clear, however, out of absolute alcohol, hence the preliminary rinsing of water-washed sections with methylated spirit to prevent dilution of the subsequent bath of absolute alcohol. Oil of cloves can also be employed, but has the disadvantage that it

[1] Absolute alcohol may of course be employed, instead of the first bath of methylated (or rectified) spirit, but methylated answers just as well and is less expensive (but see note, p. 97).

dissolves the anilin dyes, and the colour of stained sections treated with it is apt to be less permanent; it has the advantage, however, of clearing out of methylated spirit, absolute alcohol being unnecessary. The alcohol and clearing agents are conveniently placed in watch-glasses or small shallow glass capsules. The section is known to be cleared when it appears quite transparent and almost invisible when the watch-glass or capsule containing it is held over a dark surface. If after two minutes in the clearing agent the section still appears cloudy and opaque, it has not been sufficiently dehydrated, and should be returned to a fresh bath of absolute alcohol for a short time, and then transferred again to the clearing agent. Care should be taken that watch-glasses, etc., used for the absolute alcohol and clearing agent are perfectly dry. The clearing agent, especially clove oil, can be used many times before becoming useless.

For transferring the sections from one solution to another an ordinary needle, fixed in a light wooden handle, suffices, or, better still, a piece of glass drawn out at one end, the section being carefully lifted by one corner to prevent crumpling; but for the final process of mounting it is necessary to use a section lifter or cigarette paper. The section, spread out with care, is raised by means of the section lifter or cigarette-paper introduced under it, and transferred to the slide, any crinkles are removed by spreading with a needle, the superfluous clearing agent is drained off, a drop of xylol balsam put on, and it is then covered with a clean cover-glass. If clove oil has been used as the clearing agent, the sections after draining, should be blotted with two or three thicknesses of filter-paper to remove as much oil as possible before putting on the balsam. In blotting firm pressure should be used, and the section will then adhere to the glass slide and not to the blotting-paper. With

delicate sections all the processes of staining, dehydrating, clearing, etc., may be carried out on the slide.

(*b*) *Paraffin sections.*—The section fixed on the slide (p. 104) must be freed from paraffin before staining and mounting. The slides with attached sections are treated as follows : Immerse in (1) xylol for one or two minutes, drain ; (2) absolute alcohol one to two minutes to remove the xylol, drain ; (3) distilled water. They are now ready for staining, and are to be flooded with the staining solution or immersed in it, and after staining they are treated in the same manner, but in the reverse order, viz. (1) distilled water ; (2) methylated spirit ; (3) absolute alcohol ; (4) xylol. On being removed from the xylol the slide is drained for a few seconds, a drop of xylol balsam is then put on, and the section covered with a clean cover-glass. Glass pots (Fig. 22) filled with the alcohol, xylol, etc., are convenient for the treatment of paraffin sections, the slide with the section upon it being immersed in the fluid.

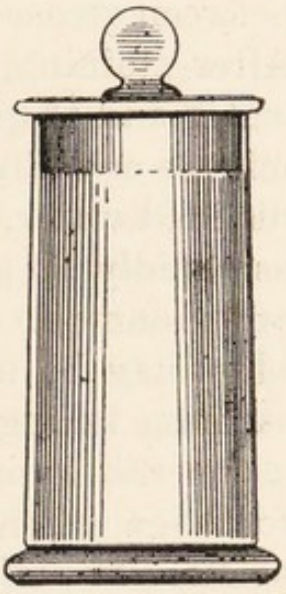


FIG. 22.—Glass pot for clearing, etc.

Section Staining

When Gram's method is applicable it gives by far the best results, and should always be employed. If, however, the organisms are decolorised in Gram's process some other method must be adopted. One of the best is to stain for from ten minutes to six or eight hours in Löffler's methylene blue. Fresh easily staining organisms will be sufficiently stained in ten or fifteen minutes, but when the organism is difficult to stain, as glanders, six to eight hours may not be too long a time. Warming intensifies the staining properties of all staining solutions ;

for frozen sections the watch-glass of stain may be warmed on a sand-bath or asbestos cardboard, or in the blood-heat incubator. Sections on the slide may be flooded with the stain and warmed on a piece of asbestos cardboard placed over a Bunsen flame, or a penny may be heated in the Bunsen and the preparation laid on it, the coin being re-heated as often as required. The stain may be prevented from flooding the slide by confining it between grease-pencil lines as described for films (p. 117). After staining, the sections are well rinsed in distilled water and then slightly decolorised by rinsing for half a minute or so in a watch-glass of 1 per cent. acetic acid in distilled water. They are then again washed and passed as rapidly as possible through alcohol, cleared in xylol, and mounted. Carbol-methylene blue or carbol-thionine blue may be used instead of the Löffler's solution, the staining taking from a few minutes to half an hour. If a contrast stain be desired the sections may be treated for a few seconds with the eosin solution after the dilute acetic. If staining be prolonged evaporation must be prevented. In the case of a section mounted on the slide and flooded with stain, the slide should be placed on a piece of wet blotting-paper on a tile and covered with the lid of a Petri dish.

The micro-organisms in sections stained with Löffler's blue are very liable to become decolorised unless the dehydration is expeditiously performed. To avoid this Unna's method may be adopted. After staining and decolorising with acidulated water as described, the sections are placed on the slide (if not already mounted thereon), gently warmed, and so dried; they are then treated with xylol and mounted in balsam. The tissue elements, however, are apt to suffer.

A better method is, after decolorising with the dilute acid, to dehydrate with anilin instead of with alcohol,

the section being treated with fresh anilin two or three times, then with a mixture of equal parts of anilin and xylol, and finally with two or three baths of xylol.

Capsule Staining

Many organisms, especially in the tissues or body fluids, are invested with a capsule of gelatinous matter, probably derived from the membrane of the bacterial cell, and differing in composition in different species. The capsule may be as thick as the bacterial cell itself, and appears, in the unstained state or after staining by the ordinary methods, as a clear halo or zone surrounding the organism. Organisms in films of albuminous matter often appear to be surrounded by a clear halo, which must not be mistaken for a capsule. As organisms frequently lose their capsules on ordinary culture media, Moor recommends cultivating in fluid serum to obtain the redevelopment of the capsule. In order to stain the capsule one of the following methods may be adopted.

1. Stain the preparations by just dipping in the following solution :

Carbol-fuchsin	1 part
Distilled water	1 part

Rinse in water, and then stain for fifteen seconds in a very weak aqueous solution of gentian violet (0·1 per cent.). Rinse in water, dry, and mount.

2. *McConkey's method.*—The following solution is prepared :

Methyl green	1·5 grm.
Dahlia	0·5 grm.
Distilled water	100 c.c.

When dissolved, 10 c.c. of a saturated alcoholic solution of fuchsin are added, and the whole is made up to 200 c.c. with distilled water. The stain should not be used for a fortnight, and should be kept in a dark place. Specimens are stained for five minutes or longer, then thoroughly washed in a stream of water, dried, and mounted.

3. *Friedländer's method* (for tissues).—Mix :

Concentrated alcoholic solution of gentian violet	50 parts
Distilled water	100 parts
Acetic acid	10 parts

Stain the sections in this solution in the warm incubator for twenty-four hours. Rinse well in 1 per cent. acetic acid, pass through alcohol and xylol, and mount in balsam.

Spore Staining

When spore-bearing bacteria are stained by the ordinary methods the spores are just tinted, or remain uncoloured with the outlines more or less stained. This seems to be due to the fact that the spores are surrounded with a slightly permeable membrane which inhibits the entrance of the staining agent. By staining by some method which causes the penetration of the stain, and then cautiously decolorising, it is possible to remove the colour from everything except the spores, the impermeable membrane of which in the same way prevents the full action of the decolorising agent.

(*a*) *Simple method.*—A film is prepared in the ordinary way. If a cover-glass, it is floated on a watch-glass of, or, if a slide, it is flooded with, carbol-fuchsin, and the stain is warmed for twenty minutes. After being washed in water the preparation is rinsed for a second or two in 1 per cent. sulphuric acid and again washed at once in water. If there is still a good deal of the red colour remaining, the film may be once more rinsed in the acid, but if nearly colourless it should be mounted in water and examined with the $\frac{1}{6}$-in. objective. If the spores alone are well stained the preparation may be counter-stained with Löffler's methylene blue for two to five minutes, washed, dried, and mounted. If, however, the bacilli as well as the spores retain the red colour, the preparation must be further decolorised in the acid, while if everything has been decolorised, it may be re-stained with warm carbol-fuchsin.

The spores sometimes stain better if the preparation be fixed by passing through the flame *twelve* times instead of three, as is

usual. To obtain good preparations and ones showing the spores *in situ*, the specimens should be made as soon as spores have definitely developed in the cultures.

Spore staining often requires a good deal of patience, and in many instances it is difficult to obtain a satisfactory preparation by this simple method, in which case that of Moeller should be made use of, and rarely fails.

(*b*) *Moeller's method.*—Prepare the cover-glass or slide specimen in the ordinary way. Treat with absolute alcohol for two minutes, and then with chloroform for two minutes. Wash in water and treat with a 5 per cent. solution of chromic acid for two minutes, wash, and then stain with warm carbol-fuchsin for ten minutes. Wash, decolorise carefully in 1 per cent. sulphuric acid, again wash and counter-stain with Löffler's methylene blue for one minute ; wash, dry, and mount. Some organisms, such as the *B. mesentericus*, stain better if treated with the chromic acid for five to ten minutes.

Flagella Staining

Many organisms possess delicate protoplasmic processes—flagella—in greater or less number ; but these are not visible when the organism is examined in the living condition (except by the use of dark-ground illumination), nor when the ordinary staining methods are employed. In order to demonstrate them it is necessary to make use of some special method, in which a mordant is essential. One of the earliest devised was that of Löffler, which with care gave fair results. It is not, however, nearly so satisfactory as some more recent ones, so is omitted.

For all methods of flagella staining the cover-glasses or slides must be absolutely clean, the cultures recent, and the growth sufficiently diluted to obtain the organisms in an isolated condition.

(*a*) *Stephen's method.*—This is a modification of the well-known Van Ermengem method,[1] and has been communicated to the writer by Dr. J. W. W. Stephens.

[1] *Centr. f. Bakt.*, xv, 1894, p. 969.

To clean slides.—Rub the slides with a clean cloth and place on a piece of *clean* wire gauze and heat with a *smokeless* flame for some minutes (by this means grease is completely removed). Remove the slides when cool, not before.

To make the suspension.—All methods are unsatisfactory. Rub a little of the culture in a small drop of tap-water in a watch-glass. Then transfer a drop with the smallest possible platinum loop to a minute drop of water on the slide. Mix and spread with the platinum wire as quickly as possible. The film thus made should dry *immediately* if a small drop only of water has been used.

Age of the culture.—A twenty-four hours' culture does quite well (a younger one is perhaps better, but flagella can be shown for a week or fortnight or more).

I.	*The mordant :*	
	Osmic acid, 2 per cent. . . .	1 part
	Tannic acid, 20 per cent. watery solution	3 or 4 parts
II.	*Silver solution :* Silver nitrate . .	0·1 per cent.
III.	Gallic acid, 2 per cent. solution . .	1 part
	Ammonia fort.	1 part

These solutions should be freshly prepared and mixed immediately before use.

To stain.—Place the mordant on the film for one or two minutes or less (time unimportant).

1. Wash in tap-water thoroughly.
2. Shake off as much water as possible.
3. Place a few drops of silver nitrate on the slide for a few seconds or longer.
4. Shake off all excess.
5. Allow one drop of the ammonia-gallic solution to fall on the *middle* of the slide from a small pipette. A wave spreads away from the centre to each end of the slide. As soon as the film is seen standing out clearly and black in the centre (in a few seconds) wash off in tap-water.
6. Add again a drop or two of the silver solution and allow it to act for half a minute or thereabouts.
7. Wash in tap-water, blot, and dry over the flame.
8. It is best not to mount in balsam or in cedar-wood oil, as the preparations rapidly fade in these.

If done with any care, the film should now appear black and distinct to the naked eye with no precipitate, and the flagella will be found to be stained distinctly and intensely with hardly any

ground substance, or at least insufficient to interfere with a clear view of them.

(*b*) *Pitfield's method.*—Two solutions are freshly prepared :

A.	Saturated aqueous solution of alum . .	10 c.c.
	Saturated alcoholic solution of gentian violet	1 c.c.
B.	Tannic acid	1 grm.
	Distilled water	10 c.c.

The solutions should be made with cold water, filtered, and preserved in separate bottles. For use equal quantities are mixed together. The specimens are flooded with the mixture and held over the flame until it nearly boils ; they are then laid aside, with the hot stain on them, for one minute, and are finally washed in water. After washing, the preparations are flooded with anilin gentian violet for one second, washed in water, dried and mounted.

(*c*) *McCrorie's method* [1] (*modified by Morton* [2]).—Prepare the following solutions :

A.	Tannic acid	1 grm.
	Potash alum	1 grm.
	Distilled water	40 c.c
B.	"Night" blue	0·5 grm.
	Absolute alcohol	20 c.c.

Mix and filter.

The prepared slides are stained with this solution (which should always be filtered before use) for two minutes, the solution being changed two or three times, washed gently in running water, and then counter-stained in anilin gentian violet for one to two minutes, washed, dried, and mounted.

Preservation of Cultures

Gelatin and agar cultures may be satisfactorily preserved by submitting them to the action of formaldehyde vapour for some hours by soaking the wool plug of the culture tube in formalin and plugging the tube with it. The tube may then be sealed with gutta-percha tissue, sealing-wax, or paraffin wax, or best of all in the blowpipe flame. Plate cultivations may also be exposed to the vapour and the lid of the dish afterwards cemented on, or the cultures may be made in the flat bottles ("Soyka's bottles")

[1] *Brit. Med. Journ.*, 1897, vol. i, p. 971.

[2] *Trans. Jenner Inst. Prev. Med.*, vol. ii, p. 242.

devised for the purpose, and after development treated like tube cultures.

Preservation of Pathological Specimens

These may be preserved in the ordinary way in spirit, but a much better method, by which the natural colour of the specimen is retained, is the following. The specimens are first washed in water, and then placed in the following solution for twenty-four to forty-eight hours :

Formalin	6 parts
Sodium chloride	1 part
Sodium sulphate	2 parts
Magnesium sulphate	100 parts

After being taken from the formalin solution the specimens are placed in methylated spirit for ten minutes, and then in a fresh bath of methylated ; in this the colour to a large extent returns, and they should be carefully watched and not allowed to remain in it for more than an hour. They are then mounted in the following mixture :

Glycerine	400 c.c.
Potassium acetate	200 grm.
Water	2000 c.c.

A trace of formalin should be added to this.

Delépine describes an "arsenious acid jelly" method for preserving pathological specimens (*Journ. Pathol. and Bacter.*, vol. xviii., 1914, p. 345).

The writer has preserved meat infected with *B. prodigiosus* very satisfactorily by the following method. Slices were cut off and placed in the formalin solution given above for a few hours. They were then well drained and placed in suitable glass capsules. Ordinary nutrient gelatin was melted and sufficient poured in to cover the specimens, and when it had set a little formalin was poured on and allowed to remain for a few days. It was then poured off and the glass top cemented down.

For further information on preparation of tissues, section cutting, staining methods, etc., see *The Microtomist's Vade Mecum*, Bolles-Lee ; *Practical Histology*, Schäfer ; *Methods of Morbid Histology and Clinical Pathology*, Walker Hall and Herxheimer ; and *Lehrbuch der Mikroskopischen Technik*, Rawitz.

CHAPTER IV

METHODS OF INVESTIGATING MICROBIAL DISEASES—THE INOCULATION AND DISSECTION OF ANIMALS—HANGING-DROP CULTIVATION—INTERLAMELLAR FILMS—THE MICROSCOPE

THE systematic study of a condition dependent on the activity of micro-organisms is in many instances no light matter. When only one or two forms are present and these are readily cultivated it may be comparatively easy, but when there are many the investigation may become exceedingly complicated. The first step to be taken is to ascertain by careful microscopical examination whether micro-organisms be present, and if so, their general characters, and their distribution, both in fresh unstained and in stained preparations, and if possible at different stages of the disease. In disease conditions, for example, the blood and secretions may be examined both before and after death, but in the latter it must be remembered that soon after the fatal event adventitious organisms rapidly make their appearance, gaining access from the air and from the intestinal tract. If organisms be detected an attempt should be made to determine whether there is any predominant form and if this is constantly present at different stages. If organisms are found, it simplifies matters, but if not, it cannot therefore be said that they are absent, for they may be relatively few in number or be in a degenerate condition,

and consequently be missed in a microscopical examination ; or they may be confined to a particular locality or tissue, or be present only at one stage of the infection. In addition to the microscopical examination, cultures must be made aërobically and anaërobically on various media, those media being chosen which will probably be suitable for the growth of the organism present in the particular condition (see p. 94). In addition, it will in most cases be advisable, and in all safer, in order to isolate the various species, to make plate cultivations, either in Petri dishes, etc. (p. 88), or by streaking several sloped tubes of agar, etc. (p. 91). Having obtained pure cultivations it will be necessary to determine the species of organism,[1] if it has been previously isolated and described, or to give a careful description of it, if it be a new one, for the use of subsequent investigators. In the identification or description of an organism all the following features should be carefully noted :

1. The morphology of the organism under various conditions, its size, form, and motility, the presence of flagella, and their number, arrangement, and character.

2. The presence or absence of spore formation, its nature, the conditions under which it occurs, and any peculiarities in the germination of the spores, and their size and location in the cell.

3. The peculiarities of staining, and the staining reaction with Gram's and the Ziehl-Neelsen methods.

4. The characters of the colonies in gelatin, agar, and other media, both surface and deep.

5. The characters of the growth on a variety of culture media at different temperatures—for example, for a pathogenic organ-

[1] The descriptions of a large number of species of bacteria have been collected and tabulated in convenient form by Chester in *A Manual of Determinative Bacteriology* (Macmillan and Co., 1901). The terms he suggests for describing bacterial growths, etc., might well be adopted by bacteriologists. A committee of the Society of American Bacteriologists has drawn up an elaborate chart for the description of species of organisms.

ism, on blood-serum, agar, and gelatin (surface and stab cultures), in broth and on potato ; liquefaction or not of the gelatin ; the growth in milk, with or without curdling, and the reaction therein ; and the fermentation reactions on carbohydrates, glucosides, alcohols, etc. ; the nature of the gas, if any, formed therefrom, and the H : CO_2 ratio.

6. The behaviour towards oxygen—is it aërobic or anaërobic ?

7. The range of growth at different temperatures.

8. The reducing power by growing in litmus broth which becomes decolorised, or by the formation of nitrites in a solution containing nitrates.

9. The production of indole with or without nitrites.

10. The production of pigment and the conditions under which it occurs.

11. The pathogenic action on various animals if it be a disease germ, or the changes which it produces if it be an organism connected with other conditions.

12. The chemical changes which it induces.

13. The thermal death-point and the action of germicides and antiseptics upon it (see Chapter XXII).

For descriptive purposes, "standard" culture media should always be employed, and the acidity or alkalinity of the medium stated (p. 61).

It must never be forgotten that under cultivation the properties of organisms may be considerably modified, and due allowance must be made for this. For example, pathogenic organisms may lose their virulence more or less completely, pigment production be lost, and fermentive action modified (see also p. 6).

To obviate these difficulties the organisms should be cultivated under as nearly natural conditions as possible and sub-cultivation avoided so far as can be. No general rule can be given as to the duration of life of cultures on artificial media. Most organisms will retain their vitality for at least three or four weeks without being transferred to a fresh soil, some for many months ; a few must be sub-cultured every week, or even every three to four days, or they will die out ; while there are still a small number

which have so far rarely or never been cultivated. On the whole, organisms retain their vitality best on gelatin.

For an organism to retain its virulence it is, as a rule, necessary to pass it through a susceptible animal at longer or shorter intervals, and to enhance the virulence recourse must be had to a succession of passages through susceptible and then less susceptible animals. In this way the virulence of organisms has been increased to a point far greater than is ever met with naturally, as in the case of the *Streptococcus pyogenes*. If an organism retains its virulence even slightly, it is generally possible, by employing large doses, to enhance this by passage through a susceptible animal. Another method may also be adopted, namely, to inject along with it some other pathogenic form, such as the *Streptococcus pyogenes* ; the combination will kill the animal, and the slightly virulent organism can be recovered and will be found to have increased in virulence. A third method is to inject the organism into a susceptible animal together with a lethal dose of toxin obtained from a virulent form of the same species, or with some substance, such as lactic acid, which lowers the vitality of the tissues. The slightly virulent organism will then be able to grow under the more favourable conditions, and a form which has become completely non-virulent can be made to regain its lost virulence.

Collodion sacks are frequently used to study the action upon animals of the dialysable products produced by micro-organisms which do not form any appreciable amount of toxin *in vitro*, for cultivating species which are difficult to grow by ordinary methods, for studying the phenomena of infection when the micro-organisms are protected from the phagocytes, and for other purposes. A glass rod or small test-tube, according to the size desired, is dipped into a beaker containing the ordinary

(*not* flexible) collodion, is then withdrawn and allowed to dry, and the process is repeated two or three times. In order to detach the collodion from the glass, the whole is dipped for a few seconds alternately into strong spirit and into water, the collodion loosens, and may be easily peeled off the glass. The sack may be sterilised by placing in a test-tube and heating to 150° C. in the hot-air steriliser.

For the inoculation of animals various methods may be adopted. Thus, after clipping the hair, the organism may be introduced by rubbing into the skin after scarification, or, a small incision having been made through the skin, a small quantity of a culture may be introduced on a platinum needle; or a broth culture or an emulsion, made with sterilised water or broth, may be injected with a sterilised syringe subcutaneously, intravenously, intraperitoneally, or into the muscular or other tissues or organs as required, since the seat of inoculation may have to be varied for the different species to produce their pathogenic effect. For injection purposes a syringe like an antitoxin syringe, *i.e.* with asbestos or metal piston and glass barrel that can be boiled, may be used. Several sizes, 1 c.c., 2 c.c., and 5 c.c. at least, are required. An all-glass syringe is a still better form, but is expensive. For accurate dosage, the piston-rod should be graduated and have a nut travelling on a screw up and down it. Before use the syringe with the needle should be boiled for ten minutes to sterilise it; after use it may be well rinsed and again boiled. The needles should be wiped dry and a wire inserted, or they may be kept in a bottle of xylol.

Guinea-pigs and rabbits are usually inoculated in the thigh or abdomen; mice in the dorsal region or at the root of the tail (dorsally), the hair being clipped, and the skin disinfected, but this is not generally necessary.

Numerous mechanical holders have been devised for animals, but are not as a rule required. Rabbits may be inoculated intra-venously by one of the large veins in the ear. The ear is shaved, and the skin is well washed with a little alcohol with vigorous rubbing; the base of the ear is lightly pinched so as to obstruct the venous but not the arterial circulation, and render the vein prominent, and the injection is made with a small syringe fitted with a fine needle, the needle being passed into the vein towards the base of the ear. After the withdrawal of the needle the wound is compressed for a little and may be dressed with some antiseptic wool and collodion.

Guinea-pigs frequently eat the carcases of their dead companions, so that the cages should be examined twice daily, and, if the carcase is required, it may be advisable to keep each animal in a separate cage.

The phenomena occurring after inoculation must be noted. Usually these are not very obvious in the rodents, but loss of appetite, sluggishness, staring coat, convulsions, etc., may be observed. The weight of the animal is a good index of what is happening. If the infection is serious, the weight rapidly falls; if the animal is to recover, its weight soon begins to increase after the preliminary fall. The temperature in the rectum may also be taken, but is not so valuable, as in the guinea-pig variations occur from mere handling or other slight causes. The temperature of the guinea-pig averages 38·6°, but varies between 36° and 39° C. (Eyre).

The examination of the dead animal should be carried out with as little delay as possible. For dissection, the body should be pinned out on the back on a board, which may stand in a shallow enamelled iron pan, by pins or nails through the feet, and the abdomen well soaked with antiseptic solution, not so much to sterilise the skin as to prevent the hair from getting into the incision; to obtain

complete sterilisation of the skin, it is preferable to clip or shave the hair and then sear with a red-hot iron. Knives, forceps, scissors, etc., should be well boiled in an enamelled iron mug or pie-dish, the water being kept boiling during the progress of the dissection and the instruments rinsed from time to time in it. A little sodium carbonate may with advantage be added to the water. A small enamelled iron fish-kettle with perforated strainer forms an excellent steriliser for instruments, or a surgical instrument steriliser may be used. An incision is made and the skin is well reflected and pinned out ; the knife and forceps should then be re-sterilised, or fresh sterile instruments taken, for the deeper incision and for opening the body cavities ; these again must be re-sterilised, or a third set of instruments employed for incising the organs.

During the progress of the dissection the condition of the tissues at the seat of the inoculation should be noted, and likewise the conditions of the serous membranes and the various organs. In many infections the organism is met with most abundantly in the spleen, in others in the blood, and in some at the seat of inoculation. When a systematic examination is made, film specimens and cultures on two or three media, aërobic and anaërobic should be prepared from the seat of inoculation, the spleen, liver, lungs, and heart-blood, and in some cases from the serous membranes, muscles, or central nervous system in addition, the carcase being in the intervals covered with a bell-jar which has been rinsed in, or with filter-paper moistened with, antiseptic solution. An assistant is often useful or even necessary. The greatest care must be taken to avoid dropping or splashing or otherwise disseminating infective material, any stains being immediately swabbed up with antiseptic solution ; and the operator must exercise every precaution to pre-

vent the infection of himself and others. It is convenient to have some efficient antiseptic solution near at hand; it may be kept in a large bottle on a wall bracket and drawn off as required by a syphon tube provided with a tap or spring clip. The most generally used antiseptics are 5 per cent. carbolic, and 1–500 corrosive sublimate, but 2 per cent. cyllin or kerol or, particularly for sporing organisms, 5 per cent. bacterol, is more efficient. The access of flies to the carcase must also be prevented, as they might carry infection. When finished with, the carcase should be efficiently disinfected and disposed of without delay, preferably by burning it, together with the board on which it has been pinned out.

If the carcase be left, especially in warm weather, for even a few hours before the examination is carried out, the tissues are liable to become invaded and infected by organisms from the respiratory and digestive tracts. In the *post-mortem* room, infection of the tissues is very common; out of fifty cases, Symes[1] found only seventeen to be sterile. Ford states that even in normal animals, killed and immediately examined, bacteria are present in 70 per cent. of the internal organs.[2]

When the blood of an animal is required several expedients may be adopted. From large animals, like the horse, sheep, and goat, it may be obtained by passing a hollow needle into the external jugular vein (which runs superficially on either side of the under part of the neck) and allowing the blood to drip into a test-tube or flask. In the case of small animals not again needed, the animal may be decapitated or the throat may be cut, and the blood collected in a porcelain dish; but if a sample only is wanted, and the animal has to be further treated, as in immunity work, it is generally possible to bleed from a

[1] *Lancet*, 1899, vol. i, p. 365.

[2] *Journ. of Hygiene*, vol. i, No. 2, 1901, p. 277.

superficial vein. In the guinea-pig the needle of a syringe may be passed into the heart and 2–3 c.c. of blood withdrawn without injury to the animal; smaller quantities may be obtained from a superficial ear vein. In the rabbit blood may be obtained by passing the fine point of a piece of glass tubing, drawn out and bent to a convenient angle, or the needle of a syringe, into one of the ear veins and aspirating the blood into it. Or the vein may be punctured and the blood allowed to drip into a small tube.

Blood may be obtained from a patient for the agglutination or the Wassermann reaction, for microscopical examination, or for culture experiments, by pricking the finger or the lobe of the ear or the thumb (outer surface in a line with the root of the nail) with a sterile needle, preferably a flat one of the "Hagedorn" type, or with half a steel pen (nib) or a glass point; for disinfection, the skin may be rubbed with a little alcohol or ether. After swinging the arm and winding a piece of rubber tubing round the finger or thumb and pricking 1–3 c.c. may generally be obtained. Larger quantities must be obtained by puncture of a vein with a hollow needle. The blood may be collected in a small test-tube, vaccine tubes, small bulbous tubes (Fig. 7, p. 53), or Wright's tubes (Fig. 37 p. 237).

When small tubes with contained blood are sealed in the flame, care should be taken that one end is not wetted with the blood, and this dry end should be sealed *first* so as to obtain a perfect seal. When centrifuging to obtain the serum this sealed end should be placed downwards in the centrifuge "bucket."

Organisms, in natural infections in man, are usually present only in small numbers in the blood, and for demonstrating them by culture methods it is necessary to withdraw 2–5 c.c. from a superficial or deep vein by

means of a sterile syringe under aseptic conditions; superficial veins may be rendered prominent by winding rubber tubing round the limb, so as to obstruct the venous, but not the arterial, circulation. Broth tubes or agar plates are inseminated each with 0·5 c.c. of the blood. Douglas and Colebrook recommend trypsin broth for this purpose, *i.e.* broth to which 5 per cent. of Allen and Hanbury's compound solution of trypsin has been added *after* sterilisation.

Although the modern methods of isolation and cultivation have rendered immense service to bacteriology, they have also had the effect of diminishing the attention paid to the exact morphology and biology of organisms. At the present time there is a tendency to investigate bacteria *en masse* rather than to study them as individual living forms. As the late Marshall Ward remarked:

"The introduction and gradual specialisation of Koch's method of rapid isolation of colonies encouraged the very dangers they were primarily invented to avoid. It was soon discovered that pure cultures could be obtained so readily that the characteristic differences of the colonies in the mass could presumably be made use of for diagnostic purposes, and a school of bacteriologists arose who no longer thought it necessary to patiently follow the behaviour of the single spore or bacillus under the microscope, but regarded it as sufficient to describe the form, colour, markings, and physiological changes of the bacterial colonies themselves on and in different media, and were content to remove specimens occasionally, dry and stain them, and describe their forms and sizes as they appeared under these conditions. To the botanist, and from the point of view of scientific morphology this mode of procedure may be compared to what would happen if we were to frame our notions of species of oak or beech according to their behaviour in pure forests, or of grass or clover according to the appearance of the fields and prairies composed more or less entirely of it, or—and this is a more apt comparison, because we can obtain colonies as pure as those of the bacteriologist—of a mould fungus according to the shape, size, and colour, etc., of the patches which grow on bread, jam, gelane, and so forth."

Examination of Living Organisms

Examination in the fresh and living condition is an essential procedure in the investigation of micro-organisms. This may be done by placing a droplet of sterile water, broth, or salt solution on the slide, inoculating with a trace of the material or growth, and covering with a cover-glass and examining microscopically. The action of stains and reagents on the organisms may be observed by the irrigation method. A drop of the stain or reagent (C, Fig. 23), is placed on the slide, A, just in contact with one margin of the cover-glass, B, and is drawn through the preparation by means of a small piece of filter-paper, D, placed on the other side, a torn margin touching the film of fluid at one edge of the cover-glass.

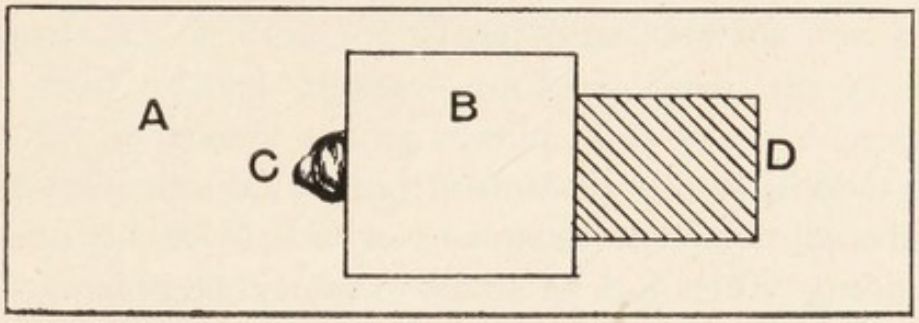


FIG. 23.—Method of irrigation.

The filter-paper absorbs the fluid from under the cover-glass, leaving the cells and other particles behind, and at the same time the reagent on the opposite side flows under the cover-glass to take the place of the absorbed fluid. The excess of the reagent or stain may afterwards be washed away by running in water under the cover-glass in a like manner. Care must be taken to keep the upper surface of the cover-glass dry and free from fluid. The advantage of this method is that it may be applied while the specimen is being examined under the microscope, and the action of the reagent on a particular cell or granule can, with a little care, be observed. If the cells

be large and it is desirable to avoid pressure of the cover-glass, a fine hair or bristle may be so placed on the slide that when the cover-glass is lowered one edge rests on it. If the specimen has to be kept for any length of time, the film of fluid will before long evaporate and the preparation become dry. To prevent this a ring of oil or vaseline may be painted round the margin of the cover-glass so as to seal it to the slide.

A simple method for keeping organisms under examination for a lengthened period of time, and of watching their growth and development, is by the use of hanging-drop preparations. To prepare a hanging-drop, a ring of vaseline is painted round the margin of the hollow of a hollow-ground slide (or other cell, see below). A cover-glass is sterilized by flaming in the Bunsen, care being taken not to heat sufficiently to melt it. A droplet of some sterile fluid medium—water, broth, wort, sugar solution, etc.—is then placed in the centre of the cover-glass with a sterile platinum loop. This droplet is then inoculated with the organism which is to be observed, care being taken not to add too many organisms—a few isolated organisms and small groups in each field is what should be aimed at. The vaselined cell is now taken and turned over, so that the ring of vaseline is downwards, and is then applied to the cover-glass, in such a way that the droplet is situated in the middle of the hollow, but not touching the slide at any point. The cover-glass adheres to the slide by means of the vaseline, and on quickly inverting the whole, so that the fluid has no time to run, it will be found that the droplet is hanging from the under surface of the cover-glass in a cell which is hermetically sealed by the vaseline, and evaporation is thus rendered impossible (Fig. 24). Such a preparation, in fact, can be kept for a week or ten days in a warm incubator without drying up. Great care must be exercised in

examining a hanging-drop specimen microscopically, especially with the immersion lenses, for the slightest pressure breaks the unsupported cover-glass. It often saves time first to centre the drop with the low power before examining with the immersion lens; an ink or pencil dot at the margin of the drop aids focussing. The light must be diminished by closing the diaphragm, lowering the condenser, etc. (p.145), and artificial light is generally preferable to daylight. The central parts of the drop only should be examined, not the margin.

Instead of hollow slides, various devices may be employed to form the cell. Metal, glass, or vulcanite rings, or rings cut out of thin sheet lead, tin-foil, cardboard, or two or three thicknesses of paper or filter-paper may

FIG. 24.—Hanging-drop preparation

be cemented on to slides with vaseline, Hollis's glue, gold size, or Canada balsam, or a thick ring of vaseline, paraffin, or plasticine may be used.

The only certain method for ascertaining whether an organism is motile or not—often an important clue to its identification—is by the use of hanging-drops. Actively motile organisms may frequently assume a non-motile resting stage, although still alive, and various factors may bring about this condition, such as old age, exhaustion of nutriment, excessive heat or cold, electric shocks, and the like. The absence of movement of an organism in a specimen prepared from an ordinary culture, particularly if more than a day or two old, does not necessarily prove that it is non-motile. A hanging-drop should be prepared with a nutrient medium (the best, perhaps, is glucose broth) and placed under conditions of temperature, etc., favourable to the growth of the organism, and

examined after an interval of an hour or so, or better still at intervals of half an hour for three or four hours. In this time the old cells will revivify, and new ones will have been produced, and if the organism be a motile one, more or less active movement of some of the cells is almost sure to be observed. It is necessary to beware of two fallacies in connection with motility—not to mistake for it the so-called Brownian movement, which is a vibratory one backwards and forwards about one point, and common to all fine particles suspended in a fluid; and not to be misled by a flotation of the cells due to currents set up in the fluid from some cause or other—all the particles then tending to move *in the same direction*.

Another purpose for which the hanging-drop cultivation may be employed is that of obtaining a permanent record of the various phases through which an organism may pass during its development. If a number of these cultivations be made, say twenty, in an exactly similar manner, and afterwards kept under identical conditions, and if at the end of every half-hour one of the preparations be taken, its cover-glass carefully removed, and the droplet dried and stained, a permanent record of the life-history of the organism is obtained extending over ten hours.

Various more elaborate forms of cells for hanging-drop preparations can be obtained, some being provided with inlet and exit tubes for the passage of various gases. For anaërobic preparations cells are made having a groove at the bottom into which a mixture of pyrogallic acid and potash is introduced.

The observation of hanging-drop cultivations at blood-heat can be carried out on some form of warm stage.

Interlamellar films.[1]—Another method of investigating the life-

[1] Delépine, *Lancet*, 1891, vol. i, June 13.

history of organisms, especially moulds and protozoa, is by means of interlamellar films. A glass slide 1½ by 3 in. is sterilised in the Bunsen flame, and while hot three small drops of sealing-wax are placed on it, so arranged that they form the apices of an equilateral triangle, the side of which measures about one inch, and a drop of sterile nutrient medium is deposited between them. A cover-glass of about 1¼ in. in diameter is then sterilised in the Bunsen flame, a droplet of a suitable nutrient medium is placed upon it and inoculated with the organism to be observed, and the prepared cover-glass is picked up with sterilised forceps, inverted, and lowered on to the slide. The nutrient medium is thus contained between the slide and the cover-glass, and by using a hot wire, and so softening the sealing-wax, it can be spread out to form as thin a layer as desired. The preparation is kept in a moist chamber to prevent evaporation, and can be studied when required.

The Microscope

A bacteriological microscope is generally of the monocular form, and should be provided with a rack-and-pinion coarse adjustment and an efficient fine adjustment. The stage, preferably of vulcanite, should be large and roomy and quite plain, with two or more holes at its margin to receive spring clips for fixing the slide. For the ordinary examination of specimens a mechanical stage is not needed, but for some purposes a mechanical stage is very useful, and for a critical survey of the whole of a specimen, *e.g.* a blood-film, it is essential. If a mechanical stage be fitted, the surface should be flat and any bar, stops or clips removable; failing this a detachable form is to be preferred (Fig. 25), so that, if required, the stage may be free for the examination of plate cultivations, etc.

New forms of binocular microscopes have been introduced by Messrs. Beck and by Messrs. Leitz which possess marked advantages over the monocular instrument.

A sub-stage condenser is essential for all work in which high powers are employed, and also enhances the value of

low powers. It consists of a system of lenses below the stage, by means of which the light is concentrated on the object. It should have a rack-and-pinion, or a screw, adjustment for focussing, and be provided with some form of diaphragm for modifying the light, preferably an "iris." The condenser must be centred—that is, adjusted so that its optical axis corresponds with the optical axis of the objective ; and for this purpose it ought to be provided with two lateral screws working at right angles to each other, by means of which its position

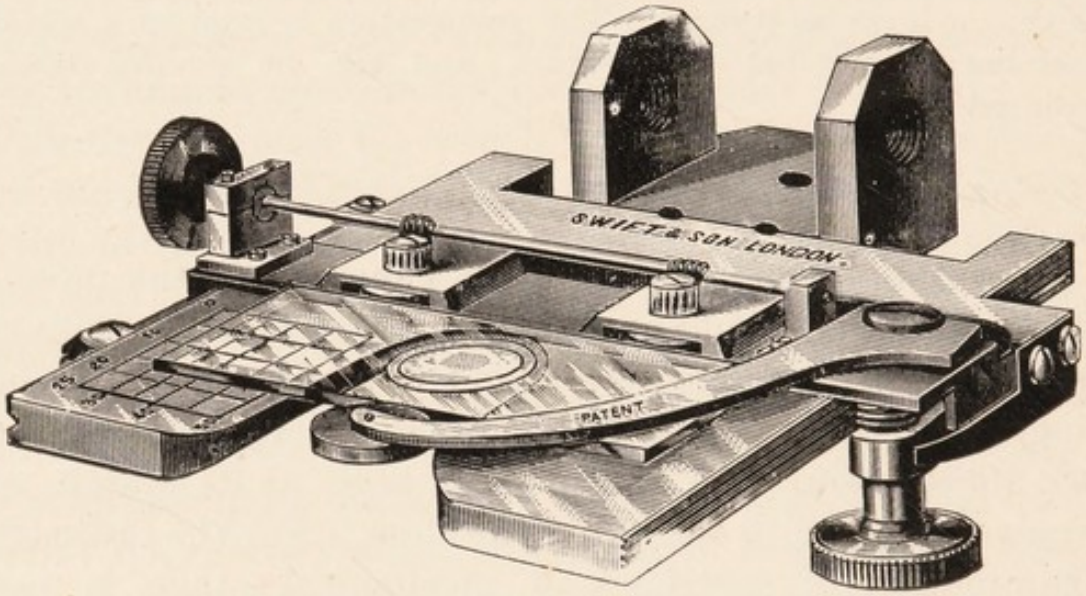


Fig. 25.—Swift's detachable mechanical stage.

relative to the optical axis can be altered. In order to centre, a diaphragm with small aperture is used, and the hole in the diaphragm is focussed with a low power ; then, by means of the lateral screws, this hole is brought into the centre of the field. Below the sub-stage condenser a mirror with concave and plane surfaces should be fitted, the *plane* surface being used with the condenser, as a general rule. The concave mirror may be used for illumination with low-power objectives, the condenser being detached or swung out of position. The necessity for

careful illumination must be insisted upon ; in fact, to obtain the best results the light should be readjusted for every specimen by mirror, diaphragm, and condenser, *i.e.* " critical " illumination should be aimed at. A good specimen may be utterly spoilt, visually, by faulty illumination ; while an indifferent one may be made to look passable by proper illumination. In the examination of micro-organisms in the fresh or living and unstained condition, it is necessary, *as a rule*, to diminish the light by means of a small diaphragm, or by racking down the condenser, or by both ; while for stained or opaque objects the full aperture of the diaphragm, or thereabouts, may generally be employed. It must be remembered, however, that the resolving power of a lens (see below) is diminished by closing the diaphragm and by throwing the condenser out of focus ; the illumination then becomes " non-critical." For fine work, if the illumination is too intense, this should be diminished by diminishing the source of light or by interposing a coloured screen, such as Gifford's, which consists of a cell containing a solution of malachite green in which is inserted a piece of green signal glass. Coloured glass may also be interposed. The microscopist should accustom himself to examine specimens both by daylight and by artificial light ; hanging-drop specimens are usually best seen with the latter. For artificial light, probably nothing surpasses a paraffin lamp with flat wick, the *edge* of the flame being always used, while to obtain the best results the mirror should be removed, and the flame used direct by elevating and tilting the microscope somewhat. For the finest work, daylight illumination is inadmissible. An admirable form of electric lamp is the " Barnard," made by Messrs. Swift and Son, the source of illumination being a Nernst lamp. For ordinary routine work, an incandescent carbon or metal filament electric lamp,

a Nernst lamp, or an argand or incandescent gas burner may be used. Various devices have been introduced for the employment of monochromatic illumination, *e.g.* the mercury vapour lamp by Barnard.

With the filament, Nernst, or incandescent gas lamps, the image of the filament or mantle is troublesome when the condenser is in focus ; this may be obviated to some extent by the use of frosted bulbs or by interposing a screen of fine ground glass, by the use of Gordon's glass rod illuminator, or by interposing a spherical flask filled with water or dilute copper sulphate solution. Incandescent bulbs may be frosted by dipping in a 15 per cent. solution of caustic soda and allowing to dry.

An admirable form of electric lamp has been introduced by the Ediswan Company in which a ball of tungsten is rendered incandescent and the source of light is almost a point and free from structure.

Two eyepieces are sufficient, and the lower-power ones are to be preferred, such as the B and C of the English, or the 2 and 3 of the Continental, makers. Although increased magnification can be obtained by the use of a high-power eyepiece, it is at the expense of definition, the image losing its sharpness, because the eyepiece magnifies the image formed by the objective, and any imperfections in the latter are made more apparent, so that the use of very high eyepieces is not to be recommended, except with the finest lenses ; moreover, as will be pointed out later, it is useless to increase the amplification beyond a certain point.

With regard to the length of the tube of the microscope, this differs in the English and Continental systems. The standard English tube-length is 8·75 in., the Continental is 6·3 in., and is usually adopted, but the longer tube gives greater amplification. The tube of the microscope is generally provided with an inner, or draw-tube, by means

of which its length can be nearly doubled; this gives increased amplification, but at the expense of definition, at least with the higher powers which are corrected or adjusted for a definite tube-length.

The lenses or objectives must next be considered.

For powers higher than the $\frac{1}{6}$-in., or thereabouts, it is advisable, for many reasons, to employ the immersion system of objectives. With these lenses a drop either of water, in the water-immersion system, or of cedar oil, in the oil-immersion one, is placed on the cover-glass, and the objective is racked down so that its front lens touches and is immersed in either the water or oil, as the case may be. It is a good plan then to raise the objective very slightly by means of the coarse adjustment, still, however, keeping it in contact with the drop of water or oil. The observer, looking down the microscope, then very cautiously and gradually racks down again with the coarse adjustment until the object comes into view, and finishes the focussing with the fine adjustment. The fine adjustment should only be used after the object has been brought into view by means of the coarse adjustment. After the examination has been concluded for the day, the lens should be carefully wiped with a soft rag, or preferably with a piece of soft Japanese paper, to remove the water or oil. If the oil should happen to dry on the lens, it may be removed by wiping with a soft rag or Japanese paper moistened with alcohol, quickly drying with another rag or paper. Instead of cedar-oil, a liquid paraffin has also been used.

The $\frac{1}{12}$ in. (2 mm.) oil-immersion lens is the one usually selected. It combines sufficient magnification for most purposes with adequate working distance for convenience in using. If expense is not an object, the Zeiss $\frac{1}{8}$ in. (3 mm.) apochromatic oil-immersion lens is a very fine one for general use. By means of the compensating

oculars sufficient magnification can be obtained, while the working distance is greater, the field is larger, and the penetrative power is greater than with the $\frac{1}{12}$ in. lens.

The immersion system of objectives has many advantages : the loss of light is less, the distance between the cover-glass and the front of the objective—the working distance, as it is termed—is greater, and more can be seen with an immersion lens than

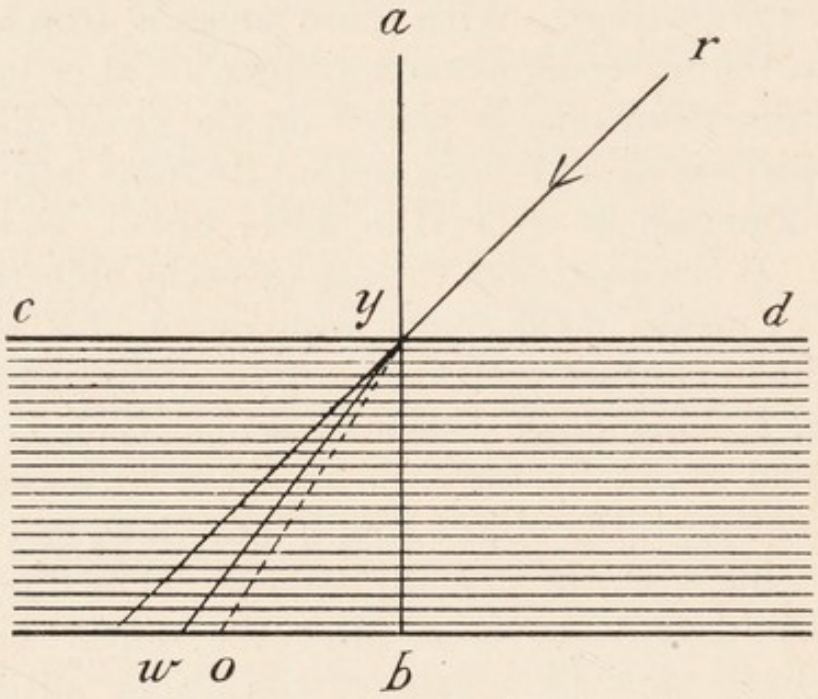


FIG. 26.—Diagram to illustrate the refraction of light.

with a dry lens of equal magnifying power. This can be best illustrated by means of two simple diagrams.

In Fig. 26 let *cd* represent the surface of a fluid, either water or oil, and let *ab* be drawn perpendicular to this surface, and cutting it at *y*. Let *ry* represent a ray of light proceeding from a rarer medium, such as air, into a denser one, water or oil. As is well known, this ray when it enters either the water or the oil does not continue in the same direction, but is " refracted " or bent nearer the perpendicular *ab*, the bending being more marked with oil than with water. Thus we may suppose that the direction of the ray in water would be represented by the line *yw*, and in oil by the dotted line *yo*. Conversely, a ray of light proceeding from a denser medium into a rarer is bent away from the perpendicular, and the rays *wy* in water, and *oy* in oil, would, on emerging into air, proceed in the direction *yr*.

In Fig. 27 (which for convenience is drawn somewhat out of proportion) let S represent an ordinary glass micro-slide, X a layer of Canada balsam in which the object is mounted, and covered with the cover-glass G, while L is the objective with its front lens. Let the object be illuminated by the ray of light *Yy* ; this on entering the glass of the slide and the Canada balsam will be refracted or bent nearer the perpendicular and will proceed in the direction *yt*. Canada balsam, and also cedar oil, produce about the same amount of "refraction," or bending of a ray of light, as crown glass, and hence these three substances—crown glass, Canada balsam, cedar oil—are said to have the same "refractive index," and, consequently, the glass of the slide, the Canada balsam, and

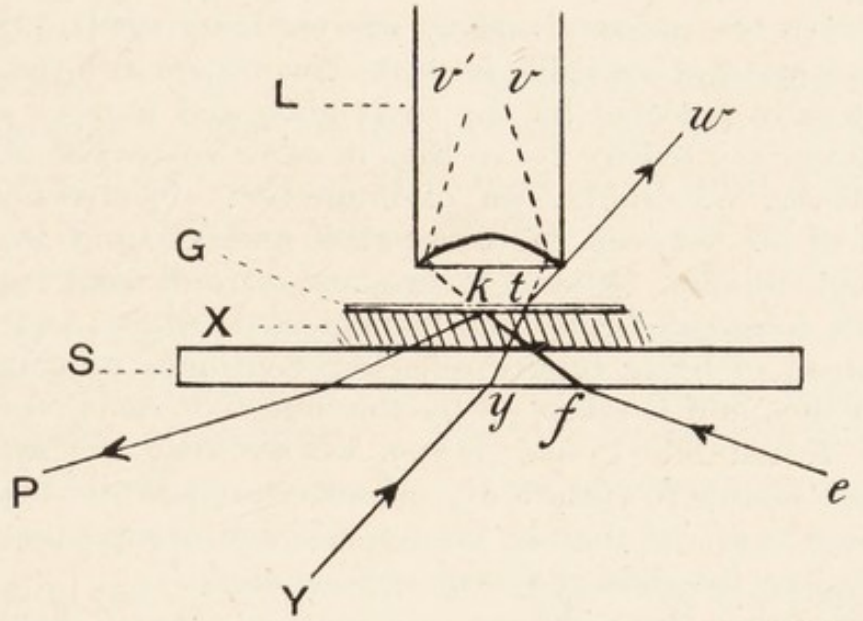


FIG. 27.—Diagram to illustrate the course of rays of light through an objective.

the cover-glass act as one homogeneous medium, and the line *yt* is a straight one. In the first place, let us suppose that the objective L is a dry one, having a layer of air between its front lens and the cover-glass ; then the ray of light, on emerging from the cover-glass into the air, is now bent away from the perpendicular and pursues a direction practically parallel to its former one, represented by the line *tw*, and misses the lens altogether—the lens is unable to take it up. If, however, we suppose that our objective is an oil-immersion one, and that a drop of cedar oil takes the place of the layer of air between the cover-glass and the front lens in the foregoing example, then the glass slide, Canada

balsam, cover-glass, cedar oil, and the front lens of the objective form practically one medium ; they all have the same refractive index and produce the same amount of refraction or bending of a ray of light. Therefore the direction of the ray forms a straight line in all these, and the ray passes into the objective as is represented by the broken line *t*—*v*. More important still, however, is that which happens to rays which fall on the slide at a very oblique angle. In the same figure (Fig. 27) let *ef* represent such a ray ; on entering the slide it will be refracted, and its passage through the slide, balsam, and cover-glass may be represented by *fk*. As before, let us suppose that in the first place our objective is a dry one, and that we have a layer of air between the cover-glass and its front lens. In this case, if the angle which *fk* makes with the perpendicular is greater than about 39° or 40°, the ray, instead of emerging from the cover-glass into the layer of air, is totally reflected by the cover-glass and pursues a course roughly represented by *k*P, so that it never enters the objective. If, however, we employ an oil-immersion objective, with oil instead of air between the cover-glass and its front lens, then, as before, the slide, balsam, cover-glass, oil, and front lens of the objective form practically one homogeneous whole, and the ray *efk*, instead of being totally reflected, continues its course in a straight line, and is taken up by the objective, as is represented by the dotted line *k*—*v*′. Hence we see that the same rays which are unable to enter a dry objective are admitted by an oil-immersion one, and that an oil-immersion lens can take up rays which fall on the slide at a very oblique angle.

In order that these oblique rays may be present, ready to be taken up by the oil-immersion objective, it is necessary to employ a sub-stage condenser. It is only by means of a sub-stage condenser that a " wide-angled cone of rays," as it is termed, is obtained. Hence to make full use of an oil-immersion objective —to " get most out of it "—it is absolutely essential to employ a sub-stage condenser, and for the finest work a special " oil-immersion condenser " is employed. It will be obvious also that although a water-immersion objective admits more rays than a dry one, it does not admit so many as an oil-immersion. It must be pointed out, however, that Canada balsam, or some medium having the same or a higher refractive index, must be used for mounting to obtain the full advantage of the oil-immersion system. The oil-immersion can of course be used for examining objects mounted in water, etc., cedar oil being still used

between the cover-glass and the lens. It is to be noted that a dry objective cannot be used as an immersion one, nor an immersion objective dry, as the construction differs in the two cases.

Of late "dark ground illumination" has been much employed, particularly for the examination of living objects. In this special condensers are used, the central rays passing through which are "stopped out," so that the object is illuminated only by very oblique rays and appears white on a dark background. A dry lens is used, or if an oil-immersion one, a stop must be introduced to reduce its aperture, and slides and cover-glasses of special thickness together with brilliant illumination are necessary.

The lenses in the objective are formed by cementing together different kinds of glass in order to correct for "spherical" and for "chromatic" aberration. The rays passing through the margin and the centre of a simple lens are not focussed at the same point, and a distorted image is the result; this is known as "spherical aberration," while the violet and red ends of the spectrum, being of different refrangibility, and a simple lens acting like a prism, coloured fringes are observed; this is termed "chromatic aberration." The apochromatic system of objectives and eyepieces has these defects very perfectly corrected by the use of special glass and fluorite, correction being partly effected in the objective, and this is completed by combination with the special eyepieces. The latter, termed "compensating oculars," are therefore essential for perfect correction with apochromatic objectives, but can also be used with ordinary lenses. For photographic purposes apochromatic lenses are far superior to achromatic ones. Apochromatic objectives are, however, expensive, and though *advantageous* are not really *necessary* for *ordinary* bacteriological work; they possess one great advantage over ordinary objectives, namely that higher eyepieces may be used.

In consequence of certain optical principles, the "diffraction" theory, for details of which the reader must

refer elsewhere,[1] it is useless to increase the magnifying power of objectives beyond a certain point ; for, although the object viewed appears *larger*, no more *details of structure* can be made out.

The use of the immersion system increases the "resolving power," or the amount of detail which can be seen. Thus, if a number of fine equidistant parallel lines be ruled on a glass plate, it is impossible to see with a dry lens, using white light, more than about 90,000 lines to the inch as isolated lines. If more are ruled they will not appear, and practically nothing is visible. Using white light with a water-immersion objective it is possible to see about 120,000 lines to the inch, and with an oil-immersion as many as 146,000 lines to the inch, as separate lines—a clear gain in resolving power in the latter case of about one half over a dry lens.[2] As it is necessary, in order to see such fine structures as lines ruled 50,000 or more to the inch must be, to have considerable amplification in addition to resolving power, not much is gained, in ordinary work at any rate, by adopting the immersion system for the lower power objectives, such as the $\frac{1}{6}$-in.

By the physical theory of microscopical visibility, it can be shown that objects having a diameter of less than about 0·16 μ cannot be seen with the best optical appliances and using monochromatic light of short wave length (by which resolution is increased to between 150,000 and 160,000 lines to the inch). If, then, a micro-organism is less in size than this it could not be seen microscopically, and this fact may explain why it is that in certain undoubted infective diseases no micro-organism has yet been isolated. Of the existence of such "ultra-microscopic" organisms we have proof. The finest porcelain filters, such as the Chamberland B, do not allow visible particles to pass through, yet in several instances, if the infective material be filtered

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[1] See *Carpenter on the Microscope*, edited by Dallinger. (Churchill.)

[2] These figures refer to lenses having a numerical aperture of 1·0 (dry), 1·33 (water), and 1·4 (oil).

through such a filter, the filtrate is still infective. This is the case with the blood-serum in yellow fever, Cape horse sickness, dog distemper, hog cholera, and swine fever, in bird and cattle plagues, and with the juice of bird molluscum. The organism of cattle pleuro-pneumonia is just on the limit of visibility. The rabic and vaccine viruses also seem capable of passing through a Berkfeld V. Some thirty ultra-microscopic viruses are now known, including, in addition to those mentioned above, those of anterior poliomyelitis, measles, molluscum, and trachoma. These organisms are sometimes termed "filter passers." These experiments do not necessarily prove that the organism *in all stages* is invisible.[1] Hort claims that many of the ordinary bacteria form minute buds which may pass through the larger-pored filters (*loc. cit.* p. 13). Siedentopf and Zsigmondy have devised a method whereby ultra-microscopical particles may be rendered visible, but inasmuch as they appear merely as luminous points, it is questionable whether the method will be of great service in bacteriology.

Resolution may be doubled by employing ultra-violet light. But this entails the use of an entire *quartz* system of lenses, glass being opaque to ultra-violet rays, and the results can only be registered photographically; they are not visible to the eye.

It is not usually necessary in bacteriological work for the immersion objective to be provided with a "correction collar." The "correction collar" is an additional screw in the objective by means of which the distance between some of its constituent lenses can be altered to "correct" for varying thicknesses of cover-glass, etc., and though necessary with the higher power dry lenses, it is theoretically unnecessary with the immersion system. Nevertheless, as slight variations do occur in the various media, glass, oil, etc., and they may not form a truly homogeneous whole, for the finest work the correction collar is still desirable. So much for the high-power objectives. As regards the lower powers, which, of

[1] See Roux, *Bull. de l'Inst. Past.*, vol. i, 1903, pp. 1 and 49. Remlinger, *ibid.* vol. iv, 1906, pp. 337 and 385; *Trans. XVIIth Internat. Cong. Med.* 1913, Sect. IV, Pt. I, pp. 35 (Löffler) and 49 (McFadyean).

course, are dry, a $\frac{2}{3}$-in. and a $\frac{1}{6}$-in. are generally selected. The $\frac{2}{3}$-in. is a more serviceable lens than the 1-in. which is often recommended. A very useful accessory is a "double" or "triple nosepiece." This consists of a light metal framework, which is attached to the lower end of the tube of the microscope, on to which two or three objectives can be screwed. The framework can be rotated, thus bringing each objective in succession into the optical axis of the instrument, and the necessity for unscrewing and screwing on each time an objective is changed is obviated. A microscope such as described, with substage condenser, two eyepieces, a $\frac{2}{3}$-in. and a $\frac{1}{6}$-in. dry and a $\frac{1}{12}$-in. oil-immersion objectives, triple nosepiece, etc., complete in case, can be obtained for about £16 (pre-war price), and it is well to add another sovereign or two for superior finish. British, American and Continental firms supply microscopes arranged as indicated, and in this department the English makers hold their own.

The measurement of micro-organisms is carried out by means of a stage micrometer, alone, or in combination with an eyepiece micrometer. The former consists of a scale of tenths and hundredths of a millimetre or hundredths and thousandths of an inch ruled in fine lines on a glass plate, by means of which the measurements can be made by focussing the scale under the microscope. The stage micrometer is placed in position on the stage and the scale is focussed with the particular ocular, objective, and tube length which are to be used. A drawing of the scale is made with a camera lucida; the micrometer is then removed and the object placed in position and a second drawing is made of the object on the scale already drawn. A simpler and less expensive arrangement is to make use of a disc of glass ruled with equi-distant fine lines, which can be placed on the diaphragm in the eyepiece after unscrewing the top

lens. The value of the divisions in this eyepiece scale is first ascertained by means of the stage micrometer. The stage micrometer is then removed and the object to be measured put in its place, and its dimensions are determined by means of the eyepiece scale. If the objective or the eyepiece be changed or the tube length altered the value of the divisions of the eyepiece scale will be altered, and must again be determined by means of the stage micrometer. The eyepiece micrometer is a more elaborate device of the same nature as the eyepiece scale. It consists of an eyepiece in which two fine filaments can be adjusted by means of screws so as just to correspond with the limits of the object. This having been done, and without altering any of the adjustments, the object is removed and replaced with the stage micrometer, and the distance between the two filaments is then determined. The unit for microscopical measurement is the micron (sometimes erroneously termed a micro-millimetre), which measures one thousandth of a millimetre, or approximately $\frac{1}{25000}$ of an inch, and is designated by the sign μ.

If a micrometer is not available, rough measurements may be carried out by comparison with a red blood-corpuscle. The majority of the red corpuscles of normal human blood measure 7·5 μ in diameter.

CHAPTER V

INFECTION—VEGETABLE AND ANIMAL PARASITES—THE INFECTIVE PROCESS—ANTI-BODIES—ANTISERA AND ANTITOXINS—IMMUNITY

Infection

By the term Infection is meant the invasion of the living tissues by living micro-organisms which grow and multiply at the expense of the host, and which may be either animal or vegetable in nature. A disease produced by the growth and multiplication of micro-organisms is termed an *infective disease*, and is transmissible in most instances by inoculation. If the micro-organisms are from time to time discharged from the body of the host, either with the excreta, secretions, desquamated particles, or in some other way, the disease becomes *infectious* or *contagious*, according to the ease with which another individual becomes *infected*, and the material which conveys the infection is often termed the *contagion* or the *virus*, though the latter is often applied to the organism itself. Thus, in scarlatina and smallpox the contagion is very readily conveyed from person to person even for a distance through the air, and these are infectious diseases. Ringworm and syphilis, as a rule, require more or less close contact for infection to take place, and these are, therefore, contagious diseases; while malaria is neither infectious nor contagious, since persons in the neighbour-

hood never directly contract the disease, though it can be conveyed by inoculation, and it is therefore infective only. But the distinction between *infectious* and *contagious* is mainly one of degree, and these terms have now to a large extent been discarded. Excluding individual susceptibility, the relative infectivity of a disease probably depends on three factors : (1) the contagion is freely given off aërially and is not destroyed thereby ; (2) the contagion gains access by the respiratory tract ; and (3) the relative virulence of the contagion ; in some instances the smallest amount of the contagion is sufficient to infect. If the contagion can gain access only through a wound or the digestive tract, the chances of infection may be largely reduced. In certain instances infection is conveyed by an intermediary, *e.g.* the mosquito in malaria, and in such cases infectivity will obviously depend on the presence and abundance of the intermediary. Infection is manifestly a part of the whole subject of parasitism, which includes the animal and vegetable parasites which develop in the animal body. If, however, the subject of parasitism is considered more closely, it will be seen that there is a vast difference between, say, a condition caused by the echinococcus or by the round worm, in which the effects are largely mechanical and in which relatively little poison is produced by the parasite, and the disease diphtheria caused by the diphtheria bacillus, in which the diphtheria bacilli have little or no action mechanically, but elaborate virulent chemical poisons which cause a general *intoxication*. Some parasites also may produce a *general* infection, *e.g.* anthrax, others only a *local* infection, *e.g.* ringworm.

Parasites may therefore be divided into infective and non-infective, though a series of links connect them, and the two groups cannot be sharply separated. The *infective parasites* are : (1) vegetable micro-organisms, chiefly

bacteria, a few yeasts and some moulds ; (2) many protozoa ; and (3) a few metazoa, generally worms. The *non-infective parasites* are the animal parasites generally, particularly many worms.

The production of the phenomena of disease by pathogenic organisms has been ascribed to (1) the using up of the oxygen which should go to the tissues ; (2) the using up of the proteins of the body and of the food ; (3) the effects of plugging of the vessels by the microbes ; and (4) the effects of substances or " toxins," having a poisonous action, formed by the microbes. Of these, the first three are quite subsidiary, embolism and thrombosis being perhaps the most important, and the toxins are the chief factors which induce the pathogenic effects. These toxins are substances of a very complex composition, probably allied to the proteins ; in some instances they seem to be of the nature of enzymes or ferments, and they are direct products of the bacterial cells. The toxins of most pathogenic organisms, *e.g.* typhoid, cholera, plague, etc., are more or less integral parts of the bacterial cells ; they are " endotoxins," and are not excreted to any extent into the surrounding medium, but may gain access to it by autolysis of some of the organisms. A few organisms, notably *Bacillus diphtheriæ* and *Bacillus tetani*, produce extra-cellular toxins which are found in the culture liquid. The toxins are classified by Sidney Martin,[1] as follows (see also p. 40) :

(1) Poisons produced by the digestive or the destructive action of bacteria on proteins in the culture medium. Examples of these are the poisons of the *Bacillus anthracis* and of the pus-producing staphylococci.

(2) Poisons which are the result of the digestive or destructive action of bacteria on proteins, but formed as

[1] *Manual of General Pathology*, p. 76.

an excretion (the toxin) of the bacterium. The *Bacillus diphtheriæ* is the best example of this. A similar combination of poisons is found in snake-venom.

(3) Poisons which are excretions only, such as those produced by the tetanus bacillus.

(4) Poisons which are typically intra-cellular, but which may also be excretory. The poisons produced by the typhoid bacillus, the *Bacillus coli*, the *Bacillus enteritidis* of Gaertner, and the cholera vibrio belong to this group.

Thiele and Embleton [1] suggest that the toxins of bacteria are really cleavage products derived from their cellular proteins under the influence of ferments present in the body of the host. These cleavage products are, however, toxic only at a certain stage of their disintegration. Given the power of existing and multiplying in the body of the host, the pathogenicity of a bacterium depends on the quantity and consequent activity of the ferments of the host. A certain degree of ferment activity renders the cleavage products of the bacterio-protein toxic, a further degree of ferment activity carries the disintegration so far that the cleavage products are no longer toxic. A bacterium may therefore be harmless to a host if the latter (*a*) has no ferments capable of digesting its bacterio-protein; (*b*) has such a poor supply of ferments that the bacterio-protein is so slowly disintegrated that toxic products never attain a sufficient concentration to be harmful; (*c*) has such a plentiful supply of ferments that the cleavage of the bacterio-protein rapidly passes beyond the toxic stage. A harmless bacterium, *e.g. B. megaterium*, may be rendered pathogenic if suitable ferments can be produced in the host to bring about the necessary disintegration of its bacterio-protein.

[1] *Lancet*, vol. i, 1913, pp. 234 and 332.

The Infective Process

With regard to the pathogenic micro-organisms, or disease germs, Koch laid down the following conditions, which have been termed "Koch's postulates," which must be complied with before the relation of an organism to a disease process can be said completely to be demonstrated :

(1) The organism in question must be present in the tissues, fluids, or organs of the animal affected with, or dead from, the disease.

(2) The organism must be isolated and cultivated outside the body on suitable media for successive generations.

(3) The isolated and cultivated organism, on inoculation into a suitable animal, should reproduce the disease.

(4) In the inoculated animal the same organism must be found.

To these may be added :

(5) Chemical products with a similar physiological action may be obtainable from the artificial cultures of the micro-organism, and from the tissues of man or animals dead of the disease.

(6) Specific serum and other reactions, agglutinative, bacteriolytic, complement fixative, etc., are generally obtainable, under certain conditions, if the blood of the infected person or animal be allowed to act on the specific organism producing the infection.

It is true that one or more of these conditions may not be fulfilled in all cases, but on general evidence the disease is classed as infective.

The modes of infection, or entrance of the infective agent into the body, are varied. The infective agent may enter by (1) the gastro-intestinal tract, *e.g.* typhoid fever. cholera, and glanders ; (2) the respiratory tract *e.g.*

pneumonia and influenza, and occasionally typhoid fever, plague, etc. ; (3) by inoculation, not necessarily only of the skin, but also of the mucous membranes, *e.g.* the septic diseases, glanders, tetanus, etc. The extreme infectivity of some diseases—*e.g.* variola, scarlatina, influenza, etc.—may be due to the fact that infection takes place by the respiratory tract. In certain instances the infection is conveyed in some special way, *e.g.* by mosquitoes in malaria and in yellow fever. Nor is infection necessarily confined to one mode of entrance ; in plague, for example, infection by the skin is commonest in some epidemics, but it is not infrequent by the respiratory, and may occur by the digestive, tract. The infecting agent may remain localised, giving rise to a *local infection*, or it may be widespread through the body, a *septicæmia* [1] or *general infection*. The absorption of chemical products from a local site of infection may produce general symptoms ; this is *intoxication*, as occurs in cholera, in which the microbe is limited to the bowel, in the early stage of diphtheria, in which the diphtheria bacillus is limited to the membrane, and in a local abscess. Fever is usually one of the results both of intoxication and of general infection.

The localisation of a particular infection may in some instances be due to a selective affinity of the strain of the organism for a particular tissue. Rosenow has given evidence of this in the case of pathogenic streptococci. He isolated streptococci from various lesions in man, and then injected cultures intravenously into rabbits and determined the site of the lesions in the inoculated animals. Fourteen strains from appendicitis produced lesions in the appendix in 68 per cent. of sixty-eight

[1] "Septicæmia" and "a septicæmia" have different meanings. The former is applied to a general infection with the so-called *septic* organisms, the latter to a general infection with *any* organism.

rabbits injected, which is in marked contrast to an average of only 5 per cent. of lesions in the appendix in animals injected with strains isolated from sources other than appendicitis. Eighteen strains from ulcer of the stomach or duodenum produced hæmorrhages in 60 per cent., and ulcer of the stomach or duodenum in 60 per cent., in contrast to an average of 20 per cent. hæmorrhages and 9 per cent. ulcer following injection of other strains. Twelve strains from cholecystitis produced lesions in the gall-bladder in 80 per cent. of forty-one animals injected, in contrast to an average incidence of lesions here of only 11 per cent. with other strains. Twenty-four strains from rheumatic fever produced arthritis in 66 per cent., endocarditis in 46 per cent., pericarditis in 27 per cent., and myocarditis in 44 per cent. of seventy-one animals injected, in contrast to an average of arthritis in 27 per cent., endocardial lesions in 14 per cent., pericarditis in 2 per cent., and myocarditis in 10 per cent. of animals injected with strains from sources other than rheumatic fever. Six strains from erythema nodosum produced lesions of the skin in 90 per cent. of 20 animals injected, in contrast to an average of 2 per cent. in animals injected with strains from sources other than erythema nodosum and herpes zoster. Eleven strains from herpes zoster produced herpetiform lesions of the skin, lips, tongue, or conjunctivae in 77 per cent. of sixty-one animals injected, in contrast to an average of only 1 per cent. of what seemed to be herpes of the skin with other strains.

Infection, if not too rapidly fatal, is frequently followed by remarkable alterations in the body fluids and tissues. One of these, if recovery ensues, is the production of immunity or insusceptibility to the same infecting agent; this will be considered later (p. 215). Another is the formation in quantity of substances which normally are

absent or are present only in small amount, such as agglutinins, bacteriolysins and antitoxins: To these the general name of "anti-bodies" is given, and these may now be considered.

Anti-Bodies [1]

Anti-bodies may and do develop as the result of a natural infection, but much larger amounts can be obtained by the injection into an animal of complex substances, such as bacterial toxins, bacteria, blood-corpuscles, cells and cellular proteins, ferments, etc. Thus an animal injected with sub-lethal doses of a bacterial toxin, *e.g.* diphtheria toxin, acquires a tolerance towards the toxin, becomes immunised, and a substance is developed in the blood that antagonises the toxin which was injected; this substance is known as *antitoxin*. If bacteria be injected, the fresh blood-serum *in vitro* has a solvent action on the bacteria (bacteriolysis), and also has an agglutinating action upon the bacteria employed; if blood-corpuscles be injected, the fresh blood-serum has a solvent action on the same kind of blood-corpuscles (hæmolysis); if cells be injected, the blood-serum has a solvent action on the cells (cytolysis), and so on. If ferments be injected, anti-ferments are formed and will prevent the specific action of the ferment. Antitoxins, bacteriolysins, agglutinins, etc., are also formed in the course of a natural infection, but not nearly to the extent that is possible after artificial inoculation. Traces of them are also present normally in the uninfected or untreated animal. With doubtful exceptions,[2] it is only complex

[1] All the subjects dealt with in the subsequent portion of this chapter are discussed in detail by Emery, *Immunity and Specific Therapy*, 1909.

[2] Ford has described the formation of an anti-body by the injection of a poisonous glucoside derived from fungi.

bodies of protein nature, or allied to the proteins, which give rise to the production of anti-bodies on inoculation; alkaloids, carbohydrates, mineral poisons, etc., do not give rise to anti-bodies, though some insusceptibility to them may be produced (see also p. 215). Any substance which gives rise to an anti-body may be termed an *antigen*. A remarkable property of anti-bodies is their extreme specificity; as a rule they react only with the antigen which has produced them.

Anti-bodies are probably formed for the most part in the spleen, lymph-glands and bone-marrow by leucocytes, or by endothelial cells, or by both.

Antitoxins.—The anti-bodies produced by the inoculation of an animal with bacterial toxins or toxic proteins (*e.g.* ricin, abrin, and snake-venom) are known as antitoxins, and are of considerable practical importance. An animal injected with increasing amounts of the toxin acquires a high degree of immunity, and its blood-serum injected into a second animal confers on the latter a similar immunity against the same toxin, but not against other toxins; the serum is specific. The anti-serum formed by the injection of toxin is antitoxic and not anti-microbic, and the diphtheria bacillus will grow and multiply in diphtheria antitoxin. Since, however, the pathogenic effects of an organism such as the diphtheria or the tetanus bacillus are caused by the toxin which it forms, the antitoxin will counteract the effects of the *micro-organism*, as well as of its toxin. The neutralisation of the micro-organism, however, may not be quite complete, a certain amount of local reaction or necrosis ensuing.

Antitoxins are prepared by injecting animals—preferably horses, but goats, rabbits, etc., may also be employed—with bacterial toxins or with cultures.

With those organisms which produce potent toxins

such as diphtheria and tetanus, it is customary to grow the organism in a fluid medium so that an active and virulent toxin is obtained. The culture is then filtered through a Berkefeld or Pasteur-Chamberland filter and the toxic filtrate inoculated subcutaneously into an animal, generally a horse, commencing with sub-lethal doses.

The dose of toxin can be gradually increased, and concurrently with the increase in insusceptibility the blood-serum acquires antitoxic properties. The treatment is tedious, and the activity of the antitoxic serum is largely dependent upon the amount and activity of the toxin injected. The requisite degree of strength having been attained, the horse is bled with aseptic precautions, the blood is allowed to coagulate, and the serum is bottled for use. Antitoxin may be obtained in a concentrated form by "salting out" the globulin constituents of an antitoxic serum (p. 182), and a dried product may be prepared by evaporating the serum to dryness *in vacuo* at 40° C. (10 c.c. serum = 1 grm. dry residue).

The mode of production of the antitoxin by the injection of the toxin has been the subject of various theories. By some it has been supposed that the antitoxin is modified toxin, the modification being brought about by the vital activities of the cells. But the amount of antitoxin produced does not necessarily bear any relation to the quantity of toxin injected. Woodhead records instances in which the amount of antitoxin formed amounted to 40,000 times the equivalent amount of toxin injected, bleeding the animal only temporarily reduces the antitoxin content of the serum, and substances which increase the secretive properties of glandular cells, such as pilocarpine, enormously increase the output, so to speak, of antitoxin.

In view of these facts Ehrlich elaborated his "side-

chain theory," a theory which, whether it be the real explanation or no, has received a considerable amount of experimental support, and has had far-reaching effects in stimulating research. Ehrlich believed that the chemical activities which are the manifestations of the vital activities of the living cell are due to a very large nucleus or chemical molecule having a ring structure, analogous to the benzene ring, and having attached to it a number of atomic groups or "side-chains." A "side-chain" is an atomic group, a carbon atom of which is linked to one

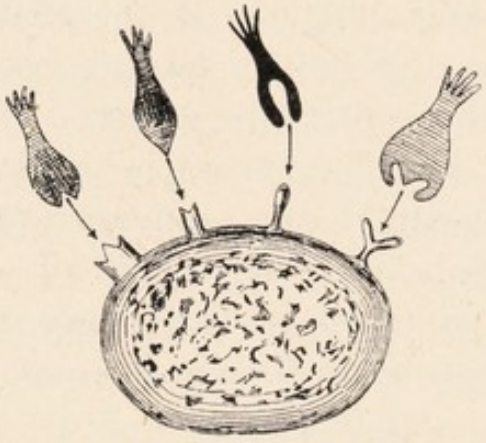


FIG. 28.—Diagram to represent the cell with its various combining groups or side-chains. (After Ehrlich.)

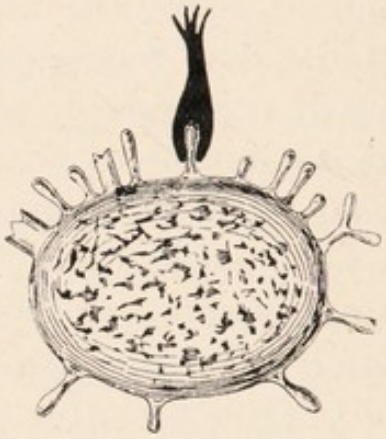


FIG. 29.—First stage in antitoxin formation. Black = toxin molecule. (After Ehrlich.)

of the carbon atoms in a ring. These atomic groups or side-chains are unstable in nature, and enter freely into combination with other suitable groups should these be presented to them, and thus the physiological activities of the cell, assimilation, nutrition, etc., are carried out (Fig. 28). Now Ehrlich supposes that antitoxin is merely an excess of certain side-chains which are normally present and subserve some of the ordinary functions of the cell and which have become free in the blood. The antitoxins being specific, by this assumption the difficulty is obviated of supposing that special chemical

groups or molecules exist preformed ready to combine with a number of different toxins on the remote chance that some one of these may at some time or other come within the particular sphere of action of one of those groups. Moreover, small amounts of anti-bodies, such as antitoxin, bacteriolysin, agglutinin, etc., are met with in normal untreated animals and in man. While some have supposed that the small amount of diphtheria antitoxin (equivalent to half a unit or so) present in human blood-serum is due to an infection with the diph-

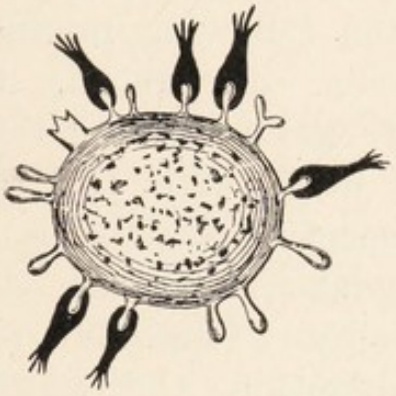


FIG. 30.—Second stage in anti-toxin formation. (After Ehrlich.)

FIG. 31.—Third stage in anti-toxin formation. Side-chains beginning to be produced in excess. (After Ehrlich.)

theria bacillus (not necessarily an attack of diphtheria), it seems more reasonable to suppose that this antitoxin is due to a natural liberation of such side-chains from the protoplasm and that artificial antitoxin production is merely a very great stimulation of this natural process.

The toxin molecule, according to Ehrlich, possesses at least two fixative atomic groups or side-chains. One of these, the "haptophore group," conditions the union of the toxin molecule with cell-protoplasm; the other, the "toxophore group," conditions its toxic action. Similarly, in order that the cell may suffer the full effect of

the action of the toxin, it also must possess two receptive groups or side-chains having a maximum affinity for the haptophore and toxophore groups of the toxin; these may be termed the "receptor" and "toxophile" groups respectively (see Fig. 33). The relationship of each fixative group of the corresponding groups—viz. that of the toxin and that of the side-chain of the cell—must be most intimate, and analogous to the relations to each other of a male and a female screw (Pasteur) or of a lock and its key (E. Fisher).

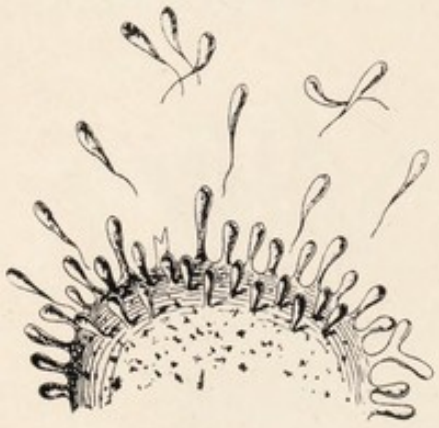


FIG. 32.—Fourth stage in antitoxin formation. Side-chains, *i.e.* antitoxin, free in the blood. (After Ehrlich.)

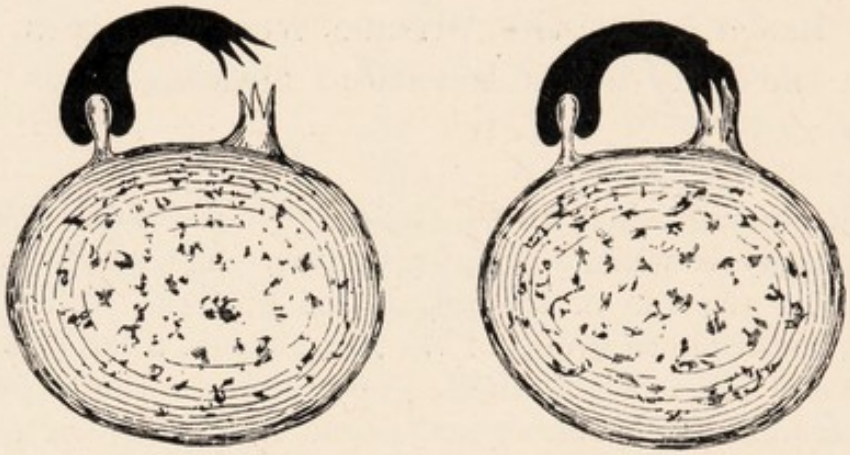
The genesis of antitoxin on the "side-chain theory" takes place in the following manner: Toxin being introduced, the haptophore groups of the toxin molecules unite with the particular receptor side-chains of the protoplasm for which they have an affinity (Fig. 29). By this combination the physiological activities of the cell are interfered with, a defect is created, the cell is damaged (it is only necessary to consider the case of one cell, or, more strictly of one molecular group of the cell-protoplasm). But through its recuperative powers the cell soon recovers by the formation of new receptor side-chains to take the place of those which have been put out of action. On injecting more toxin, this combines with these new receptors and a defect is again created (Fig. 30). Once more the cell responds, and a fresh series of receptors is developed (Fig. 31). But by this continual stimulation, as it were, the cell commences to form the particular receptors *in excess of that needed to repair the defect created*, and ultimately these receptors are reproduced in

such numbers that they no longer all remain attached to the cell but some become free in the plasma (Figs. 31, 32). *These receptor side-chains, detached from the cell and floating free in the blood-stream, constitute the antitoxin.* This excessive production of side-chains after stimulation by repeated injections of toxin is not a phenomenon confined to anti-toxin formation, but is a general physiological law enunciated by Weigert ; as a result of repeated stimulation, over-production or hyper-compensation is the rule and is met with in various pathological processes. Ehrlich has termed the diverse free receptors which occur in the body fluids in various circumstances " haptines."

Ehrlich distinguished three classes of receptors. *Receptors of the first order*, to which belong antitoxins, which possess a haptophore group capable of saturating the affinities of the corresponding haptophore group of the homologous toxin ; *Receptors of the second order*, which have, in addition to the haptophore group, a second or ergophore group which, after union with the antigen has taken place, is able to bring about the changes peculiar to the particular type of antibody. The precipitins and agglutinins belong to this class. *Receptors of the third order* : In addition to the haptophore group the antibodies possess a complementophile group which unites with complement which is necessary to complete the lysis of corpuscle or bacterium (p. 199). The opsonins and the antibodies concerned in complement fixation belong to this class.

The existence of both haptophore and toxophore groups in the toxin molecule is suggested by the following experiments. Tetanus toxin injected into the blood-stream of an animal rapidly disappears, within a few seconds of the injection, and even if the animal be at once bled, the blood withdrawn being replaced by fresh blood, tetanus ensues, but not until after the lapse of an incubation period of some hours. The tetanus toxin, therefore, immediately becomes fixed or anchored to the tissues

of the central nervous system. Evidently the toxin molecules by means of their haptophore groups enter at once into combination with the nerve-tissues; this after a time brings the cells within the sphere of influence of the toxophore groups, and after a certain incubation period toxic symptoms ensue (Fig. 33). The affinity of tetanus toxin for nerve tissues may be shown in another way. If fresh guinea-pig brain be emulsified with tetanus toxin, the emulsion will be found to be innocuous on injection,



A B

FIG. 33.—Diagrammatic scheme to represent the union of toxin (black) with the cell. In A the toxin is attached to the protoplasm by the union of the haptophore and receptor groups. In B the toxophore and toxophile groups have also united, and poisoning now ensues.

owing to a combination between the two having taken place. The cerebral cortex of a highly susceptible animal (*e.g.* mouse) has a marked neutralising power, of a less susceptible animal (*e.g.* rabbit, fowl) a feebler, and of an insusceptible animal (*e.g.* frog, tortoise) no neutralising power.[1] Moreover, both diphtheria and tetanus toxins may be converted into non-toxic modifications ("tox-

[1] The combination of brain matter with tetanus toxin seems to be specific and of the same order as that between antitoxin and toxin. See Noon, *Journ. of Hyg.*, vol. vii, 1907, p. 101, and Besredka and Bordet, *Ann. de l'Inst. Past.*, xvii, 1903.

oids ") which to some extent retain the power of immunising and of producing antitoxin on inoculation, and of combining with antitoxin : that is to say, according to Ehrlich, the toxophore groups have been destroyed while the haptophore groups remain unaffected. It is the presence of the haptophore group which conditions the union of toxin with antitoxin. Thus, if toxin be injected into blood containing antitoxin, the haptophore groups of the toxin unite with the free receptor groups, *i.e.* with the antitoxin (Fig. 34), and therefore the toxophore groups cannot exert their influence because the toxin is now unable to unite with the protoplasm, its haptophore or binding groups being already occupied.

FIG. 34.— Neutralisation of toxin by antitoxin in the blood. (After Ehrlich.)

In a poisonous toxin, such as diphtheria or tetanus toxin, the toxophore group is more readily destroyed than the haptophore group, and by heating a toxin for some time to 60°–70° C. its toxicity is destroyed, but it still retains an affinity for antitoxin. If some antitoxin be mixed with such heated toxin it will be found that the capacity of the former for neutralising active toxin is much diminished—in other words, although the toxophore groups of the heated toxin have been destroyed, the binding or haptophore groups still remain. Toxin which has been kept for some time decreases in toxicity, but retains the power of combining with antitoxin, again showing that haptophore or binding groups are present (such derivatives of toxin possessing haptophore groups are termed " toxoids "). Wassermann and Bruck have obtained presumptive evidence of the existence of the

second stage in antitoxin formation, viz. the increased production of receptors by the cells. Using tetanus toxin which had been kept for some time and had lost its toxicity, but which still combined with antitoxin—that is, toxoids with haptophore groups were still present—they found that on injecting it into animals *no* antitoxin was formed as a result of the injection. They then performed some experiments based on the following line of reasoning: If the old non-poisonous tetanus toxin containing these toxoids be first injected into an animal, and after a short interval, some fresh, actively poisonous tetanus toxin, more of the active toxin ought to be required to kill this animal than a normal one, because, owing to the previous toxoid injection, part of the cell receptors susceptible to tetanus toxin are already occupied. Provided Ehrlich's theory be correct, so that this binding of the toxoid really occurs, the conditions should be entirely different, when, instead of injecting the toxin shortly after the toxoid, a longer time elapsed—one to three days—before the injection of the active tetanus toxin. For in that case Weigert's law should come into play and the receptors should have increased in number—*i.e.* the animal would now possess *more* sensitive groups than before. This should be manifest by the fact that, in contrast to the first experiment, the fatal dose of active tetanus toxin ought now to be smaller than previously; in other words, a smaller dose should now tetanise the animal in a shorter time. The experiments yielded results which were exactly in accordance with these theoretical considerations. A guinea-pig was injected with some of the non-poisonous toxoid, and then, one hour later, with the active tetanus toxin. It was found that much more toxin was required to kill this animal than a normal guinea-pig of equal size. If, on the contrary, an interval of one to three days were allowed to elapse, it was then

found that a dose of tetanus toxin which would not even tetanise a normal guinea-pig was sufficient to kill the treated one.

The fact that no antitoxin was formed—*i.e.* no receptors are thrust off—by the single injection of the non-poisonous toxin, or toxoid, Wassermann ascribed to the lack of stimulus which he suggests resides in the toxophore groups.

The slow combination of the haptophore and receptor groups has been proved by Wassermann in another way. The researches of Meyer and Ransom have shown that tetanus toxin is absorbed by the nerve-trunks, not by the blood and lymph-channels, while tetanus antitoxin is absorbed by the latter—the blood and lymph-channels. Adrenalin is a substance which strongly contracts the capillaries, and thus tends to block absorption in a particular area. The following experiment was devised: Tetanus toxin and antitoxin were mixed in such proportions that the mixture was innocuous to animals, *i.e.* it was just neutral. If this mixture be injected into the hind paw of a guinea-pig no tetanus develops. When, however, some adrenalin is injected into the hind paw of a similar-sized guinea-pig, and a few minutes are allowed to elapse so that the capillaries may contract, and then the mixture of toxin and antitoxin is injected, typical tetanus ensues. The explanation of this is that the channel of absorption for the tetanus *antitoxin*, the vessels, is blocked by the adrenalin, while that for the *toxin*, the nerve path, remains open. The toxin and antitoxin had not yet combined, or such combination as had occurred is a loose one and becomes dissociated, and, therefore, the toxin travelled along the nerves to the central nervous system with the production of tetanus.

The experiment, however, succeeds only within a certain period, not exceeding an hour after mixture of the

toxin and antitoxin, because after this the toxin-antitoxin combination becomes a stable one.

If a longer time—say three or four hours—is allowed to elapse, it will be found that, even in the adrenalin animal, no tetanus is produced, because by this time the combination, previously a loose one, has become so stable that the substances can no longer be dissociated. This union can be hastened by employing more tetanus antitoxin, for with an excess of antitoxin, even after only half an hour, it is impossible by means of adrenalin to free the tetanus toxin. This experiment, therefore, shows that the combination of tetanus toxin with antitoxin takes place slowly and is at first a loose one, and that the union becomes firmer and firmer with lapse of time. It also suggests the possibility of hastening the combination by increasing the amount of antitoxin—a point of considerable practical value in serum therapy.

The above considerations are of importance in the antitoxin treatment of disease. Antitoxin, in the strict sense, is not antimicrobic, and therefore antiseptic treatment of the throat in diphtheria, and of the wound in tetanus, should be pursued. The fact that the toxophore group of the toxin does not come into action as a rule for many hours at least (an exception is snake-venom) is a fortunate coincidence, for the antitoxin may, therefore, act before tissue damage has occurred. Antitoxin cannot repair tissue damage if this has been produced by the toxin, but it can, and does, prevent the occurrence of further damage by neutralising any fresh amounts of toxin that may be absorbed. Hence the necessity for early treatment. Toxin already anchored to the tissues by its haptophore group may for some time be dissociated from them if a *multiple* of the simple neutralising dose of antitoxin be injected, and the quantity necessary to accomplish this rises rapidly as the interval between the introduction of the toxin and of the antitoxin increases ; hence the necessity for the use of antitoxin in large excess. Probably the union between tissue and toxin at first is a loose one, and a large amount of antitoxin by mass action transfers the affinity of the toxin from the tissues to itself It must be clearly recognised that

colloidal reactions (to which category that between antitoxin and toxin, anti-body and antigen, belongs) differ considerably from ordinary chemical reactions.

An essential condition in antitoxic treatment is the administration of a sufficient amount of anti-serum, and this does not depend on the actual volume of serum injected. The anti-serum may be regarded as a solution containing a variable amount of the antitoxic or anti-microbic constituent, and for therapeutic use its strength must be ascertained, and is for convenience described in arbitrary units.

The dose of antitoxin is dependent upon the gravity of the disease, and not on the age of the patient, for evidently just as much toxin may be formed in a child as in an adult. The antitoxins are strictly specific ; diphtheria antitoxin, for example, has not the slightest influence in tetanus.

To obtain an immediate reaction to antitoxin it should be administered intra-venously. A subcutaneous injection may not be completely absorbed in less than thirty-six hours, an intramuscular injection is much more rapidly absorbed.

In cases of mixed infection, *e.g.* where diphtheria bacilli are associated with streptococci or staphylococci, the diphtheria antitoxin will have no influence on the streptococcic or staphylococcic infection.

The complications and accidents of antitoxin treatment are few and usually unimportant. Abscess and other local troubles at the seat of inoculation should not occur if proper antiseptic precautions be taken. Urticaria or other rashes and joint pains are by far the most troublesome complications. These are due to the injection of foreign serum, and not to the antitoxin, for the serum of an untreated horse produces a like effect. Repeated injections of serum at short intervals may be continued for a long period without inducing more disturbance than that caused by one or two or a few injections, but if twelve days or more elapse between two injections a condition of "supersensitation," due to anaphylaxis, ensues (see p. 182). This consists in the rapid appearance of rashes, joint pains, pyrexia, etc., or even of grave symptoms, faintness, vomiting, dyspnœa, convulsions, collapse, etc. This, however, is preventable (see p. 189).

Anti-sera may be used as prophylactics, but the immunity produced by them does not last more than three weeks.

Various hypotheses have been advanced to explain

the manner in which toxin is neutralised by antitoxin. Roux and Buchner suggested that the antitoxin in some way renders the cells and tissues insusceptible to the toxin, and Buchner performed experiments showing that while mice are more susceptible than guinea-pigs to tetanus toxin, a tetanus toxin-antitoxin mixture which is just neutral for mice is distinctly toxic for guinea-pigs.

To explain this Ehrlich suggested that there may be present in a toxin solution several toxic substances, some of which exert a toxic action on the guinea-pig but not on the mouse. Madsen and Dreyer showed that a mixture of diphtheria toxin and antitoxin which is innocuous to guinea-pigs on subcutaneous inoculation is lethal to rabbits on intra-venous injection, and in order to explain this Ehrlich made a similar assumption. Morgenroth, however, found that the difference in the latter case depends on the mode of injection. The reaction between the toxin and antitoxin takes time to complete : there is an interval probably of some hours at 20° C. before equilibrium is reached (see also next page). When a recently prepared mixture of toxin and antitoxin is injected subcutaneously, absorption is slow, and in the meanwhile the toxin and antitoxin combine, but when the mixture is injected into the veins, the toxin is fixed by the tissues before it has had time to combine with the antitoxin, and poisoning ensues. If the mixture be kept for some hours before injection, intravenous injection is then innocuous.

Ehrlich concluded that diphtheria toxin is neutralised by diphtheria antitoxin much in the same way as a strong base is neutralised by a strong acid, and that the course of neutralisation suggests the presence in the toxin of several toxic and atoxic substances (toxoids and toxones), all of which combine with, though they have different affinities for, the antitoxin.

Arrhenius and Madsen, however, believe that the toxin-antitoxin reaction is analogous to the action of an acid on an alcohol, and that the chemical laws of mass action apply equally to the two. The chief reaction is considered to be between two substances only, toxin and antitoxin, that it is reversible, and that when the system has reached equilibrium, a fraction of toxin and also of antitoxin remain free, this fraction of toxin producing the "toxone effect" (see p. 179). If equivalent quantities of acetic acid and alcohol are mixed, the reaction is never complete; the acid and alcohol never entirely disappear, because the water formed reacts with the ethyl acetate, re-converting it into acid and alcohol. Such a reaction is termed reversible, and this particular case could be thus represented:

$$CH_3.COOH + C_2H_5.OH \rightleftarrows CH_3.COOC_2H_5 + H_2O.$$

Bordet has suggested that the fixation of toxin by antitoxin is an adsorption phenomenon, similar to the fixation of a dye by a tissue.

These hypotheses may now be examined more in detail. Ehrlich's experiments [1] on diphtheria toxin seemed to show that the neutralisation of toxin by antitoxin follows the laws of simple chemical combinations, such as the neutralisation of a strong base (NaOH) by a strong acid (HCl). If so, it would be expected that antitoxin would neutralise proportionate amounts of toxin; but this is not so, and Ehrlich was forced to the conclusion that toxin is a complex mixture of proto-, deutero-, and trito-toxin, and toxone, with different toxicities and different avidities for antitoxin. Moreover, when toxin is kept it decreases in toxicity, though still retaining much of its avidity for antitoxin. Ehrlich assumed, therefore,

[1] See *Trans. Jenner Inst. Prev. Med.*, vol. ii, p. 1; *Croonian Lect. Roy. Soc. Lond.*, 1900; and p. 314.

that the toxin becomes transformed into substances termed toxoids, which are non-toxic but retain their affinity for antitoxin (see also section on the standardisation of diphtheria antitoxin). This he explained as due to destruction of the unstable toxophore groups, with the retention of the more stable haptophore groups. That the neutralisation of toxin by antitoxin is due to some sort of union between the two, though not necessarily chemical combination in the strict sense, seems to be proved by the work of Martin and Cherry. Brodie,[1] and Martin and Cherry,[2] making use of a Chamberland filter, the pores of which had been rendered very fine by saturating with gelatin, found that toxin would pass through such a filter but that antitoxin would not, presumably because the molecule of the latter is larger. By mixing diphtheria toxin and antitoxin in such proportion that the latter was in sufficient quantity to neutralise the toxin, and subjecting the mixture to filtration through a gelatin filter, the filtrate was found to be non-toxic. Now since toxin can pass through such a filter, the inference is that the toxin has united with the antitoxin. Using snake-venom and its anti-serum or anti-venin, another method was employed. The anti-venin is destroyed by heating to 68° C. for ten minutes, while the toxic properties of the venom are unaltered by this treatment. By making mixtures of venom and antivenin, and, after a certain time has elapsed for the interaction to take place, heating to 68° C. for ten minutes, it was found that the mixture is non-toxic, pointing to the union of the toxin (venom) with the antitoxin (anti-venin). Calmette had performed the same experiment but with a different result, finding his mixtures still toxic after heating. Calmette, however, treated his solutions almost

[1] *Journ. of Path. and Bact.*, 1897, p. 460.
[2] *Proc Roy. Soc. Lond.*, vol. lxiii, 1898, p. 420.

immediately after mixing, and Martin and Cherry point out that a certain *time* must be allowed to elapse for the interaction to take place, and noted that moderate warming hastens it, as is the case with all chemical interactions. For instance, they found that one mixture of venom and anti-venin allowed to interact for two minutes, five minutes, and ten minutes before heating, killed the animals in thirteen hours, fifteen hours, and twenty-three hours respectively (the control animal with the same dose of venom died in nine hours), but after fifteen minutes the same mixture rendered the animal ill but it survived, while after thirty minutes no toxic symptoms ensued.

At one time it was stated that by electrolysis of toxins small amounts of antitoxin are formed, but this is very questionable. Electrolysis destroys the toxicity of toxins by the production of acids, chlorine, and hypochlorites.

Ehrlich's views have been opposed, principally on physico-chemical grounds. Thus, Danysz, observed that if ricin or diphtheria toxin be brought into contact with its corresponding antibody, the degree of neutralisation depends on the manner of mixture. If the toxin be added to the antitoxin in two fractions, allowing a considerable time to elapse between the additions, the mixture contains a much larger amount of free toxin than is the case when the whole (and same) amount of toxin is added at once to the antitoxin. This phenomenon, known as the "Danysz or toxone effect," seems inexplicable if toxin and antitoxin have relations the same as a strong base and a strong acid.

Arrhenius, Dreyer, and Madsen maintain that the phenomena observed in the toxin-antitoxin reaction are explicable on the hypothesis that the rate of reaction—avidity—of the toxin decreases as antitoxin is added, that the interaction is a slow one, and that different fractions of the toxin are progressively neutralised by the added antitoxin, but more and more slowly. On these grounds they consider that there is no reason to regard the diphtheria poison as a highly complicated body. Whereas Ehrlich considers the toxin and antitoxin to combine with great avidity, analogous to the combination of a strong base with a

strong acid, *e.g.* NaOH with HCl, these critics believe the avidity of antitoxin to be feeble, analogous to the combination of ammonia with boric acid, in which as more and more acid is added, the amount of free ammonia decreases, but more and more slowly, in correspondence with a hyperbolic curve. The phenomena can be calculated according to the law of "mass action," there being an equilibrium between

$$\frac{\text{Free } NH_3.}{\text{vol.}} \quad \frac{\text{Free } H_3O_3B}{\text{vol.}} = K \frac{(NH_4H_2O_3B)^2}{\text{vol.}}$$

where K is the constant of dissociation. The curve of the neutralisation of tetanolysin by anti-tetanolysin corresponds almost exactly to the ammonia-boric-acid curve.

Whereas on Ehrlich's views the combination of toxin and antitoxin would be represented by a straight line, and the crude toxin seems to be composed of a whole series of different toxins and substances having an avidity for antitoxin, on this hypothesis, although the greater part of the toxicity of toxin is removed by the antitoxin, the latter must be added in large excess before the toxicity completely disappears, and the course of neutralisation would be represented by a hyperbolic curve. In fact, as the antitoxin is added, the amount of free toxin diminishes but never completely disappears. There comes a point, of course, when the amount of free toxin is so small as to be negligible and cannot be recognised by the ordinary indicators (blood-corpuscles, animal tests, etc.). This hypothesis would explain the fact that while a certain amount, V, of a mixture of toxin and antitoxin is innocuous to an animal, a multiple of the dose, n V, of the *same* mixture may be toxic; it would also explain Buchner's experiments alluded to above (p. 176), and Roux's experiments in which a toxin-antitoxin mixture innocuous to normal guinea-pigs was toxic to guinea-pigs whose resistance had been reduced by injections of the Massowah vibrio.

Nernst has questioned from the mathematical standpoint the validity of the views of Arrhenius, and so did Craw from much experimental work on agglutination and on the interaction between megateriolysin and anti-megateriolysin; Craw also considered that there is some doubt attaching to Arrhenius's calculations. According to Craw, the two substances most thoroughly investigated by Arrhenius and Madsen, diphtheria toxin and tetanolysin, do not admit of sufficiently exact determin-

ation, the former because of the uncertainty attaching to animal experiments, the latter because tetanolysin is a most unstable body. Working with a more stable substance, megateriolysin, he held that the Arrhenius and Madsen equation does not apply. Again, on the addition of a small amount of antitoxin to toxin there is no decrease in toxicity (as noted by Ehrlich and attributed by him to the presence of toxoid) as there should be, and Arrhenius was thus forced to the conclusion that a second substance, epitoxonoid, is present with the toxin in diphtheria toxin. Craw denies that the toxin-antitoxin reaction is reversible, believes that antitoxin must be regarded as a colloid (and is not in true solution), that the mixture therefore is heterogeneous, not homogeneous, and that the chemical law of mass action is not applicable.

On the other hand, Craw maintains that the phenomena of the toxin-antitoxin reaction, including the Danysz effect, have their counterpart in adsorption phenomena, such as occur in the staining of paper, porcelain, etc., with anilin dyes, in the " adsorption " of substances by colloids, etc.,[1] and this view is supported by Bordet, Gengou and H. R. Dean. Thus, when solutions of arsenious acid are shaken up with colloidal ferric hydroxide, a portion of the arsenic is ta ken up by the ferric hydroxide and a portion remains in solution. Moreover, proportionately more arsenious oxide is taken up by ferric hydroxide from dilute than from concentrated solutions ; this has its counterpart in agglutination. Again, when an antitoxin is added to a toxin in just sufficient amount to produce a non-toxic solution, the amount of toxin which must then be added to constitute a fatal dose is greater than the minimum lethal dose without antitoxin. This is also found to be the case with ferric hydroxide and arsenious acid ; if ferric hydroxide and arsenious acid are mixed so as to form just a non-toxic mixture, the amount of arsenious acid which must then be added to render the mixture toxic is greater than the toxic dose of arsenious acid.[2]

If pieces of filter-paper be placed in a dilute solution of stain at sufficiently long intervals, the pieces first immersed will become coloured while those last immersed will remain colourless. On

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[1] " Adsorption " is physical in nature and mainly due to surface condensation.

[2] See Findlay, *Physical Chemistry and its Applications in Medical and Biological Science*, 1905.

the other hand, if all the pieces be simultaneously placed in the solution they all become coloured to the same degree. This is exactly comparable to the Danysz effect. All the phenomena of the toxin-antitoxin reaction seem best explained on the adsorption hypothesis of Bordet. Specificity, it is true, is not completely explained thereby, nor is it explained by any other hypothesis.[1]

The antitoxic constituent of antitoxin seems to be a protein body, probably allied to globulin, and, as already mentioned, the globulin content of the blood of an animal treated for antitoxin production increases in some cases. Tizzoni, by precipitating the antitoxic serum by saturation with magnesium sulphate at 30° C., obtained the antitoxin in the precipitate. By partial saturation of antitoxic serum with ammonium sulphate, the antitoxin is carried down with the second precipitate, that is with the pseudo-globulin fraction. It is thus possible to concentrate antitoxic serum and to make use of a weak serum, which would otherwise be inconvenient on account of the volume necessary to inject in order to introduce the requisite amount of antitoxin. For this purpose various salts have been employed for saturation, ammonium sulphate (Pick and others), magnesium sulphate (Dieudonné), mixtures of sodium and potassium chlorides (Atkinson), etc.

Dzergowski and Predtéchensky [2] have elaborated a very exact method by which they state that the whole of the antitoxin can be concentrated and recovered from a comparatively weak serum by means of precipitation with ammonium sulphate. Homer describes another method (*Journ. of Hygiene*, vol. xv, 1916, p. 388).

ANAPHYLAXIS.—An animal usually becomes more and more tolerant to injections of an antigen, *e.g.* to diphtheria and tetanus toxins in the preparation of the corresponding antitoxins. Sometimes, however, the opposite

[1] On the toxin-antitoxin reaction see Craw, *Proc. Roy. Soc. Lond.*, B, vol. lxxvi, 1905, p. 179; *Journ. of Hyg.*, vol. vii, 1907, p. 501; and *ibid.* vol. ix, 1909, p. 46; Arrhenius, *Immuno-chemistry*, 1907, and *Journ. of Hyg.*, vol. viii, 1908, p. 1; Madsen, *Brit. Med. Journ.*, 1904, vol. ii, p. 567; Bordet, *Ann. de l'Inst. Pasteur*, xvii, p. 161; McKendrick, *Proc. Roy. Soc. Lond.*, B, vol. lxxxiii, 1911, p. 493; Gengou, *Journ. of State Med.*, xx, 1912, pp. 65 and 141 (Bibliog.); Dean, *Lancet*, 1917, vol. i, p. 45 (Bibliog.).

[2] See Hewlett's *Serum Therapy*, 1910, p. 68.

effect is produced, viz. increased sensitiveness. This has been noticed in the preparation of tetanus antitoxin ; after the animal has received a few doses of the toxin without ill-effect, a smaller dose of toxin may cause fatal tetanus. The tuberculin reaction is, probably, another example ; tubercle toxins circulating in the tuberculous individual render him peculiarly sensitive to a minute dose of tuberculin (*i.e.* tubercle toxin) which in a normal person produces no effect. Sensitisation may be obtained, though with difficulty, by administration by the mouth, and this may be the explanation of the urticaria, etc., produced in some individuals by certain foods, *e.g.* shell-fish. This condition of hypersensitiveness is known as "anaphylaxis" (*i.e.* the opposite of "prophylaxis"). Probably any antigen under appropriate conditions may induce anaphylaxis, but the phenomenon has been especially studied in connection with serum injections, though any protein, *e.g.* egg-white or bacterial cells, similarly causes it. The injection of an anti-serum usually produces no ill-effect other than the rashes, joint pains, and pyrexia already mentioned, even if large amounts of the serum be given extending over days or even weeks, but a second injection of serum given after a first injection with an interval of twelve days or more between the two series of injections is liable to be followed by effects which may be more or less serious, constituting the so-called "anaphylactic shock" or "serum disease." Minor disturbances in the form of immediate or accelerated reactions, "supersensitisation," may at other times ensue (see p. 175).

The symptoms of anaphylactic shock are nausea and vomiting, small and rapid pulse, faintness or more serious heart failure, dyspnœa with rapid and shallow respiration and feeling of suffocation, collapse, rigors, convulsions, and even coma. The severity of the symptoms varies in

different cases, and the symptoms usually pass off in the course of an hour or two ; but a few fatal cases have been recorded. Death is easily produced experimentally, and, post-mortem, scattered ecchymoses are found and a distended condition of the lungs due to spasm and contraction of the bronchioles, to which the fatal event is due.

The ordinary sequelæ of serum injections, the rashes, etc., never appear before the seventh day after the first dose of serum, but in the immediate reaction, rash, pyrexia, joint pains, vomiting, rigors, and occasionally convulsions and collapse occur, either almost immediately or within six hours after the second injection of serum. In the accelerated reaction, these phenomena appear between the eighteenth hour and the fifth day after the second injection of serum.

The immediate and accelerated reactions may occur a long time after the first course of serum treatment if more serum be given. Goodall records one case in which over four years elapsed between serum treatments for first and second attacks of diphtheria, an accelerated reaction occurring after the reinoculation for the second attack.

The Arthus phenomenon occurs when a guinea-pig receives several doses of normal horse serum at intervals of some days. Another injection of horse serum then causes an œdematous mass, an aseptic abscess, or an area of necrosis at the site of the new inoculation, which may be far removed from the region of the previous inoculations, and the animal becomes cachectic and dies.

The Theobald Smith phenomenon occurs when a guinea-pig has been sensitised by a very small single dose of normal horse serum, 0·01 c.c., 0·001 c.c., or even 0·000001 c.c. ; if, then, after an interval of twelve to fourteen days a somewhat larger dose of serum, 0·1 c.c., be given, the serious symptoms of hypersensitiveness develop within a

few minutes, viz. respiratory failure, paralysis, clonic spasms, and frequently death. At one time it was believed that a small sensitising dose is more effective than a large one in producing anaphylactic shock, but it has been shown that this is not the case, a large dose merely lengthens the incubation period (up to, it may be, forty days). The reason for this may be that the toxic substance slowly formed by the sensitising dose combines as it is produced with a part of the antigen injected, so that the ultimate result is as though a small sensitising dose had been injected. The first dose, or series of doses, of antigen inducing the sensitive state is known as the sensitising dose, the second dose of antigen causing the effect is known as the reacting or toxogenic dose.

The cardinal features of anaphylaxis are—(1) Any antigen will cause it, *e.g.* serum, egg white, toxins, etc. ; (2) the sensitising and toxogenic doses must be of the same kind of antigen, *e.g.* horse serum and horse serum ; thus a first dose of horse serum followed by a second dose of sheep serum would *not* induce anaphylaxis. Closely allied proteins may cause some reaction ; (3) the amount of antigen given may vary within wide limits without definitely influencing the severity of the effect ; (4) sensitisation is usually produced in from twelve to fourteen days after the administration of the sensitising dose, never before ; with massive sensitising doses it may be delayed for a period up to six weeks ; (5) sensitisation once induced may exist for years ; (6) reaction having once occurred, the animal is no longer sensitive ; (7) extremely small doses of antigen will sensitise, even 0·000001 c.c. of serum, though apparently there is no limit in the other direction ; (8) anæsthetisation when the toxogenic dose of antigen is given prevents the development of symptoms.

Animals vary greatly in the ease with which they are sensitised. The guinea-pig, horse and goat are very

readily sensitised, the rabbit, dog and probably man are less susceptible, the mouse is almost or completely refractory. Sensitisation may be induced by any route which ensures the penetration of the antigen in a relatively intact state. The digestive tract, therefore, usually fails unless large quantities of the antigen are given. In the case of the toxogenic dose, antigen administered by the mouth or rectum rarely induces anaphylactic shock ; the most certain methods are intracerebral and intravenous injections, next intramuscular, then intraperitoneal, intrapleural and subcutaneous, and finally intrathecal.

A theory to explain all the phenomena of anaphylaxis is difficult to formulate. The sensitising dose of antigen undoubtedly produces some anti-body which reacts with, or in the presence of, the second dose of antigen. The necessity for an incubation period after the sensitising dose points to this, as well as the fact that "passive" anaphylaxis may be induced by injecting an animal with the serum of a sensitised one ; the treated animal suffers from anaphylactic shock on being injected with the antigen. The substance which gives rise to the anaphylactic shock is termed "apotoxin" by Richet and "anaphylatoxin" by Friedberger, and it may be akin to a precipitin.

Discussion has taken place as to whether the reaction occurs in the cells, the cellular theory, or in the body fluids, the humoral theory. Experiments by Dale and others show that reaction does take place in tissues free from plasma. Dale [1] sensitised guinea-pigs, then excised the uterus and washed it free from blood and suspended it in Ringer's solution. On flooding the uterus with the homologous antigen contraction occurs. Specificity is shown by the fact that only the homologous antigen

[1] *Journ. Pharmacol. and Exper. Therapeutics*, iv, 1913–14, p. 167.

causes contraction, and an animal may be sensitised to two or three different antigens and the uterus contracts when it is flooded in turn with the homologous antigens. Once the reacting dose has been given and the uterus has contracted, the muscle is no longer sensitive to the antigen. But the substance produced by sensitising is also present in the blood, as is shown by the fact that passive anaphylaxis may be produced. Probably anaphylaxis is both cellular and humoral and can occur in the blood or in the tissues, usually in both ; all that is essential is the presence of the requisite amount of anti-body. The cells will take up a certain amount of the anti-body and any excess remains in the blood.

There is a striking analogy between peptone poisoning and anaphylactic shock and protein cleavage products may be the substances concerned in the production of the latter.

Gay and Southard believe that every antigen consists of two distinct portions and suppose that during sensitisation one of these, a toxic portion, is eliminated, and that the other, the sensitising, is retained. The latter acts in some way upon the body cells, rendering them capable of being affected by the toxic moiety of the antigen, whereas previously they are incapable of being so affected. This, however, does not explain *humoral* anaphylaxis.

Besredka also assumes that there are two distinct elements in antigen, one thermostable and having the properties of an antigen (see p. 164), which he terms "sensibilisogen," and which on injection produces its anti-body, "sensibilisin." The other substance is thermolabile, and is termed "anti-sensibilisin," and combines with sensibilisin whenever it meets with the latter. Sensibilisin is particularly fixed by the cells of the nervous system, and, according to Besredka, it is the violent

reaction between anti-sensibilisin, and sensibilisin in the nerve tissues which causes the serious disturbance characteristic of anaphylaxis. When, therefore, a small dose of serum ($\frac{1}{100}$–$\frac{1}{30}$ c.c.) is administered, the sensibilisogen slowly forms sensibilisin. If a second dose of serum is given twelve days or more after the first injection, the anti-sensibilisin in it combines with the sensibilisin formed by the first injection, and disturbance results. Anæsthetisation prevents the symptoms of anaphylaxis because the anæsthetic renders the nerve cells insensitive to the reaction between the sensibilisin and antisensibilisin.

According to Richet, a "toxigen" is formed in the blood or cells at the end of the incubation period and persists for a long period. A toxic apotoxin or precipitin is formed as a result of the interaction of toxigen with antigen, the toxicity of which is further increased by combination with the alexin of the blood.

Bordet suggests that the union of anti-body and antigen creates a complex which by adsorption monopolises certain principles in the blood plasma which then becomes toxic. Thus Wassermann and Reysser found that if guinea-pig serum and kaolin, an inert powder, be mixed and then centrifuged, the intravenous injection of the fluid is followed by symptoms closely resembling those of anaphylaxis. A weak agar jelly (0·05 per cent.) acts similarly. The serum must be fresh and active; serum heated to 56° C. is inert. It is doubtful, however, whether colloids do produce typical anaphylactic death; when death occurs it is probably due to intravascular clotting.

A remarkable feature of anaphylactic shock is the almost complete disappearance of complement, but this is not the immediate cause of the condition. Friedberger found that complement is able to form from antigen a toxic body which on injection into a normal animal gives rise to all the phenomena of anaphylactic shock. But

while complement alone is capable of effecting this change to a certain extent, the toxic product appears far more rapidly and in much greater quantity if the specific anti-body be present as well. The condition necessary for maximum toxicity, *i.e.* for the formation of the largest amount of *anaphylatoxin*, depends upon three factors, (*a*) the relative quantities of complement, antibody and antigen present together, (*b*) the time during which these substances interact, and (*c*) the temperature at which the reaction occurs. As regards the quantities of reacting substances, for a given amount of antigen there is a definite range within which complement and antibody give rise to anaphylatoxin, but outside which, *i.e.* if either be in excess or deficient, the toxin does not appear. If the time be insufficient, the mixture is non-toxic ; if the time be extended beyond certain limits, the mixture is also non-toxic, because protein-cleavage proceeds so far that the toxic substances are split up into simpler non-toxic bodies. The lower the temperature, the slower the formation of anaphylatoxin ; the optimum temperature is 37° C. This theory not only accounts for the production of anaphylaxis, it also explains why anaphylaxis does not occur after repeated injections of antigen (*e.g.* anti-toxic serum) at shorter intervals than twelve to fourteen days. In the latter case, anti-body is present in so large amount that antigen is subjected at once to such a degree of cleavage that the toxic stage is rapidly passed and the non-toxic stage reached, and thus there is never sufficient toxin present to cause symptoms. Anaphylaxis is of considerable importance in serum treatment, *e.g.* where a patient has had a previous course of serum treatment and has again to be treated with serum. If this be the case, anaphylaxis may be prevented by several procedures. If 5–10 c.c. of antigen (*i.e.* the serum) be given per rectum, this having been well washed out, the individual

is rendered refractory in from ten to twelve hours and subcutaneous or intravenous injections of serum may then be given with impunity. In man sensitisation rarely if ever attains such a degree as to react with 1 c.c. of serum. If, therefore, 0·5–1 c.c. be injected subcutaneously, the ordinary dose may be injected with impunity five or six hours later. If immediate treatment is necessary, such a preliminary injection may be followed five or ten minutes later by a larger dose and every five minutes or so afterwards by steadily increasing amounts. In this way large amounts of serum may be given in a comparatively short time without giving rise to anaphylactic shock.

In the case of prophylactic doses of tetanus antitoxin, these will usually amount to 3–5 c.c. of serum and sensitisation will not be induced thereby until an interval of over five weeks.

On the serum disease, supersensitisation, and anaphylaxis, see Hewlett, *Serum Therapy*, ed. 2, 1910; Rosenau and Anderson, *Journ. Amer. Med. Assoc.*, 1906, p. 1007; Von Pirquet and Schick, *Die Serum-Krankheit*, 1905; Richet, *Ann. de l'Inst. Pasteur*, xxi, p. 497, and *Anaphylaxis* (Constable and Co., 1913. Bibliog.), Besredka, *Ann. de l'Inst. Pasteur*, xxi, p. 950, and *Bull. de l'Inst. Pasteur*, vii, 1909, p. 721; Currie, *Journ. of Hygiene*, vol. vii, 1907, pp. 35, 61, and vol. viii, 1908, p. 457; Grünbaum, *ibid.* vol. viii, 1908, p. 9; Goodall, *ibid.* vol. vii, 1907. p. 607; Bordet, *Journ. State Med.*, 1913, p. 449; *Trans. XVIIth Internat. Cong. of Medicine*, 1913, Sect. IV, Pt. I, pp. 1 (Besredka) and 13 (Richet), and *ibid.* Pt. II. (Friedberger); Wyard, *Lancet*, 1917, vol. i, p. 105.

Anti-microbic Sera.—If an animal be injected with increasing doses of bacteria, care being taken to keep below a lethal one, the animal gradually becomes accustomed to the microbe, and ultimately acquires a high degree of immunity, so that it is unaffected by amounts which would infallibly kill an untreated animal. More-

over, the blood-serum of such a treated animal, if injected into a second animal, will protect the latter against a few lethal doses of the microbe, but not against a large amount. Nor is the protection afforded proportional to the amount of serum injected ; for example, if 0·005 c.c. of anti-cholera serum will protect against 5 mgrm. of living cholera culture, three times as much, or 0·015 c.c. of the serum, will not protect against 15 mgrm. of cholera culture, and when a certain dose of the culture is reached no amount of serum will save the animal. The mode in which the serum acts may be studied microscopically. If cholera anti-serum and cholera culture be injected into the peritoneal cavity of a guinea-pig, and the peritoneal contents be examined at short intervals afterwards, it will be found that the vibrios lose their motility, become distorted and globular, undergo solution, and finally disappear. The protection afforded by the anti-serum is therefore due to the destruction of the microbes by solution, the process being known as bacteriolysis, and the bodies which bring it about being termed, " bacteriolysins." The reaction is known as " Pfeiffer's phenomenon " or reaction, from its discoverer. If the serum and the microbes be mixed *in vitro* the latter are unaffected ; apparently, therefore, some constituent of the *living* body in addition to the anti-serum is necessary for the solution of the microbes. But in 1895 Metchnikoff showed that the reaction will take place *in vitro* provided that some of the *fresh* peritoneal exudate of a normal guinea-pig be added to the mixture of anti-serum and microbes. The same year Bordet found that the addition of the peritoneal exudate is unnecessary provided the anti-serum be perfectly fresh. These experiments prove that the solution of the microbes is brought about by the interaction of at least two substances, one of which is present in all fresh serum and in the living body,

but is unstable, disappearing on keeping or heating the serum, the other is a relatively stable body produced during the process of inoculation. The former, the unstable normal body present in all animals, is usually termed "complement" (Ehrlich and Morgenroth), "alexin" (Buchner and Bordet), or "addiment"; while the stable constituent produced by immunisation is known as the "amboceptor" (Ehrlich), "immune body," "intermediary," "preparer" (Gruber), "fixateur" (Metchnikoff), or "substance sensibilisatrice" (Bordet).

These considerations suggest an explanation why anti-microbic serum neutralises only a limited amount of living culture, viz. the amount of complement present in the body at one time is limited, and when this has been used up bacteriolysis ceases. Anti-microbic sera are relatively inefficient in practice, insufficiency of complement being suggested as the reason. Attempts have been made to supplement the complement present by injecting *fresh normal* serum with the anti-serum, but without success, and some anti-microbic sera, *e.g.* anthrax serum, are not bacteriolytic; this explanation is, therefore, unsatisfactory. Deflection of complement may occur in some instances, or the complement may not be of the right kind. In other cases, the organism in certain situations may be inaccessible to the blood-stream and to the anti-serum, *e.g.* the vibrios, in the bowel in cholera.

Another reason advanced is the extreme specificity of anti-serum and the variability of bacteria so that many races or strains of an organism may exist; this has been proved in the cases of the pneumococcus and the meningococcus. Hence the anti-serum prepared with one race may not neutralise another race. Attempts have been made to overcome this factor by preparing the anti-serum by the injection of many races and so obtaining a "polyvalent serum."

The amboceptor or immune body seems to link the complement to the bacterium (Fig. 35); complement remains free if the appropriate amboceptor or immune body be not present, and bacteriolysis does not ensue. Complement is *thermolabile*, *i.e.* it is destroyed by heating to 56° C. for thirty minutes; while the

amboceptor is *thermostable*, *i.e.* it is not destroyed by this treatment.

According to Ehrlich, fresh serum contains numerous complements which are more or less specific for different amboceptors (see also note, p. 199). When the complement is destroyed by heating it is converted into "complementoid" (analogous to toxoid). Both complement and complementoid on injection give rise to anti-complement. The amount of complement in different sera varies considerably; horse serum contains very little, guinea-pig serum much. Complement itself probably consists of two portions, as it is generally accepted that it can be split into a "mid-piece" and an "end-piece" by the action of dilute hydrochloric acid, carbon dioxide, and dialysis. The mid-piece is thought to be in the globulin fraction, the end-piece in the albumin fraction. Noguchi, however, considers that the whole complement is present in the albumin fraction and that inactivation of the complement by acid, etc., is due not to splitting into two fractions, but to inactivation of the whole complement.

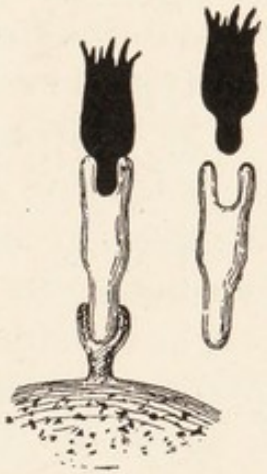

FIG. 35.—Diagram to show the union between complement (black) and protoplasm of cell by means of the amboceptor (white). (After Ehrlich.)

Pfeiffer's reaction is of considerable value in practical bacteriology for the exact recognition of bacterial species. A mixture of a suspension of the organism to be tested with a small quantity of serum from a highly immunised animal is injected into the peritoneal cavity of a normal guinea-pig. The fluid in the peritoneal cavity is then examined microscopically half to one hour after the injection, and if the reaction be positive the organisms will be found in all stages of degeneration, being mostly

converted into spherules. In this case, according to Pfeiffer, the organism is to be regarded as belonging to the same species as that by means of which the immunisation of the animal, from which the blood-serum was obtained, was carried out. If, on the other hand, the reaction be negative, the organisms are unaffected after being in the peritoneal cavity for an hour or so, and the organism is then considered to be a species different from that used for the immunisation. Thus, Pfeiffer's reaction may be made use of to differentiate the cholera-like vibrios from true cholera vibrios and the members of the typhoid-colon group from one another.

The destruction of the bacteria by bacteriolysis is regarded by some as being brought about by osmotic changes, by others by processes analogous to digestion. During bacteriolysis the specific immunising substances and anti-bodies are used up, and for the lysis of a given quantity of bacteria a certain amount of immune serum is necessary, while after lysis has taken place the latter loses the power of dissolving bacteria. The same holds good for hæmolysis, and the facts relating to bacteriolysis and hæmolysis are almost interchangeable.

Anti-endotoxic sera.—The comparative inefficiency of anti-microbic sera, particularly typhoid, led Macfadyen to attempt to prepare sera with microbial endotoxins, and the work has been continued by Südmersen and the writer. The method was to immunise horses with the endotoxin obtained by the method described on p. 42. With a typhoid serum so prepared Goodall and the writer obtained promising results.[1]

Method of applying Pfeiffer's reaction.—For Pfeiffer's test, the organism must be virulent, and a high-grade immune serum is necessary. If the organism is not virulent, it is spontaneously destroyed in the peritoneal cavity without the addition of immune serum. The method may be best explained in the case of a vibrio supposed to be the cholera vibrio. The cholera-immune

[1] *Proc. Roy. Soc. Med.*, vol. ii, 1907–8, Med. Sect., p. 245 *et seq.*

serum (obtained from a horse repeatedly injected with cholera culture) should possess a titre of not less than 0.0002 c.c., *i.e.* this amount of serum mixed with one loop (2 mgrm.) of an eighteen-hour agar cholera culture (virulent), suspended in 1 c.c. of broth, and injected into the peritoneal cavity of a small guinea-pig should cause granular degeneration and bacteriolysis of the vibrios within one hour.

Four mixtures are made—(*a*) one loop of an eighteen-hour agar culture of the vibrio to be tested, 0·001 c.c. cholera-immune serum, suspended in 1 c.c. of broth; (*b*) the same as (*a*), but 0·002 c.c. cholera serum; (*c*) the same as (*a*), but 0·001 *normal* serum of an animal of the same species as that furnishing the cholera serum; (*d*) one quarter loop of the vibrio in 1 c.c. of broth, as a control of the virulence of the culture. These mixtures are then injected into the peritoneal cavities of four guinea-pigs each of about 250 grm. weight. At intervals of thirty and sixty minutes hanging-drop preparations are made of the peritoneal fluid of each animal, the fluid being obtained by inserting a capillary pipette through a minute incision in the skin. In the guinea-pigs injected with (*a*) and (*b*), if the organism be cholera, the vibrios should show marked degenerative changes within sixty minutes, while (*c*) and (*d*) will show plenty of active vibrios. If the organism be non-virulent, two methods may be adopted for applying the Pfeiffer reaction. The first, a microscopical or *direct* method, is carried out by microscopical examination of hanging-drop specimens of the organism suspended in a drop of the immune serum to which a trace of fresh peritoneal fluid (complement) is added. If the organism is homologous with the immune serum the bacteria are soon transformed into granules. Controls are put up at the same time with a known strain of the organism with (1) its homologous immune serum + complement; (2) non-immune serum of the same animal + complement; also of the organism being tested with non-immune serum of the same animal + complement. The peritoneal fluid may be obtained by injecting 3–4 c.c. of broth into the peritoneal fluid of a guinea-pig and four hours later withdrawing the fluid (now turbid with leucocytes) and centrifuging, or allowing it to stand on ice for twenty-four hours.

In the second, *or indirect,* method, the organism is used to prepare an immune serum by injecting an animal (*e.g.* a rabbit) with it, and the immune serum so prepared is tested on a known virulent stain in the peritoneal cavity of guinea-pigs in order to

ascertain whether or no it brings about bacteriolysis, *i.e.* the Pfeiffer phenomenon.

Deflection, deviation,[1] *diversion or blocking of complement.*—Pfeiffer in 1895 observed that a *large* amount of immune serum might not protect an animal from the cholera vibrio, while a smaller amount with the same dose of vibrio did so. In 1901 Neisser and Wechsberg demonstrated an analogous reaction *in vitro*. They studied the effect of a bacteriolytic immune serum when varying amounts of the inactivated serum were employed. The quantity ranged from 0·0005 c.c. to 1 c.c. To each of these amounts constant volumes of normal serum and bacterial suspension were added. No bacteriolysis occurred when large and small amounts of immune serum were used but with medium amounts bacteriolysis was complete. They explained this anomalous reaction, the absence of bacteriolysis with *large* amounts of immune serum, as follows: When the amboceptors are in large excess, a portion combines with the complement, leaving some amboceptors free, and these free amboceptors then unite with the receptors before the activated amboceptors (amboceptors + complement) do, and thus the complement-amboceptor groups are rendered inert. The reaction is represented diagrammatically in Fig. 36. Arrhenius, however, does not accept this explanation. He says: "If we have the compounds *ea* and *ab* which may combine to form the compound *eab*, the formation of the latter depends wholly upon whether *e* has a greater affinity for *ab* than for *a*. If not, then *eab* is not formed, even if *a* is not present in excess." (*a* = amboceptor, *e* = microbe, *b* = complement.) The phenomenon may

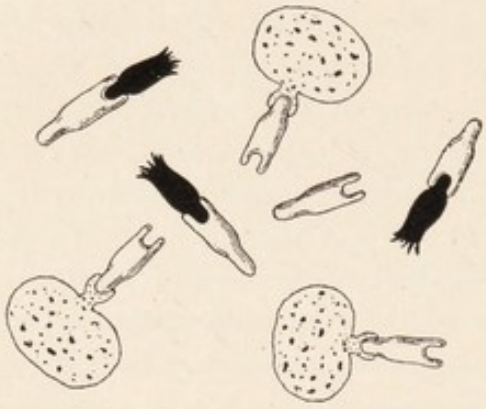

FIG. 36.—Diagram to represent the condition of the blood in which there is an excess of amboceptors (Neisser-Wechsberg phenomenon). The amboceptors (white) unite with both complement (black) and receptors (dotted), so that the receptors cannot combine with the amboceptor-complement groups.

[1] "Fixation of complement" (p. 201) is frequently erroneously termed "deviation of complement."

be quite analogous with the inhibition met with in agglutination (p. 206).

Aggressins

Bail has discussed the question of the relationship between bacteriolysis and immunity. He argues that there is apparently little relationship between the bactericidal properties of the body fluids and the immunity of an animal to infection through bacteriolytic processes ; and points out that in rabbits immunised against anthrax there is no bacteriolytic power, the bacteria disappearing gradually as the result of phagocytic action of cells, chiefly marrow-cells ; that a comparison of the sera of sheep, rabbits, and cattle shows great variation in their content of immune body, though the animals are almost equally susceptible to anthrax ; and that in test-tube experiments a bacteriolytic serum is blocked when the conditions are approximated to those in the body by the addition of body cells to the mixture ; the bactericidal properties of the serum disappear or are greatly inhibited. Kruse suggested that for infection to take place the invading bacteria must elaborate chemical substances which so act on the cells and fluids of the invaded animal that they overcome its natural resistance against infection. These substances are considered by him and Bail to be distinct from the toxins, and are termed by these writers "aggressins." [1] The aggressins are supposed to be secreted by the living uninjured bacteria and not to be extracts, nor derived by solution, of the bacteria ; they occur particularly in the fluids of pathological œdemas and exudates, and may be obtained from these by centrifugation and sterilisation at low temperatures. Bail believes that the aggressins cannot be anti-complements, anti-immune bodies, etc., but are substances heretofore unrecognised and the active substances of the infection, and he considers that in order to produce true immunity in disease anti-aggressin sera must be prepared. The following are some of the properties of these supposed aggressins : (1) Sterilised aggressin with a non-lethal dose of the corresponding organism renders the latter fatal ; (2) aggressin alone is only slowly toxic, producing a prolonged illness with emaciation preceding death ; (3) inoculation of aggression with bacteriolytic serum into the

[1] See *Centr. f. Bakt.*, Orig., xlii, 1906, pp. 51, 139, 241, 335, 437, and 546. Also an excellent summary by Marshall, *Philippine Journ. of Science*, vol. ii, 1907, p. 352.

peritoneal cavity suspends the action of the latter ; (4) aggressin with bacteria blocks phagocytosis. Bail believes that the aggressins promote infection by interfering with the protective mechanism of the infected animal, particularly, if not solely, by inhibiting phagocytosis. Upon the power to produce aggressin Bail has classified bacteria into (1) true parasites which always produce aggressin, *e.g.* anthrax and chicken cholera ; (2) half parasites, the aggressin-producing power of which is variable, *e.g.* typhoid, cholera, dysentery, and plague ; (3) saprophytes. The virulence of an organism does not coincide with aggressivity, and extremely virulent bacteria may be half-parasites.

Bail's hypotheses have been much criticised, and Wassermann and Citron believe that the supposed aggressins are derivatives of the bacterial protoplasm which have the power of combining with the specific protective substances of the animal and so inhibit the action of the latter ; they are, in fact, endotoxins of feeble toxicity.

Hæmolysis.[1]—Some blood sera possess the power of dissolving the red blood-corpuscles of another species, and of setting free their contained hæmoglobin (*e.g.* goat serum dissolves rabbits' and guinea-pigs' corpuscles, and ox and human sera usually dissolve sheep's corpuscles), and if an animal be injected with the blood-corpuscles of another species its blood-serum generally acquires the property of dissolving the blood-corpuscles with which it has been injected. For example, the serum of a normal rabbit has no, or only slight, hæmolytic action upon the red corpuscles of the sheep ; but if a rabbit receive a few injections of defibrinated sheep's blood, its blood-serum acquires hæmolytic properties and dissolves the red corpuscles of the sheep. This solution of the blood-corpuscles is termed " hæmolysis," and the substances which produce hæmolysis are " hæmolysins." While a natural hæmolytic

[1] See Bulloch, *Practitioner*, December 1900, p. 672, and *Trans. Path. Soc. Lond.*, vol. lii, Part 3, 1901, p. 208 ; Gruber, " Harben Lectures," *Journ. State Med.*, 1902, February, March, and April ; Ehrlich, *Collected Studies on Immunity ;* Muir, *Studies on Immunity.*

serum loses its power on diluting five to ten times, an artificial hæmolytic serum may be so potent that it will still act in a dilution of 1 in 2000 or more. If the active serum be heated to 56° C. it is "inactivated" and loses its hæmolysing power, but can again be rendered hæmolytic or "activated" by the addition of *fresh* normal serum; normal serum, however, rapidly loses its activating properties on keeping. It will thus be seen that there is an almost complete analogy between bacteriolysis and hæmolysis, the latter being brought about by the interaction of two substances, one specific and stable produced by the injections, the hæmolytic "amboceptor" or "immune body," and the other an unstable body present in fresh normal serum, the "complement" or "alexin."

Hæmolysin formed by the injection of corpuscles of another species is termed "heterolysin." If corpuscles of the *same* species be injected, hæmolysin is formed ("isolysin"), but the injection of the animal's own corpuscles does *not* give rise to hæmolysin, *i.e.* "autolysin" is *not* formed.

Blood-corpuscles are more tangible entities than bacteria, and are far easier to work with than the latter, and hæmolysis has been the subject of a large amount of experimental work by Bordet and Gengou, Ehrlich, Morgenroth, Gruber, Bulloch, Muir, and others, and the results obtained have shed considerable light upon the complex phenomena of immunity and of the actions of anti-bodies in general. Moreover, the globulicidal material in hæmolysis *seems* to be identical with the bactericidal one in bacteriolysis—that is to say, it is the complement or alexin.[1] According to Ehrlich's view,

[1] As previously stated (p. 193), numerous complements undoubtedly exist, yet bacteria will absorb both bacteriolytic and hæmolytic complements. Bordet and Gengou suppose that while a particular amboceptor has a maximum avidity for its homologous complement (which may be termed *dominant*), it is also able to take up other

whether it be normal or "immune" serum (*i.e.* serum of a treated animal), bacteriolysis or hæmolysis takes place only when the complement and amboceptor unite (Fig. 35, p. 193), complement by itself having little affinity for the bacterium or erythrocyte, the combination forming the "lysin," which then acts. According to Gruber, however, neither bacteriolysin nor hæmolysin exists as a chemical entity, the specific bacteriolytic or hæmolytic action being due to the fact that the cells first absorb the amboceptor and so become accessible to the complement, for the two substances do not combine in definite proportions—the more the blood-corpuscles are laden with the amboceptor the smaller the quantity of complement required to bring about their solution.

Many bacteria—*e.g. B. pyocyaneus*, *B. typhosus*, staphylococci and streptococci—besides other products directly form hæmolysins in the culture, and the hæmoglobin staining occurring in septic diseases, etc., is probably partly due to the action of bodies of this nature elaborated by the infecting organisms. These bacterial hæmolysins are, however, substances quite different from the immune hæmolysins obtained by injecting an animal with blood corpuscles.

Practical Uses of Hæmolysis, etc.

1. *Hæmolysis test.*—Some micro-organisms produce non-specific hæmolysins in culture, others do not; this may constitute a difference between allied organisms. For instance, as a rule true cholera vibrios do not hæmolyse, while many cholera-like vibrios do. The test can be applied in two ways: (*a*) Defibrinated rabbits' blood may be mixed with melted agar cooled to 45° C. The mixture is poured into Petri dishes, allowed to set, and when cool inoculated with the organism to be tested in such a manner

"non-dominant" complements, and thus bacteriolytic amboceptor is able to absorb both bacteriolytic (dominant) and hæmolytic (non-dominant) complements.

that separate, well-defined colonies are obtained. After twenty-four hours' incubation at 37° C., colonies when hæmolytic are surrounded with a clear, well-defined halo contrasting sharply with the dark opaque colour of the agar. If blood-agar is not available, a substitute may be devised by smearing some sterile human or rabbits' blood on a sterile agar plate. (*b*) A young agar culture is emulsified in 4–5 c.c. of physiological salt solution; 0·1 c.c. of this suspension is mixed in a tiny test-tube with 0·9 c.c. of sterile salt solution and one drop of a sterile suspension of well-washed rabbit or other corpuscles. After twelve to twenty-four hours hæmolysis will be apparent if the organism forms hæmolysins.

2. *Fixation or absorption of complement test.*[1]—A hæmolytic serum may be used as a delicate reagent for complement, and may thus serve as a test for an organism or an immune serum. As an example take the case of a supposed cholera vibrio. If an immune serum (previously heated to 56° C. so as to destroy complement)—hæmolytic for the corpuscles of an animal, or bacteriolytic for a given micro-organism, *e.g.* cholera vibrio—be mixed with the red corpuscles of the same animal, or with the cholera vibrio, the corpuscles or the vibrios respectively absorb the corresponding amboceptor or immune body.

Bordet showed that if corpuscles or microbes that have absorbed the corresponding amboceptor be added to fresh non-heated complement (*e. g.* fresh guinea-pig serum), the corpuscles or the microbes absorb the complement, so that none remains free in the liquid.

But if fresh guinea-pigs' serum be added to cholera vibrios which have not absorbed any cholera amboceptor, the complement will not be absorbed and remains free in the liquid. The proof of this is that if "sensitised" corpuscles (*i.e.* corpuscles which have taken up hæmolytic amboceptor) be added to such a mixture, the globules are quickly hæmolysed. If, on the other hand, vibrios which have already taken up the cholera amboceptor be added to the same quantity of fresh serum, the microbe-amboceptor complex absorbs the complement; and, provided the amount of fresh serum is not too great, the complement is absorbed so completely that "sensitised" corpuscles when added to the mixture are not dissolved. If vibrios other than cholera be added to cholera serum, the amboceptor is not fixed, the complement added remains free, and the sensitised corpuscles

[1] Often termed "deviation of complement" test.

are dissolved. These facts constitute the "Bordet-Gengou phenomenon." The mixture of an inactivated hæmolytic serum (*i.e.* heated to 56° C.) with the homologous corpuscles (*i.e.* those with which the hæmolytic serum was prepared) is known as a "hæmolytic system." The following example illustrates the method of carrying out the test: The cholera-immune serum is heated to 56° C. for half an hour. An eighteen hours old agar culture of the organism to be tested is suspended in 2 c.c of sterile physiological salt solution. The complement is fresh guinea-pig serum; a portion of this is also heated to 56° C. (= non-immune serum). The following mixtures are prepared in three small test-tubes:

Tubes 1 *and* 2 each contain 0·2 c.c. microbic suspension + 0·6 c.c. heated immune serum + 0·1 c.c. complement.

Tube 3 contains 0·2 c.c. microbic suspension + 0·6 c.c. heated non-immune serum + 0·1 c.c. complement.

These are well shaken to mix their contents, and are kept for half to one hour at 37° C. At the end of this time 0·1 c.c. of the following mixture is added to tubes 1 and 3: two volumes of heated (to 56° C. for half an hour) serum hæmolysing sheep's red corpuscles + one volume of washed sheep's corpuscles. To tube 2 is added 0·1 c.c. of a mixture of two volumes of physiological salt solution + one volume of washed sheep's corpuscles. The tubes are kept for a further hour or so at 37° C., and at the end of that time the occurrence of hæmolysis is noted. If the organism is homologous with the immune serum, the immune body will fix the complement in tube 1 and *no* hæmolysis will occur; in tube 3 hæmolysis will occur because the complement remains free. Tube 2 serves as a control, and should show no hæmolysis in three hours (though if kept for eighteen to twenty-four hours hæmolysis will occur *if the organism produces hæmolysins*, apart from any action of complement). If the organism is not homologous with the immune serum, hæmolysis will occur in tube 1, because the complement does not become fixed, tubes 2 and 3 being the same as before.

It is not even necessary to use the living organism; the dead organism or extracts thereof, and, in cases where the organism cannot be cultivated, a dried and pulverised organ or an extract thereof, has been employed. Certain non-specific substances may sometimes be used as in the Wassermann reaction for syphilis (see "Syphilis").

The hæmolytic serum may be obtained by injecting rabbits with

a 10 per cent. suspension of well-washed sheep's red corpuscles. The sheep's blood should be obtained as aseptically as possible from the slaughterhouse; the blood, as it runs, is caught in a sterile wide-mouth bottle containing a coil of fine wire with which it is defibrinated by shaking. The fluid blood is then mixed with sterile physiological salt solution (0·85 per cent.) and centrifuged, and the deposited corpuscles are again washed with salt solution two or three times. Three doses of 1 c.c., 2 c.c., and 3 c.c. respectively are given intravenously on successive days, and after an interval of 5–7 days the rabbit's serum should be strongly hæmolytic. Very active hæmolytic sera may be purchased. The serum may be collected aseptically, inactivated by heating to 56° C. for half an hour, and preserved in sealed ampoules. The activity of the hæmolytic amboceptor must be tested and the appropriate dose of it, complement, and corpuscles ascertained. (For manner of testing, see the "Wassermann Reaction" under "Syphilis.")

Cytotoxins.—Anti-sera, analogous to the hæmolysins or hæmotoxins, may be prepared which have a destructive action upon cellular elements; these are termed "cytotoxins." If a rabbit be injected with bull's semen, its serum ("spermotoxin") acquires the property of immobilising the spermatozoa of the bull. The reaction is specific, but spermatolysis does not seem to occur. Similarly, by injecting ciliated epithelium into the peritoneum of a guinea-pig an anti-epithelial serum, or "trichotoxin," is developed. With liver, kidney, and nerve cells anti-bodies having a destructive action upon these cells are developed as a result of their injection. Nephrotoxin, the serum of an animal inoculated with an emulsion of kidney, when injected into a second untreated animal, produces albuminuria and uræmia with disintegration of the epithelium of the convoluted tubules; hepatotoxin, the serum of an animal treated with emulsions of liver, produces fatty and inflammatory changes in the liver resembling phosphorus poisoning; neurotoxin, the serum of an animal treated with emulsions of nerve tissues, produces paresis, paralysis, depression, convulsions, etc.; a leucotoxic serum obtained by injecting leucocytes agglutinates and dissolves the leucocytes and so on. The formation and mode of action of these cytotoxins resemble those of the hæmolysins. It was hoped that the study and preparation of cytotoxins would open up possibilities in the way of treating such diseases as carcinoma and sarcoma, but so far this hope has not been realised.

AGGLUTINATION.—If an animal be injected with cultures of typhoid or cholera-bacilli, its serum soon acquires the property of agglutinating or of aggregating into clumps the typhoid bacilli or cholera vibrios respectively when mixed with a broth culture of these organisms. The reaction may be observed microscopically in a hanging-drop preparation; the organisms first lose their motility and soon become aggregated into large masses or clumps. Macroscopically, the reaction may be followed in a narrow test-tube into which the mixture of culture and serum has been introduced; after some hours the micro-organisms become aggregated into masses so large as to form visible flocculi. The substances which bring about this agglutination are known as agglutinins. Agglutinins seem to be present in small amount in normal serum; for instance, most normal human sera up to a dilution of 1 in 2 or 1 in 4 will agglutinate the typhoid bacillus and still more powerfully the glanders bacillus. They are also present in bacterial cultures; if an old broth culture of typhoid be filtered, the filtrate agglutinates the bacilli in a fresh broth culture; hence *young* cultures should always be used for agglutination tests. Specific agglutinin is formed by the action of antigen derived from the bacterial cell, and agglutination is brought about by the action of the agglutinin on the antigen. The agglutinin first unites with the antigen, and this may occur at 0° C., and afterwards exerts its specific action, which takes place only at higher temperatures and in the presence of certain salts. The agglutinable substance is known as agglutinogen. Agglutinin is converted into agglutinoid at 70°–75° C.; the latter does not agglutinate, though it unites with bacteria and then prevents the subsequent action of agglutinin.

The agglutination of organisms by anti-sera, though hardly specific, is usually very special; given proper

precautions as to dilution, time-limit, condition of test culture, etc., an anti-serum will generally only agglutinate the homologous organism or closely allied species—that is, it is a group reaction. If agglutination of allied organisms takes place, it is not nearly so marked as that of the homologous organism. Anti-typhoid serum, for example, may agglutinate not only the typhoid bacillus, but also, though to a much less degree, members of the paratyphoid group. As the result of infection or of inoculation with an organism, agglutinins may similarly be produced which agglutinate not only the organism of the infection, but also other organisms—*e.g.* typhoid serum may agglutinate *B. paratyphosus* and *B. coli* as well as the *B. typhosus* and typhus serum *B. typhosus* and *M. melitensis*; the agglutination is, however, always much more marked with the homologous organism. The agglutinins acting on the infecting organism may be termed primary or homologous, those acting on other organisms secondary or heterologous. In a case of double infection each organism may produce its own primary agglutinin, so that the agglutination of two species by a serum may be due to the presence either of a primary and a secondary agglutinin or of two primary agglutinins. Castellani,[1] by applying the saturation test (p. 213), found that an organism would absorb both its primary and secondary agglutinins, but would not absorb two different primary agglutinins. This test, therefore, would distinguish a double infection from a single one. Thus, if a typhoid serum agglutinated both the *B. typhosus* and the *B. coli*, and the serum after saturation with typhoid culture still agglutinated the *B. coli*, this would point to an infection with the latter as well as with *B. typhosus*. The formation of primary and secondary agglutinins may be brought about as follows: In the bacterial cell there

[1] *Zeitschr. f. Hyg.*, xl, 1902, p. 1.

are several substances, each of which forms its own agglutinin. The cells of two bacterial species we can imagine both contain three or four substances capable of producing agglutinins, and it may happen that one of these in each species is approximately the same and will produce almost the same agglutinin—the secondary agglutinin—and, therefore, the serum produced by each bacterium will agglutinate the other.

The agglutination reaction is made use of in bacteriological tests and in clinical diagnosis. The "Bordet-Durham" reaction consists in testing an unknown organism with a specific anti-serum prepared by injecting an animal with a known microbe; if the organism tested becomes agglutinated, it is regarded as being of the same species as that with which the anti-serum was prepared. With certain precautions the "Bordet-Durham" reaction is one of the most delicate and certain for the recognition of bacterial species. The converse of this is the agglutination reaction proper (frequently termed the Widal reaction), and consists in testing an unknown serum upon a known microbe. It is especially used in the diagnosis of microbial diseases; for example, in typhoid fever the blood of the typhoid patient powerfully agglutinates the typhoid bacillus, that of Malta fever the *Micrococcus melitensis*, that of bacillary dysentery the dysentery bacillus, etc.

A remarkable phenomenon observed in connection with agglutination, which the writer has particularly noticed in the case of Malta fever, is the occurrence of what may be termed a zone of no reaction or of inhibition with some particular dilution. Thus, dilutions of 1 in 10 and 1 in 20 may agglutinate strongly, a 1 in 30, however, may hardly agglutinate at all, while dilutions of 1 in 40 and upwards to 1 in 100 or more may agglutinate well. A similar phenomenon has been observed with non-specific

agglutinating agents, and also in the action of coagulating agents on colloid emulsions. Thus orthophosphoric acid agglutinates a certain volume of a suspension of *B. coli* when present to the extent of between 118 cgrm. and 4 cgrm., and between 1·1 mgrm. and 0·001 mgrm., but not in intermediate amounts between 40 and 1·1 mgrm.

Anti-serum, prepared by injecting erythrocytes, also agglutinates the red blood-corpuscles, and in certain diseases, *e.g.* pneumonia, chromocyte clumping may be a marked feature.

Various theories have been propounded to account for the phenomena of agglutination :

1. Pfeiffer and Emmerich and Loew regarded agglutination as a vital paralysis of the bacilli due to the action of a bacteriolytic enzyme. Agglutination, however, is not a vital phenomenon, for dead bacilli agglutinate, and bacteriolytic enzymes seem to be destroyed by temperatures at which agglutinins remain unaffected.

2. Gruber, Dineur, and Nicolle supposed that a glutinous substance, "glabrificin," is absorbed from the serum by the bacilli causing the cell membranes or the flagella to become adhesive ; but this explanation will hardly account for the aggregation of non-motile organisms.

3. Paltauf and Duclaux considered that a precipitate is produced in the medium, which during flocculation mechanically carries the bacilli with it ; but there is no demonstrable evidence that such precipitation occurs.

4. Bordet separated the mechanism of agglutination into two stages—(1) fixation of agglutinin, and (2) aggregation. The fixation of agglutinin by the organisms he considers to be analogous to the adsorption of a dye by a tissue ; and once the agglutinin is fixed, the organisms obey the laws of inert particles, aggregation being caused by changes in surface tension, in the molecular attraction, between the organisms and the surrounding medium, a

view supported by Craw.[1] Ohno,[2] however, believes that the union of agglutinin and agglutinable substance is not analogous to the fixation of a dye by a tissue, but that it is a chemical combination, as maintained by Ehrlich.

Priestley[3] regards the agglutination and precipitin reactions as being essentially the same in nature, the agglutination of bacteria by specific sera being probably due to the formation of altered serum protein in and around the bacteria, and the subsequent flocculation, by electrolysis, of this altered protein and the bacteria. The altered protein is probably altered serum globulin. Inactivation by heat and the production of "zones of no reaction" he regards as being most likely due to the development of inhibitory substances.

Agglutinated bacteria are not injured by agglutination; they will, in fact, grow and multiply in an agglutinating serum. The amount of agglutination does not bear any constant ratio to the intensity of an infection; on the whole, if the patient is reacting satisfactorily to an infection, the agglutination reaction tends to be marked; if not, it may be feeble or absent. Thus, in severe typhoid infections with fatal issue, agglutination may be absent. Ruffer and Crendiropoulo regard the agglutinins as being formed in the polymorphonuclear leucocytes.

In order to obtain reliable results by the agglutination method in the diagnosis of disease, and particularly to compare the intensity of agglutination at different stages of an attack and in different individuals, it is necessary to employ the same method, the same dilutions, and cultures of the same agglutinability. In order to standardise these factors, Dreyer[4] has introduced what is termed

[1] *Journ. of Hygiene*, vol. v, 1905, p. 113. See also Joos, *Zeitschr. für Hyg.*, xxxvi, p. 422, and *ibid.* xl, p. 203.

[2] *Philippine Journ. of Science*, vol. iii, 1908, p. 47.

[3] *Journ. of Hygiene*, vol. xv, No. 4, 1917, p. 485.

[4] See *Journ. Roy. Army Med. Corps*, September, 1916 (Refs.), *Lancet*, 1917, vol. i, pp. 365 and 568 (Refs.).

the "*Standard Agglutination* Method" for the diagnosis and comparison of degree of agglutination in typhoid and paratyphoid fevers and bacillary dysentery. "Standard" dead cultures of the respective organisms are employed, and the test is a macroscopic one done in small tubes (for method of carrying it out see section on "Typhoid Fever").

The following details and terms are used in connection with it:

1. *Standard Agglutinable Cultures* are prepared of definite opacity and measured agglutinability from strains of the bacilli concerned which have been specially selected for their high specificity. In successive batches of standard agglutinable culture the relative sensitiveness to agglutination of the bacilli contained is indicated by a figure—the so-called *Reduction Factor*—the original standard agglutinable cultures having been arbitrarily given the figure 2·5 as a reduction factor. The reason for this will appear immediately in connection with the *Standard Agglutinin Unit.*

2. *Standard Agglutination* is the degree of agglutination present in the highest serum dilution in which marked agglutination without sedimentation can be seen by the naked eye.

3. The *Standard Agglutinin Unit* is that amount of agglutinating serum which when made up to 1 c.c. volume with normal saline solution causes standard agglutination on being mixed with 1·5 c.c. of the original standard agglutinable culture and maintained at 55° C. for 2 hours (in the case of dysentery agglutination 4½ hours) in a water-bath, followed by 15 to 20 minutes at the room temperature.

4. The *Reduction Factor.*—The total volume in which the reaction occurs being 2·5 c.c. (1 c.c. of serum added to 1·5 c.c. of standard culture) the original standard agglutinable culture was given the reduction factor of 2·5 to express the sensitiveness to agglutination of that particular culture. All subsequent batches of culture have been given reduction factors calculated on this basis, thus securing constancy in the agglutinin unit. For example, if a batch of standard culture proves to be twice as sensitive to agglutination as the original standard, so that half the amount of serum produces standard agglutination under test conditions, the new standard culture is given a reduction factor of double the size of the original factor—i. e. 5.

The Agglutination Reaction

A. *For Clinical Diagnosis ("Widal" Reaction)*

This is principally made use of in typhoid and paratyphoid fevers, Malta fever, and bacillary dysentery.

Collection of blood.—Blood is collected (p. 137), preferably in a Wright's capsule (Fig. 37, *d*, p. 237), or in a capillary bulbous pipette (Fig. 7, p. 53), or in a vaccine tube. The ends of the tube are sealed, the *dry* end always being sealed first; the blood is allowed to coagulate (which may be hastened by placing in the blood-heat incubator), and then centrifuged to separate the serum, care being taken that the dry sealed end of the tube, which will be perfectly sealed, is distal when spinning.

If tubes are not available, the blood may be spotted on to a piece of glass, cover-glass, or slide, glazed paper, tinfoil, etc., and allowed to dry. For use, a drop of distilled water is placed on the dry blood to dissolve it, and the solution used like serum.

The culture.—For the *microscopic* test a *young* broth culture is to be preferred. A hanging drop should be examined to ascertain that clumps are absent; this specimen is kept as a control. If clumps are present they may be removed (in the case of typhoid) by filtering the culture through filter-paper. A suspension of an agar culture may also be used, likewise dead cultures: a broth culture or suspension of an agar one being heated to 65° C. for ten minutes and preserved in sterilised glass pipettes; dead cultures are, however, unsatisfactory in tropical climates. For the *macroscopic* test a thickish suspension of an agar culture in salt solution is to be preferred, the suspension being allowed to sediment for half to one hour before use. Some strains of an organism are better than others, and old laboratory strains are generally much more sensitive to agglutination than recently isolated ones.

Dilution of the serum.—This may be carried out in various ways, with the hæmocytometer pipette, with a pipette with rubber teat as used for opsonin work (Fig. 37, *a*, p. 237), with a throttled pipette, by the drop method (see pp. 54, 55) or with a platinum loop. With the pipette a little serum is aspirated up so as to occupy $1\frac{1}{2}$–2 cm. of the stem, and the upper limit is marked with a grease pencil or ink. A bubble of air is then admitted so that an air-space is left between the end of the pipette and the lower end of the column of serum. The end of the pipette is then immersed in a watch-glass of salt solution, and the salt solution is aspirated up to the mark, another bubble of air is admitted, and the process

is repeated again and again ; so that, finally, the pipette contains 1 volume of serum and 4–14 volumes of salt solution, each volume being separated from the next one by an air-bubble. The contents of the pipette are then expelled into a watch-glass and thoroughly mixed, and further dilution of this dilution is performed in the same manner. Two or three dilutions are usually made—*e.g.* 1 in 25, 1 and 50, and 1 in 100. A platinum loop may also be employed as a measure ; a loopful of the serum is deposited in a watch-glass, and by spotting round it nine or fourteen loops of salt solution a dilution of 1 in 10 or 1 in 15 is prepared, or any other dilution in a similar manner.

The microscopic test.—Two or three hanging-drop slides are vaselined, and two or three cover-glasses cleaned. One loopful of a dilution of serum is placed on each cover-glass, and to each is added a loopful of the broth culture of the organism—*e.g.* typhoid—and well mixed up, and the specimens are mounted as hanging drops. Starting with three dilutions of serum—*e.g.* 1 in 15, 1 in 25, and 1 in 50—the dilutions in the specimens will be 1 in 30, 1 in 50, and 1 in 100 respectively.

Care should be taken that the hanging-drop preparations are quite sealed with the vaseline, so that evaporation is prevented. The hanging drops are then examined microscopically, a $\frac{1}{6}$-in. objective sufficing for typhoid. In the case of typhoid the following phenomena will be observed : The motility of the majority of the bacilli is very quickly arrested, and in a few minutes they begin to aggregate together into clumps, and by the end of the half hour there will be very few isolated bacilli visible. In less marked cases the motility of the bacilli does not cease for some minutes, while in the least marked ones the motility of the bacilli may never be completely arrested, but they are always more or less sluggish as compared with the control hanging drop made from the culture, while clumping ought to be quite distinct by the end of one hour (with a 1 in 30 to 1 in 50 dilution).

The central portions of the drop should be examined, not the margins. With blood which has been dried and dissolved organisms may become entangled in *débris*, and must not be mistaken for clumps.

In all cases two or three different dilutions should be made to exclude the possibility of a " zone of no reaction " with some particular dilution (*see p.* 206).

Macroscopic, or sedimentation method.—The *serum*, having been diluted by means of a pipette with saline solution, is mixed

with five to twenty times its volume of culture suspension containing plenty of micro-organisms in the same manner as described in the previous section. The mixture is sucked up into a fine, but not capillary, bore tube. This is sealed at the lower end and allowed to stand in the upright position for eight to twenty-four hours at 20° C., or six hours at 37° C.; the reaction is often distinct within an hour at 37° C. When the reaction is positive the organisms become agglutinated and form flocculi, which are easily seen with the naked eye or with a hand-lens and stick to the sides or sink to the bottom of the tube. The dilutions usually employed are 1 in 30 to 1 in 200, and two or three different dilutions should always be put up. Whole blood is not suitable for the sedimentation test; clear serum should always be used. It is well to set up at the same time a control tube with saline solution, or, preferably, with normal serum.

If sufficient serum is available the mixture may be put up in little test-tubes, such as the inner tubes of Durham's culture tubes (p. 73) The Dreyer "Standard" method is now much used for the diagnosis of typhoid and paratyphoid fevers and bacillary dysentery (see "Typhoid Fever").

B. *For the Recognition of Bacterial Species*

1. *Bordet-Durham reaction.*—This is carried out in much the same manner as for clinical diagnosis, but an immune serum of high agglutinating value or high "titre" (at least 1 : 1000) is required, and the serum from a patient is not applicable. The immune serum may be obtained from a horse or other animal immunised with killed cultures (and living also if a high titre is required). In the laboratory the serum may be prepared by giving a rabbit three to five intravenous injections at intervals of seven days of killed culture of a virulent strain of the organism, *e.g.* typhoid or cholera. The culture is killed by heating to 60°–65° C. for half an hour, and the dose is increased from one loop to ten loops of an agar culture. Seven days after the last dose the animal is bled from an ear vein, and the serum obtained. The agglutinating value of the serum must be determined, and controls should always be put up with normal serum of an animal of the same species as that from which the immune serum has been obtained. A series of dilutions of both sera is made with salt solution and a twenty-four hour agar culture of the organism to be tested used. Both the macroscopic and microscopic

methods should be employed. The dilutions may be made with a 1 c.c. pipette graduated in hundredths, with the hæmocytometer pipettes, or by the method used clinically

2. *Saturation test.*—Castellani noticed that a suspension of a microbe added to the homologous agglutinating serum absorbs most, if not all, the specific agglutinin, whereas an organism not homologous with the serum absorbs little or only a small portion of the agglutinin. The test may be carried out as follows :

Ten loopfuls of a young agar culture of the organism to be tested are mixed with 10 c.c. of a 5 per cent. solution of a highly agglutinating serum. After incubating for two or three hours, the mixture is centrifuged, the clear supernatant fluid decanted, and the agglutinating power of the decanted liquid is then tested on the organism with which the serum was prepared. If the organism tested is homologous with the organism with which the agglutinating serum was prepared, the decanted fluid will have lost most, or a considerable proportion, of its agglutinating power for the latter.

The Meiostagmin Reaction.—Ascoli has found that if an immune serum be mixed with an alcoholic extract of the homologous antigen and the mixture incubated at 37° C. for two hours the surface tension is reduced ; if the serum and antigen extract are not homologous the surface tension is unaltered. For example, in the case of typhoid the following is the procedure. An alcoholic extract of typhoid bacilli is prepared ; this is diluted with saline solution to 1–1000—1–1,000,000. The typhoid serum is similarly diluted, 1–10. To 9 c.c. of the diluted serum 1 c.c. of the diluted antigen extract is added. By means of some form of viscosimeter or stalagmometer the number of drops yielded by a given volume of the mixture is ascertained, immediately after the mixture is made and after the mixture has been incubated at 37° C. for two hours. If the surface tension has been reduced, the number of drops counted in the second determination will be greater than in the first.[1]

Anti-ferments.[2]—By the injection of rennin or other enzyme the blood-serum of the treated animal acquires the property of neutralising the action of the enzyme with which the inoculation has been performed. Thus if rennin and antirennin (the serum of an animal injected with rennin) be mixed with milk no curdling

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[1] Ascoli and Izar, *Münch. med. Woch.*, lvii, 1910, pp. 62, 182, 403.

[2] See Dane, *Trans. Path. Soc. Lond.*, vol. lii, 1901, Part 2, p. 127.

takes place. Similarly, the serum of an animal inoculated with pancreatin inhibits the action of this ferment, and if coagulated egg-albumen, pancreatin, and anti-pancreatin be mixed, the egg-albumen undergoes no digestion.

Precipitins.[1]—Kraus was the first to demonstrate the presence of specific precipitins in blood by adding typhoid, cholera, and plague anti-sera to filtrates of the cultures of the corresponding microbes. If to such a filtrate in a test-tube a little of the corresponding anti-serum be added by running in carefully, so that it forms a layer at the bottom, an opalescent ring makes its appearance at the line of junction of the two fluids. So also if an animal be injected with milk, its serum, when added to milk of the same kind as that with which it has been injected, causes precipitation of the casein. This reaction is specific, and it is thus possible to distinguish various milks from one another. Similarly, anti-sera which produce precipitates, each with the homologous substance, are obtained by the injection of peptone, of egg-albumen, blood-serum, and other proteins. The latter reaction has an important medico-legal application, for by means of it the blood and flesh of different species of animals can be distinguished. Thus the presence of horseflesh in sausages can be detected. The method employed is to inject a rabbit intraperitoneally with four to six injections of defibrinated blood or of blood-serum (or with a solution of the particular substance, *e.g.* horseflesh), commencing with about 5 c.c. and increasing to 10 c.c. at intervals of a few days. After treatment the animal is bled from an ear vein, and the serum is obtained. The blood to be tested may be dried on filter-paper, pieces are then cut up, a solution is made in 1·6 per cent. sodium chloride solution, and to this the specific serum is added. Tested in this way human blood anti-serum reacts—*i.e.* forms a precipitate—markedly with human blood, less so with ape's blood, not at all with other blood; ox blood anti-serum reacts with ox blood, less so with sheep, feebly with horse, hardly at all with dog. Mixtures of bloods may also be tested. Precipitins are also formed naturally *in vivo*. Thus the serum of a patient the subject of hydatid disease gives a precipitate with hydatid fluid, and the reaction

[1] See Nuttall, *Journ. of Hyg.*, vol. i, 1901, p. 367 (Bibliog.), also *Brit. Med. Journ.*, 1902, vol. i, p. 825; Welsh and Chapman, *Journ. of Hygiene*, vol. x, 1910, p. 177; *ibid.*, *Australasian Med. Gazette*, December 12, 1908 (hydatid disease).

may be used diagnostically. The production of the anti-body seems to be due to the globulin constituent of the injected serum.

It will thus be seen that the anti-bodies which result from the injection into an animal of different substances are extremely numerous and have varied properties, their most notable characteristics being their extreme specificity and the extraordinary delicacy of the interactions produced by them. It is important to note that these anti-bodies are produced only as the result of inoculation with complex compounds allied to the proteins. The tolerance established by the ingestion or inoculation of simpler compounds, such as arsenious acid and morphine, is of a different nature, and is not coincident with the development of anti-bodies. According to Ehrlich, the latter kind of tolerance may be due to the exhaustion or using up of certain receptors (" chemo-receptors ") of the protoplasm (see p. 229).

Immunity[1]

No fact in biology is more striking than the differences in susceptibility to infection exhibited by different races and different animals. For example, the natives in many parts of the world are comparatively insusceptible to yellow and typhoid fevers and malaria, the dog and goat are rarely affected with tuberculosis, animals do not suffer from typhoid or cholera, and tetanus is never met with in the fowl ; and to come nearer home, while some individuals are lucky enough to escape most of the commoner infectious fevers, others seem to contract them on every possible occasion, and to suffer from all the ills that flesh is heir to. These instances show that there is

[1] See Metchnikoff, *Immunity in Infective Diseases*, 1905. Also *Brit. Med. Journ.*, 1902, vol. i, p. 784 ; 1904, vol. ii, pp. 557–582 ; and 1907, vol. ii, pp. 1409–1425 ; *Journ. of Hygiene*, vol. ii, 1902 ; Emery, *Immunity and Specific Therapy*, 1909.

often a natural insusceptibility to infective disease, or a natural immunity, as it is termed. This may be complete or partial, or it may appertain only to a race—"racial immunity"; or, varying in different individuals and at different ages, it constitutes "individual immunity," as in the case of diphtheria and scarlatina, which become more and more rare as age advances.

Still more striking, perhaps, is the fact that an insusceptibility may be acquired after an attack of infective disease or be conferred in certain instances by inoculation. Thus second attacks of smallpox and scarlatina are rare, inoculated smallpox and vaccinia protect against variola, and bacterial vaccines confer considerable protection.

With regard to the immunity of native races to certain diseases, this is partly due to natural selection and heredity; during long periods of time, the individuals being all exposed to the same risks, the susceptible ones are weeded out, while the survivors transmit their insusceptibility to their descendants; but this, of course, does not explain the reason for the relatively greater immunity of the insusceptible individuals. In some instances immunity of the adult is due to recovery from an infection in childhood; this is frequently the case with malaria among native races. Immunity is generally not absolute either to infection or to intoxication; that is, susceptibility may be present under particular conditions. Thus fowls, which are highly refractory to tetanus and tolerate considerable doses of tetanus toxin with impunity, can be tetanised with large doses of an active toxin; white rats, which are insusceptible to anthrax, become susceptible after fatigue, or when fed on an exclusively vegetable diet. Immunity is therefore either (1) natural, or (2) acquired, and it is evinced against either (*a*) toxins, or (*b*) micro-organisms, and these different phases must be considered.

1. *Natural immunity against toxins.*—Toxins cannot enter the body through the intact skin, and frequently not through intact mucous membranes, hence many toxins may be swallowed with impunity. If the toxin does get into the blood or tissues, there are various non-specific reactions in the body by which it may be eliminated or destroyed. Thus the dilatation of the vessels and the acceleration of the blood-stream which take place in an inflamed area dilute and eliminate the toxin, and the proteolytic enzymes produced by the organisms and as a result of tissue disintegration may have a destructive action on the toxins. Oxidation, hydration and dehydration, and various analytic and synthetic processes which go on in the body, and particularly in the liver, are other agencies whereby toxins may be destroyed. These non-specific processes by which toxin is destroyed or eliminated, though of the greatest importance, can probably deal with only *small* amounts of toxin ; if *large* amounts are present, specific reactions have to be evoked.

Another cause of natural immunity to toxins may be the absence of suitable receptors for the toxin. As already stated (p. 167), in order that a bacterial toxin or endotoxin may produce intoxication, it must become anchored to the cells by its haptophore group, and that this may occur the cell molecules must possess atomic groups or side-chains (" receptor groups ") which have a special affinity for the haptophore groups of the toxin. Should these be wanting the toxin cannot become anchored to the cells, its toxophore groups cannot exert their influence, and natural immunity is the result.

This has been proved to be the case in several instances. Thus in the lizard and turtle, if tetanus toxin be injected no effect is produced, but the toxin is not eliminated and remains in the body for months, as may be proved by

withdrawing a little of the blood and injecting it into a mouse ; the animal dies of tetanus.

In other instances, for some reason or other, the cells of the animal are insusceptible to the toxophore group of the toxin. Thus, if an alligator be injected with tetanus toxin, no effect is produced, but the toxin rapidly disappears from the blood. If the animal be kept at ordinary temperature (20° C.), although the toxin disappears, antitoxin is not formed, but if it is kept at 30°–37° C. antitoxin is rapidly produced. The two experiments together suggest that the toxin is fixed by the cells, but has no effect upon them ; if the toxin were not fixed, it would be possible to detect it, and presumably it would not produce antitoxin.

2. *Natural immunity against micro-organisms.*—A number of factors are doubtless concerned in preserving the body from invasion by micro-organisms, and while non-specific reactions may suffice when the number of organisms is small, specific reactions have to be evoked if the number of organisms be large. The unbroken surfaces of the body have a considerable protective action in preventing the entrance of micro-organisms. Infection is an active process quite different from the mere presence upon the skin or a mucous membrane of the parasite capable of causing disease. A whole host of potentially infective bacteria are constantly present upon the skin and mucous membranes which for the most part do no harm whatever, and possibly, by preoccupation of the soil, tend to ward off other more definitely injurious organisms. The surfaces of the body indeed seem to possess a high degree of insusceptibility to ordinary infections, they have a local immunity. Thus wounds of the mouth and rectum generally heal well in spite of their septic condition. In some cases this local immunity depends upon factors which operate only so long as they

are intact. In the young the mucous membrane of the digestive tract is easily affected so as to become the seat of slight pathological conditions that depress their protective power and hence the prevalence of tuberculous cervical and mesenteric glands and of microbic infections of the stomach and intestine in the young. Typhoid fever ceases with the anatomical changes, coming on in the fourth decade of life, that reduce the absorbing power of the intestine.

The mere presence of infective and invasive bacteria upon a mucous surface is, again, not tantamount to infection. In every epidemic disease, we know or have reason for believing that many more persons carry the germ of the disease than actually contract it. Thus diphtheria bacilli and meningococci are found in the throat and nasopharynx of many well persons during an epidemic who never develop the disease, and similarly during prevalence of cholera, dysentery, and enteric fevers, the bacilli causing them are present in the intestinal tract of persons in health. And these healthy "carriers," while they themselves may escape infection, are frequently the means of infecting others. The reason for the phenomenon is to be sought in an adequate defensive mechanism in the one group who escape infection, and in a defective mechanism in the other acquiring it. That this is the explanation is suggested by the fact that in the case of a cholera carrier, the ingestion of irritating substances may transform the well carrier into a case of cholera.

Infants frequently exhibit a more marked resistance to some of the diseases of childhood, *e.g.* measles, than do older children. This may be, and probably is, due to the transference of protective substances from the mother to the child first by the placental circulation and later by the milk. The immunity, being passive (p. 228), tends to disappear so that the child of a few years becomes

susceptible. Not all individual parasites of the same species, whether bacterial, protozoal or ultramicroscopic, are potentially equal as agents of infection. The quality of virulence, so-called, is of high importance. Not a few of the common parasites vary greatly in virulence, *i.e.* in capacity for infecting, from degrees that make them almost harmless to degrees that make them inconceivably potent. This state of virulence in some instances is determined by races of the parasite of particular quality, so that what is virulent for one species is not necessarily virulent for another. Thus strains of pneumococci are known of which a single organism will set up a fatal septicæmia in the rabbit, but many millions of which may not infect the guinea-pig. By successive transfers through a susceptible animal a slightly virulent strain may be rendered incredibly virulent. The changes take place sometimes slowly and sometimes quickly ; in the latter instance, they correspond to or suggest the appearance of " sports " or " mutants " among the higher plants or animals. It may well be that epidemic prevalence of a disease is related to some such acquisition by the germ of heightened virulence or capacity for infecting.

On the other hand, certain parasites under particular conditions acquire the power of resistance to factors inimical to their existence. Under the influence of specific germicidal serums and drugs, they undergo a subtle change through which they acquire a capacity of effective resistance to the germicidal agent. This state is known as " fastness " and seems to be equivalent to the development of sports or mutants among higher forms. Our knowledge of this condition has been chiefly derived from a study of trypanosomes and spirochaetes, but it also occurs among the bacteria. Among the protozoal trypanosomes it persists only so long as they continue to multiply asexually in the blood of the host and disappears when the organisms multiply sexually in the

intermediate host. Bacterial fastness tends to disappear when the organisms are cultivated artificially outside the body. This acquirement of a resistant state by the parasite may be accountable in some instances for the relapses which occur in the course of some infective diseases, *e.g.* typhoid fever.

The flushing-out action of accelerated circulation will exert some action in eliminating organisms from a localised focus of infection just as it does with toxins. The body temperature may be of some importance, and the febrile condition so generally induced by infection is probably to some extent protective and curative. Thus frogs, fish, and chickens are naturally immune to anthrax. In the one case the body temperature is low, 18° C. or thereabouts; in the other it is high, 40° to 41° C., and this may influence the growth of the anthrax bacillus, preventing the full and rapid development which may be necessary for the production of the disease. That such is the case would seem to be shown by experiments in which when the temperature of the medium is raised or lowered, infection takes place; frogs and fish kept in water raised to a temperature of 35° C., and chickens refrigerated so as to reduce their temperature, all perish from anthrax after inoculation. It is clear, however, that this is not necessarily the only factor, for sparrows, which have a temperature as high as that of the chicken, can be infected with anthrax without refrigerating. Behring would ascribe the immunity of white rats to anthrax to the high alkalinity of their blood, and claims to have shown experimentally that a vegetable diet reduces this, and fatigue is said to act similarly.

In some cases the animal, after invasion by the organism, becomes gradually tolerant to its presence (*immunitas non sterilisans*). This is particularly the case in protozoan infections, *e.g.* piroplasmosis. The animal,

after a period of ill-health, gradually recovers, though the organisms may still be present, as can be demonstrated by injecting some of its blood into a susceptible animal. Conceivably the receptors necessary for the intoxication become gradually used up, and when this state is attained the animal becomes insusceptible.

The blood, lymph, and other fluid and tissue juices undoubtedly exert a more or less germicidal action on bacteria experimentally *in vitro*, and to some extent probably also in the body. But in this respect there is often a marked difference between the circulating blood and the blood *in vitro*.

Lewis and Cunningham (1872), Traube and Gscheidlen (1874), Fodor (1877,) and Wysokowicz showed that bacteria injected into the circulation rapidly disappear, and were inclined to attribute this result to the bactericidal properties of the blood. In the main, however, this disappearance is due to lodgment in the capillaries, phagocytosis, and excretion by the excretory glands.

Hankin found that Halliburton's cell-globulin β (really a nucleo-protein) had marked germicidal properties, and concluded that it was probably the germicidal constituent of the blood-serum. Bitter, who repeated Hankin's experiments, failed, however, to confirm them. To the germicidal constituents of the cells and body fluids Buchner gave the name "alexins."

Grohmann performed the first experiments with extravascular blood. He found that anthrax bacilli, after being kept in plasma, became less virulent. Fodor, adding anthrax bacilli to blood and plating at intervals, found there was a progressive diminution in the number of organisms.

Nuttall, in 1888, used the defibrinated blood of several animals, rabbits, mice, pigeons, sheep, and found that it destroyed the *B. anthracis*, *B. subtilis*, *B. megaterium*,

and *M. pyogenes* var. *aureus*. He confirmed Fodor's results, which also showed that after a while the blood loses its germicidal properties and becomes a suitable culture medium. The blood or serum similarly loses its bactericidal properties on heating, and serum that has once been used loses its bactericidal properties. Nissen continued this work, and also found that fresh serum is germicidal for a variety of organisms.

In 1890, Buchner with Voit, Sittmann, and Orthenberger came to the conclusion that the germicidal action of cell-free serum is due to the protein constituents.

Christmas prepared a germicidal substance from the spleen, and Bitter, who examined the method, in the main confirmed Christmas.

Behring and Nissen, however, found that the serum of the white rat, dog, and rabbit destroys the *Bacillus anthracis*, but serum from the mouse, sheep, guinea-pig, chicken, pigeon, and frog has no action. Thus, while the rabbit is highly susceptible to anthrax, its serum is germicidal; the chicken, on the other hand, is immune to anthrax, but its serum is inactive. Hence there is a considerable difference between the action of circulating and of extra-vascular blood.

Vaughan, Novy, and McClintock, in a series of papers, ascribed powerful bactericidal properties to the nucleins, and surmised that in serum the nucleins set free by the disintegration of leucocytes and other cells are the germicidal agents. Forrest and the writer [1] found, however, that all the germicidal properties ascribed by Vaughan to the nucleins are probably due to the weak alkali in which the nucleins were dissolved, and came to the conclusion that Vaughan's results are at least not proven.

Gengou also found that the *plasma* collected in vaselined tubes is often almost devoid of bactericidal power,

[1] *Journ. Roy. Army Med. Corps*, 1904.

whilst the corresponding *serum* may be capable of destroying large numbers of micro-organisms.

We therefore see that while the blood, lymph, and other fluids and tissue juices undoubtedly exert more or less germicidal action on bacteria experimentally *in vitro*, there is often a marked difference in this respect between the circulating blood and the blood *in vitro* and it may be doubted if this factor is of great importance in the production of natural immunity. At the same time, it is to be noted that directly infection has started more or less cellular disintegration and serous exudation occur, and thus the germicidal action of the body fluids and tissues may be exerted *in vivo*, though such substances may act rather by stimulating the leucocytes or by rendering the bacteria more phagocytosable, as will be referred to later (p. 232).

Thus Kanthack and Hardy found that the coarsely granular oxyphile leucocytes in the frog are first attracted to the site of a bacterial invasion, there discharge their oxyphile granules, the bacteria then show signs of degeneration, and polymorphonuclear leucocytes and other "phagocytic" cells now approach and ingest the degenerate bacteria. The observations, however, do not seem to have been confirmed. Wooldridge also protected animals from anthrax by injections of "tissue fibrinogen" (nucleoprotein). For some micro-organisms a bacteriolytic mechanism exists, the amboceptor-complement complex, whereby they may be digested and got rid of. Thus normal serum has a marked bacteriolytic action on *B. typhosus* and *B. coli*. In many cases, however, *e.g.* for staphylococci, such a bacteriolytic mechanism does not naturally exist, but may be evoked as a result of infection.

The hypothesis which ascribes immunity to the germicidal and bacteriolytic action of substances in the fluids of the body has been termed the "humoral theory."

Another important theory of immunity is the doctrine of phagocytosis, so ably supported by Metchnikoff. This is the " cellular " theory of immunity. It has as its basis the following fundamental facts : Firstly, the leucocytes in the circulating blood ingest and destroy any foreign particles present therein ; secondly, an injury to the tissues is immediately followed by an inflammatory reaction, in which the leucocytes emigrate from the vessels and congregate at the injured spot. Similarly, in many instances the leucocytes rapidly congregate at the seat of a bacterial infection, and approach and engulf the bacteria in the same manner as they do other foreign particles, and so rid the body of the unwelcome guests (Plate I., *a* and *b*).

The migration of the leucocytes towards the scene of action is explained by Metchnikoff on the hypothesis that the chemical substances elaborated by the bacteria attract the latter and exert what he termed " positive chemotaxis." In this case the bacteria are removed by the leucocytes, and general infection and death do not occur. But, unfortunately, in other cases the bacterial chemical products repel, or perhaps it is more correct to say do not attract, the leucocytes, and " negative chemotaxis " occurs, so that the bacteria are free to grow and multiply, and general infection ensues. Positive and negative chemotaxis can be shown to occur by a simple experiment. If a fine capillary tube containing some peptone solution be introduced into a suspension of bacilli, *e.g. B. fluorescens liquefaciens*, under a cover-glass, and watched microscopically, the bacilli will be attracted to the tube and soon invade its lumen. If, however, a weak acid be substituted for the peptone water, the bacilli will be repelled. The process by which the bacteria are ingested by the leucocytes can be similarly watched. The leucocytes which act in this manner are termed phagocytes, and they are of two classes—the

macrophages, the large mononuclear leucocytes, and the smaller microphages, or polymorphonuclear leucocytes. Certain of the tissue cells and endothelial cells also possess phagocytic properties. The importance of phagocytosis is also shown by the fact that, while in ordinary susceptible rabbits infection with anthrax is followed by a feeble phagocytosis and the animals succumb, in rabbits vaccinated against anthrax phagocytosis is very active. Moreover, in an animal refractory to anthrax, such as the frog, anthrax bacilli grow and multiply if they be enclosed in paper or collodion sacs, so as to prevent the access of the phagocytes.

Phagocytosis, *in vitro*, and probably also in the normal body, is extraordinarily active, so that it might be expected always to be sufficient to deal with any number of bacteria that might be introduced. If, however, the bacteria be virulent, negative chemotaxis will occur. Moreover, the presence of substances which render the bacteria phagocytosable, "opsonins," is necessary, and it seems likely that the amount of opsonin becomes diminished in infection (see p. 234).

Metchnikoff admits that the destruction of bacteria in phagocytosis is brought about by chemical bacteriolytic substances, which he terms "cytases," and which he regards as being derived from the leucocytes, and as identical with the alexins. He believes that there are two kinds of cytases, one "macrocytase," obtainable from tissues, such as the spleen and lymph-glands, rich in macrophages, which acts specially on elements of animal origin, the other "microcytase," derived from the microphages, and which acts principally on micro-organisms. He considers the alexic action to be of the nature of a digestive process (but this is doubtful), and as regards the complex nature of a cytolytic serum, which contains amboceptor and complement, believes that the ambo-

ceptor is formed within the macrophages in intra-cellular digestion, and that a portion of it escapes from them into the serum. All the facts point to the leucocytes and leucocytic tissues being the great defensive mechanisms against parasitic invasion, either by the production of alexins, or of bacteriolysins, or by phagocytosis, or probably by a combination of these (the "cellulo-humoral" hypothesis of immunity). It is probable that the greater part of phagocytosis takes place in the spleen, and dogs deprived of the spleen become susceptible to anthrax. This organ acts as a sort of filter, and phagocytosis may be active in it when none can be discerned in the blood. Phagocytosis is also active in the bone-marrow.

Although small amounts of antitoxin may occasionally be met with in the normal animal (*e.g.* diphtheria antitoxin in man and in the horse, see pp. 167 and 306), this substance plays little or no part in natural immunity against either toxin or micro-organism. Thus the blood-serum of the fowl, which is highly refractory to tetanus, does not exert the slightest antitoxic or neutralising action on tetanus toxin.

3. *Acquired immunity.*—Acquired immunity may be induced in several ways :

(1) By an attack of the disease ending in recovery.

(2) By vaccinating with a modified and less virulent form of the living infective agent (Pasteur's method).

(3) By treatment with sterilised cultures, or with bacteria-free toxins.

(4) Occasionally by treatment with sterilised cultures or toxins of a different species. Thus, *B. pyocyaneus* protects from anthrax, and Klein [1] showed that an injection of one of the six following organisms— (1) Koch's comma, (2) Finkler-Prior's comma, (3) *B. coli*, (4) *Proteus vulgaris*, (5) *B. prodigiosus*, (6) *B. typhosus*

[1] *Trans. Path. Soc. Lond.*, 1893, p. 220.

will protect an animal against any one of the remaining five. He therefore concluded that there is an immunising agent common to all these six organisms, and that this substance is intra-cellular and a constituent of the bacterial cells themselves. In this case, however, the immunity is probably one against certain bacterial proteins and not against the specific endotoxins of the organisms.

(5) By injection of the blood-serum derived from an animal treated or immunised by method (3)—that is to say, antitoxins or other anti-bodies (*e.g.* amboceptors) are introduced.

The immunity acquired by methods (1)–(4) is known as "active immunity," because the animal's cells and tissues are altered by the process, so that they are no longer susceptible to the microbe or its toxin. The immunity conveyed by method (5)—the injection of an immune serum, is known as "passive immunity," because the immunity lasts only so long as the anti-bodies remain; there is no active participation of the animal's cells and tissues in the process. Active immunity is generally of long duration—some months at least—and is not transmissible to the fetus; but passive immunity is of short duration—two to four weeks—and is transmissible to the fetus and nursling. Acquired immunity to toxins may be due to the elimination of the receptors concerned in the fixation of the toxin by the cells, or to the production of the neutralising antitoxin. The leucocytes are probably the active agents in destroying and eliminating toxin, whether neutralised by antitoxin or not.

Various explanations have been given of the production of acquired immunity against the organisms. Pasteur suggested that the organism, by its growth in the body, exhausts some specific pabulum necessary for its develop-

ment, so that it cannot again grow in the animal which has been attacked. This hypothesis, therefore, presupposes that in the body there is some nutrient material necessary for the growth of each species, which is difficult to believe, and is negatived by the fact that an organism will grow in the blood and tissues removed from an animal vaccinated against, and insusceptible to, the disease produced by itself.

Pasteur's "exhaustion" theory has been revived by Ehrlich [1] in a modified form, under the name of "atrepsy," to explain certain cases of immunity. Thus, for a chemical poison to act, Ehrlich assumes that particular receptors in the protoplasm for binding the poison are necessary; these he terms "chemo-receptors." Bird-pox, virulent for both fowl and pigeon, if passed through the pigeon becomes completely avirulent for the fowl. To explain this Ehrlich suggests that the parasite in passing through the pigeon has to assimilate substances different from those assimilated during its passage through the fowl; therefore that part of the receptors which deals with the nutritive substances of the fowl's organism is not in use during the passage through the pigeon, and may become atrophied, so that on the parasite being transferred back to the fowl it will not be able to thrive owing to the loss of the receptors necessary to assimilate the fowl's nutritive substances. Ehrlich suggests that the majority of non-pathogenic micro-organisms, if introduced into the animal body, perish by this mechanism. In the case of mouse carcinoma inoculated into rats, the tumour-cells proliferate for a few days, then atrophy and disappear. Ehrlich suggests that some specific substance is necessary for the proliferation of mouse carcinoma-cells which is not present in the rat, and as soon as the traces of this specific substance carried over by the inoculation are used up, the cancer-cells cease to proliferate and finally atrophy and disappear. These are examples of Ehrlich's "atrepsy" and "atreptic immunity."

Chauveau, in his retention theory, suggested that the bacteria during their growth in the tissues form substances which ultimately inhibit their growth, and, if

[1] "Harben Lecture," ii, *Journ. Roy. Inst. Public Health*, 1907.

the animal recovers, prevent a subsequent development of the organism. The same objections may be urged against this hypothesis as against Pasteur's exhaustion hypothesis.

Bacteriolysis and phagocytosis are probably the two principal factors which bring about the refractory condition in acquired immunity against bacteria, as well as recovery from an infection. After immunisation it may be shown that phagocytosis is increased, and that positive chemotaxis takes place towards the organism, whereas previously negative chemotaxis occurred ; the leucocytes have been " educated," as it were, to be attracted, instead of repelled, by the bacterial invasion. According to Andrewes,[1] the defence against the pyogenic cocci is not only essentially phagocytic, and dependent upon the polynuclear leucocytes, but is also, in the main, opsonic. In tuberculosis and syphilis the polynuclear leucocyte takes little part in bodily defence, which is essentially a function of the endothelial and fixed tissue-cells. With the colon group of organisms certain humoral responses, notably agglutination and bacteriolysis, are better marked than with most other bacteria, and polynuclear phagocytosis seems subsidiary.

Antitoxin formation probably plays little or no part in acquired immunity, or even in recovery from infection. In diphtheria, for instance, antitoxin is not found until the disease has subsided. Possibly, in chronic infections, antitoxin formation does play a subsidiary *rôle* in recovery.

To sum up, natural immunity is probably due to a number of factors, some or all of which may be operative in particular instances, and it is impossible to state with certainty any general law. In most cases phagocytosis is the principal means of defence, the germicidal, inhibitory, or bacteriolytic actions of the body-fluids aiding,

[1] " Croonian Lectures," *Lancet*, June 25 *et seq.*, 1910.

though of subsidiary importance ; in others the cells and tissues are unaffected by the bacterial toxins, sometimes because the cells are lacking in the particular side-chains or receptors which fix the toxin ; sometimes because, for some unknown reason, the cells are unaffected by the toxophore group of the toxin.

As regards the immunity acquired after an attack of disease, this may be due to the " education " of the leucocytes, whereby they are attracted, whereas formerly repelled, by the products of bacterial development, or to substances which stimulate the action of the leucocytes. The germicidal, inhibitory, and bacteriolytic actions of the body-fluids may also be enhanced. It seems probable also in certain instances that the side-chains or receptors having an affinity for the toxin become in some way destroyed or used up, so that further fixation of the particular toxin cannot take place.

It is to be noted, as Metchnikoff has pointed out, that immunity is much more rapidly acquired against micro-organisms than against their toxins. In Nature, it is principally against micro-organisms that the body requires protection.

Adaptability seems to be one of the innate properties of protoplasm, and immunity is but an instance of adaptability. It might be expected, therefore, that immunity towards infection will become established, more or less completely, when the need for it arises ; and we find that this is the case, however difficult it may be to explain the mechanism by which it is attained.

The Rôle of the Serum in Phagocytosis

The fact that in an immunised animal, no sooner does the virulent organism gain access than the leucocytes migrate to the site of infection, surround the invaders, ingest and so destroy them, was at one time ascribed by

Metchnikoff to "education," *i. e.* modification, of the leucocytes; but since the serum of the immunised animal injected into a non-immunised one causes the leucocytes in the latter to behave in the same manner as they do in the immunised animal, the effect must be due to something in the plasma or serum, and Metchnikoff ascribed the action to substances, "stimulins," which heighten the activity of the leucocytes. Later work has not confirmed this view, and no certain proof of the existence of stimulins is forthcoming, although Leishman attributed a stimulin action to thermostable substances in the serum in typhoid and Malta fevers. Subsequently Metchnikoff conceived the serum as acting, not on the leucocytes, but on the microbe, causing it to become positively chemotactic and no longer to repel, but to attract the phagocytes. Considerable support was given to this view by the work of Wright and Douglas, who, by a modification of Leishman's ingenious method for quantitatively estimating phagocytosis, emphasised the importance of the serum in the mechanism of phagocytosis.

Neufeld and Rimpau also concluded that substances, "bacteriotropines," are produced in the course of immunisation which promote the phagocytosis of bacteria.

Leishman's method for estimating phagocytosis.[1]—A thin suspension of some micro-organism, *e.g. M. pyogenes*, is mixed with an equal volume of blood from the finger; a droplet of this mixture is placed on a clean slide, and covered with a cover-glass, and the preparation is at once placed in a moist chamber in the incubator at 37° C. for half an hour. At the end of this time it is taken out, the cover-glass slipped off, and the films on slide and cover-glass are dried, fixed, stained, and examined microscopically, and the number of microbes ingested by the polymorphonuclear leucocytes is counted.

Wright and Douglas[2] found that washed leucocytes

[1] *Brit. Med. Journ.*, 1902, vol. i, p. 73.

[2] *Proc. Roy. Soc. Lond.*, B. lxxii, 1903, p. 357; B. lxxiii, 1904, p.

without serum are non-phagocytic, but become so on the addition of normal serum. If, however, the serum be first heated to 60°–65° C. before being added to the mixture of leucocytes and microbes, phagocytosis does not take place ; but if the unheated serum is mixed with the bacteria, the mixture kept at 37° C. for fifteen minutes and then heated to 60° C. for fifteen minutes, phagocytosis can still take place, thus demonstrating that the serum acts in some way on the bacteria, rendering them suitable prey for the phagocytes. This thermolabile serum feast preparer is termed "opsonin" by Wright and Douglas (from a Greek word meaning "to cater for").

They have also shown that during the process of active immunisation the opsonic value of the serum is increased, and they have succeeded in demonstrating this opsonic immunity for a number of infections, such as the staphylococcic, Malta fever, pneumococcic, and tuberculous. If it be desired to measure the quantity of opsonins present, say in a case of furunculosis, which is almost always caused by the *M. pyogenes*, var. *aureus*, the following are required : (1) a drop or so of the patient's serum ; (2) a drop of serum from a normal person ; (3) a suspension in salt solution of a culture of *M. pyogenes*, var. *aureus* ; (4) leucocytes washed free from the plasma. Equal volumes of the patient's serum, leucocytes, and suspension are mixed, drawn up in a capillary tube, incubated for fifteen minutes at 37° C., and films are then prepared and stained. As a control a similar mixture is prepared and treated in the same way, but using the *normal* serum instead of that of the patient. The films are then examined, and the number of cocci taken up by, say, fifty leucocytes is counted in the two specimens, and

128 ; B. lxxiv, 1905, pp. 147, 159 ; B. lxxvii, 1907, p. 211. Also in *Practitioner*, May, 1908 ; various papers in *Lancet* and *Brit. Med. Journ.* ; Wright, *Studies in Immunity*, 1909.

a ratio obtained. Taking the figure for the normal serum as 1, that for the patient's serum will probably be 0·5 or 0·6, and this is termed the "opsonic index" (see below, p. 242).

In subacute and chronic local infections the opsonic value of the serum is usually diminished, occasionally increased. In acute infections the index will, as a rule, be low; in chronic infections which are not strictly localised, *e.g.* tuberculosis, the index will sometimes be low, sometimes high. A low index generally indicates an infection, or a low power of resistance to the particular organism, or that a chronic but quiescent infection exists; a high index may indicate that the person has had an infection but has overcome it, or has a quiescent infection. The normal index for healthy persons varies only within narrow limits, from about 0·8 to 1·2 as extremes; an index above or below these values is therefore probably pathological.

By injecting small quantities of a vaccine consisting of a killed culture, tuberculin, etc., the opsonic index can usually be raised, and coincidently the infection tends to be cured. The first effect of the injection is to cause a fall in the opsonic index, the "negative phase" of Wright, which is usually afterwards followed by a rise, and by properly spacing the injections a considerable rise in the opsonic value may ultimately result. If too much vaccine be given the effect may be to depress the index for a long period and cause harm instead of good, hence the desirability of controlling all injections by determinations of the opsonic index. This, however, renders the treatment very laborious, and generally by employing small doses and allowing at least a week to elapse between the doses, determinations of the opsonic index are unnecessary (for dosage, etc., see p. 244). By movement, massage, etc., applied at or about the seat of a local

infection, bacterial products are disseminated which may alter the index ; a process of auto-inoculation may thus result.

The opsonic index may be used for diagnostic purposes ; a low or high opsonic value towards a particular organism suggests that an infection by this organism exists or has recently existed.

Bulloch came to the conclusion that the normal blood contains a number of specific opsonins, one for tubercle, another for *M. pyogenes*, and so on. Simon, Lamar, and Bispham,[1] however, from a number of carefully devised experiments, conclude that specificity of normal opsonins does not exist, and suggest that opsonins may be a constant quantity, and that the number of organisms taken up by the leucocytes is influenced by a second unknown and variable factor.

Russell [2] also concludes that in *normal* serum the opsonins are "common" and not specific, and can be removed by a number of bodies. In immune serum, on the other hand, both "common" and "immune" opsonins are present, the latter being quite specific. That is to say, in the process of immunisation specific opsonins are formed and the increase of opsonins following injection of a vaccine is probably due to the formation of immune opsonins which react specifically.

Muir and Martin [3] believe that in immune serum a specific thermostable opsonin is present, together with a normal thermolabile opsonin.

Wright considers the opsonins to be substances distinct from all others, but Metchnikoff, Dean, and other observers suggest that they are identical with the "substance sensibilisatrice."

[1] *Journ. Exper. Med.*, vol. viii, 1906, p. 651.
[2] *Johns Hopkins Hosp. Bull.*, vol. xviii, 1907, p. 252.
[3] *Proc. Roy. Soc. Lond.*, *B.* lxxix, 1903, p. 187.

It is doubtful if opsonins are present in more than traces in the unaltered blood *plasma* : like alexins, they seem to develop as a result of coagulation. The *rôle* of opsonins in immunity and in recovery from infection is therefore a complex problem.

The opsonic method has been much criticised. Thus Moss [1] says : "None of the present methods of estimating the opsonic content of the blood seem sufficiently accurate to be of practical value" ; Fitzgerald, Whiteman, and Strangeways,[2] in an elaborate investigation, concluded that the method is unreliable. Whereas Wright takes into account the serum only, Shattock and Dudgeon [3] state that "the cells (*i.e.* the phagocytes) vary in value like the serum." Many of the criticisms have been based on an imperfect technique. On the whole, it may be said that Wright's method, with careful technique and in practised hands, gives information previously impossible to obtain, and the proper dosage of, and treatment by, vaccines has been largely elaborated by means of it.

Method of Determining the Opsonic Index

The requisites are :

1. Several Wright's pipettes with india-rubber teats.
2. The serum of the patient to be tested.
3. The serum of a healthy person for a control.
4. A suspension of the organism for which the determination is to be made.
5. A suspension of living leucocytes.

1. *Wright's pipettes with india-rubber teats.*—These are made of glass tubing of the form shown in *a*, Fig. 37, which is about two-thirds full size. Glass tubing must be chosen which properly fits the teats. A piece of glass-tubing about 4 inches in length is taken, heated in the blowpipe flame until quite soft, then it is taken out of

[1] *Johns Hopkins Hosp. Bull.*, vol. xviii, 1907, p. 237.

[2] *Bull. Committee for the Study of Special Diseases* (Cambridge), vol. i, 1907, No. 8.

[3] *Proc. Roy. Soc. Med.*, vol. 1, 1908, "Medical Section," p. 169.

the flame and the two ends are drawn steadily apart; the more they are drawn apart the finer will be the bore of the tube—about $\frac{1}{16}$ in. is a suitable size. The middle of the capillary part should then be introduced into a small white gas-flame and drawn apart so as to form two pipettes. By filing off the sealed end at a suitable spot the open extremity may be slightly contracted as shown in *b*; this prevents the column of fluid in the tube moving so quickly. A throttled pipette (p. 56) may be used.

2 and 3. *The Sera.*—These two specimens should be

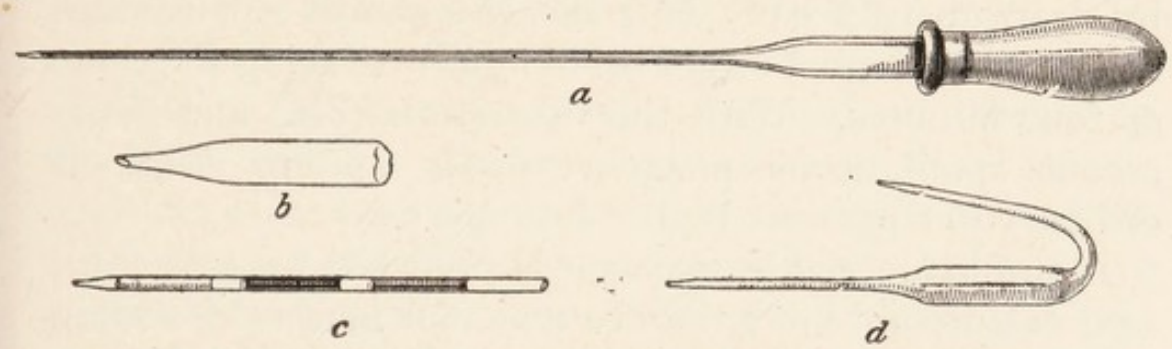

FIG. 37.—*a*. Glass pipette, with india-rubber teat for opsonic determinations, etc.; *b* shows (enlarged) the contracted extremity of the pipette; *c* shows the step of the pipette, containing the equal volumes of serum, leucocytic suspension, and bacterial suspension, before mixing; *d* is the Wright's capsule for collecting blood.

taken at about the same time, and the determination should be made as soon as possible.

The blood is preferably collected in a Wright's capsule (Fig. 37, *d*). Both ends of the pipette are broken off, and the blood is collected by immersing the *bent* end in the blood as it runs from a prick made with a Hagedorn or triangular needle in the ear or finger. The capsule, which should be at least one-third filled, is then sealed in the flame, the dry or straight end being sealed first. After coagulation, which may be hastened by placing in the warm incubator for half an hour, the capsule is hung by the curved end in the centrifuge and centrifuged to obtain

clear serum. Little change in the serum ensues for two to three days if the capsules are kept sealed. The capsules may be stuck into a lump of plasticine until required. Plasticine is useful for many such purposes, for temporarily plugging tubes, etc.

4. *Suspension of the organism.*—In the case of tubercle, suitable dead cultures can be purchased. To prepare the suspension from these, a small portion of the growth (about as big as a grain of rice) is ground up in a small agate mortar, 1·5 per cent. salt solution being added drop by drop up to 2 c.c. This suspension will still contain clumps, which must be got rid of by centrifuging for three or four minutes. With the tubercle bacillus and gonococcus spontaneous phagocytosis is apt to occur if ordinary (0·8 per cent.) salt solution is used.

A staphylococcic suspension is prepared by taking an agar culture not more than twenty-four hours old, adding salt solution (0·8 per cent)., and shaking gently so as to wash off the growth. When the suspension is made it must be pipetted off into a small tube and centrifuged for a few minutes. The suspension must not be too thick, otherwise the leucocytes will take up an unaccountable number of cocci ; the proper density can be judged by experience alone, but the suspension should be only faintly opalescent. Suspensions of pneumococci and other organisms are made in the same way. Variations in the number of bacteria ingested may occur according as recently isolated or old strains are employed.

Instead of centrifuging, the suspensions may be filtered through a double thickness of filter-paper.

5. *Suspension of living leucocytes.*—To prepare this, take about 10 c.c. of physiological salt solution containing $\frac{1}{2}$ per cent. of sodium citrate, to prevent the coagulation of the blood. This must be freshly prepared (or kept sterile, which is inconvenient), and the simplest method

is to use " soloids " prepared for the purpose by Burroughs and Wellcome ; one of these dissolved in 10 c.c. of distilled water will yield the solution required. This is put into a centrifuge tube and warmed to blood-heat. A healthy person is then pricked in the ear or finger, and his blood is allowed to drop into the fluid until 1 c.c. or more has been collected. The tube is then centrifuged until all the corpuscles have come to the bottom and the supernatant fluid is left clear. If the deposit is closely examined the red corpuscles will be seen to be at the bottom, whilst above them there is a thin grey layer of leucocytes. The whole of the clear fluid is then pipetted off, as close as possible to the leucocyte layer, but without disturbing the latter, with a pipette armed with an india-rubber teat, or with a syringe. The tube is again filled with saline solution, the blood and fluid are mixed, the mixture is centrifuged, and the clear fluid pipetted off, and this process of washing is repeated. Next, the leucocyte layer with the upper layer of red corpuscles (which also contains leucocytes) is pipetted off into a small tube, and the whole is thoroughly mixed by repeatedly sucking into, and expelling from, the pipette. The result is a suspension of living leucocytes mixed with red corpuscles.

The process. (1) Make a pipette and place an india-rubber teat on the thick end. With a grease pencil or with ink, make a transverse line about an inch from the point ; the volume of fluid contained between the point and this mark is spoken of as the unit.

(2) Having the patient's serum and the suspensions of leucocytes and of bacteria ready to hand, take the pipette between the index finger and thumb of the right hand and compress the nipple. Immerse the point beneath the surface of the suspension of bacilli, and relax the pressure on the nipple until the suspension has risen exactly to the mark so that one unit has been drawn up ;

then remove the point from the fluid and relax the pressure again so that a *small* volume of air is sucked up. This will be quite easy if the point is a good one, otherwise it will be difficult or impossible, as the column of fluid will either refuse to stir or will oscillate violently. Next immerse the point in the suspension of leucocytes and draw up one unit. This will be separated from the suspension of bacteria by the bubble of air. Remove the point from the suspension and draw up a second volume of air.

Lastly, draw up one unit of the serum. There will now be in the pipette (counting from the nipple towards the point) one unit of bacterial suspension, a bubble of air, a unit of leucocytes, a bubble of air, and lastly a unit of serum (*c*, Fig. 37).

(3) Put the point of the pipette on to a clean hollow-ground slide or an artist's porcelain sunk palette, and express the whole of its contents, and mix well together, aspirating them repeatedly into the pipette and expelling without causing bubbles. If bubbles form, a hot wire brought near will quickly dispel them. When thoroughly mixed, aspirate the mixture into the pipette, suck up a short volume of air, and seal the tip in the flame.

Then place the pipette point downwards in the incubator, or better, in a water-bath at 35° to 37° C., noting the time exactly, and proceed to prepare a second pipette in precisely the same way, using the same suspensions of bacteria and leucocytes, but the *control serum* instead of the patient's. Place this in the incubator or water-bath, by the side of the other, noting the time at which this is done. When each pipette has been incubated for a quarter of an hour it is removed from the incubator or water-bath, the end broken off and the nipple fitted to the thick end; then the contents are expelled on to a hollow slide or porcelain palette and mixed thoroughly

together. Films are then prepared. This may be done by depositing a drop in the middle of a large cover-glass (1-inch squares, No. 2), dropping on to it another cover-glass and drawing the two apart. Or the films may be made on slides, for which Wright recommend roughing the slides with fine emery paper and spreading the film with the sharp edge of a broken slide (see below). The films then have to be stained. For staphylococci, streptococci, pneumococci, *B. coli*, etc., the films may be fixed with formalin and stained with carbol-thionine blue or borax-methylene blue (see "Malaria"), or they may be stained without previous fixing with the Leishman stain. For tubercle, the films may be fixed in a saturated solution of mercuric chloride (one or two minutes), stained in warm carbol fuchsin, decolorised with $2\frac{1}{2}$ per cent. sulphuric acid in methylated spirit, and counter-stained with methylene blue. (Plate I, *a* and *b*).

Wright now uses the whole blood instead of the leucocyte layer only. After the blood has been drawn into the citrated salt solution it is centrifuged, washed twice with salt solution, the fluid is pipetted off, and finally the corpuscles are well mixed. The various mixtures—washed corpuscles, bacterial suspension, and serum—are made and incubated as previously described. In order to make the film for staining and counting, the contents of the pipette are discharged on to one end of a slide roughed with fine emery paper and the mixture is spread by means of a slide which has been broken across after notching with a file or glass cutter. The object is to obtain a broken edge having a very slight concavity, and many slides may have to be sacrificed to attain this. The film is spread by drawing (not pushing) along, the leucocytes adhere to the edge of the spreader, and finally are deposited mostly at the end of the preparation, the red corpuscles being left behind.

Lastly, the films after staining are examined with the oil-immersion lens, preferably with the aid of a mechanical stage, and the number of organisms contained in not less than fifty polymorphonuclear leucocytes is counted. Parts of the film in which the cells are broken down or not well stained, or cells containing obvious clumps of organisms, should be avoided. The ratio between the number in the control and the number in the specimen prepared with the patient's serum gives the *opsonic index*. Thus, if in the control there are 125, while in the patient's specimen there are 75, the index would be $\frac{75}{125} = 0{\cdot}6$, *i.e.* not much more than half the normal.

Preparation of vaccines for treatment, etc.—The vaccine used for treatment is a sterilised, standardised suspension of the infecting organism, except in the case of tuberculosis, for which tuberculin (TR or BE) or an analogous preparation is employed. In certain instances a mixture of organisms is used—*e.g. M. pyogenes*, var. *aureus* and var. *albus*, with or without the acne bacillus in some cases of acne—and the strain of organism isolated from the lesion is generally to be preferred.

The vaccine is prepared by growing the organism under appropriate conditions, the staphylococcus on agar, the streptococcus, pneumococcus, and gonococcus on blood-agar, etc. The growth is then made into a suspension by adding a few drops of sterile saline solution and well rubbing up with a sterile glass or aluminium rod. Two or three tubes are treated in this way; the suspension is poured into a sterile tube or small flask of stout glass, the culture tubes are rinsed out with a little more of the salt solution, and the washings added to the suspension, two or three sterile glass beads are added, and the vessel, sealed or corked, is shaken vigorously for some time, preferably in a shaking machine, so as thoroughly to break up the masses of organisms. The contents of the vessel, which should measure 5 c.c. or thereabouts, are then centrifuged for some minutes, the suspension is poured off from the deposit into a second sterile flask and is now ready for standardisation.

Standardisation is carried out by Wright's method. Two or three volumes of citrate solution are sucked up into a pipette such as that used for opsonic determinations, the finger is pricked and

one volume of blood is taken up in the pipette, separated from the citrate solution by an air-bubble, and finally one volume of the bacterial suspension, also separated from the blood by an air-bubble, is taken up. The whole contents of the pipette are then well mixed by expelling on to a clean slide and sucking up three or four times. About one-third of the mixture is then transferred to each of three clean slides, and the drops are spread with the edge of a slide so as to obtain thin uniform smears. These are allowed to dry, stained with Leishman's stain, and the number of red corpuscles and bacteria is counted in a number of microscopical fields. Assuming that there are 5,000,000 red cells in a cubic millimetre of blood, it is easy to calculate approximately the number of bacteria contained in the suspension. Suppose that 500 red cells have been counted, and with these 1500 bacteria are admixed. Since equal volumes of blood and suspension have been taken, one cubic millimetre of bacterial suspension will contain $\frac{5{,}000{,}000 \times 1500}{500} = 15{,}000{,}000$ bacteria. But one cubic centimeter contains 1000 cubic millimetres, therefore the suspension contains $15{,}000{,}000 \times 1000 = 15{,}000{,}000{,}000$ bacteria per cubic centimetre, and by appropriate dilution any bacterial content of the suspension may be obtained. Thus, if 1,000,000,000 organisms per cubic centimetre is desired, 1 c.c. of the suspension must be diluted with 14 c.c. of salt solution. To the prepared dilution of the bacterial suspension 0·5 per cent. of carbolic acid, or 0·2 per cent. of trikresol, is added, and the flask is placed in a water-bath at 56° to 60° C. for one or one and a half hours, according to the resistance of the organism. The proper dilution may subsequently be introduced into small sterile glass ampoules of 1–2 c.c. capacity, which, after sealing and standing for twenty-four hours, may again be sterilised for an hour at 60° C. to ensure the destruction of the organisms: cultures may be made from the sterilised vaccine to ascertain that this is the case. The lower the temperature and the less the heating, consistent with sterilisation, the more active will be the vaccine.

The annexed Table [1] gives an idea of the dosage of vaccines, their toxicity, and frequency of inoculation.

The smaller doses are given at the commencement of the treatment, and the doses are gradually increased.

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[1] See Harris, *Practitioner*, May 1908, p. 647.

Vaccine	Relative toxicity.	Doses	Frequency of inoculation.
Tuberculin.	Very toxic	$\frac{1}{1000000}$ – $\frac{1}{10000}$ – $\frac{1}{2000}$ mgrm.	Every 10–14 days.
B. coli . .	Very toxic	5–15 millions	Every 2, 5, or 10 days.
Pneumococcic	Less toxic than *B. coli*	10–50 millions	Every 36–48 hours in pneumonia ; every 10 days in chronic infections.
Streptococcic	More toxic than pneumococcic	20–60 millions	Every 7–14 days.
Staphylococcic	Less toxic than streptococcic	100–1000 millions	Every 7–10 days.
M. melitensis	—	$\frac{1}{10}$ sq. cm. of surface agar culture (because very difficult to count)	Every 7–14 days.
Gonococcic	Slightly toxic	100–500 millions	Every 7–14 days.

The writer has employed endotoxin solutions as vaccines and believes they are very efficient.

Prophylactic vaccines.—In addition to the therapeutic vaccines for the *treatment* of the declared disease, vaccines are also employed for *prevention* of disease. The preventive or prophylactic vaccines may be :

(1) Living, but attenuated, cultures, *e.g.* anthrax and cholera. This method has also been proposed for plague, and vaccinia must be regarded as being of this nature (this is the " Pasteurian method ").

(2) Killed cultures, autolysed cultures, and endo-toxins.—The first and second are used for typhoid, plague and dysentery, and Hewlett has suggested endo-toxins for typhoid, cholera, plague and diphtheria. Castellani has introduced mixed bacterial vaccines.

(3) Immune sera give protection for a limited time.

(4) Besredka has suggested " sensitised vaccines," *i.e.* cultures saturated with the homologous immune body derived from an immune serum. Some sensitised vaccines are now upon the market. The sensitised streptococcic vaccine frequently acts very satisfactorily in septic wounds with a streptococcic infection.

(For further particulars, see Hewlett's *Serum Therapy*, ed. 2. J. and A. Churchill, 1910.)

CHAPTER VI

THE PATHOGENIC MICROCOCCI AND STREPTOCOCCI. B. PYOCYANEUS. S. VENTRICULI. CEREBRO-SPINAL FEVER AND GONORRHŒA

Suppuration and Septic Conditions

SEPTIC infection and suppuration are of great practical importance, and a knowledge of their etiology is one of the principal factors which have conduced to the great progress made during the Victorian era in the treatment of wounds, whether accidental or due to the surgeon's knife.

Ogston in 1881 and Rosenbach in 1884 demonstrated that micro-organisms are almost invariably present in the pus of acute abscesses, and these observations were repeatedly confirmed by subsequent investigators. Experiments were then initiated in order to ascertain whether these organisms bear a causal relation to suppuration or are merely accidentally present; they showed that a large number of organisms can produce suppuration. These and a number of other observations, clinical and experimental, have proved that the suppurative and septic conditions met with spontaneously, or occurring after surgical interference, are due to the action of micro-organisms. In the "cleaner" wounds and abscesses the chief of these are several species of micrococci (commonly known as *staphylococci*, and the infections which they produce, as *staphylococcic infections*) and streptococci and

occasionally and in special circumstances and localities the pneumococcus, the colon bacillus and the typhoid bacillus. In foul and dirty wounds anaerobic bacilli, alone or associated with the foregoing, play a prominent *rôle*.

Under the terms " suppuration " and " septic diseases " are included such varied conditions as abscesses, boils and carbuncles, cellulitis, osteomyelitis, erysipelas, gonorrhœa, infective endocarditis, pyæmia, septicæmia and sapræmia, puerperal fever, suppurative wounds and gas gangrene.

Although such organisms as the tubercle bacillus, *Actinomyces* and *Entamœba histolytica*, induce suppuration, this is not usually regarded as a *septic* condition ; pathologically, it is only an incident, as it were, in the course of the infection.

There is overwhelming evidence that micro-organisms produce suppuration and septic conditions. Another question of importance is whether aseptic mechanical injuries and sterile chemical agents are able to produce suppuration. Aseptic mechanical injury alone does not seem to be capable of inducing pus production, but it is otherwise with regard to sterile chemical agents. The capacity of a chemical substance to induce suppuration depends partly on the substance and partly on the animal treated with it.

Mercury produces suppuration in the dog, but not in the rabbit ; silver nitrate (5 per cent. solution) has a similar action. Ammonia fails to produce pus ; it is either absorbed without damage, or if in stronger solution produces necrosis of the tissues. Turpentine produces large sterile abscesses in carnivora, and Brieger's cadaverine is likewise stated to set up suppuration.

Sterilised cultures (above a certain amount) of the *Micrococcus pyogenes* and a crystalline body, phlogosin, obtained by Leber from its cultures, produce abscesses

on inoculation. Buchner was also able, by warming various bacteria with 0·5 per cent. caustic potash, to obtain a solution containing protein which was powerfully pyogenic, and Nannotti found that sterilised pus had a similar property. It thus seems certain that a number of sterile chemical agents can set up suppuration. At the same time, it must be clearly recognised that suppuration and suppurative complications, as they occur naturally, are to be regarded as due to the activity of micro-organisms in almost every instance.

Of so-called "septic" diseases, sapræmia, septicæmia, and pyæmia must be mentioned. By "sapræmia" is meant the constitutional condition arising from the absorption of the toxic products elaborated by micro-organisms, the latter being localised and absent from the general circulation. In the acute form it is not a common condition, the best example being that which occurs after parturition; by simply clearing and washing out the uterus the symptoms rapidly abate. In septicæmia not only is there usually (though not necessarily) a local site of infection, but in addition micro-organisms are present in the general circulation. It is true they are not abundant in the latter situation, and Cheyne [1] believes that they are to a large extent arrested in the capillaries. Micrococci and streptococci are the commonest forms. Pyæmia is characterised by the presence of micro-organisms, most frequently streptococci, in the general circulation, and in addition by the formation of abscesses in various situations. These arise usually from suppurative phlebitis with the formation of septic emboli and thrombi. The sequence of events, according to Cheyne,[2] is (*a*) phlebitis in direct connection with the wound; (*b*) a thrombus impregnated with micro-organ-

[1] *System of Medicine*, Clifford Allbutt, ed. 2, vol. i, p. 876.

[2] *Loc. cit.* p. 881

isms is formed in the vein ; (*c*) this softens and disintegrates, and particles or emboli are carried to distant parts ; (*d*) these lodge in the capillaries, with the formation of infarctions and abscesses. Suppurative pylephlebitis is a pyæmia affecting the portal system of vessels. As regards the so-called chronic pyæmia or multiple abscesses, Cheyne considers that it differs from true pyæmia in that embolism plays no part. Organisms gain access to the blood-stream, settle in any spot where the vitality of the tissues is depressed, grow and multiply and there produce an abscess.

The mere presence of micro-organisms does not always suffice, however, for they may be present without producing suppuration ; and the same organism, for example, the *Streptococcus pyogenes*, may at one time produce a localised abscess, at another diffuse cellulitis, and at a third pyæmia ; a number of factors control and modify the occurrence and the particular form of septic disease.

As already mentioned (p. 222), many micro-organisms when injected into the blood-stream are rapidly disposed of ; so when moderate quantities of the *Micrococcus pyogenes* are injected into the circulation of a rabbit, abscesses, as a rule, form only in the kidney. If, however, the organisms be attached to gross particles, so that they cannot pass through the capillaries, embolism occurs and abscesses form about the embolic foci. The virulence of the infecting organism, which varies much, is another factor of great importance. The effect of inflammation and injury in making a part " susceptible " is also very marked. Inject the *M. pyogenes* into animals in which the endocardium or a bone has been damaged, and in all probability an endocarditis or an osteomyelitis will ensue. Damaged tissues, as occur in lacerated and contused wounds and in gunshot and shrapnel injuries are particularly vulnerable to infection. Bacterial associa-

tions are also important. Thus the *B. perfringens* grows much more profusely in the presence of micrococci and streptococci and *vice versa*. The dose and concentration of the organisms are other important factors. Watson Cheyne found that 250,000,000 cocci (*M. pyogenes*) injected into the muscles of a rabbit produced a circumscribed abscess, but 1,000,000,000 caused a general septicæmia and death. So, probably, while the cells in a healthy wound can dispose of a few organisms, if the latter are abundant or in masses they may gain the mastery.

In this Chapter the pathogenic micrococci and streptococci and *B. pyocyaneus* are dealt with. The anaerobic bacilli which play such a part in dirty wounds and gunshot injuries are discussed in Chapter XIII.

Micrococcus pyogenes, var. aureus (Staphylococcus pyogenes aureus)

Morphology and biology.—A minute spherical organism measuring about 0·75 μ in diameter. It generally occurs in more or less irregular groups, but may be met with singly or in pairs (Plate I. *c*). It is non-motile, does not form spores, and stains well with all the anilin dyes and also by Gram's method. It is aërobic and facultatively anaërobic, will develop *in vacuo*, and grows well and rapidly on all the usual culture media at temperatures from 18° to 37° C. On agar-agar it forms a thickish, moist, shining growth, cream-coloured at first, but after a day or two developing a characteristic orange-yellow colour. It grows in the same manner on blood-serum without liquefaction of the medium. Gelatin is rapidly liquefied, the liquefied gelatin being at first somewhat turbid from yellowish masses of organisms; these later on subside and form an orange-yellow sediment (Plate

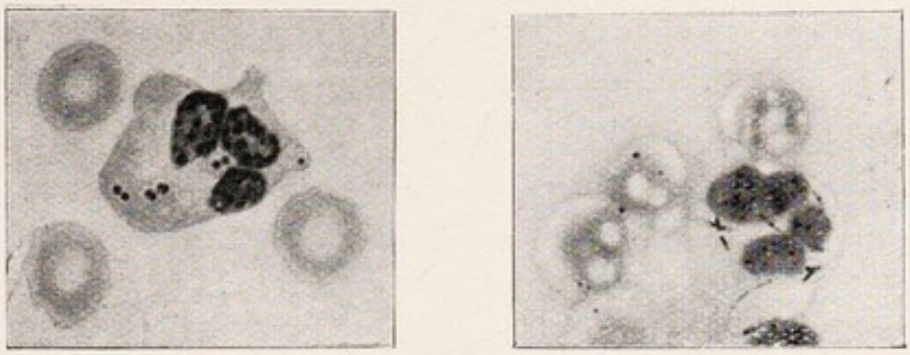
I. *d*). In gelatin plates the colonies form at first small whitish, granular points, developing in two or three days into circular areas of liquefaction with yellowish masses of the organism floating in them. On potato it forms a growth similar to that on agar. When grown in milk it produces coagulation. Acid production (lactic and butyric acids) can be demonstrated by growing on a neutral litmus glucose-agar. When grown in broth or peptone water it gives the indole reaction with the addition of a nitrite, but not without.

The rate of liquefaction of gelatin and the pigment production vary; the latter is sometimes much deeper than at others, recently isolated cultures show it better than old ones, and the presence of oxygen also seems to be necessary. The amount of acid production appears to vary directly with the virulence, which is likewise very variable.

Pathogenicity.—The *Micrococcus pyogenes*, var. *aureus*, is by far the commonest of all organisms met with in suppurative processes. Ogston found it alone in thirty-four, and associated with the *Streptococcus pyogenes* in sixteen, out of sixty-four cases of abscess. It occurs in acute abscesses, boils, and acne, in some cases of puerperal fever and infective endocarditis, and is almost invariably found in osteomyelitis, but only occasionally in pyæmia. The organism injected under the skin of man or animals produces an abscess, and injection into the blood-stream under certain conditions is followed by infective endocarditis or by pyæmia. Impetigo pustules are produced by inunction into the skin.

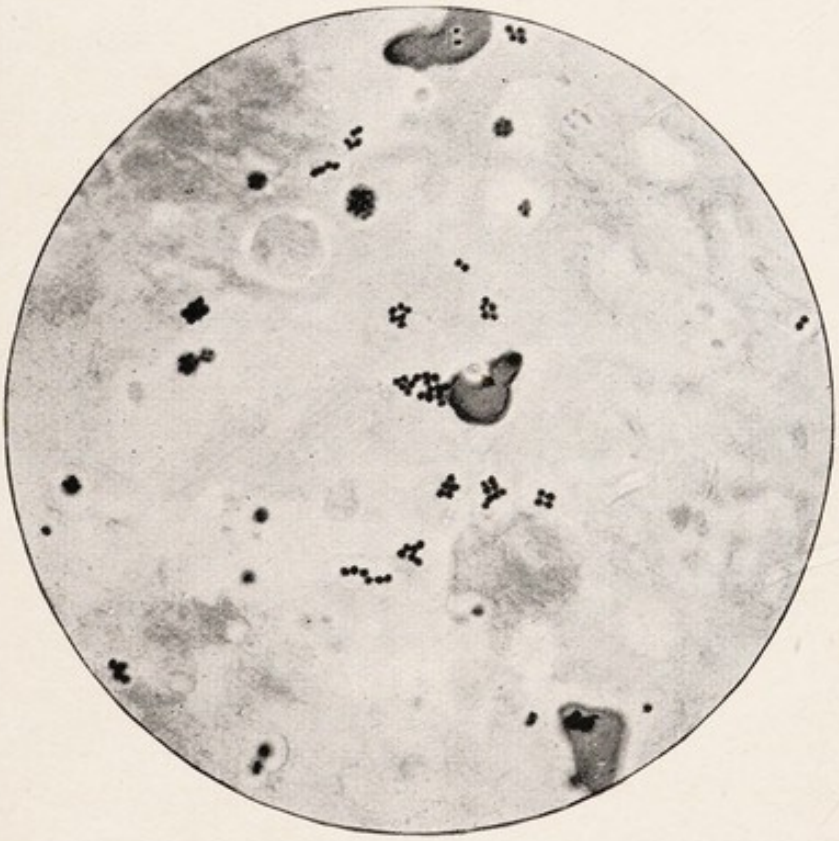
It may be said to be universally present on all parts of the skin, and in the mouth, and is frequently met with in the air. According to Sternberg, recent cultures in gelatin are destroyed by an exposure to a temperature of 56° to 58° C. for ten minutes; but when dried much

PLATE I.



a *b*

Phagocytosis by polymorphonuclear leucocytes. *a. M. pyogenes*, var. *aureus*. *b. B. tuberculosis*.



c. M. pyogenes, var. *aureus* in pus. Smear preparation. × 1000.

d. M. pyogenes, var. *aureus*. Gelatin stab-culture, four days old.

higher temperatures, 90° to 100° C., are required, and in the dried state (on a cover-glass) it retains its vitality for more than ten days. According to different experimenters, from five to fifteen minutes are required to destroy it with a 1–1000 mercuric chloride solution; but it is evident that much depends on the state of aggregation of the organisms, and Abbott has shown that while most of the cocci in a culture are destroyed in five minutes, a few may survive much longer.

Toxins.—In a case of infective endocarditis examined by Sidney Martin, due to the *M. pyogenes*, var. *aureus*, a large amount of an albumose and of a basic body was extracted from the blood and spleen. The albumose produced fever and wasting, and retarded the coagulation of the blood.

Leber extracted a crystalline body, which he termed phlogosin, from cultures of the *M. pyogenes*, var. *aureus*, and Brieger also obtained a crystalline base.

The decomposition products of the action of the *M. pyogenes*, var. *aureus*, on egg-albumen are, according to Emmerling, phenol, indole, and skatole, many volatile and non-volatile acids, betaine, and trimethylamine.

Anti-serum.—Attempts have been made to prepare an anti-serum by the injection of cultures, but the serum is of no practical value. A *vaccine*, prepared by heating a suspension of an agar culture to 65° C. for half an hour and standardising, has been used with much success in chronic staphylococcic infections, such as acne and boils.

Micrococcus pyogenes, var. albus, and var. citreus. Micrococcus epidermidis. Micrococcus cereus

These organisms are of rarer occurrence than the preceding one. In morphology and cultural characteristics the first two agree with the *Micrococcus pyogenes*, var.

aureus, except that the *albus* produces a white, shining, porcelain-like growth, and the *citreus* a lemon-yellow growth, on agar. They are said to be less pathogenic than the *aureus*, and are only occasionally found alone, being usually associated with the *aureus*. Cheyne, however, states that in his experience the *albus* is more virulent than the *aureus*, and mixed infections with the *aureus* are regarded as more severe than infection with the *aureus* alone. The *albus* has been found in some cases of panophthalmitis, and is said by Flügge to be commoner than the *aureus* in the lower animals.

Chief Types of Human Micrococci

Organism.	Broth culture.	Pigment. on agar.	Clot in milk.	Liquefaction of gelatin.	Reduction of neutral red.	Reduction of nitrate.	Acid formation from				Pathogenesis.
							Maltose.	Lactose.	Glycerin.	Mannitol.	
Micrococcus pyogenes	Turbid	Orange, yellow, or white	+	+	0	+	+	+	+	+	+
Micrococcus epidermidis	Turbid	White	+	+	+	+	+	+	+	0	Feeble.
Micrococcus salivarius	Clear	White	0	0	0	+	+	0	+	0	0
Scurf micrococcus	Turbid or clear	White	0	0	0	+	0	0	0	+	0

Andrewes and Gordon [1] regard the *aureus*, *albus*, and *citreus* merely as variants of a single species, the *Micrococcus pyogenes*. They found that every variety of colour, from orange, through yellow to white, might be obtained by cultivation. The *Micrococcus flavescens*, met with by Babes in abscesses, may probably be placed in the same category. On the other hand, the *Micrococcus epider-*

[1] *Rep. Med. Off. Loc. Gov. Board* for 1905–06, p. 543.

midis (*albus*), first described by Welch as occurring on the skin, in stitch abscesses, etc., and feebly pathogenic compared with the *M. aureus*, is stated by these authors to be perfectly distinct from the foregoing. Other organisms which are occasionally met with in abscesses, the *Staphylococcus cereus albus* and *S. cereus flavus* of Passet, form shining waxy growths on agar, and do not liquefy gelatin, and are probably variants of another species, which may be termed the *Micrococcus cereus*. There may be many other varieties of micrococci not yet properly differentiated.[1] Well-defined micrococci occur in the saliva (*M. salivarius*), and in the scurf from the scalp. Andrewes and Gordon give a differential Table (see p. 252) of some of these micrococci.

Micrococcus zymogenes

Isolated by MacCallum and Hastings[2] from a case of acute endocarditis. A minute micrococcus, non-motile, and staining by Gram's method. On surface agar it forms a thin, slightly elevated, moist, glistening, greyish-white growth. In gelatin stab-cultures the growth is somewhat opaque and granular, with slow liquefaction. Blood-serum is slowly liquefied. On potato a thick, moist, dirty-white growth develops, becoming dry and brownish after three days. Broth becomes slightly cloudy after twenty-four hours' growth, but in three to four days the organisms settle to the bottom, leaving the medium clear. Neither indole nor gas is formed. In neutral litmus milk the litmus is decolorised after a few hours, and in twenty-four hours the milk is firmly curdled. Somewhat later liquefaction of the curd ensues from above downwards; at first the turbid fluid is reddish in the superficial layer and yellowish below; ultim-

[1] See Gordon, *Rep. Med. Off. Loc. Gov. Board* for 1903–04, p. 388.
[2] *Journ. Exp. Med.*, vol. iv, 1899, p. 521.

ately the whole curd is transformed into a turbid liquid with a reddish colour throughout. These changes in milk are characteristic of the organism. It is pathogenic to white mice, hardly so to guinea-pigs and white rats, and moderately so to rabbits; intravenous inoculation into the latter sometimes sets up an endocarditis. Harris and Longcope [1] have reported five more instances of the occurrence of this organism (once from a cesspool, four times as secondary invasions at autopsies), and Birge [2] has isolated a similar but less virulent organism from the larynx of crows. Braxton Hicks [3] has also isolated this organism from a case of malignant endocarditis.

Micrococcus neoformans

This organism was isolated by Doyen from malignant growths, and was supposed by him to be the causative organism of malignant disease. It is a typical Gram-positive coccus, giving a white growth on agar and liquefying gelatin in three to four days. According to Dudgeon and Dunkley,[4] it gives all Gordon's fermentation tests for the *M. pyogenes*, var. *albus*, except that it does not acidify mannitol.

The serum of patients suffering from malignant disease does not give any marked agglutination with the *M. neoformans*, nor does it contain opsonins specific for the organism. The *M. neoformans* is non-pathogenic for rats and mice.

Botryomycosis

This condition is met with in the horse in the form of fibroid granulomata in the organs and in the abdomen, which may break down and discharge a yellowish pus.

[1] *Centr. f. Bakt.* (1te Abt.), vol. xxx, 1901, p. 353.
[2] *Johns Hopkins Hosp. Bull.*, vol. xvi, 1905, p. 309.
[3] *Trans. Roy. Soc. Med.*, vol. v, 1912, Path. Sect., p. 126.
[4] *Journ. of Hygiene*, vol. vii, 1907, p. 13.

Groups of micrococci embedded in a gelatinous matrix are present in the granulomata and in the pus (*Ascococcus equi*).

The Streptococci

Many streptococci of very variable virulence occur in man and animals. Formerly only one pathogenic species was described, *Streptococcus pyogenes*, now several varieties, if not species, are recognised.

Morphology.—The streptococci are non-motile cocci, each cell measuring about 1 μ in diameter. They stain well with anilin dyes and are Gram-positive.

Fission takes place in one direction only, so that chains of cocci are formed (Plate II, *b*). A cell here and there in a chain is often somewhat larger than its fellows, and some authors have considered these enlarged individuals to be arthrospores. The length of the chains is very variable and may be modified by cultivation, and occasionally branch-chains form. In pus, etc., the chains are usually not very long (Plate II, *a*).

Von Lingelsheim distinguished two varieties, *brevis* and *longus*, the former rendering broth turbid, growing in short chains, and being non-pathogenic to mice and rabbits, the latter leaving the broth clear, growing in long chains, and always pathogenic to these animals.

Gordon [1] divided the streptococci into four varieties, viz. (1) the *S. longus*, isolated from the mouth, restricted to an organism forming exceptionally long chains ; (2) *S. medius*, including the majority of streptococci from pus, sepsis, and erysipelas, and Lingelsheim's *longus* ; (3) *S. brevis*, including Lingelsheim's *brevis* and the *Diplococcus pneumoniæ* ; (4) *S. scarlatinæ* or *conglomeratus*, isolated from scarlatinal angina. The *Diplococcus pneumoniæ* is

[1] *Rep. Med. Off. Loc. Gov. Board* for 1898–99, p. 482.

a short streptococcus; it and the *S. mucosus* are described under "Pneumonia."

Cultural reactions.—The streptococci can be cultivated on the ordinary culture media, and usually grow both aërobically and anaërobically. On agar, or better, glycerin agar, minute whitish, semi-transparent, more or less isolated colonies form in twenty-four to forty-eight hours (Plate II. *c*). On gelatin the growth has much the same characters, and is better seen, as this medium is clearer than agar, but it takes some days to attain the maximum. In stab-cultures minute spherical colonies develop all down the line of the stab but without invading the surrounding medium; the gelatin is not liquefied. In broth a flocculent deposit forms, the fluid sometimes remaining clear, sometimes becoming turbid. There is no growth on potato. Litmus milk is usually acidified and sometimes coagulated, and acid is generally produced from glucose. The indole reaction can be obtained in broth cultures in seven to fourteen days on the addition of a nitrite, but not without. It is the only organism with which the writer is acquainted that does not reduce a weak solution of methylene blue.

The thermal death-point of the streptococci is 53° to 55° C., the time of exposure being ten minutes, and they are destroyed by weak solutions of disinfectants, *e.g.* 1–100 phenol, in ten minutes.

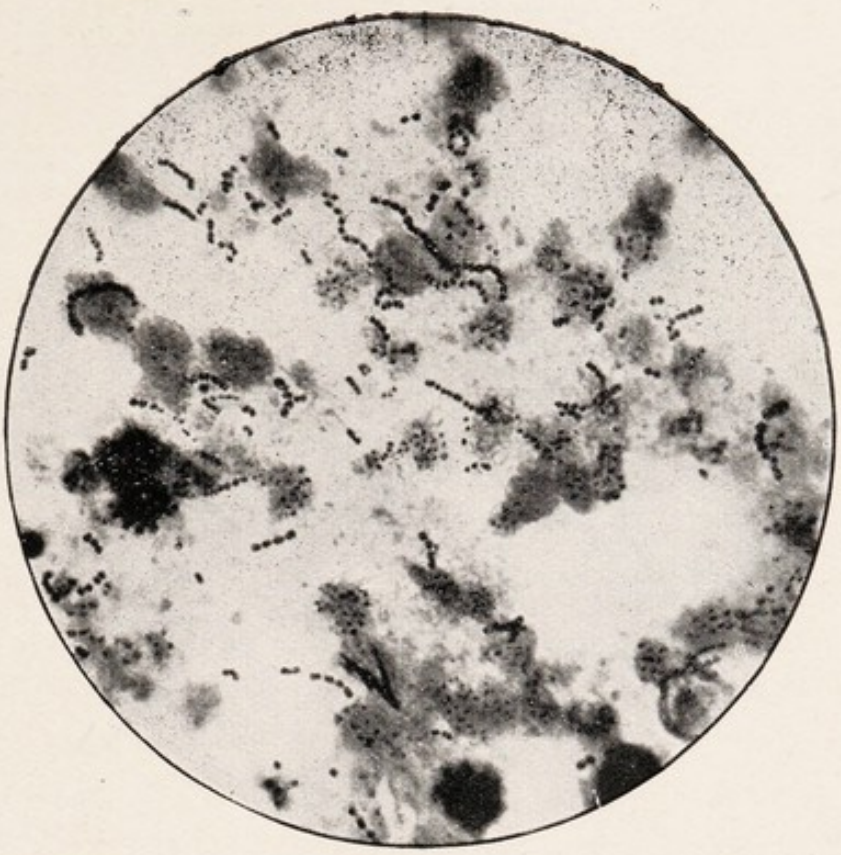
Considerable attention has been directed to the differentiation of streptococci by Houston,[1] Andrewes,[2] Andrewes and Horder,[3] Gordon,[4] and Besredka. Considerable differences are found in the fermentation re-

[1] *Rep. Med. Off. Loc. Gov. Board* for 1902–03, p. 511, and 1903–04, p. 472.

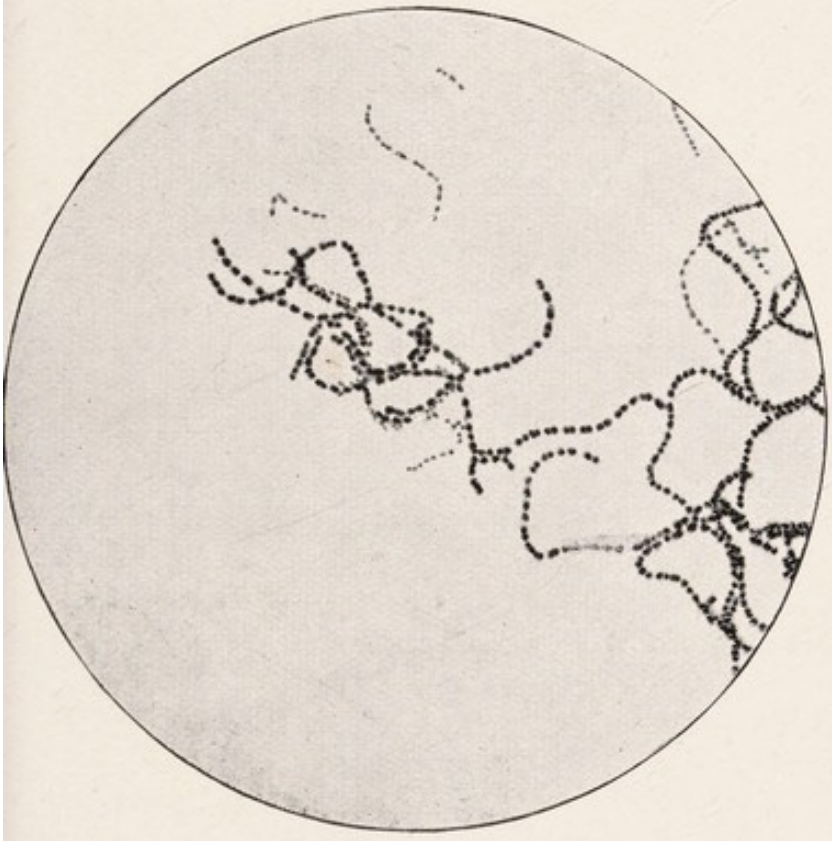
[2] *Lancet*, November 24, 1906.

[3] *Ibid.* 1906, vol. ii, pp. 708, 775, 852.

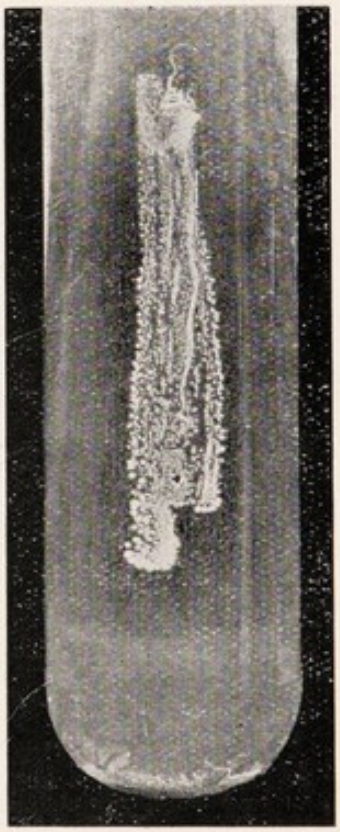
[4] *Ibid.* November 11, 1905, and *Rep. Med. Off. Loc. Gov. Board* for 1903–04, p. 388.



a. Streptococcus pyogenes in pus. Smear preparation. × 1000.



b. Streptococcus pyogenes. Film preparation of a broth culture. × 1500.



c. Streptococcus pyogenes. Pure culture on glycerin agar.

actions of various strains of streptococci, and Andrewes and Horder distinguish (1) *Streptococcus pyogenes* from pus, erysipelas, cellulitis, pyæmia and septicæmia, endocarditis, etc. (2) *S. salivarius*, the common type in the saliva. Also met with, probably as a "terminal" infection, in endocarditis and septicæmia. Shades into the *S. fæcalis* and *S. anginosus*. (3) *S. anginosus*, from inflamed and scarlatina throats, endocarditis, and rheumatism. (4) *S. fæcalis*, abundant in fæces, air, and dust. Met with also in endocarditis, meningitis, cystitis, and suppuration. Two strains of the *Diplococcus rheumaticus* proved to be this organism. (5) The pneumococcus. (6) *S. equinus*, present in the intestine of herbivora. They do not assert that these are absolutely defined species; at the most they seem to be species in the making, and are connected by transitional forms. Walker [1] does not consider that these reactions afford a means of distinguishing definite varieties among human streptococci.

Andrewes and Horder give the following Table summarising the characters of the various streptococci:

Name.	Milk clot.	Reduction of neutral red.	Cane sugar.	Lactose.	Raffinose.	Inulin.	Salicin.	Coniferin.	Mannitol.	Growth on gelatin at 20° C.	Morphology.	Pathogenicity to mouse.	Hæmolysis.
Streptococcus pyogenes .	—	—	+	+	—	—	±	—	—	+	*longus*	+	+
Streptococcus salivarius .	+	±	+	+	±	—	±	—	—	±	*brevis*	—	0
Streptococcus anginosus .	+	±	+	+	—	—	—	—	—	±	*longus*	+	+
Streptococcus fæcalis . .	+	+	+	+	—	—	+	+	+	+	*brevis*	—	0
Streptococcus equinus .	—	—	+	—	—	—	+	+	—	+	*brevis*	—	..
Streptococcus pneumoniæ		—	+	+	+	±	—	—	—	—	*brevis*	+	0

\+ = Positive or acid-production. — = Negative or no acid-production.
± = Acid-production sometimes present, sometimes absent.
(These differences are not constant; with various strains one or other reaction may be lacking.)

Crowe [2] makes use of Dorset's egg-medium with the

[1] *Proc. Roy. Soc. Lond.*, B, vol. lxxxiii, 1911, p. 541.
[2] *Proc. Royal Soc. Med.*, vi, 1913 (Path. Sec.), p. 117.

addition of 0·005 per cent. of neutral red for the purpose of differentiating streptococci.

The *Streptococcus pyogenes* is found in some 16 per cent. of acute circumscribed abscesses. It is, however, especially frequent in spreading inflammations, lymphangitis, cellulitis, and progressive gangrene, and is a common cause of septicæmia, pyæmia, and puerperal fever. It is met with in about one-third of the cases of infective endocarditis, occasionally in acute osteomyelitis, and seems to be the cause of the septic pneumonia so often observed after operations about the mouth and throat.

A streptococcus (*S. viridans*) producing a green growth on blood-agar and belonging to the *S. salivarius* group has been isolated by Major [1] and others from several cases of sub-acute infective endocarditis. It is probably not a distinct form but only a variant of the *S. salivarius*.

In erysipelas, streptococci are present in the lymphatics at the margin of the zone of redness. These were first isolated by Fehleisen, who described the organism as the *Streptococcus erysipelatis*, and by inoculation experiments on man and animals demonstrated its causal relation to the disease. The experiments on man were made in cases of extensive and inoperable carcinoma and sarcoma, as it had been noticed that malignant tumours were frequently benefited after an attack of erysipelas. Several cases were inoculated, and in all but one typical erysipelas developed (see Coley's fluid, p. 281). Jordan,[2] however, produced typical erysipelas in a rabbit's ear not only with the streptococcus, but also with staphylococci, pneumococci, and *B. coli*, and although human erysipelas is generally caused by the streptococcus, this disease may, therefore, occasionally be produced by staphylococci, and

[1] *Johns Hopkins Hosp. Bull.*, xxii, 1912, p. 326.
[2] *Münch. med. Woch.*, August 27, 1901.

possibly by the pneumococcus, *B. coli*, and even the *B. typhosus*.

At one time the *Streptococcus erysipelatis* was considered to be different from the *Streptococcus pyogenes*, but the two organisms are now regarded as identical, the differences in cultural characters being slight and not constant. A typical erysipelas in the human subject may be induced by inoculation with a pure culture of a streptococcus derived from a case of suppurative peritonitis, and an animal immunised against a streptococcus derived from a case of erysipelas is also immune against a streptococcus isolated from an abscess.

Rosenow maintains the essential identity of the members of the streptococcus-pneumococcus group of organisms, and claims in some cases to have transformed one form into another. He says: "The apparent positions of the various members of the streptococcus group may be illustrated by the position of the fingers in a partially flexed hand, in which the hæmolytic streptococcus occupies the position of the little finger, the pneumococcus the place of the index finger (the opposite extreme), *Streptococcus viridans* (representing the group of more or less saprophytic, non-hæmolyzing streptococci) the middle finger, the streptococci from rheumatism the ring finger, and *Streptococcus mucosus*, having some of the properties of both pneumococci and streptococci, the thumb. In this grouping there is in general an increase in parasitism and virulence as we approach the thumb (*Streptococcus mucosus*)."

Broadhurst [1] subjected over 700 strains of streptococci derived from various sources from man and animals to an exhaustive series of cultural tests. She considers that fermentation tests are not definitely helpful in indicating the origin of streptococci. Human throat strains gener-

[1] *Journ. Infectious Diseases*, vol. xvii, 1915, p. 277.

ally fail to ferment mannitol, while a large proportion of human fæcal strains ferment this substance. Raffinose fermenters are common in the human throat and also in bovine fæces, but are strikingly lacking in milk. Almost the commonest lactic ferment in milk is a streptococcus (*S. lacticus*) : it is non-pathogenic and does not hæmolyse.

Epidemic sore throat may be caused by streptococci derived by milk infection. This form is virulent for the rabbit, is hæmolytic, and ferments salicin, but does not ferment raffinose and inulin.[1]

The different effects produced by the *Streptococcus* —abscess in one case, erysipelas in another, cellulitis or pyæmia in a third—are attributable partly to differences in virulence, partly to the site of infection and mode of entrance into the body, partly to real differences existing between different races of streptococci, and partly to the selective affinity of streptococci for particular tissues which has already been referred to (p. 161).

Streptococci have been described in a number of diseases about which we know little, such as variola, scarlatina (*S. scarlatinæ* or *conglomeratus*), and vaccinia, but in most instances they are probably not causal. Strangles, a disease of horses, seems to be due to streptococci.

Anti-serum.—Streptococcal infections are of considerable importance, and an efficient anti-serum would, therefore, be valuable. For its preparation, the virulence of the streptococcus has to be increased by passing it through a series of rabbits, and it is only by growing it in serum media that satisfactory cultures for the inoculation of the horses can be prepared. Human serum is the best, but is difficult to obtain ; a mixture of asses' serum and peptone beef-broth comes next. The cultures are grown for about a fortnight and are then inoculated into

[1] Smillie, *Journ. Infect. Diseases*, vol. xx, 1917, p. 45.

horses, first killed and then living cultures being used, and after a time the blood acquires anti-microbic properties. It is customary now to make use of a " polyvalent " serum, *i.e.* one prepared by the injection of many strains of streptococci. The streptococcus anti-serum has been employed in erysipelas, cellulitis, puerperal fever, and pyæmia, in many cases with success. Cheyne suggested its use before operations about the mouth and throat as a preventive of septic pneumonia, but a *vaccine* would probably be better for this purpose.

A *vaccine* prepared by sterilising cultures with heat has been used with benefit in streptococcic infections which do not run too rapid a course, *e.g.* infective endocarditis. Sensitised streptococcal vaccine (p. 244) seems to be very useful in wounds with streptococcal infection.

Bacillus pyocyaneus

This is the organism found in green and blue pus, and it also occurs on the surface of the body. Its presence in wounds greatly retards healing, and occasionally a general toxæmia may result from it. It has been met with in otitis media and in the green pus of the pleural and pericardial cavities. It is a slender bacillus measuring 3 to 4 μ, frequently united in pairs and forming filaments. It is actively motile, does not form spores, and is aërobic and facultatively anaërobic. It does not stain by Gram's method. On gelatin it grows freely with rapid liquefaction, a greenish, fluorescent colour developing in the liquid, while whitish flocculi of growth sink to the bottom. On agar a whitish, moist layer develops, and the medium is stained a greenish or bluish colour. On potato the growth is dirty brown or sometimes greenish.

Milk is coagulated and a greenish colour develops. Broth becomes turbid, and there is a slight film formation

with a yellowish-green colour. Oxygen is necessary for the development of the pigment, which is generally a mixture of a blue pigment, pyocyanin, and a yellow one, pyoxanthose. Pyocyanin ($C_{14}H_{14}N_2O$) is said to be an anthracine derivative; it is soluble in chloroform, and on oxidation yields pyoxanthose.[1] Various races of the organism exist, differing in their pigment production.

Subcutaneous inoculations of a small amount of a culture produce local abscesses; larger amounts cause œdema with purulent infiltration of the tissues and death. Animals can be vaccinated by means of small quantities of living cultures or by sterilised cultures. Sterilised cultures will prevent infection (experimentally) by anthrax if used early—that is to say, if an animal be inoculated with anthrax, and shortly afterwards injected with a broth culture of the *Bacillus pyocyaneus*, a fatal result is averted. Emmerich and Loew [2] isolated from cultures a ferment-like body, "pyocyanase," which they state has preventive and curative properties towards anthrax and diphtheria infections. Dry pyocyanase has been used as an application in diphtheria to dissolve the false membrane.

B. pyocyaneus sometimes occurs in diarrhœa with green stools and may be isolated from the blood in some cases of marasmus in young children. A form of epidemic dysentery seems occasionally to be caused by this organism (see "Dysentery"). A few cases of general infection with this organism have also been recorded. It has also been isolated from conditions of dermatitis and bullous eruptions.[3] The *B. pyocyaneus* has been found in water, dung, soil, and in the effluent from filter beds. Lehmann and Neumann state that, with the exception of patho-

[1] See *Centr. f. Bakt.*, xxv, p. 897. *Journ. Exp. Med.*, September–November, 1899.

[2] *Zeitschr. f. Hyg.*, 1899; *Centr. f. Bakt.*, xxxi (*Originale*), p. 1.

[3] See Pernet, *Brit. Med. Journ.*, vol. ii, 1904, p. 992.

genicity, there is no essential difference between this organism and the *B. fluorescens liquefaciens* so frequently met with in water.

The *B. pyocyaneus* seems to be of more frequent occurrence and of greater pathogenicity in the tropics than in this country. A disease in dogs bearing a remarkable similarity to rabies may be caused by it (see " Rabies ").

Clinical Examination

In many cases some idea can probably be formed as to the organisms likely to be present in the pus or discharge, etc., from the clinical characters of the disease, in which case the examination may be more particularly directed towards the isolation of the suspected organism. For example, in a urethral discharge the gonococcus will be especially looked for, in an empyema following pneumonia the *Diplococcus pneumoniæ*, in a meningeal exudate the *D. pneumoniæ* or the *D. intracellularis*, *B. tuberculosis* or cocci or streptococci, and in a tropical abscess of the liver the *Entamœba histolytica*. In buboes, cocci and streptococci, Ducrey's bacillus, and the plague bacillus may occur. In the eye certain special organisms may be met with. Suppurating glands are frequently due to the tubercle bacillus. In all cases the pus or discharge should be collected with aseptic precautions in sterile capillary pipettes or in sterile test-tubes at the time of operation. The discharge from opened abscesses and from wounds is liable to become contaminated and the original infection to be masked. In septic wounds the infection may be a mixed one.

In all cases the examination should be commenced as early as possible.

(1) Make several smears from the pus or discharge.

(2) Stain one or two of these with Löffler's blue and one or two by Gram's method. Mount and examine microscopically.

(*a*) If Gram-positive staphylococci only are detected, the presence of the ordinary pyogenic cocci may be suspected. Proceed as in 3, 4, and 5.

(*b*) If encapsuled Gram-positive diplococci are detected, suspect the presence of the *Diplococcus pneumoniæ*, and proceed as in 3, 5, and 6.

(*c*) If diplococci and tetracocci are present, note whether they are in groups within the pus-cells; if so, and if Gram-negative,

suspect the presence of either the gonococcus or *Diplococcus intracellularis meningitidis* (see pp. 275 and 265).

(*d*) If free Gram-positive tetracocci are detected, suspect the presence of the *Micrococcus tetragenus*, and proceed as in 3 and 4 (rare).

Staphylococci not infrequently appear in pairs and fours.

(*e*) If streptococci are present, proceed as in 3, 4, 5, and 6.

(*f*) If bacilli are present they may be the colon bacillus, the *Bacillus perfringens*, the bacillus of malignant œdema, the bacillus of Hibler, the tetanus bacillus, the typhoid bacillus, the *Bacillus pyocyaneus*, or putrefactive bacilli of the *Proteus* group (which see). The result of Gram-staining and the clinical history of the case will be some guide.

α. The colon bacillus, especially frequent in suppurative peritonitis and in diseases of the urinary organs. (See p. 441.)

β. The *Bacillus perfringens*, the bacillus of malignant œdema and the bacillus of Hibler are met with in foul wounds, especially gunshot wounds and gangrenous conditions, with development of gas. (See Chapter xiii.)

γ. The tetanus bacillus is found in the wound in cases of traumatic tetanus. (See Chapter XIII.)

δ. The typhoid bacillus is rare ; it may occur in suppurative conditions complicating or following typhoid fever. Proceed as in 3 and 4. (See also p. 414.)

ζ. When the *Bacillus pyocyaneus* is present the pus or discharge may be blue. Proceed as in 3 and 4.

(*g*) If yellow granules, having a rosette-like structure microscopically, are present, actinomycosis may be suspected and examined for by the methods given in Chapter XV.

(*h*) If thread forms be present, *streptothrix* or *aspergillar* infection may be suspected (see Chapters XV and XVII) : if large round or ovoid cells or yeast-like forms, *Blastomycetes* or *Sporotrichon* (Chapter XVI).

(*i*) If a mixture of organisms be present, agar and gelatin plate cultivations should be prepared and further examined by sub-cultures from the colonies.

(*j*) If no organisms can be detected microscopically, proceed as in 3 and 6. In the pus of ordinary abscesses micro-organisms can generally be detected, unless caused by the tubercle or glanders bacillus, the pneumococcus, or the *Entamœba*. In broken-down granulomata, *e.g.* gummata, if unopened, no organisms may be present.

(3) Make several cultivations on agar and gelatin (anaërobic if required), and examined microscopically and by subcultures when the growths have developed.

(4) Make two or three sets of agar and of gelatine plate cultivations. Examine the colonies microscopically and by subcultures.

(5) Stain two or three of the cover-glass preparations by Gram's method, and counter-stain with Bismarck brown.

(6) Inoculate guinea-pigs or mice subcutaneously and intraperitoneally with the material.

(7) Organisms can rarely be detected in the blood by a microscopical examination of stained films. Therefore 2–5 c.c. of blood should be withdrawn and cultivated (p. 137).

(8) If the abscess be probably a tropical abscess of the liver, the pus or scrapings from the wall of the abscess should be examined for the presence of the *Entamœba.* (Chapter XVIII.)

Diphtheroid bacilli are frequent in wounds.

Micrococcus meningitidis

Weichselbaum in 1887 isolated from cases of cerebro-spinal fever (epidemic cerebro-spinal meningitis, spotted fever) a coccus which he named the *Diplococcus intracellularis meningitidis,* and further research has confirmed the accuracy of Weichselbaum's discovery and the etiological relationship of the organism to the disease.

While tending to be epidemic, the disease also occasionally occurs sporadically. The organism is met with in the exudate on the meninges, in the cerebro-spinal fluid, occasionally in the blood, rarely in the urine, and in other situations, *e.g.* eye, ear, joints. It is also present in the naso-pharynx of carriers and sometimes (perhaps always at an early stage) in the same situation in cerebro-spinal fever.

Cerebro-spinal fever varies much in severity and presents every degree of duration from acute fulminating cases, which may die within twenty-four hours of the first onset, to cases running a lingering course of weeks or months. In the acuter cases a hæmorrhagic septicæmia

may be present with hæmorrhages in the skin, hence the name "spotted fever" sometimes applied to it (typhus fever has also been termed "spotted fever").

Morphology, etc.—The meningococcus, as it may be termed, occurs as single cocci and diplococci in groups within the leucocytes (Plate III. *a*); in grouping and general appearance, in fact, it closely resembles the gonococcus, and, like the last-named, is Gram-negative, though staining well with the ordinary anilin dyes and with the Leishman stain. The cerebro-spinal fluid is generally turbid from the presence of numbers of polymorphonuclear leucocytes, many of which contain the cocci. Some of the cocci may also be free in the fluid. At an early stage and in some of the very acute and fulminating cases the fluid may be almost or quite clear, almost free from cells, and the cocci may be very scanty. In cultures the organism occurs as cocci, diplococci, and occasionally as tetrads. Swollen involution forms may appear and the cocci of different strains may vary in size.

Cultural characters.—The meningococcus is an obligatory aërobe, and does not usually grow at a temperature below about 23° C. It will not grow on ordinary agar or in broth and many culture media have been employed for its cultivation. One of the best is blood-agar, or ordinary agar smeared with blood, human or rabbit. Serum or ascitic-fluid agar or broth are also good soils. Nutrose ascitic agar ("nasgar") was formerly recommended by Gordon but has not fulfilled expectation:

Ascitic fluid	15 c.c.
Distilled water	35 c.c
Nutrose	1 grm.

The mixture is placed in a flask, brought to the boil with constant shaking, and filtered. It is then mixed with double the volume of ordinary nutrient agar, steamed for thirty minutes, filtered, and filled into tubes.

Gordon and Hine have devised another medium—legumin trypagar—made with Douglas's trypsin agar with the addition of a saline extract of pea-flour and preferably also of some serum. It is quite a good medium, though not so good as blood agar, but has the advantage that it can be made in any quantity and stored for use.

The colonies of the meningococcus on blood or legumin agar after twenty-four hours' incubation at 37° C. appear as moist, grey, translucent, circular or oval discs with regular outline; after a further twenty hours' growth they may attain a diameter of 3 to 4 mm. The colonies never exhibit any yellowish coloration as do those of some other Gram-negative cocci (see p. 272).

The vitality of the meningococcus is brief on blood and legumin agar—twenty-four to forty-eight hours. In trypsin broth with the addition of a little serum the meningococcus will live for two to three weeks at 37° C. Vines employs a starch medium consisting of beef-broth with the addition of 1 per cent. starch and 1·5 per cent. agar; stab-cultures (but *not* surface cultures) in this medium kept at 37° C. live for two, three or even four weeks.

Absence of growth at 23° C. has been put forward as a valuable diagnostic feature of the meningococcus, and so it is up to a point. Gordon found that of thirty-five strains, thirty-two did not grow at this temperature in ten days, the remaining three showed some growth on legumin agar in forty-eight hours.

The meningococcus is exceedingly intolerant of drying; desiccation kills it in two to five minutes. Hence swabbings for culture from carriers should be inoculated on the spot and the cultures be kept warm until placed in the incubator.

Small quantities of nasal mucus enhance the growth of the meningococcus, while saliva has the contrary effect.

This action of saliva is due to the salivary bacteria, chiefly streptococci.

Fermentation Reactions.—These are best obtained in liquid media tinged with litmus and containing 1 per cent. of the fermentable substance, *e.g.* Hiss's medium (p. 325) or veal broth with some added serum. The meningococcus forms acid from glucose, maltose and mannose, but not from lactose, galactose, saccharose, inulin, mannitol, dulcitol and a number of glucosides. It was formerly stated to ferment galactose but this seems to be an error due to the galactose used undergoing partial decomposition during sterilization of the medium. Mannose is acidified in three to four days, but in six to seven days the medium returns to its original slight alkalinity and colour. Dextrin is stated by Symmers and Wilson [1] to be acidified. The fermentation reactions are liable to some variation; thus Gordon met with three strains which failed to ferment glucose. If an atypical result is obtained, if for instance what appears to be a typical meningococcus fails to ferment glucose, the culture should be examined by subculture to ascertain that the organism is alive, or if a sugar is fermented which should not be, the culture should be examined to ascertain if other cocci or streptococci are present in addition.

Agglutination and Races.—An agglutination reaction is given in some cases with the patient's serum, but is neither constant nor marked enough to form a sure means of diagnosis. The macroscopic method is to be preferred and the mixtures of serum and culture should be kept at 55° C. for twenty-four hours and the readings then taken.

Symmers and Wilson [2] found that the blood of cerebro-spinal fever cases may occasionally agglutinate the *B.*

[1] *Journ. of Hygiene*, vol. ix, 1909, p. 9.
[2] *Ibid.* vol. viii, 1908, p. 314.

typhosus and *B. coli* in comparatively high dilutions.

Agglutination is a valuable means for the recognition of the meningococcus. The immune serum is best prepared, according to Hine, by giving a young rabbit intravenously a dose of 1000 million killed cocci, followed one hour later by a dose of 500 million, and on the sixth day by a dose of 3000 million. The serum is tested on the eighth day, and if satisfactory the animal is bled to death on the ninth day and the serum preserved: such a serum will probably have a titre of 1–1200 or thereabouts.

For agglutination tests, the organism is grown on a legumin agar plate for twenty-four hours at 37° C., the growth is emulsified in saline and the saline suspension for the test is standardised to contain 2000–4000 million cocci per c.c. For the test, the macroscopic method is employed, the tubes containing the mixtures are kept at 55° C., and the readings taken at the end of twenty-four hours.

By the application of agglutination and saturation tests the meningococcus has been differentiated into at least four types or races. The method employed was as follows: A series of meningococci from the cerebro-spinal fluid of thirty-two cases of cerebro-spinal fever having been collected, an agglutinating serum was prepared with one of them and all the thirty-two strains were tested as to agglutination with it. The result was that nineteen of the strains agglutinated well and three only slightly. They were all further tested by the saturation test, with the result that all the nineteen strains which agglutinated well absorbed agglutinin while the remaining thirteen, including the three which agglutinated only slightly, failed to absorb agglutinin. The nineteen strains which agglutinated and absorbed were grouped together as type I.

Type II was differentiated by taking one of the thirteen

strains which were excluded by the first test, preparing a second agglutinating serum with it and testing all the thirty-two strains with this second serum. Although twenty-one of the strains showed some agglutination, this was well marked in the case of seven of them only. On applying the absorption test, all of these seven cocci, and also one of the cocci that had agglutinated only slightly with the second serum, were found to absorb agglutinin. These eight cocci formed, therefore, type II. By the same procedure two more types were differentiated among the remaining strains, viz. types III and IV. The final result was that of the thirty-two strains, nineteen formed type I, eight formed type II, four formed type III, and one formed type IV. One specimen was amphoteric, qualifying for both types I and III. It is noteworthy that no less than twenty-seven of the thirty-two meningococci (84 per cent.) were included in the first two types. Further work has confirmed these results with the addition of a few strains with anomalous reactions, these have been termed para-meningococci.

Similar types of meningococci are also present in the nasopharynx; moreover, if a case of cerebro-spinal fever has meningococci in the nasopharynx, the cocci in this situation are of the same type as in the cerebro-spinal fluid. Further, only one type is present in the cerebro-spinal fluid of a case. The different types maintain their serological characters and breed true.

Pathogenesis.—Monkeys may be infected by intracerebral or intrathecal injection with the production of a typical cerebro-spinal meningitis. Injected into the peritoneal cavity of mice and guinea-pigs, or intravenously in rabbits, the meningococcus produces a fatal septicæmia.

Carriers.—As already mentioned, the meningococcus is present in the nasopharynx of a certain number of con-

tacts and others. The greater the closeness of contact, the larger the proportion of infected contacts. Of 4,667 healthy non-contacts Bassett-Smith found the meningococcus in fifty-three = 1·124 per cent.; of fifty-two healthy contacts, it was present in three = 5·77 per cent. These figures have been much exceeded in other investigations; 10–13 per cent. of non-contacts have sometimes been found to carry the meningococcus. The duration of the carrier state is uncertain; a majority clear in from two to three weeks, but others may persist for ten weeks or longer, and all treatments seem more or less unsatisfactory. The proportion of carriers who become cases of cerebro-spinal fever is not known, but a considerable number escape. The presence of the meningococcus in the nasopharynx suggests that infection of the meninges is derived from this source via the cribriform plate of the ethmoid.

Anti-Serum and Vaccine.—Flexner first prepared an anti-serum by the injection of horses with cultures. The method recently employed at the Rockefeller Institute for preparing consists in beginning with small and increasing doses of living meningococci injected intravenously daily for three days, followed by a period of rest of seven days. A second, and sometimes a third, series of similar injections with increased amounts are given. Then another strain is taken and the same procedure is adopted and in this way a polyvalent serum for three or four strains may be prepared in nine to twelve weeks.

In some instances the anti-meningococcal serum has been found very beneficial, markedly reducing the mortality, in others little or no result has followed its use. This difference probably depends on whether the serum is strictly homologous for the infecting organism or no. The serum should be given intrathecally. Vaccine treatment has also been tried in doses of 25 to 100

million to commence with, but the general experience is not favourable.

Vaccine has also been used for prophylaxis, dose 50 to 150 million intravenously. Sufficient data as to its value are not available.

The Gram-negative Cocci of the Upper Respiratory Tract

It may be of service here to describe certain Gram-negative cocci which are met with in the nasopharynx and to contrast them with the meningococcus.

Micrococcus pharyngis siccus.—Common. Grows freely, forming white and adherent colonies. Develops at 23° C. and rapidly ferments glucose and saccharose.

Micrococcus flavus I.—Common. The colonies, first whitish, soon become yellow, and on touching them they tend to slide about and may be picked up whole. Develops at 23° C. and ferments glucose and saccharose in three to four days.

Micrococcus flavus II.—Rare. More delicate in growth than the preceding. Colonies become yellow and are sticky and stringy. Easier to sub-culture than the preceding. Ferments glucose and saccharose slowly and the change is often earlier in saccharose.

Micrococcus flavus III.—Not common. Colonies of a canary-yellow and of the consistency of paint. Two forms are described, one growing strongly at 23° C. in twenty-four hours, the other failing to grow at this temperature. Ferments glucose in four days but fails to ferment saccharose.

Micrococcus catarrhalis.—Not uncommon. Colonies whitish and like paint, easily emulsified. Growth at 23° C. is feeble. No sugars are fermented (see also p. 280).

Meningococcus.—Colonies whitish, or with the faintest yellowish tinge, of consistency like paint and easily emulsified. Rarely grows at 23° C. Ferments glucose, but not saccharose. Mannose first becomes acid and then alkaline. The *Diplococcus crassus*, which is Gram-positive, and the *D. mucosus*, which is Gram-negative and grows on gelatin, may also occur in the nasopharynx (Arkwright, *loc. cit.*).

The following table summarises the fermentation reactions :

Organism.	Glucose.	Saccharose.	Mannose.	Galactose and Inulin.
M. pharyngis siccus . .	+[1]	+[1]	+[1]	
M. flavus I.	+[1-2]	+[2]	+[3]	No
M. flavus II. . . .	+[4]	+[4]	+[4]	change
M. flavus III. . . .	+[3-4]	0	+[3-4]	with
M. catarrhalis . . .	0	0	0	any
Meningococcus . . .	+[4]	0	+[3-7]	

+ = acid, — = alkaline, 0 = no change. The figures indicate the days on which the change occurs (after Gaskell)

Still observed in simple posterior basic meningitis of infants a diplococcus closely resembling the meningococcus but growing more freely on agar, etc. By some it is regarded as an attenuated form of the latter. According to Arkwright it does not liquefy gelatin, and grows on this medium at 22° C., fails to produce acid from glucose, maltose, and galactose, and is not agglutinated by a meningococcus serum. It is in these respects very like the *M. cinereus* of Lingelsheim. Wollstein [1] failed to find any reliable criteria of difference between strains of the *D. intracellularis* and several cultures obtained from cases of posterior basic meningitis. Houston and Rankin [2] found that ten Gram-negative cocci isolated from cases of sporadic cerebro-spinal meningitis differed from the *D. intracellularis* in respect of their opsonins and agglutinins, though eight of them were identical with the meningococcus in fermentative power.

Literature on the Meningococcus.—Gordon, *Rep. Loc. Gov. Board*, 1907 (Bibliog.) ; Arkwright, *Journ. of Hygiene*, vol. vii, 1907, p. 193 and vol. ix, 1909, p. 104 ; *ibid.* vol. xv. 1916, pp. 405, 446, and 464 (Eastwood, Griffith, Scott) ; Medical Research Committee, *Rep. of the Special Advisory Committee upon Bacteriological Studies of Cerebro-Spinal Fever during the Epidemic of* 1915, *and Bacteriological Studies in the Pathology and Preventive Control of Cerebro-Spinal Fever* (Special Report Series, No. 3) ; various papers in the *Lancet* and *British Med. Journ.* for 1915, 1916 and 1917.

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[1] *Studies from the Rockefeller Inst.*, vol. x, 1910, No. 13.
[2] *Brit. Med. Journ.*, 1907, vol ii, p. 1414.

Clinical Examination

1. In a case of suspected cerebro-spinal fever, no time should be lost in obtaining aseptically some cerebro-spinal fluid by lumbar puncture. The fluid should be examined as soon as possible and should be kept warm in the incubator, in the pocket, or in a thermos flask with warm water, until finished with.

a. The fluid will probably be thickly turbid. Smears should be made with the deposit, obtained by allowing the fluid to stand for a little while or by centrifuging lightly. Some of the smears may be stained with Leishman (see "Malaria") or with Löffler's or thionine blue, others by Gram's method, counterstaining with Bismarck brown. The presence of diplococci and groups of diplococci which are Gram-negative within the polymorphonuclear leucocytes, which form the majority of the cells in the fluid, is practically diagnostic (the gonococcus may cause a cerebro-spinal meningitis but this condition is so rare that it may be neglected). A few cocci and diplococci may be free in the fluid. At an early stage and in some of the fulminating cases the fluid may be nearly free from cells and the meningococcus difficult to detect microscopically. It can, however, generally be found after centrifuging and by careful examination.

If the cocci are not found microscopically, they may sometimes be demonstrated after incubating the fluid at 37° C. for twenty-four hours.

At a late stage in the disease, the cocci may disappear from the cerebro-spinal fluid and the polymorphs be largely replaced by lymphocytes.

b. Cultures should be made from the fluid by smearing some of the fluid on to plate of blood, serum or legumin, agar, p eferably the two former, and incubating at 37° C. The plates are examined after twenty-four and forty-eight

hours' incubation, suspicious colonies being examined microscopically with Gram-staining, and subcultured on to blood agar, etc., some tubes being incubated at 37° C., others at 23° C., and into litmus glucose and litmus saccharose serum broth (also mannose, if available). Cultures are best made both before and after incubation of the fluid; the latter sometimes succeeds when the former has failed. Hope of growth need not be abandoned until the culture has been incubated for four days: the medium should always be liberally inoculated.

The coccus dies in the cadaver in thirty-six to forty hours, sometimes earlier.

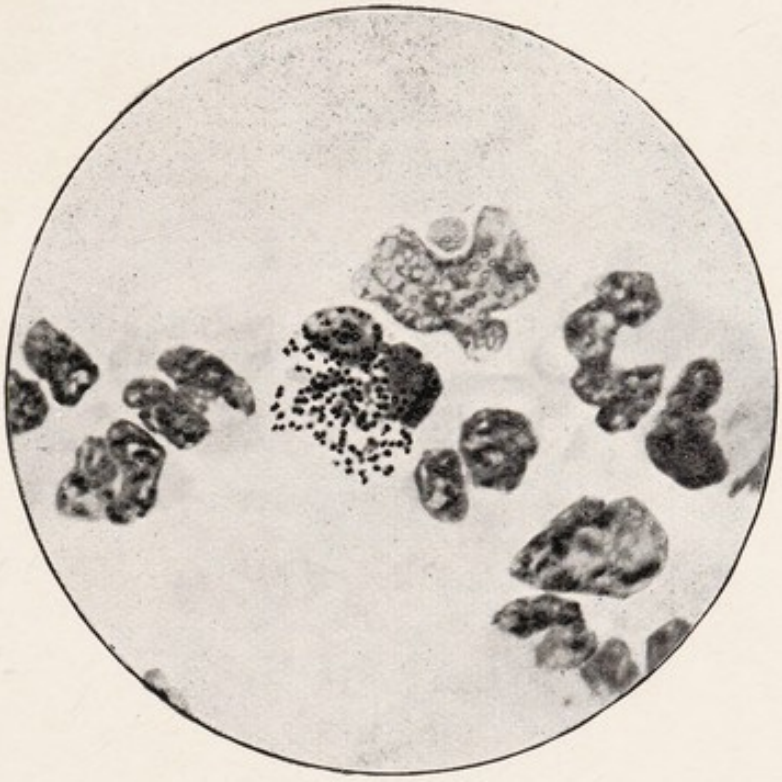
2. Carriers. These may be examined by plating swabbings taken from the nasopharynx in the same manner as cerebro-spinal fluid. The swabbings should be cultured on the spot if possible, or as soon as practicable, the swabs in the meanwhile being kept warm and moist or the coccus may die. It is important also to avoid soiling the swab with saliva; this may be accomplished by the use of West's swabs which consist of a piece of large glass tubing of suitable length curved at one end. Within the tube is a piece of flexible wire having the swab at one end. For use, the swab is withdrawn into the curved end of the tube, the tube is then introduced into the mouth and the curved end turned up at the back of the palate. The swab is then pushed out of the tube, rubbed over the nasopharynx and withdrawn again into the tube, which is then removed from the mouth. By this means contamination with saliva is prevented.

Micrococcus gonorrhœæ

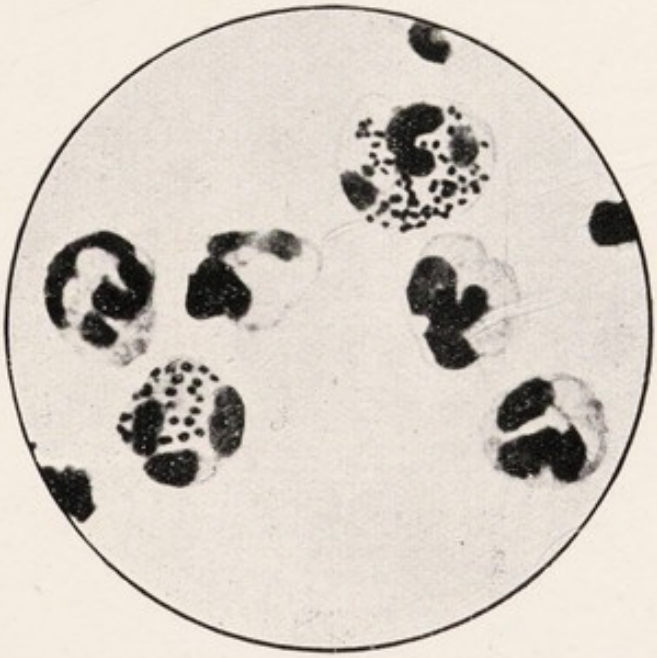
The *Micrococcus gonorrhœæ* was discovered by Neisser in 1879 in cases of gonorrhœal urethritis. In gonorrhœal pus it occurs usually in pairs, occasionally in tetrads, the

elements of which are somewhat ovoid in shape, their opposed surfaces being flattened. The organism has a characteristic arrangement: it occurs in groups *within* the pus-cells (Plate III. *b*). The individual cocci vary somewhat in size, the average being about 0·7 μ in the long and 0·5 μ in the short diameter. It stains readily with the ordinary anilin dyes, Löffler's blue being perhaps the best, but is decolorised by Gram's method—an important practical distinction from many other cocci.

Cultural characters.—The gonococcus is difficult to cultivate, and usually soon dies out under cultivation—within a week, unless transferred to fresh soil—but it does not seem to lose its virulence. Growth takes place between 25° and 38° C., but the optimum temperature is between 35° and 37° C. It is aërobic, and possibly facultatively anaërobic, and will develop on a feebly alkaline or acid soil. The ordinary agar and gelatin media are useless for the cultivation of the gonococcus; it will grow only on a medium containing "native" protein. Blood-serum agar gives fair results, but the ordinary Löffler's blood-serum is of no use. The best medium is agar smeared with blood. Ordinary sloping agar tubes or small agar plates may be employed. Blood obtained by pricking the finger, with antiseptic precautions, is taken up in a sterile capillary tube and deposited on the agar. A trace of gonorrhœal pus, collected with aseptic precautions, is taken up on a small sterile camel's-hair brush, and is rubbed up with the drop of blood and smeared over the surface of the agar. The cultures are incubated at 37° C., and in twenty-four hours the colonies of the gonococci appear as transparent greyish specks, which increase in size up to the end of three days. At this stage the colony measures 1 to 2 mm. in diameter, is raised, brownish, and finely granular in appearance, and roundish with a crinkled margin. The cocci from cultures



a. The meningococcus. Smear of cerebro-spinal fluid. × 1000.



b. The gonococcus. Smear of gonorrhœa pus. × 1500

resemble those in the pus, but tetrads are more frequently met with. Egg-broth also gives good results. The fermentation reactions and comparison with other Gram-negative cocci will be found in the Table, p. 279. The specific virulence of gonorrhœal pus is destroyed by exposure to a temperature of 60° C. for ten minutes.

Pathogenicity.—The gonococcus is a strictly parasitic organism, and seems exclusively to attack man. From inoculation experiments on the human subjects it appears to be the specific organism of gonorrhœal urethritis and vulvitis. In the female it is most frequent in the urethral or vulvar discharge, less so in that from the cervical canal, and is rarely or never seen in a purely vaginal one. It is generally, even at an early stage, associated with other organisms, particularly other diplococci (see Table, p. 279) which have to be distinguished from the gonococcus. The features which serve to identify the latter are its shape and size, its non-staining by Gram's method, its arrangement in *groups within* the pus-cells, absence of growth on ordinary media, the characters of the colonies, and the fermentation reactions.

The gonococcus is associated with a variety of lesions besides those already mentioned, viz. epididymitis, ovaritis, salpingitis, cystitis, peritonitis, arthritis, and conjunctivitis. It has been met with in the blood, and occasionally produces endocarditis, pericarditis, and meningitis. The gonococcus is fatal to guinea-pigs and mice by intraperitoneal inoculation.

Toxin, anti-serum, and vaccine.—Christmas [1] found that the blood-serum of the rabbit, fluid or coagulated, is an excellent culture medium for the gonococcus. By cultivating the gonococcus for ten days in an ascitic bouillon mixture he succeeded in obtaining a toxin which, when injected intravenously into rabbits in large doses, caused

[1] *Ann. de l'Inst. Pasteur*, xi, 1897, p. 609.

death, in smaller doses fever and loss of weight, while precipitated with alcohol and injected into the anterior chamber of the eye it produced severe inflammation. By injecting rabbits with small doses of the toxin immunisation was produced, and the blood acquired antitoxic properties. A vaccine may be prepared by sterilising cultures with heat, and has proved of service in chronic gonorrhœal infections.

Clinical Diagnosis

The diagnosis of gonorrhœa is very important, not only in clinical but also in medico-legal cases. For this purpose microscopical examination and culture methods are made use of. In a chronic gleet the material must be examined carefully and repeatedly.

(1) *Microscopical examination.*—Several thin smear specimens of the pus or discharge should be prepared. If the best results are desired the films should be air-dried, and then fixed by placing in a mixture of equal parts of alcohol and ether for fifteen minutes. After fixing, a couple of the films are stained in Löffler's blue for five to ten minutes, washed in water, dried and mounted. Leishman's stain also gives good results, the films being merely air-dried and not fixed. The preparations are then examined with a $\frac{1}{12}$-inch oil-immersion ; a lower power lens is useless. The ovoid cocci in pairs, and occasionally in tetrads, occurring within the pus-cells in groups of not less than four pairs are very characteristic. Diplococci situated outside the pus-cells should be neglected (it is to be noted that the nuclei of the pus-cells are deeply, the cytoplasm only faintly, stained with methylene blue). The next step is to ascertain the staining reaction by Gram's method. Stain two more films for fifteen minutes in anilin gentian violet, dip in water, place in Gram's iodine solution for two minutes, decolorise in absolute alcohol until the drainings fail to stain white filter paper, and counter-stain for forty-five seconds in an aqueous solution of Bismarck brown. The gonococcia are decolorised, and take up the brown stain. In chronic urethritis the urine may be centrifuged, and preparations are made from the deposit and threads and stained; it may be necessary to massage the prostate in order to obtain secretion.

(2) *Culture methods.*—Whenever a diagnosis is of great im-

portance an attempt should be made to cultivate the organism. Plate cultures of agar smeared with blood as described (p. 276) and another set with agar only should be prepared and incubated at 37° C. In forty-eight hours colonies of the gonococcus should be recognisable on the blood-agar, but not on the plain agar.

If cultures are obtained, the fermentation tests (see below) may be applied.

In gonorrhœal vaginitis, etc., in the female, the discharge generally contains large numbers of other organisms, and the gonococcus is usually difficult or impossible to detect.

N.B. *The greatest caution must be exercised in declaring a case free from infection on the ground of* NEGATIVE *results of the examination.*

The Characters of the Chief Gram-negative Cocci (Gordon)

Organism or source.	Growth on nutrose ascitic agar at 37° C.	Growth on gelatine at 20° C.	Pathogenicity.	Glucose.	Galactose.	Maltose.	Saccharose.
M. catarrhalis. Nasal and pharyngeal discharge	Opaque, granular	Positive (grows on ordinary agar at 37° C.)	Mice and guinea-pigs by intraperitoneal inoculation only	0	0	0	0
M. intracellularis (meningococcus). Cerebro-spinal meningitis	Clear, smooth or no growth.	Negative	In some cases monkeys, mice and guinea-pigs by intraperitoneal inoculation only	+	—	+	0
M. gonorrhœæ (gonococcus). Urethral discharge	No growth unless blood added.	Negative	*Ib.*	+	+	0	0
From nasal discharge from Hertford case of influenza-like epidemic (see "Influenza")	Clear, smooth, later becomes yellowish	Negative at first, positive later grows on ordinary agar at 37° C.	Mice and guinea-pigs by intraperitoneal inoculation	+	0	+	0
Ib.	Opaque, granular	Negative	*Ib.*	+	+	+	+
From urethra . .	Opaque, somewhat granular, smooth edges	Positive	—	+	+	+	+
M. melitensis. Malta fever	Creamy and slightly yellowish	Positive	Monkeys. Also rabbits and guinea-pigs by intracerebral inoculation	—	0	0	0

+ = acid. — = alkali. 0 = no action.

Micrococcus catarrhalis [1]

This organism occurs in the nose and throat in cases of catarrh, and particularly in the "influenzal cold" (see "Influenza") in bronchial catarrh, and occasionally in other conditions and in well people. Morphologically it occurs in pairs and tetrads, often within the polymorphonuclear leucocytes. It is Gram-negative. The primary generation develops feebly on agar, but subsequent generations grow fairly well, forming whitish translucent colonies. Blood or ascitic media should be used for isolation. Some of the fermentation reactions and a comparison with other Gram-negative cocci are given in the tables on pages 273 and 279. A vaccine prepared with it is frequently of service in catarrhal affections of the respiratory tract.

Micrococcus tetragenus

This organism is frequently met with in phthisical cavities and may be expectorated in the sputum, and has also been found in the pus of acute abscesses. The cells occur singly (diameter 1), in pairs, or in fours, and are enclosed within a capsule. It stains with the ordinary anilin dyes and also by Gram's method. On gelatin it develops slowly, with the formation of a thick, white, shining growth without liquefaction. On agar the growth has much the same characters, and on potato is white and viscous. Inoculated into animals, particularly mice, a local abscess may form, but usually a fatal general infection ensues, and the organism is found in the blood and organs.

A few cases of general infection in man have been described, which may assume a typhoid type, and two cases of tetragenus cerebro-spinal meningitis are recorded by Ramond and Resibois.[2]

Sarcina ventriculi

An organism occurring in the contents of the stomach, especially in cases of dilated stomach. Originally described by Goodsir in 1842.

It occurs as a large ovoid cell, several of which are grouped together quadrilaterally so as to form more or less cubical masses,

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[1] See Gordon, *Brit. Med. Journ.*, 1905, vol. ii, p. 423; Arkwright, *Journ. of Hygiene*, vol. vii, 1907, p. 145.

[2] *Le Progrès Médical*, September, 1915, p. 463.

the so-called "woolpacks." According to Falkenheim, it forms on gelatin in thirty-six to forty-eight hours roundish, prominent colonies of a yellowish colour, and in neutral hay infusion a brownish film and flocculi. It produces an acid reaction.

Other sarcinæ also occur in the stomach.

Clinical examination.—1. The organism can be detected in the vomit, etc., most readily by examination in the fresh state, a little of the material being placed on a slide, diluted with water if necessary, irrigated or not with iodine solution, covered with a cover-glass, and examined.

2. Film preparations may be stained with weak carbol fuchsin, or by Gram's method.

Other Organisms met with in Suppurative and Septic Conditions

Many other organisms may be met with in various suppurative and septic processes, *e.g.* :

a. The *B. coli* in cystitis and pyelitis, ischio-rectal abscess, peritonitis associated with perforation and intestinal obstruction, and puerperal fever (see Chapter X).

b. The *Diplococcus pneumoniæ* in abscesses, empyema, arthritis, meningitis, pericarditis, peritonitis, etc. (see Chapter XII).

c. The *B. typhosus* in abscesses, cholecystitis, empyema, and osteomyelitis (see Chapter X).

d. Anaërobic bacilli, such as *B. œdematis* and *B perfringens*, in foul, gangrenous wounds (see Chapter XIII).

e. The *B. tuberculosis and B. mallei* (see Chapter IX).

f. The *actinomyces* and *streptothrix* forms (see Chapter XV).

g. Blastomycetes, *Sporotrichon* (see Chapter XVI) and *Hyphomycetes* (see Chapter XVII).

h. The *Entamœba histolytica* (see Chapter XVIII).

i. Capsulated bacilli (see note, pp. 291 and 391).

Coley's Fluid

This preparation consists of the toxins of the streptococcus of erysipelas and the *B. prodigiosus*. It was devised by W. B. Coley, of New York, as a cure for inoperable malignant tumours, particularly sarcoma. The treatment is based on the undoubted fact that malignant growths may decrease, or even disappear

completely, after an attack of erysipelas (p. 258). Originally prepared by growing a virulent streptococcus obtained from a fatal case of erysipelas in bouillon for about ten days ; the culture is then inoculated with the *B. prodigiosus* and the two are allowed to grow together for another week or ten days. The culture is finally heated to from 58° to 60° C. for one hour, and a piece of thymol added to preserve it. The fluid is now prepared by growing the organisms separately and then mixing the two sterilised cultures in proper proportions.

The fluid is injected subcutaneously in the vicinity of the tumour. The primary dose recommended is $\frac{1}{4}$ minim of the fluid. The dose is gradually increased each day until there is a temperature reaction of 103° to 104° F.

Full particulars will be found in Coley's paper (*Proc. Roy. Soc. Med.*, vol. iii. 1909–10, Surg. Sect., p. 1).

CHAPTER VII

ANTHRAX

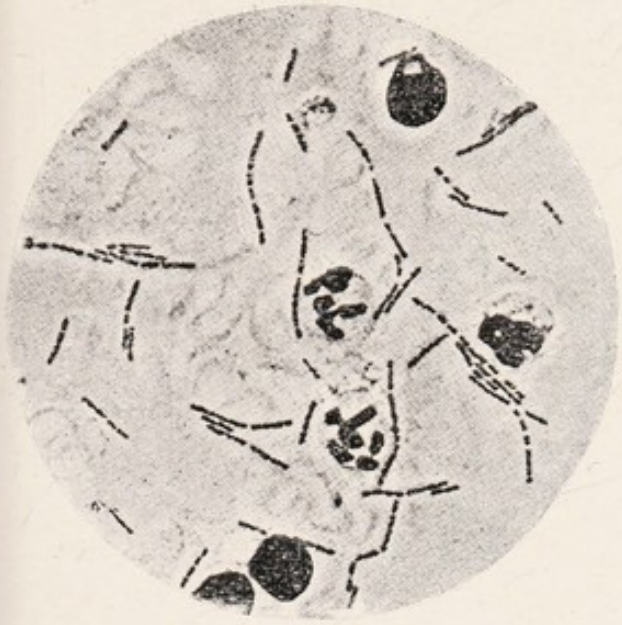
ANTHRAX is essentially a disease of cattle known as splenic fever, which occurs in England only sporadically, or in small outbreaks, but in some parts of the world assumes serious proportions—as in Siberia, where it is termed the Siberian plague. In France also at one time it ravaged the sheep to such an extent as to threaten them with extinction. In Great Britain 795 animals died of anthrax in 1914. Man is also occasionally attacked.

Anthrax was the first disease to be definitely associated with a specific micro-parasite, for the organism was observed as glassy homogeneous rods and filaments in the blood of infected animals so long ago as 1849 by Pollender and 1850 by Davaine, and the latter also claimed in 1863 to have demonstrated by inoculation experiments the causal relation of the organism to the disease. Davaine's experiments were made by inoculating an animal directly with the blood from an infected animal, and were, therefore, hardly conclusive, as they did not comply with the second and third of Koch's postulates, which declare that the micro-organism must be cultivated outside the body, and the cultivated organism must produce the disease on inoculation, and the objection was raised that infection was due, not to the bacillus, but to something else in the blood. This objection was subsequently removed by the work of Pasteur and of Koch, who obtained pure cultures of the organism, the *Bacillus*

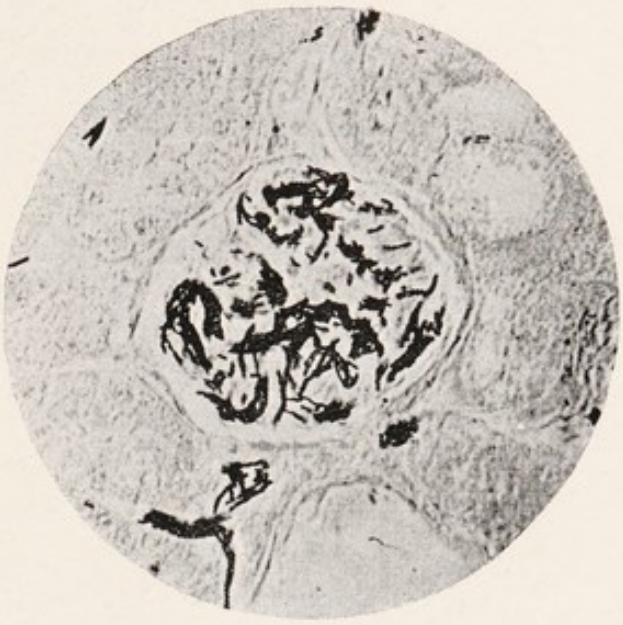
anthracis, and with these produced results the same as had previously been obtained by inoculation with the blood of an infected animal.

Morphology.—The *Bacillus anthracis* is a rod-shaped organism varying slightly in size in different animals and under cultivation; in the blood it measures from 5 to 20 μ in length and 1 to 1·25 μ breadth (Plate IV. *a*), but in cultures long filaments develop. Examined in the fresh and living condition in a hanging-drop preparation, these rods and filaments appear homogeneous or slightly granular; in stained preparations, however, they are seen to be made up of a series of segments with unstained interspaces, each segment measuring about 4 to 5 μ in length, and the ends of the segments appear cut off square, provided care has been taken not to overheat in fixing and to stain with an aqueous solution; they also appear to be encapsuled (Plate IV, *c* and p. 295). In the blood the filaments never exceed about five or six segments in length, except perhaps in swine, in which animals they may be somewhat longer. In cultures, however, the filaments may be of almost unlimited length, and lie parallel to one another or in more or less tangled masses. In the animal body during life, and for some hours after death, spores never occur; but in cultures more than a day or so old, and from which oxygen has not been excluded, they are always present, almost every segment containing one. The spores are ellipsoidal, measuring about 1 μ by 1·25 μ, and are centrally placed in each segment, the long axis corresponding with the long axis of the segment.

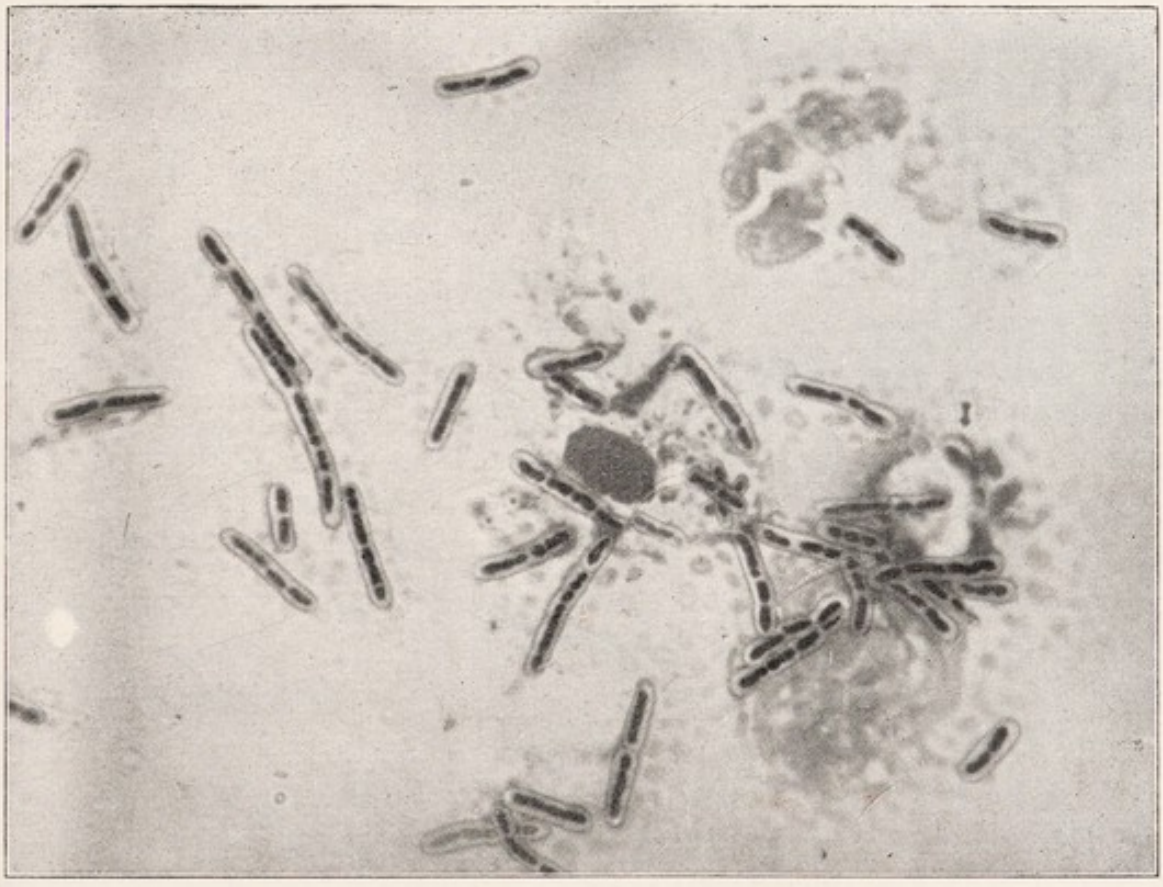
Cultural reactions.—The anthrax bacillus is aërobic and facultatively anaërobic; it is non-motile, and stains well with the ordinary anilin dyes, and especially so by Gram's method. It grows readily on all culture media at from 20° to 37° C., the latter being the optimum. Develop-



a. Bacillus anthracis. Smear of blood of inoculated guinea-pig. Leishman stain. × 750.



b. Anthrax. Section of kidney through glomerulus. Gram and Eosin. × 500.



c. Bacillus anthracis. Smear of blood showing capsules around the bacilli. McFadyean's method. × 1000.

ment ceases at temperatures below about 15° and above 5° C. Small, cream-coloured, granular colonies develop in a gelatin plate in about thirty hours, and in two to three days appear as small, roundish, cream-coloured pasty masses in little pits in the gelatin, due to liquefaction. Microscopically the colonies are somewhat characteristic; each consists of a mass of wavy, tangled filaments like a tiny wad of cotton-wool. In gelatin streak-cultures development is slow, and in four or five days a creamy, pasty growth forms in a trough of liquefaction. In a gelatin stab-culture (preferably 5 per cent. gelatin) lateral branches spread from the central growth, longer in the upper layers, shorter below, so that at the end of a week the culture is like an inverted fir tree (Fig. 38), and the gelatin becomes gradually liquefied from above downwards. The colonies on an agar plate develop in twenty hours at 37° C. as cream-coloured points. The surface colonies microscopically consist of little masses of wavy, tangled filaments (Plate V. *a* and *b*); "they are not circular but run to a point in two or three directions, with gracefully curved margins" (Reichel), and the growth is sticky. The young deep agar colonies, which Eurich [1] considers most characteristic, consist of inter-

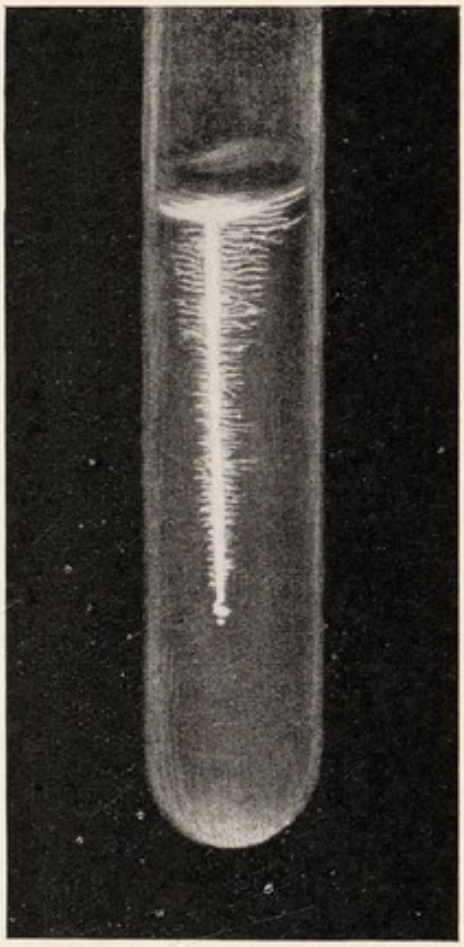


FIG. 38.—Anthrax. Gelatin stab-culture. Seven days old.

[1] *Journ. Path. and Bact.*, xvii, 1912, p. 249.

lacing knotted coils of fine filaments. On an agar surface culture at 37° C. there is a copious development in eighteen hours of a thick, cream-coloured, slimy growth, which at this early stage has a finely granular, ground-glass appearance. On blood-serum a thick creamy layer forms, with slow liquefaction of the medium. On potato the organism grows freely as a dry greyish layer, with an abundant formation of spores. In broth it forms a somewhat scanty flocculent deposit, the broth remaining clear and giving the indole reaction.

In old cultures various involution forms are met with; the rods lose their regular shape and become swollen, producing the so-called torula forms, while the homogeneous appearance of the protoplasm changes and becomes granular. Ultra-violet rays are stated by Mme. Henri to produce marked mutations of the anthrax bacillus (see p. 6). Spores are found in all culture media when there has been free access of oxygen, as in surface cultures on potato and agar, but in a deep broth culture, where the supply is limited, spore-formation is absent or very scanty. Spores are never met with in the living animal; they only appear some hours after death, or when matter containing the bacilli comes in contact with air, as in the bloody discharge from the nostrils. It has therefore been supposed that oxygen is necessary for spore-formation to take place, but this does not seem to be the whole explanation, for spores form in an atmosphere of nitrogen, though they do not do so in one of hydrogen. The life-history of the organism and the development of spores can be well watched in a hanging-drop specimen prepared by inoculating a droplet of broth with the blood of an infected animal. The preparation can be observed on a warm stage, or examined at stated times, being kept in the intervals in the blood-heat incubator. At the end of twenty-four hours the short fila-

PLATE V.

a. Bacillus anthracis. Impression preparation of a surface colony. × 40.

b. Bacillus anthracis. Impression preparation of a surface colony. × 750.

ments, which alone are present in the blood, will have grown so long that they stretch across the field, while the protoplasm has become granular, and minute shining points are visible here and there. In another twenty-four hours the filaments extend, the protoplasm becomes still more granular, and the shining spots are now well-marked ovoid, highly refractile bodies—the mature spores. In old cultures the rods and filaments almost disappear, numbers of spores alone remaining. These spores, when placed under favourable conditions of moisture, warmth, and nutriment, again produce rods and filaments; a little bud appears at the extremity of the long diameter, which grows in length and ultimately becomes a mature rod, often with the empty spore capsule embracing one end. Sporeless varieties of the anthrax bacillus have been obtained by cultivating under unfavourable conditions, as at a high temperature (44° C.) or in the presence of minute quantities of antiseptics (1 : 1000 carbolic acid).

The spores are of considerable practical importance, for they are highly resistant forms, requiring at least some minutes' boiling and three hours in dry air at 140° C. for their destruction, whereas the bacilli without spores are destroyed in ten minutes in the moist condition by a temperature of 54° C. The same resistance occurs towards various germicidal substances. While 1 per cent. carbolic acid solution quickly destroys bacilli without spores, the spores resist 5 per cent. carbolic for days, and at least 5 per cent. solutions of high-coefficient phenoloid disinfectants, acting for not less than twenty-four hours at 20° C., are required to kill the spores. The resistance of the spores is stated to increase with their age, but the writer has not found this to be the case. Formalin and a formalin-containing disinfectant, "Bacterol," seem to have a selective action on anthrax spores and are

efficient disinfecting agents for them. Reichel and Gegenbauer recommend for the purpose a mixture of 10 per cent. salt and 1 per cent. hydrochloric acid at 30° C., acting for twenty-four hours. Anthrax spores may retain their vitality unimpaired for years in a dried condition.

Certain anthrax-like bacilli have been described and have to be distinguished from *B. anthracis, e.g. B. pseudo-anthracis, B. anthracoides, B. anthracis similis.* These are non-pathogenic and are hæmolytic for rabbit, sheep, horse, and ox corpuscles, while the *B. anthracis* is non-hæmolytic.[1] The former form no capsule in the animal nor when cultivated in an inactivated serum, anthrax forms a capsule in such circumstances.

Pathogenicity.—The anthrax bacillus is pathogenic for man, cattle, sheep, goats, rabbits, guinea-pigs, and mice. The horse and the pig are also susceptible ; but adult white rats are partially,[2] and dogs, cats, and Algerian sheep are said to be completely, immune. Inoculated anthrax is rarely fatal to cattle in India (Holmes). The chief Veterinary Officer states, however, that a dog and a cat died of anthrax in 1914.

Young white rats, or rats fatigued by muscular work, can be infected, and frogs and fish, though immune under ordinary conditions, can be rendered susceptible by raising the temperature of their environment. Birds, such as fowls and pigeons, are also almost insusceptible, but may be rendered susceptible by lowering their temperature ; smaller birds, such as sparrows, are more susceptible. The virulence varies considerably and may be artificially modified in many ways : by passing through a series of susceptible animals it is heightened, by growing in the body of an insusceptible animal it is lowered, and the latter result is also obtained by cultivating for two or

[1] Jarmai, *Centr. f. Bakt.*, Abt. I (Orig.), lxx, 1913, p. 72.
[2] Hall, *Ibid.* lxvi, 1912, p. 293.

three weeks at a temperature of 42° to 45° C., or by the addition of certain chemical substances to the culture medium—for example, 0·01 per cent. of potassium bichromate. These methods of "attenuation," as it is termed, are practically applied in the preparation of the anthrax vaccine.

Symptoms of the disease in cattle are not very marked. A beast may appear a little out of sorts and the next day be found dead, or after suffering for a day or two with general malaise, fever, and rigors, and with a sanguineous discharge from the nostrils and bowel, it dies suddenly. Post-mortem, the chief feature that attracts attention is enlargement of the spleen; the organ may be two or three times larger than normal, is highly congested, and very soft and friable. Microscopically, the bacillus is found in enormous numbers in the spleen, somewhat less numerously in the blood, and still less so in the liver, kidney, and other organs.

Swine do not often suffer from this disease, unless fed with the offal of an infected animal, in which case the chief clinical sign is great enlargement about the throat; this is almost pathognomonic, while the chains of bacilli tend to be somewhat longer than in other animals.

Mice inoculated subcutaneously usually die in about twenty-four hours, and enlargement and congestion of the spleen are very noticeable. An infected guinea-pig generally dies in about thirty-six hours and usually shows no symptoms until the last, when it may suffer from rigors, with high temperature, convulsions, and staring coat. Post-mortem, the muscular tissue is found to be pale and œdematous, the spleen is enlarged to two or three times its normal size and is highly congested and very soft, and minute hæmorrhages may occur in the serous membranes. Microscopically, bacilli are found throughout the spleen, and are often so numerous that in a stained preparation

there appear to be more bacilli than tissue. Large numbers are also present in the blood and lungs, fewer in the liver and kidney; in the latter organ they are almost confined to the glomeruli (Plate IV. *b*). Immediately after death, however, comparatively few bacilli may be met with in the blood, the heart, and great vessels.

The spread of the disease in nature seems to result from the ingestion of spores while the animals are feeding. Although the bacilli without spores would be destroyed by the acid gastric juice, this is not the case with the spores, which are probably generally developed from the organisms present in the bloody discharges of a stricken animal, and are distributed by wind and flood, and in this way may infect large tracts of pasture. Crows and foxes may also serve to spread the disease by feeding on infected material and disseminating the spores by the excreta.[1] Pasteur suggested that earthworms might bring the spores to the surface in their casts from the buried carcases of infected animals, but some experiments by Koch negatived this. The non-sporing bacilli rapidly degenerate and die in a buried carcase.

Man seems to be relatively insusceptible to anthrax. The disease is generally met with among butchers, veterinary surgeons, shepherds, etc., and among those who sort wool or hair or work with, or carry, hides, *e.g.* glove-makers, tanners, porters, etc. Several cases in which infection was derived from shaving brushes have been reported. The disease occurs in two forms: the so-called "malignant pustule," a cutaneous infection, not unlike an angry carbuncle, occurring at the seat of inoculation, on exposed parts of the body, such as the back of the neck, the face, wrists, and hands; and "wool-sorters' disease," a general infection, severe and fortunately rare, through the lungs or stomach. A cerebro-spinal meningitis,

[1] Mollet, *Centr. f. Bakt.*, Abt. I (Orig.), lxx, 1913, p. 19.

simulating cerebro-spinal fever, may occur, but is very rare. Rag-sorters are likewise sometimes attacked by anthrax, but there is also a distinct "rag-sorters' disease" which is stated to be due to a non-motile, non-sporing, non-liquefying, capsulated bacillus, the *Proteus capsulatus hominis* [1] of Bordoni Uffreduzzi.

Under the Factories and Workshops Act 1895 all cases of anthrax contracted in connection with various industries have now to be reported to the Home Office. In 1909, 56 cases, in 1910, 51 cases were thus reported, with mortalities of 21·5 and 17·6 per cent. respectively. In addition, in 1910 there were 31 other cases in England and Wales. 101 cases of Anthrax occurred in 1913 with 10 deaths as follows:

Industries	Cases	Death
Wool	43	4
Horsehair	5	1
Hides and Skins	19	2
Other Industries	3	—
Not reportable	31	3
	101	10

Industrial anthrax has also been exhaustively dealt with by Legge.[2] It is particularly Persian wool, Chinese hides, and Russian hair which are dangerous, while Argentine, Australian, and New Zealand wools are almost innocuous. The sorting and exclusion of wool derived from infected animals seem to be impracticable, and the

[1] Capsulated bacilli have been met with in many septic processes. This group includes Friedländer's pneumo-bacillus, *P. capsulatus hominis*, *B. mucosus capsulatus* of Fricke, and the *B. coli immobilis*. They are met with in conditions with sepsis, pus production, broncho-pneumonia, ulcerating stomatitis, etc. They are shortish, non-motile, non-sporing rods, usually Gram-negative, easily cultivated and not liquefying gelatin, and in the tissues surrounded with a capsule (p. 391).

[2] *Brit. Med. Journ.*, 1905, vol. i, pp. 529, 589, and 641.

efficient sterilisation of the thousands of bales that are imported an impossibility. As regards hides and skins, Legge points out that it is doubtful if there is any way in which hides to be afterwards tanned can be effectively disinfected, and to be of real benefit it would have to be done before the material is opened in the warehouse ; but to secure this would be impossible. A method introduced by Seymour Jones has been favourably reported on [1] ; it consists in soaking the skins for twenty-four hours in a mixture consisting of 1 per cent. formic acid and 1 in 5000 mercuric chloride. After this treatment the skins are soaked in a strong brine solution. The writer, however, has found that for horsehair the solution, to be efficient, must be two or three times stronger than this. As regards horsehair, Webb and Duncan [2] carried out a number of experiments on its disinfection, from which it would seem that, leaving out of consideration white or grey hair, which is liable to change colour, no injurious effect is produced on hair by steam disinfection provided the temperature does not exceed 218° F. ; but this is a comparatively low temperature for efficient disinfection, and success can then be obtained only with minute care in the construction and regulation of the apparatus. Legge concludes that to secure certain destruction of all anthrax spores in horsehair absolute reliance cannot be placed on either steam disinfection (within the limits in which it can be applied) or simple boiling. Adoption of one or the other is a very material safeguard, but risk must always be run by those who prepare the hair for disinfection. Disinfection has been attempted by subjecting the material to the action of certain phenoloid disinfectants, but from experiments by Hall and the

[1] Ponder, *Report to the Worshipful Company of Leathersellers*, 1911.

[2] *Ann. Rep. of Chief Inspector of Factories*, 1900, p. 472, and 1902, p. 278.

writer, a modified Seymour-Jones method or formalin or bacterol seem to be the only efficient ones.[1]

Steam disinfection at 215°–230° F. can be applied to wool, but the fibres are materially damaged by the process.[2]

A number of cases of anthrax, resulting in many deaths, have been reported in various parts of the United States from tanneries dealing with hides imported from China. Also a number of cattle have been infected as the result of drinking water from rivers and creeks receiving the waste liquors from these works.

Houston [3] detected the anthrax bacillus in a catch-pit in a hide factory at Yeovil, and in sewage and effluents and in the mud of the Yeo. It has also been met with in linseed cake and oats.

Toxins.—From pure cultures of the *Bacillus anthracis* Hoffa obtained small quantities of a ptomine, which produced fall of temperature and hæmorrhages, and Hankin isolated a proteose which in large amounts was fatal, but in small amounts conferred immunity to subsequent inoculation with living bacilli. Brieger and Fränkel obtained a tox-albumin from animals dead of anthrax. Marmier, by growing the anthrax bacillus in a solution of peptone, glycerin, and salts, and subsequently precipitating with ammonium sulphate, obtained a toxin which he states is neither protein nor basic, and is contained within the bacterial cells.

Sidney Martin,[4] by growing the anthrax bacillus in alkali albumen for ten days, obtained from the culture albumoses and an alkaloidal substance. From the

[1] In disinfection experiments with anthrax, agar should be used for the subcultures, broth for some unexplained reason being inefficient. See Hewlett and Hall, *Journ. of Hygiene*, xi, 1911, p. 473.

[2] See Eighth Rep., Anthrax Investigation Board.

[3] *Second Rep. Commis. on Sewage Disposal*, 1902, p. 31.

[4] *Brit. Med. Journ.*, 1892, vol. i, p. 641.

bodies of animals which had died of the disease, chiefly from the spleen and blood, he obtained similar substances, the amount of alkaloid being more than double that of albumose. The mixed products produced fever in animals followed by coma and death. The albumose was proved to be the fever, and the alkaloid the coma, producer; the latter also caused a spreading œdema at the seat of inoculation.

Anti-serum.—An anti-serum for anthrax was prepared by Marchoux by immunising sheep by vaccination and then inoculating with progressively increasing doses of virulent anthrax cultures. Sclavo has prepared an anti-serum by first immunising asses with a vaccine and then inoculating them with increasing doses of virulent cultures over a prolonged period. This serum has been used successfully in a number of cases of anthrax in man, and should always be employed, 60–80 c.c. being injected intravenously. Salvarsan also seems to be an efficient drug for the treatment of anthrax. As already mentioned (p. 262) *B. pyocyaneus*, and pyocyanase obtained therefrom, is antagonistic to anthrax infection. Louis and Fortineau [1] state that they have treated 50 cases of anthrax infection in man by injections of 10 c.c.–20 c.c. of sterilised broth cultures of *B. pyocyaneus* with a mortality of 10 per cent.

Vaccine.—An attenuated virus has been extensively employed for the *prophylactic* vaccination of cattle and sheep. Cultures are attenuated by growing at 42°–43° C. (Pasteur, Chamberland, and Roux). A weak vaccine is first injected, followed after ten to twelve days by an injection of a stronger vaccine. The mortality as a result of the vaccination is small and the animals are subsequently protected for some months against the virulent disease. Sobernheim has applied a combined method,

[1] *Comp. Rend. Acad. Sc.*, vol. 158, No. 14, 1914, p. 1035.

5–15 c.c. of anti-anthrax serum being inoculated on one side of the animal, and the vaccine on the other. This practically eliminates all danger from the vaccine.

Clinical Examination

(1) *In veterinary practice.*—If an animal is suspected to have died from splenic fever, an extensive post-mortem is inadvisable because of the risk of distribution of material containing bacilli and subsequent development and dissemination of spores, with infection of pasture, etc. The abdomen should be opened and the spleen examined. If this is found to be much enlarged, and so soft that it can hardly be handled without rupture, there is a high probability of splenic fever, which the history of sudden death, with or without symptoms, coupled with a sanguineous discharge, increases. To confirm the diagnosis, some smear preparations should be made from the spleen and blood,which can be stained and examined on arriving home. If slides or cover-glasses are not available, the ear or a small piece of the spleen may be removed and taken home, where the specimen may be examined. When material is sent from a distance for examination the ear should be forwarded.

The smears may be stained with Löffler's blue and by Gram's method with eosin. Methylene-blue staining gives the most characteristic appearances, according to McFadyean. A smear preparation is made, not too thin, is air-dried, and then fixed by passing *once* through the Bunsen flame. The film is stained in a 1 per cent. aqueous solution of methylene-blue for ten minutes and then lightly rinsed and dried. The anthrax bacilli appear as blue rods surrounded by a pale violet capsule (Plate IV, *c*). If the post-mortem has been made shortly after death no spores are visible. *Unless the material be quite fresh large saprophytic bacteria somewhat resembling anthrax are always present and must not be mistaken for that organism;* by the McFadyean method of staining these saprophytes do *not* show the violet capsule. If a hanging-drop preparation can be made early enough, anthrax bacilli are non-motile, while many saprophytes resembling them are motile.

The stained preparations can be kept and produced in a court of law if necessary. Cultivations can also be made from the spleen, but the necessary culture media are not of course usually forth-

coming. Finally, a guinea-pig or mouse may be inoculated subcutaneously in the abdomen with a particle of the spleen, and after death examined microscopically and by culture methods.

As regards the disposal of the carcase of an animal dead from anthrax, this should be burned if possible, but, failing this, it may be buried in a deep pit, preferably with plenty of lime. All traces of blood and discharge must be carefully mopped up with a strong lime-wash or solution of chloride of lime, or other reliable disinfectant.

(2) *In man.*—In malignant pustule, smear specimens should be prepared from the fluid of the vesicles or with the scrapings from the incised pustule, or sections of the excised pustule may be made, and stained, some with Löffler's blue, others by Gram's method with eosin. The bacilli are not often met with in the blood, except shortly before or after death. Examination of the blood-serum of the case by the opsonic method, using anthrax spores, may be of value. At the same time cultivations on agar and gelatin should be prepared, and may yield positive results when the microscopical examination has been negative. In the later stages of the disease the bacilli may be difficult to find, even in sections.

In all cases of doubt a guinea-pig or mouse should be inoculated subcutaneously with the material, and if the animal dies the diagnosis of anthrax may be confirmed by the characteristic appearances, by a microscopical examination, and by cultivation. The animal experiment is by far the most certain method of diagnosis, a negative result being nearly as valuable as a positive one.

N.B.—It must be noted that both cultivation and inoculation experiments may fail to give positive results if the material be old or putrid.

(3) *In wool, hair, etc.*—Eurich (*loc. cit.*) recommends a suitable quantity of the material to be placed in a flask with 50 c.c. to 100 c.c. of boiled water to which 3–5 c.c. of 5 per cent. solution of caustic potash is added. If much blood-stained, the mixture is allowed to stand at 37° C. for several hours. It is then poured into a flat dish and the wool or hair is well teased. The mixture is then heated to 80° C. for two to three minutes. Tubes of melted agar (6–9 c.c.) at 80° C. are then inoculated with $\frac{1}{2}$ c.c. of the wash and poured into Petri dishes (4 inch). The characteristic deep-lying colonies (p. 285) should then be searched for after twenty hours' incubation. Animals may be also inoculated.

CHAPTER VIII

DIPHTHERIA [1]

Diphtheria in England — The Diphtheria Bacillus — The Pseudo-Diphtheria Bacillus—Clinical Diagnosis—The Xerosis Bacillus—Diphtheritic Affections of Birds and Animals

DIPHTHERIA seems to have been known from the earliest ages, being recognised by the classical (medical) writers, and it was epidemic in England and on the Continent during the Middle Ages. Bretonneau [2] experienced an outbreak at Tours, 1818–1821, and gave to the disease the name "Diphtérite" (afterwards changed to "Diphtérie") from the formation of membranes which is so marked a feature in it. In England the diphtheria deaths have only been separately scheduled since 1855. Since 1881 until recently there has been a steady increase in the prevalence of diphtheria, particularly in the large towns, but latterly the prevalence seems to be decreasing.

As regards croup, it is universally admitted that the vast majority of cases of membranous croup are cases of diphtheria.

Diphtheria is distinctly a disease of the young, especially at the ages from two to ten, and this holds good both for London and for England and Wales.

That diphtheria is an infective disease is amply proved

[1] See *The Bacteriology of Diphtheria.* Cambridge University Press, 1908.

[2] See *Memoirs on Diphtheria,* New Sydenham Soc., 1859.

by the history of epidemics, and by the recorded cases where the disease has been conveyed from one individual to another.

The disease occurs in all grades of severity, from the classical ones with wash-leather-like membrane and great prostration, to those which present a mild tonsillitis or angina.

The bacteriological study of diphtheria was commenced as long ago as 1882 by two German investigators, Klebs and Löffler. Klebs especially investigated the pathological histology, and ascribed the disease to small rod-shaped organisms, which he observed in the membrane. It was reserved for Löffler to place this observation of Klebs on a firmer basis by the isolation and cultivation of the bacillus from the membrane, and by the production of certain phases of the disease by inoculation with the isolated organism. The cause of diphtheria is, therefore, this diphtheria bacillus, which, from its discoverers, is frequently known as the Klebs-Löffler bacillus.

The isolation of the specific organism was by no means an easy matter, as a number of other species of bacteria is frequently associated with it in the membrane, but was accomplished by Löffler by the use of a special culture medium now known as Löffler's blood-serum, which consists of a mixture of blood-serum (ox serum was that originally used) 3 parts and glucose bouillon 1 part, the whole being coagulated (see p. 69). On this medium the diphtheria bacillus grows and multiplies exceedingly well, while the other organisms associated with it in the membrane are to a large extent inhibited in their growth. By rubbing a small piece of membrane from a case of diphtheria over the surface of two or three tubes, or of a plate of Löffler's serum, and incubating at 37° C. for twenty to twenty-four hours, colonies of the diphtheria bacillus will be found more or less isolated according to the number

of organisms present in the membrane, and by subculturing from these pure cultures may be obtained.

Characters of the Diphtheria Bacillus

Morphology.—The *B. diphtheriæ* is a small, delicate bacillus, with rounded ends, measuring 3 μ or 4 μ in length. It is non-motile and does not form spores. The size varies somewhat even on the same medium, and three varieties of the bacillus have been described, viz. long, medium, and short, according to the length. These varieties tend to be constant and to breed true. Some of the rods both in cultures and in the membrane have a swollen end, the so-called clubbing, and parallel grouping, both in the membrane and in cultures, is almost universal, the bacilli lying parallel side by side (Plate VI. *a*). This parallel arrangement arises from the peculiar mode of division of the bacillus. If a cell be observed upon a warm stage it first elongates, then becomes constricted at about its middle, and then suddenly *one* side of the cell-membrane seems to rupture and one half of the cell bends over to the other, so that the two halves form a V. This mode of division, occurring in contiguous cells and being repeated, and the cells thus becoming more and more crowded together, leads to the arrangement in parallel series. The bacilli are generally joined end to end in pairs, and distinct thread and branching forms, though of rare occurrence, may be met with. On different media the same strain exhibits considerable variation in size. On blood-serum and on gelatin the bacilli are of medium length and on the whole fairly regular in shape; in broth they tend to be short and stunted; while on agar, especially glycerin agar, they are much larger than on the former media, and long club-shaped, spindle-shaped and barred or segmented involution forms are abundant; on blood-serum club-shaped

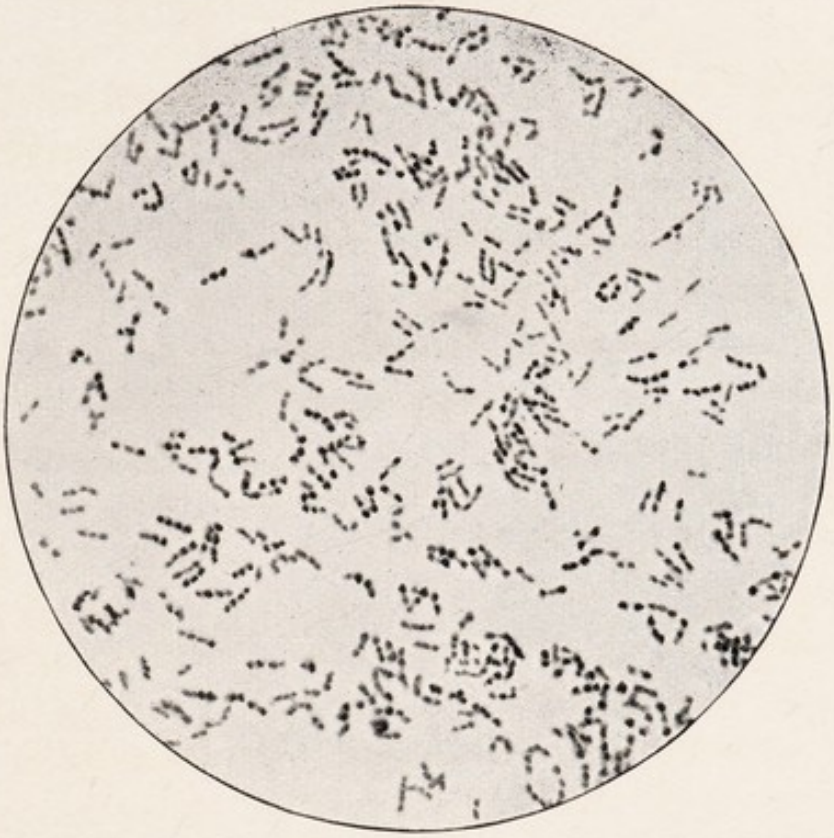
involution forms also occur,. but sparsely in a young, eighteen to twenty hours' culture, in a forty-eight hours' culture more numerously.

Staining reactions.—The *B. diphtheriæ* stains well with the ordinary anilin dyes and is Gram-positive. With Löffler's methylene blue the coloration is usually somewhat irregular, more deeply stained portions alternating with paler intervals, the so-called segmentation, and especially marked with agar cultures. The ends of the organisms are also frequently deeply stained, the so-called polar staining, while the phenomenon known as "metachromatism" is often marked both at the poles and also in the rod, appearing as granules of a purplish tint and contrasting with the blue of the methylene blue. With Neisser's stain (p. 327) deep inky coloured dots, appearing somewhat larger in diameter than the rods, occur at the poles of the organism and occasionally at the centre.

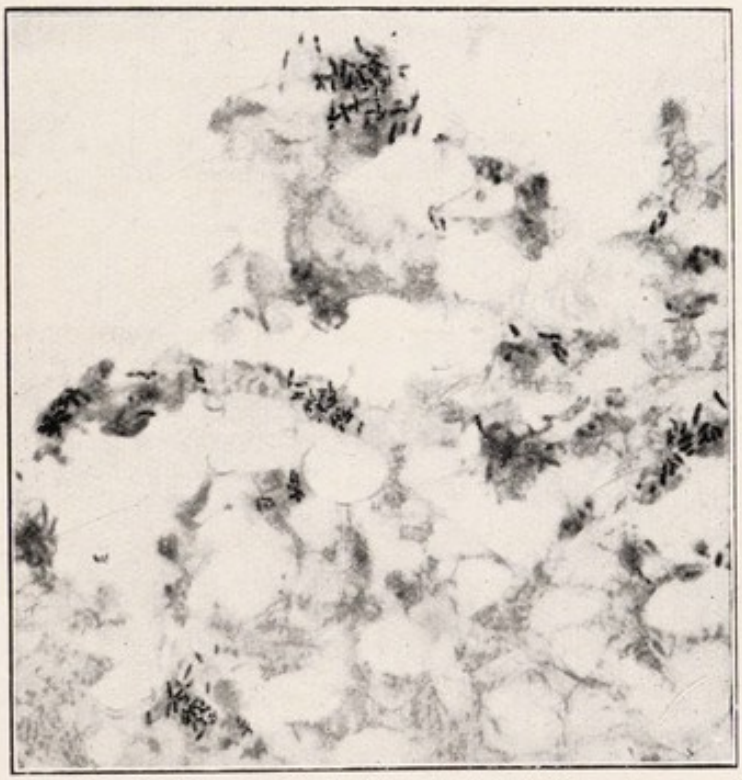
Cultural reactions.—The diphtheria bacillus is an aërobic and also a facultatively anaërobic organism, and grows well on all the ordinary culture media, forming cream-coloured growths or colonies, the latter on serum tending to be somewhat flattened, with regular margins. It grows slowly on gelatin, forming a raised whitish growth without liquefaction of the medium, and flourishes in milk, with the production of an acid reaction, but without curdling. In broth some strains give a granular growth on the sides and at the bottom of the tube, the broth remaining clear, sometimes with a thin surface pellicle; other strains may render the broth turbid throughout. On potato the growth is slight and invisible.

The indole reaction can be obtained in peptone-water cultures either with or without a nitrite, but the writer has shown that this reaction is due, not to indole, but to skatolecarboxylic acid (see below, p. 322).

PLATE VI.



a. The Klebs-Löffler or diphtheria bacillus. Film preparation of a serum culture. × 1500.



b. Section of diphtheritic membrane with Klebs-Löffler bacilli. Gram and eosin. × 1000.

The diphtheria bacillus attacks glucose and lactose with the formation of acid only, no gas (see Table, p. 326). As regards the production of acid, Neisser found that during the first nine hours there is little or none ; at the end of twenty-four hours a considerable quantity has been formed, and the amount increases until the end of the second day, after which the production ceases.

The *B. diphtheriæ* is distinguished from all other diphtheroids by giving acid in glucose and dextrin but not in saccharose (Hine).

The *B. diphtheriæ* is agglutinated by the serum of patients and by a diphtheria serum, but the test is difficult to apply on account of the coherence of the growth, is somewhat erratic with different strains, and is of no practical value in the diagnosis of the disease. For the same reasons, the agglutination reaction is of little use for the recognition of the organism and for distinguishing it from the so-called " pseudo-diphtheria " bacilli.

The Klebs-Löffler bacillus retains its vitality in cultures for a month, and when dried for three or four weeks. According to Welch and Abbot, it is destroyed in ten minutes by a temperature of 58° C. It is readily destroyed by antiseptics when in culture, but in the membrane it is difficult to find an agent which will penetrate and kill the bacilli beneath the surface.

The diphtheria bacillus and its characters under cultivation have been described somewhat fully, because of the importance of the identification of the organism as a means of clinical diagnosis. As mentioned at the commencement of this chapter, the clinical diagnosis of diphtheria presents many difficulties, and considerable assistance may be derived from a bacteriological examination. The diagnosis is based on the presence or absence of the Klebs-Löffler bacillus, either in smears or in cultivations made from the membrane or secretion (see p. 325). This

method is of very real assistance in doubtful, and especially in mild, cases, which clinically it may be very difficult to decide whether they be diphtheritic or no. The mild cases are those which it is of the greatest importance to identify, especially in schools, for if not recognised the patients may go about and prove a source of infection to all around. The method also affords valuable evidence as to when a case can be considered free from infection; so long as bacilli are present in the throat infection must be possible, and the length of time for which they may occasionally persist is remarkable. In half the cases the bacilli disappear within three days of the disappearance of the membrane, in a few cases they linger for as long as three weeks, but occasionally they persist much longer. The writer isolated them for so long as five months (and virulent to the last); and a case is recorded in which they persisted for no less than fifteen months after the attack. In all cases two or three examinations should be made at short intervals with negative results before the bacilli can be pronounced to be absent, and no case should be discharged from hospital until the absence of bacilli has thus been proved. When bacilli persist, treatment with antiseptic sprays or gargles, combined with irrigation of the nose, may be tried. Irrigation of the nose is important, for the bacilli probably extend to the post-nasal space, where they are untouched by a throat spray or gargle. Another mode of treatment has also been adopted. A polyvalent *anti-microbic* agglutinating anti-diphtheria serum has been prepared, dried, and compressed into tablets, one of which is dissolved in the mouth every two hours, and fifteen minutes after solution the naso-pharynx is flushed with physiological salt solution. While this treatment sometimes succeeds, it often fails. The writer has tried the use of subcutaneous inoculations of diphtheria endotoxin (2·5, 5·0, and 7·5 mgrm.)

at intervals of seven to ten days. Of 32 cases treated in this way, 25 cases (78 per cent.) cleared up after the second or third dose.

With regard to the value to be attached to the bacteriological examination for diphtheria, while the finding of the bacilli is proof positive of the diphtheritic nature of the affection and of its infective nature, their apparent absence is not of so much value, as various circumstances modify the result. For example, an unskilled person may not happen to touch the right spot with the swab, or from struggling, etc., on the part of the patient even a skilled operator may fail to reach any but a small portion of the mucous membrane, instead of obtaining a good mop from all over, especially when there are no definite patches of membrane. The use of antiseptic gargles or paints shortly before the swabbing is taken will likewise prevent the growth of the bacilli. It sometimes happens that a very mixed growth is obtained in the cultures, and in such cases the Klebs-Löffler bacillus may be missed. Bearing such sources of fallacy in mind, and making due allowances for them, the negative result of a bacteriological examination may have considerable value in those cases which clinically are doubtful. *In no case where there is a reasonable suspicion of diphtheria should treatment with antitoxin be delayed until the bacteriological report is obtained.*

The bacilli from the throat are frequently associated with other organisms, especially micrococci and torulæ; and those cases in which the temperature tends to be high and the throat fetid are usually a mixed infection of diphtheria bacilli with the *Streptococcus pyogenes* or *Micrococcus pyogenes*, var. *aureus*. The fact of such mixed infection cannot, however, be definitely decided from the cultures, as these organisms may be present in the mouth or throat without necessarily taking part in the infective

process. Nor can the severity of the disease be gauged from the characters or numbers of the diphtheria bacilli and other organisms present, though perhaps in a series of cases those which yield practically pure cultures will probably be more severe than those which yield cultures with few bacilli. It has been stated that the long form of the diphtheria bacillus is the most, and the short form the least, virulent, the medium being intermediate in virulence, but this is by no means a universal rule. Westbrook [1] has divided all forms of the diphtheria bacillus into three groups, distinguished by their staining reactions with methylene blue. Those with deeply staining granules he calls "*granular forms*," those with transverse bands "*barred forms*," and those staining evenly "*solid forms*." Each group is further divided into seven types according to shape and size, the types being designated by the letters A to G and being progressively smaller from A to G.

It is sometimes stated that a microscopical examination, unless controlled by inoculation of the isolated bacteria, is unreliable. Such a statement is extremely misleading. If the bacilli which have been cultivated from a suspicious throat possess all the characters of diphtheria bacilli, inoculation experiments are not needed, and if they were performed with a negative result (*i.e.* the bacteria are not virulent) would prove little, for the bacilli from different parts of a culture from a throat often possess different degrees of virulence. Occasionally, it is true, even the expert may be in doubt about a particular bacillus, but such cases are the exception. Here an inoculation experiment may help, but would be of little value if a negative result were obtained. It is absolutely essential in the microscopical examination for diphtheria to use a good lens, proper illumination,

[1] *Rep. Minnesota State Board of Health*, 1899–1900.

and sufficient amplification, not less than 800–1000 diameters.

The Schick test may sometimes be of value in elucidating the nature of doubtful cases (*see* "Clinical Diagnosis," p. 330).

Pathogenicity.—The diphtheria bacillus is pathogenic for man, the horse, ox, rabbit, guinea-pig, cat, chicken, pigeon, and finches, all of which are more or less susceptible, while mice and rats are immune. In man the respiratory tract is usually affected, though the conjunctiva and other mucous membranes, as of the vagina and stomach, and wounds may be attacked. A pseudo-membrane usually forms, consisting of laminæ of fibrin entangling a few leucocytes and other cells, and here and there small effusions of blood, together with coagulative necrosis of the underlying mucous membrane, and the bacilli are for the most part located in the superficial layers of this pseudo-membrane (Plate VI, *b*), though in all cases in which the disease has lasted for any time they are found in the lungs, spleen, and kidneys, and may occur even in the blood. If the patient recovers from the diphtheritic attack, paralytic sequelæ are not uncommon and are due to a peripheral neuritis. Pseudo-membranes may be formed by other organisms, *e.g.* by the streptococcus and pneumococcus, also by the pneumobacillus, and occur in Vincent's angina (p. 331), but it is doubtful whether paralytic sequelæ follow any but a diphtheritic infection. They are certainly excessively rare in non-diphtheritic infections.

Some remarkable skin affections of an eczematous or ecthymatous nature have been found by Hare [1] and others to be due to the diphtheria bacillus.

Another affection which seems to be generally diphtheritic is membranous rhinitis. Whereas true nasal

[1] *Lancet*, 1908, vol. i, p. 282.

diphtheria is a serious condition, membranous rhinitis is seldom, if ever, attended with any risk to life, sequelæ do not occur, and it is rare to obtain a history of infection from cases of it. This is extraordinary and very difficult to explain, for virulent diphtheria bacilli are abundant in the nose and nasal secretion.

Diphtheroid organisms can occasionally be isolated from well people and those not known to have been in contact with diphtheria cases. The Klebs-Löffler bacillus can be isolated from the throats of nearly 7 per cent. of the presumably healthy population ; [1] in the throats of contacts the percentage rises to 33 or more. Murray and the writer [2] found diphtheria-like bacilli in 58 out of 385 children (15 per cent.) admitted into the Victoria Hospital, Chelsea.

Ford Robertson believes that diphtheroid organisms—possibly the Klebs-Löffler bacillus itself—may play an important part in the production of general paralysis of the insane. His views have not gained general acceptance, and Eyre (*loc. cit.*) found that the percentage incidence of all diphtheroid organisms and of the Klebs-Löffler bacillus in the throats of the insane was not greater than in well persons, and was unable to isolate the *B. diphtheriæ* post-mortem from cases of general paralysis.

Traces of antitoxin can be detected in the blood after an attack of diphtheria, usually at the end of the first week of convalescence : this antitoxin has probably little to do with the actual recovery from the disease (see p. 230). A small amount of antitoxin has also been occasionally found in well people and in untreated horses. It has been suggested that in such cases there has been a latent infection with the *B. diphtheriæ*, but on Ehrlich's

[1] See Eyre, *Brit. Med. Journ.*, 1905, vol. ii, p. 1104.

[2] *Brit. Med. Journ.*, 1901, vol. i, p. 1474. See also Graham-Smith, *Journ. of Hygiene*, vol. iii, 1903, p. 216,

side-chain hypothesis it seems more likely that in such cases an excess of the receptors which constitute antitoxin happens to be naturally free in the blood.

By the Schick test (see p. 330) it has been shown that a majority of infants up to two years of age have a considerable content of anti-bodies in their blood, hence their comparative insusceptibility to diphtheria, while children between 2 and 5 years of age have much less; adults generally have a high content of anti-bodies. Diphtheria carriers, who are simply carriers and not cases of diphtheria, may give a negative Schick test, while true cases will give a positive Schick test.

Guinea-pigs are the animals generally employed for experimental work on diphtheroid organisms. In order to compare the effects and virulence of various bacilli it is customary to make the inoculation with a measured volume of a forty-eight hours' broth culture. From 0·1 c.c. to 2 c.c. of such a culture, according to the virulence, inoculated subcutaneously, is usually required to kill a 250-grm. guinea-pig within three days. At the seat of inoculation hæmorrhagic œdema forms, hæmorrhages occur in the serous membranes, and especially in the adrenals, while the renal epithelium and the liver-cells undergo cloudy degeneration.

Inoculated into the trachea of the guinea-pig, rabbit, and chicken, pseudo-membranes form, and the same occurs with the superficially injured conjunctiva and vagina. It is stated by some that the diphtheria bacillus does not develop on a normal mucous membrane—this must first be injured, and the staphylococcus and streptococcus, so often associated with the diphtheria bacillus in the human subject, may play a part in preparing the way for infection by damaging the cells and tissues. Rabbits usually live somewhat longer than the guinea-pig after inoculation, and paralysis frequently develops

if life is prolonged, simulating the post-diphtheritic paralysis of man.

The question of the occurrence of the Klebs-Löffler bacillus in the lower animals is of considerable importance with regard to the spread of the disease and the conveyance of infection. The so-called diphtheritic affections of pigeons, poultry, and calves (referred to more in detail below, p. 332) are as a rule diseases quite distinct from human diphtheria, and are not communicable to man. A number of observers assert, however, that cats may suffer from the disease, which in these animals runs a chronic course, and is associated with bronchitis, lobular pneumonia, nephritis, and wasting. Klein [1] points out that not only are cats liable to the disease in houses where diphtheria has occurred, but that a similar infectious disease exists naturally among cats, and symptoms similar to this natural disease may be produced by inoculating healthy cats with the Klebs-Löffler bacillus. The diphtheria bacillus has also been isolated from the horse.[2]

Several epidemics of diphtheria have been traced to an infected milk supply. In some instances the infection has undoubtedly been derived from contamination from a human source, *e.g.* in an outbreak in Lambeth, Priestley traced the infection to a particular dairy in which a dairyman with an ulcerated thumb was employed and the ulcer was infected with virulent diphtheria bacilli, but in others this mode of infection has not been demonstrated, and it has been suggested that certain eruptive conditions on the teats and udder of the cow may be caused by the Klebs-Löffler bacillus and the milk become infected therefrom. Klein [3] made experiments with a view of determining this point. He inoculated healthy cows

[1] *Rep. Med. Officer Loc. Gov. Board* for 1889, p. 162.
[2] Cobbett, *Centr. f. Bakt.*, xxviii, No. 19, p. 631.
[3] *Rep. Med. Officer Loc. Gov. Board* for 1889 and 1890.

in the shoulder with a bouillon culture of the diphtheria bacillus. This caused fever and local swelling, and in about a week a papular and vesicular eruption appeared on the udders and teats. The *B. diphtheriæ* was isolated from the contents of the vesicles and also from the milk on the fifth day, but not subsequently. The cows died in two to four weeks, and the *B. diphtheriæ* was obtained from the local lesions. Abbott [1] obtained somewhat different results, but Klein [2] points out that these experiments were not performed under exactly the same conditions as his own.

Klein, Eyre, Dean, and Marshall [3] have isolated the diphtheria bacillus from milk. It is to be noted that diphtheria-like, *but non-pathogenic*, bacilli are often to be found in milk and cheese (see section on " Milk ").

Toxins.—Diphtheria toxin has not been obtained in a state of purity and its exact chemical nature is unknown. Löffler first investigated the chemical products formed by the diphtheria bacillus, and by precipitating bouillon cultures with alcohol obtained a white toxic substance which he classed among the enzymes.

Roux and Yersin precipitated the toxin from filtered broth cultures by means of absolute alcohol, and also by the addition of calcium chloride. They found that 0·4 mgrm. was sufficient to kill eight guinea-pigs or two rabbits, and considered it to be an enzyme.

From the blood and spleen of cases of diphtheria Sydney Martin [4] isolated albumoses (chiefly deutero-albumose) and an organic acid, but no basic body. Injected subcutaneously the albumose produces much œdema and irregularity of temperature; in larger doses depression

[1] *Journ. Path. and Bact.*, vol. ii, 1894, p. 35.
[2] *Ibid.* p. 428.
[3] *Journ. of Hygiene*, vol. vii, 1907, p. 32 (Refs.).
[4] *Brit. Med. Journ.*, 1892, vol. i, p. 641.

of temperature with paralysis and coma. Small multiple doses, not sufficient to destroy life, may give rise to some fever, and in two or three days to paralysis of the hind legs in rabbits, with general weakness and loss of weight. Post-mortem, the nerves are found to have undergone degeneration—breaking up and disappearance of the myelin and interruption of the axis cylinder, while the heart is fatty. The organic acid is also a nerve poison, but is not so toxic as the albumose. From diphtheritic membrane, extracted with a 10 per cent. salt solution, only traces of albumose and organic acid were obtained, but the extract was highly toxic, producing fever and paralysis. Sidney Martin suggests that a substance of the nature of a ferment may be present, and that the ferment in the membrane on absorption may perhaps form the albumose in the body. From cultures of the diphtheria bacillus in alkali-albumin, albumose and organic acid, with similar actions to those isolated from the body were obtained.

Brieger and Fränkel (1890) were unable to find any basic substance in cultures, and concluded that the toxic substance was a protein body, which they designated a "tox-albumin." It was destroyed by a temperature of 60° C. but not by one of 50° C., even in the presence of an excess of hydrochloric acid, and hence is probably not an enzyme. The tox-albumin is non-dialysable, is precipitated by saturation with ammonium sulphate but not with magnesium sulphate, and hence is neither a peptone nor a globulin, contains a large amount of sulphur, and gives the biuret and Millon's tests. A curious property of this substance is that small quantities (2·5 mgrm. per kilogramme of the body-weight) do not produce their effects until the lapse of weeks. Brieger and Boer in a later research prepared the diphtheria tox-albumin by precipitating a bouillon culture with a 1 per cent. solution

of zinc sulphate or chloride. The precipitate of the zinc double salt was washed with slightly alkaline water and decomposed with a stream of carbonic acid gas. The purified tox-albumin gives the xanthoproteic, biuret, and Adamkiewicz's reactions, and the red coloration on heating with Millon's reagent.

According to Ehrlich the toxin broth is a complex mixture of toxic constituents belonging to the proteins, but this is denied by Madsen and Arrhenius (see p. 177). Its poisonous property gradually diminishes on keeping, and is destroyed by boiling in five minutes, at lower temperatures more slowly, and also by light.

Diphtheria antitoxin.—By the injection of sub-lethal and increasing doses of the toxin into an animal an antitoxin is generated. For the preparation of a potent antitoxin for therapeutic use the first essential is a highly toxic toxin, and for obtaining this a diphtheria bacillus of high virulence is required, and few strains possess the necessary virulence. The virulent bacillus is grown in an alkaline broth (rendered alkaline to the extent of about 5·7 c.c. of normal caustic soda solution per litre beyond the neutral point to litmus) in Erlenmeyer flasks containing half to one litre for eight to twelve days at 37° C. Various small details have to be attended to in order to obtain toxin of maximum toxicity ; it is important that growth should occur upon the *surface* of the broth. The use of meat some days old has been advocated, or of acid beef-broth in which *B. coli* has been grown for twenty-four hours, in order to eliminate the glucose (p. 28). L. Martin makes use of " peptone " prepared by the autodigestion of a pig's stomach with dilute hydrochloric acid. The cultures are then filtered through a Berkefeld or Pasteur-Chamberland filter to remove the bacilli. The filtrate is germ-free and very toxic, and a little carbolic acid may be added to preserve it. In New York 10 per

cent. of a 5 per cent. solution of carbolic acid is added to the culture, the bacilli are allowed to deposit by standing for forty-eight hours, and the culture is filtered through paper; in this way filtration through a filter-candle is dispensed with. Less than 0·01 c.c. of the toxin should kill a 250-grm. guinea-pig in three to four days. Selected horses which have been tested with mallein and tuberculin, and kept under observation for some time to ensure that they are healthy, are then inoculated with this filtrate, commencing with a dose of 0·01 to 0·1 c.c. according to the toxicity of the toxin, or 20 c.c. of the toxin together with 10,000 units of anti-toxin may be given for the first three doses. Individual horses vary very much in their susceptibility to the toxin, so that care has to be exercised with the first injections. The injections are given subcutaneously over the shoulder, and produce a local swelling and some rise of temperature and general disturbance, lasting two or three days. When this has passed away the inoculation is repeated, a larger dose being administered provided the reaction due to the former one was not too severe. The treatment is continued for five to six months, the dose of toxin administered being gradually increased until it may attain 500 c.c. or more. Cartwright-Wood found that by growing virulent diphtheria bacilli for three or four weeks in ordinary peptone broth, with the addition of 10 or 20 per cent. of blood-serum or plasma, subjecting the culture to a temperature of 65° C. for an hour and filtering before injection, much larger initial doses can be given and some degree of immunisation attained, and subsequently the ordinary broth cultures may be injected in large doses. Individual horses vary much in their capacity to yield antitoxin: on the whole those that are moderately sensitive to the toxin seem to produce most antitoxin; a horse to be of value should after three months' treatment yield an anti-

toxic serum containing not less than 300 units per c.c. The required potency having been attained, as shown by the test described below, the horse is bled with aseptic precautions, the blood is allowed to coagulate, and the serum is drawn off and filled into sterile bottles each containing a dose of the antitoxic serum. A small amount of antiseptic, such as trikresol, is generally added as a precautionary measure to prevent the multiplication of any stray germs that may have gained access during the various manipulations.

Standardisation of antitoxin.—The potency of diphtheria antitoxin is always described in "units" and is estimated by ascertaining the quantity of antitoxin required just to neutralise a certain amount of a standardised toxin when both are injected into a 250-grm. guinea-pig. Formerly, by Roux's method, the minimal lethal dose of the toxin is first ascertained, and then the number of grammes of guinea-pig which 1 c.c. of antitoxin will protect against this minimal lethal dose is determined. If 0·01 c.c. of antitoxin protects a 300-grm. guinea-pig against the minimal lethal dose, 1 c.c. will protect $300 \times 100 =$ 30,000 grm. of guinea-pig, and the immunising value of the antitoxin would be described as 30,000. This method is open to the fallacy that if only a portion of the lethal dose be neutralised the guinea-pig may survive, and a fictitious value be given for the potency of the antitoxin. Behring later adopted ten minimal lethal doses as the test dose of toxin, and he termed ten times the amount of antitoxin which protects a guinea-pig against the ten minimal lethal doses a *unit* (the Behring unit, which therefore = 100 minimal lethal doses of toxin), from which the Ehrlich unit, now universally adopted, is derived. Though this method eliminates to a large extent the objections to the Roux method, Ehrlich found that by it the same antitoxin tested with different toxin

broths yielded different values. This he explained by assuming that diphtheria toxin broth contains not only toxin but also other substances which combine with antitoxin. These substances, though non-toxic, or comparatively so, vary in amount in different toxin broths, and variable results, therefore, may be obtained by the simple method of testing. These substances, having an affinity for antitoxin, are toxoids and toxone. There are several varieties of toxoids, viz. (1) those having a greater affinity for antitoxin than toxin itself, *protoxoids*; (2) those having the same affinity, *syntoxoids*; (3) and those having a less affinity, *epitoxoids*.[1] Toxoids are probably derivatives of toxin; they increase in quantity in old toxin broth which has been kept, and which at the same time decreases in toxicity. The toxones also combine with antitoxin, having a less affinity for it than toxin, are *primary* secretory products of the diphtheria bacillus, and while not acutely lethal, induce induration, necrosis, and paralysis. The toxoids are comparatively scanty in a fresh toxin broth and are negligible, but it is otherwise with the toxone, which is always present in appreciable quantity. Owing to the fact that toxone has less affinity for antitoxin than toxin has, if an exactly neutral mixture of toxin broth and antitoxin be prepared, considerably more than the minimal lethal dose of the toxin broth must be added to render the mixture lethal, because the first portion of the added toxin simply displaces the toxone from its combination with the antitoxin, and is neutralised by the antitoxin so set free.

Thus, suppose a certain amount of a toxin broth contains 90 units of toxin and 10 units of toxone, and to this amount 100 units of antitoxin are added so as to form a physiologically neutral mixture, the combination which

[1] See pp. 176–181 for other views on the constitution of diphtheria toxin.

occurs is shown by the following " equation " : 90 toxin-antitoxin + 10 toxone-antitoxin = L_0 (*i.e.* neutrality). If an amount of the toxin broth be now added, corresponding to 11 units of toxin, the effect will be as though only *one* unit of toxin has been added, as is shown by the following " equation " : 90 toxin-antitoxin + 10 toxone-antitoxin + 11 toxin = 100 toxin-antitoxin + 10 toxone (free) + 1 toxin (free) = L_+ (*i.e.* just acutely lethal). Thus although the equivalent of eleven minimal lethal doses of toxin has been added to the physiologically neutral mixture of toxin broth and antitoxin, only *one* minimal lethal dose of toxin remains free and active, because ten toxin units displace the ten toxone units from the toxone-antitoxin complex and are neutralised by the antitoxin thus set free. Ehrlich, therefore, devised a method of standardisation which eliminates irregularities due to the variable proportions of toxone and toxin in the toxin broth by adopting antitoxin and not toxin as the standard. In order to standardise an antitoxin, a virulent toxin broth is employed and its minimal lethal dose is approximately ascertained—*i.e.* that amount which is just sufficient to kill a 250-grm. guinea-pig on the fourth or fifth day. A solution of accurately standardised antitoxin, formerly obtainable from the Serumsprüfung Institut, Frankfort-on-Maine, is then prepared, containing one " unit " of the antitoxin in 1 c.c., and the toxin is standardised with this by mixing with one unit various quantities above and below one hundred minimal lethal doses. It is required to ascertain the amount of the toxin broth which, when mixed with one unit of antitoxin, just suffices to kill a 250-grm. guinea-pig on the fourth or fifth day after the injection of the mixture ; this amount of toxin is known as the L_+ dose. The L_+ dose may be defined as that amount of a given diphtheria toxin broth which is not completely

neutralised by one "unit" of standard antitoxin to the extent that *exactly* one simple lethal dose of toxin remains unneutralised; it corresponds usually to 105–120 minimal lethal doses. For example, suppose 0·003 c.c. of the toxin was found to be the minimal lethal dose, with separate "units" of standard antitoxin, 0·2, 0·3, 0·4, and 0·5 c.c. respectively of the toxin might be mixed, and each mixture injected into a guinea-pig; probably the guinea-pigs receiving the "unit" of antitoxin *plus* 0·2 and 0·3 c.c. of toxin would remain alive, while the animal receiving the 0·4 c.c. of toxin would die in twenty-four to forty-eight hours. The death in the last case is too rapid; more than a simple lethal dose has remained unneutralised, and therefore the L_+ dose of toxin lies between 0·3 and 0·4 c.c., and further experiments would have to be performed with amounts of toxin between these limits in order to ascertain the exact dose. Death of the guinea-pig on the fourth or fifth day has been chosen because it has been found that if the dose of toxin be diminished ever so little below that producing this result, death does not ensue under nine or ten days. That is to say, an *acute* intoxication is fatal at the latest on the fourth or fifth day, a fatal result after then being due to a *chronic* intoxication. The amount of toxin which is *exactly* neutralised by one "unit" of the standard antitoxin is known as the L_0 dose. By exact neutralisation is meant absence of any reaction, general or local, at the seat of inoculation, in the inoculated guinea-pig. If toxin broth were a single substance, containing only toxin, then $L_+ - L_0 = D$, the simple lethal dose, would be equal to the minimal lethal dose. But because of the presence of toxone, which also has an affinity for antitoxin, D, the difference between the L_+ and the L_0 doses, is usually a multiple (8–12) of the minimal lethal dose.

From these considerations we are now in a position to

define the unit of antitoxin ; a " unit " is that amount of antitoxin which will neutralise about 100 minimal lethal doses for the guinea-pig of diphtheria toxin. From certain considerations Ehrlich considers that the unit would exactly neutralise 200 minimal lethal doses of a theoretical toxin, containing only toxin and neither toxoid nor toxone, but, inasmuch as such a toxin is unknown practically, the unit corresponds usually to 105–120 minimal lethal doses of a toxin broth, the extremes which Ehrlich has found being 16 and 136 lethal doses. Having standardised a specimen of toxin by means of standard antitoxin, this standardised toxin is in its turn used to standardise the antitoxic serum which has been prepared for therapeutic use. The toxin is preserved by the addition of toluol, and is kept in a cool, dark place ; it needs to be restandardised every few weeks.

In standardising antitoxin, the L_+ dose of the standardised toxin is mixed with varying amounts of the antitoxin, the mixtures are injected into guinea-pigs, and the amount of the antitoxic serum which neutralises the L_+ dose of toxin is thus ascertained. If, for example, it were found that 0·05, 0·04, and 0·03 c.c. of the antitoxic serum neutralised the L_+ dose of toxin, but that the guinea-pig receiving 0·025 c.c. suffered from some local necrosis, wasted, and died in a few days, and the animal receiving 0·02 c.c. died in two or three days, 0·03 c.c. of this antitoxin would be about equivalent to one unit of standard antitoxin and the antitoxic serum therefore contains 33 units per c.c. For all the experiments the conditions must be kept as constant as possible, guinea-pigs weighing 250 grm. or thereabouts employed, and to eliminate irregularities a number of animals must be used. The antitoxic constituent of diphtheria antitoxin is globulin in nature, or is intimately associated with the globulin content of the serum. Thus Atkinson found that

if the serum is precipitated by saturation with magnesium sulphate, the whole of the antitoxin is carried down with the precipitate, and also that the globulin content of the blood-serum of antitoxin horses is increased. His results were confirmed by Ledingham.[1]

There can now be no doubt as to the value of the antitoxin treatment of diphtheria. Since the introduction of antitoxin treatment, which was commenced about the middle of 1894, there has been a steady decline in the case mortality from diphtheria, especially in London, where probably the majority of the cases are injected with antitoxin. From 1891 to 1894 the case mortality from diphtheria in the hospitals of the Metropolitan Asylums Board averaged about 30 per cent., in 1895 it was 22·8 per cent., and afterwards steadily fell, until during the last five years it has ranged between 6·2 and 7·9 per cent.

Not less than 2000 units should be injected for a dose, and early treatment is of paramount importance. As soon as there is a reasonable probability that the case is one of diphtheria the antitoxin should be used, and treatment should not be delayed for the result of the bacteriological examination. The statistics show that in cases treated on the first day of the disease the case mortality is 3·3, on the second day it is 6·5, on the third day 10·6, on the fourth day 12·9, and on the fifth day and afterwards 14·8 per cent.

In bad cases, and in those coming under treatment at a late stage of the disease, the dose may be increased to 10,000, 20,000 or even 30,000 units with advantage, and to bring the patient under the influence of the antitoxin as rapidly as possible the first dose may be administered intravenously. The dose may have to be repeated once or twice in mild cases, in bad cases perhaps every six or twelve hours until several doses have been given, the guide being the general condition of the patient and the rapidity of the separation of the membrane. In addition to antitoxin, the recumbent posture and general and local treatment should be pursued as usual.

In cases of mixed infection, in which the diphtheria bacilli are associated with streptococci or staphylococci, diphtheria antitoxin may prove of less value, as it has no influence on the streptococcic

[1] *Journ. of Hygiene*, vol. vii, 1907, p. 65. See also Homer, *ibid.* vol. xv, 1916, p. 388.

or staphylococcic infection, and injections of anti-streptococcic serum, or, better, of sensitised streptococcic vaccine, may be given in addition.

Diphtheritic paralysis seems to be rather more frequent after the use of antitoxin than in the cases not treated with it, probably because a greater number of cases survive.

The antitoxin has also been employed as a prophylactic in schools or other places where susceptible individuals are congregated together, and where cases of diphtheria have occurred, with excellent results.

The procedure in such circumstances should consist of a bacteriological examination of the throats of *all* the inmates in the institution, isolation of those in whom the *B. diphtheriæ* is found, and the injection of every one, or at least of all the young contacts, with a prophylactic dose, repeated if considered desirable, ten days later. For this purpose a dose of about 500 units should be given. The immunity so produced does not last for more than three weeks.

The objection to the use of antitoxin for prophylaxis is that should the patient subsequently develop diphtheria, treatment with antitoxin may induce serious symptoms due to supersensitisation or anaphylaxis. To obviate this, an antitoxin prepared in the *ox* has been placed on the market for prophylactic use. The writer believes that all the advantages of antitoxin without its disadvantages may be obtained by the use of a vaccine consisting of diphtheria endotoxin, and that it is of service in the treatment of carrier cases.[1] Behring[2] suggested the use of a toxin-antitoxin mixture for prophylactic use and the treatment of carrier cases. This, although non-toxic for the guinea-pig, engenders the formation of a large amount of antitoxin in the recipient which persists for a long time.

Some clinicians assert that antitoxin exerts its effect when administered by the mouth or the rectum. Hewlett was unable to detect any absorption of tetanus antitoxin from the stomach or rectum, nor Sternberg of diphtheria antitoxin from the rectum, of rabbits. Blumenau and Dzerzhgovsky could in no instance secure immunity in animals by oral administration of diphtheria antitoxin, nor could any antitoxin be detected in the blood of animals so treated (*Roussky Vratch*, March 9, 1913).

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[1] *Lancet*, July 20, 1912, and June 28, 1913.
[2] *Deut. Med. Woch.*, May 8, 1913.

Pseudo-diphtheria and Diphtheria-like Bacilli

Diphtheria-like bacilli are not uncommon in wounds and in pathological exudates, as from the urethra, etc. Cleland notes the frequent presence of a diphtheroid organism in surgical wounds in children. It resembles a small diphtheria bacillus, but is non-virulent to the guinea-pig, and ferments glucose, lævulose, cane-sugar and galactose. It produces no change in milk and does not ferment lactose, maltose, mannitol and dulcitol.

In lymphadenoma or Hodgkin's disease, by cultivation on an egg-medium, Bunting [1] has grown a pleomorphic diphtheroid bacillus which is Gram-positive and non-acid-fast. By inoculation into monkeys apparently typical Hodgkin's disease can be produced with this organism.

In connection with diphtheria an important question must be discussed, viz. the occurrence and nature of the so-called pseudo-diphtheria bacilli. The term was originally used by Löffler, and by the rule of priority should be reserved for the organism described by him under this name. The pseudo-diphtheria bacillus of all authors is an organism occurring in the throat in various anginal conditions, scarlet fever, etc., and occasionally in the throats and noses of well persons, and is non-pathogenic to guinea-pigs. Park and Beebe met with it in twenty-seven out of 330 healthy throats examined by them. Roux and Yersin, Abbott and Fränkel describe it as morphologically resembling the Klebs-Löffler bacillus, while Löffler, von Hofmann, Koplick, Park and Beebe, Peters, and Hewlett and Miss Knight,[2] consider that an organism differing somewhat from the Klebs-Löffler bacillus should alone be termed the pseudo-diphtheria

[1] *Journ. Amer. Med. Assoc.* lxii, 1914, p. 516.
[2] *Trans. Brit. Inst. of Prev. Med.*, vol. i, 1897.

bacillus ; to avoid confusion it is best to designate it the Hofmann bacillus.

Morphology.—Typically, the Hofmann bacillus is a shortish rod tapering towards the ends, which are rounded, the average length being from 1·5 μ to 2 μ, and it occurs in pairs, resembling two suppositories placed base to base. It is non-motile, does not form spores, is arranged in a parallel grouping like the Klebs-Löffler bacillus (due to the same mode of division), and involution forms are, as a rule, not met with (Plate VII, *a*). It is Gram-positive, and stains deeply and regularly with Löffler's methylene blue, segmentation and polar staining usually being absent. With Neisser's stain no inky granules are perceptible, as is the case with the diphtheria bacillus.

Cultural reactions.—The Hofmann bacillus develops well at temperatures from 20° to 37° C., and is almost a strict aërobe ; there is no growth anaërobically in hydrogen. On serum, agar, and gelatin it forms cream-coloured colonies or growths, barely distinguishable from those of the Klebs-Löffler bacillus ; gelatin is not liquefied. On ordinary potato it hardly grows at all, what growth there is being quite invisible. On alkaline potato,[1] however, it forms distinct cream-coloured colonies, usually visible by the second day. In stab-cultures in gelatin and glucose-agar no gas is formed, and the growth is confined to the upper part of the stab. In broth it forms sometimes a granular deposit, sometimes a general turbidity. On neutral litmus glucose-agar and in litmus milk a blue colour is developed, indicating the production of alkalinity ; milk is not curdled. Cultivated in peptone water an indole-like reaction with sulphuric acid alone can be obtained after a variable time, three to four weeks, while the diphtheria bacillus gives it in about a week ; with a

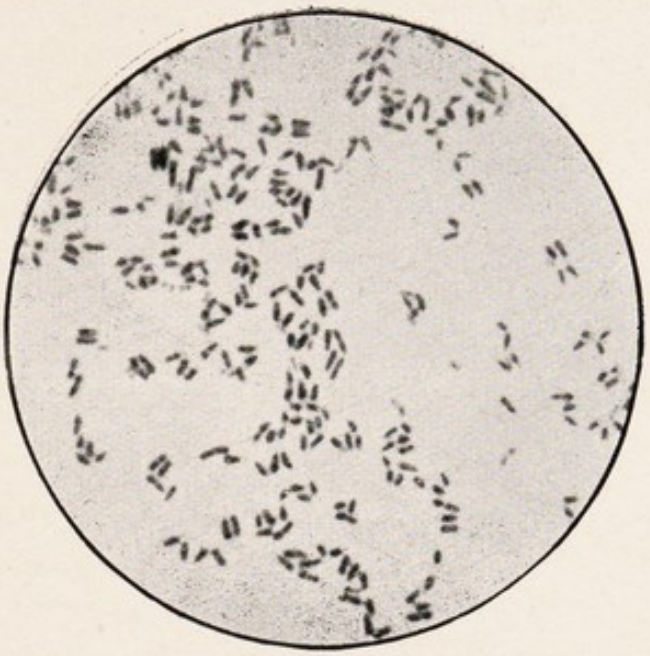
[1] Ordinary potato rendered alkaline with a 10 per cent. solution of sodium carbonate before sterilisation.

nitrite and sulphuric acid the indole-like reaction can be obtained with both the pseudo- and diphtheria bacilli in about a week. The substance giving this indole-like reaction is not indole, but skatole-carboxylic acid.[1] A broth culture reduces a weak solution of methylene blue. The Hofmann bacillus is non-pathogenic to guinea-pigs in doses of 5 c.c. or more of a forty-eight hours' broth culture, but is virulent to certain birds (see below, p. 324). Mandelbaum and Heinemann[2] state that if a glycerin-agar plate be smeared with human blood and inoculated, the diphtheria bacillus produces colonies surrounded by a yellow zone, while the colonies of the Hofmann and xerosis bacilli do not change the red colour of the blood. In addition, the Hofmann bacillus does not ferment any sugar, etc. (see Table, p. 326).

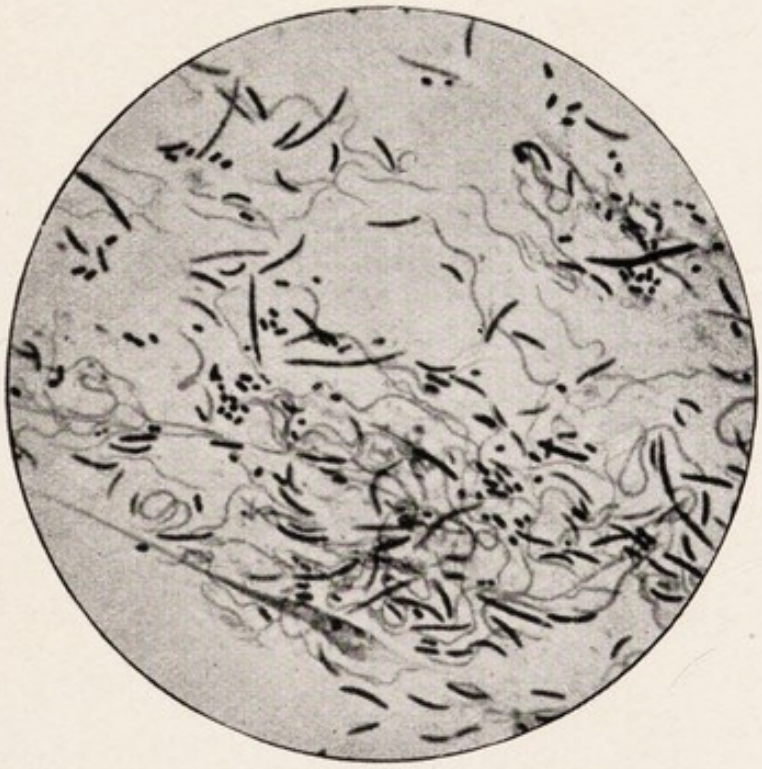
The histories of several cases investigated by Miss Knight and Hewlett seemed to show that the Hofmann bacillus is associated with mild anginal conditions, which are free from complications, end in recovery, and are not followed by sequelæ. In many of the cases the anginal condition was associated with distinct patches of membrane, and in two symptoms were present suggestive of the toxæmia which is met with in diphtheria.

In a long series of experiments Hewlett and Miss Knight believed that some evidence was obtained of the conversion of the Hofmann into Klebs-Löffler bacillus and *vice versâ*. Moreover, the Hofmann bacillus seemed in many instances to replace the Klebs-Löffler bacillus in the throat during convalescence, and it is possible in a large series of cultures to obtain connecting links between the Klebs-Löffler bacillus on the one hand and the Hofmann bacillus on the other. Cobbett,[3] however, suggests that these

[1] Hewlett, Trans. *Path. Soc. Lond.*, vol. li, 1900, p. 187; **vol. lii,** 1901, p. 113. [2] *Centr. f. Bakt.* (Orig.), liii, 1910, p. 536.
[3] *Journ. of Hygiene,* vol. i, 1901.



a. The pseudo-diphtheria or Hofmann bacillus. Film preparation of a serum culture. × 1500.



b. Vincent's angina. Smear from exudation showing fusiform bacilli (dark) and spirilla (light). × 2000.

facts are capable of another explanation, viz. that during the acute stage, diphtheria bacilli being readily found, the Hofmann bacillus is likely to be overlooked, whereas at a later stage a more careful search may be necessary to detect the diphtheria bacillus, and in the course of that search the Hofmann bacillus is therefore more frequently seen.

Miss Knight and Hewlett came to the conclusion that in some cases, at least, the Hofmann bacillus is a modified Klebs-Löffler bacillus, and the view taken of its relation to the Klebs-Löffler bacillus was, that it is a very attenuated Klebs-Löffler bacillus, *i.e.* one far removed from virulence. It would therefore seem wise to treat anginal cases in which the pseudo-diphtheria bacillus is found as possibly infective, though it would probably be inexpedient to admit to a general diphtheria ward (unless a prophylactic dose of antitoxin or of an endotoxic vaccine be given), nor would antitoxin be needed in the majority.

Most authorities have been unable to convert the pseudo-bacillus into a virulent Klebs-Löffler bacillus, or *vice versâ*, and many are of opinion that it has probably nothing to do with diphtheria (Park and Beebe, Peters, Washbourne, Cobbett, Clark). A few fatal cases have been recorded (*e.g.* by Stanley Kent) in which a careful search has failed to reveal any but Hofmann bacilli. Boycott [1] found that the seasonal prevalence of the Klebs-Löffler and Hofmann bacilli does not correspond, the former prevailing during September, October, and November ; the latter is more frequent from May to August.

Priestly records an outbreak of what he terms " pseudo-diphtheria," in which the Hofmann bacillus seemed to be the causative organism, and expresses the opinion that this bacillus is not related to the Klebs-Löffler bacillus.[2]

[1] *Journ. of Hygiene*, 1905, vol. v, p. 223.
[2] *Public Health*, July, 1903.

Salter [1] claimed to have found that the Hofmann bacillus is virulent to many small birds (goldfinch, chaffinch, canary, etc.), and that by successive passages it becomes converted morphologically into a Klebs-Löffler form with feeble virulence for the guinea-pig. He also found the filtered broth culture of the Hofmann bacillus, though harmless to guinea-pigs, to be toxic to small birds, and that it contains a non-toxic substance (toxoid) which has the power of combining with, and neutralising, diphtheria antitoxin. Salter concluded, therefore, that diphtheritic organisms are to be met with of every grade of virulence, the weakest, known as Hofmann's or the pseudo-diphtheria bacillus, representing the most attenuated form of the Klebs-Löffler bacillus. The writer,[2] Cobbett,[3] Petrie,[4] Williams,[5] and Clark[6] have, however, quite failed to confirm Salter's results. Thiele and Embleton also claim to have effected the transformation of a typical Hofmann bacillus into a virulent Klebs-Löffler bacillus by massive intra-peritoneal inoculation of guinea-pigs with Hofmann culture suspended in 30 per cent. gelatin and after death of the guinea-pig, injection of the peritoneal exudate with a smaller amount of living bacilli into a second guinea-pig, and repeating this method of inoculation. Finally the bacillus became Klebs-Löffler in morphology and 1 c.c. of its toxin killed a guinea-pig in forty-eight hours, and this toxin was neutralized by diphtheria antitoxin.

To sum up : the Klebs-Löffler-like avirulent bacilli met with in the throat, the pseudo-diphtheria bacilli of Roux and Yersin, are probably modified and avirulent diphtheria bacilli. As regards the Hofmann bacillus, the general

[1] *Trans. Jenner Inst. Prev. Med.*, vol. ii, p. 113. (Bibliog.)
[2] *Brit. Med. Journ.*, Sup., July 9, 1904.
[3] *Journ. of State Med.*, vol. xi, p. 609.
[4] *Journ. of Hygiene*, vol. v. p. 134.
[5] *Journ. Med. Research*, 1902, p. 83.
[6] *Journ. Infect. Diseases*, vol. vii, 1910, p. 335.

trend of opinion at present is to consider it as quite distinct from the Klebs-Löffler bacillus. Another view is to regard it as in reality including several species, of which one may be a modified Klebs-Löffler bacillus, the others having no relation with this organism. The Klebs-Löffler-like avirulent bacilli might, therefore, be regarded as true diphtheria bacilli *slightly* removed from virulence, the Hofmann bacillus, if derived from the Klebs-Löffler, as a diphtheria bacillus *far* removed from virulence.

In determining the fermentation reactions of the diphtheria-like bacilli, the organisms should first be grown in broth until they become acclimatised to this medium, or should be grown in a medium which suits them, *e.g.* broth with the addition of serum or of ascitic fluid. Hiss's serum-water medium is satisfactory—serum 1 part, water 3 parts, with 1 per cent. of carbohydrate or other substance, tinged with litmus and sterilised in the steamer on three consecutive days. Graham-Smith [1] gives the following Table of fermentation tests (see page 326).

Hine [2] remarks that he has yet to find an organism of the diphtheria group, giving acid with glucose and dextrin but not with saccharose, which proves to be other than the *B. diphtheriæ*.

Clinical Diagnosis

(*A*) *In man and animals*:—I. In some cases the diphtheria bacillus can be identified in the membrane or discharge, and the diagnosis established thereby.

Films are made with the exudation, or with a fragment of the membrane teased up as finely as possible on a slide, a droplet of water being added if necessary. One of these films should be stained with Löffler's methylene blue, another by Gram's method. The bacilli will be found lying parallel to one another in larger or smaller groups, together with involution forms. Films stained with Neisser's or Pugh's stain (see below) may also be of assistance. Another method is to stain the films for five seconds in

[1] *Journ. of Hygiene*, vol. vi, 1906, p. 286.
[2] *Journ. Path. and Bacter.*, vol. xviii, 1913, p. 75.

Organism.	Hiss's medium (10 days' growth).								
	Glucose.	Lactose.	Sucrose.	Galactose.	Maltose.	Lævulose.	Mannitol.	Dextrin.	Glycerin.
B. diphtheriæ, virulent and avirulent . .	C A	C A	0	C A	C A	C A	0	C A	C A
Hofmann bacillus * .	0	0	0	0	0	0	0	0	0
Xerosis bacillus * . .	C A	0	0	0	0	C A	0	0	C A
B. coryzæ *.	C A	0	0	C A	0	C A	0	0	0
Diphtheria-like bacilli :									
From the ear * .	0	0	0	0	0	0	0	0	0
From the urethra *	— A	0	0	— A	— A	— A	0	0	0
From the throat *	C A	0	0	C A	— A	C A	0	0	0
From the fowl * .	— A	0	0	— A	— A	..	0	0	0
(* Avirulent to the guinea-pig)									

C = coagulation ; — = no coagulation ; A = acid ; 0 = no reaction. Slight variations were occasionally noted ; for example, four out of twenty diphtheria bacilli gave no acid with lactose, and the amount of acid production and of coagulation was somewhat variable.

dilute carbol-methylene blue (seven drops to 10 c.c. water), rinsing and drying, and counter-staining in dilute carbon-fuchsin (ten drops to 10 c.c. water) for one minute, rinsing and drying (Higley).

II. Frequently the membrane is so crowded with different forms of organisms that it is extremely difficult to recognise the diphtheria bacilli with any degree of certainty. Recourse must then be had to cultivation.

For this purpose sloping blood-serum tubes, or tubes of serum-agar, must be employed ; simple agar is unsuitable.[1]

[1] Various selective media have been devised, *e.g.* potassium sulphocyanide neutral-red glucose blood-serum (Rankin, *Journ. of Hyg.*, xii, 1912, p 60)

A piece of membrane or a swabbing from the throat is rubbed over the surface of one or two serum tubes, care being taken not to break up the medium. The tubes are incubated at 37° C. for eighteen to twenty hours, and are then examined microscopically whether there is any visible growth or not. If there be no visible growth a scraping is taken by means of a sterilised platinum needle from the whole surface and a film is prepared. If there is a visible growth the film should be prepared from the most likely colonies, or, if the growth be confluent, from the upper half inch or so. A microscopical examination must always be made, for some colonies—certain staphylococci and torulæ, for example—simulate those of the diphtheria bacillus very closely. The films may be stained with Löffler's methylene blue for five to ten minutes, or by Pugh's method, then washed and dried. If the films are made on a slide, after staining, washing, and drying, a drop of cedar oil may be put on the stained patch, which is then examined directly without a cover-glass. If, however, there is very little growth, it is better to make a cover-glass specimen, as the position of the material is so much more easily located. The preparations are examined with a $\frac{1}{12}$-in. oil-immersion lens magnifying not less than 800–1000 diameters, and the Klebs-Löffler bacillus is identified from the description given in the text.

Prausnitz considers that if negative results are obtained after eighteen to twenty-four hours' incubation the tubes should be incubated for a further twenty to twenty-four hours and re-examined, and undoubtedly occasionally a positive result may be obtained by this longer incubation.

Löffler's methylene blue gives much more characteristic preparations than Gram's method.

Although eighteen to twenty hours is recommended for incubating the cultures, a microscopical examination will sometimes reveal the bacilli at a much earlier period. The writer has found them in as short a time as six hours, but if bacilli are then *not* found the tubes must be incubated for the longer period.

Neisser's method of staining is as follows :—

(*a*) One gramme of methylene blue (Grübler's) is dissolved in 20 c.c. of 96 per cent. alcohol, which is then mixed with 950 c.c. of distilled water and 50 c.c. of glacial acetic acid.

(*b*) Two grammes of Bismarck brown are dissolved in one litre of boiling distilled water and the solution is filtered.

The preparations are stained in (*a*) for half a minute, rinsed,

and stained in (*b*) for one minute, washed in water, dried, and mounted. The bacilli are stained brown, and contain two, rarely three, inky-blue dots. This is a valuable confirmatory stain for the diphtheria bacillus (Neisser recommended staining for three seconds in *a* and five seconds in *b*, but the longer time is better). Cocci and streptothrix forms frequently show similar inky dots, and this appearance must not be mistaken for diphtheria bacilli. Tanner treats with Gram's iodine solution for half a minute after the blue. The staining solutions usually keep well.

Pugh's stain is also a very good one. It is a mixture containing 1 grm. of toluidine blue dissolved in 20 c.c. of absolute alcohol and added to 1000 c.c. of distilled water and 20 c.c. of glacial acetic acid. The mixture is applied for two minutes. The protoplasm of the bacilli is stained a pale blue and the polar bodies are deeply stained and stand out in marked contrast; by artificial light they appear a reddish purple.

In the majority of cases, after a little experience, the Klebs-Löffler bacillus will be readily recognised if present. Occasionally, however, bacilli may be present which resemble the Klebs-Löffler very closely, and of which it is difficult to be certain. In such a case the following points should be noted in attempting to arrive at a decision:

1. The character of the growth on the medium.
2. The depth of staining with Löffler's blue, and the presence or absence of segmentation or polar staining: the Klebs-Löffler bacillus usually stains somewhat deeply, while the bacilli resembling it stain but feebly.
3. The presence or absence of involution forms, clubbing, etc.
4. The presence or absence of thread forms: the Klebs-Löffler bacillus does not form threads.[1]
5. The presence or absence of spores: the Klebs-Löffler bacillus does not form spores.
6. Motility in a hanging drop: the Klebs-Löffler bacillus is non-motile.
7. Gram's method of staining: the Klebs-Löffler bacillus stains well.
8. The grouping of the organism: the parallel grouping of the

[1] Klein and others have described thread and branched forms in cultures of the Klebs-Löffler bacillus in certain circumstances, but these are not likely to be observed under the conditions mentioned.

Klebs-Löffler bacillus is somewhat characteristic. The bacilli when lying side by side do not seem quite to touch, while the bacilli which resemble the Klebs-Löffler and show a parallel grouping frequently lie much closer together than the Klebs-Löffler bacillus ever does.

9. The reaction with Neisser's or Pugh's stain (*the culture must be a young serum one*) : the pseudo-bacillus and other bacilli do not give the diphtheritic reaction (polar staining).

10. The final test of virulence may be applied. For this purpose the organism must be isolated in pure culture by plate cultivations. Two guinea-pigs, of 250–300 grm. weight, are each inoculated with 2 c.c. of a forty-eight hours' broth culture, one receiving at the same time 1 c.c. of diphtheria antitoxin. If the guinea-pig inoculated with culture only dies, while the one receiving culture and antitoxin lives, this is complete proof that the organism is the diphtheria bacillus ; if both live no inference can be made except that the organism is non-virulent ; if both die it shows that the organism is virulent, but that it is not neutralised by antitoxin, and therefore is not the diphtheria bacillus. In cases in which bacilli persist, the test of virulence is frequently applied. If the organism proves to be non-virulent, presumably the patient is non-infective. Such a presumption, in the writer's opinion, however, is not necessarily true.

11. Agglutination tests are unsatisfactory and not of service.

It occasionally happens that a conclusion cannot be arrived at without an extended investigation.

If serum tubes are not available an egg may be used. It is boiled hard, the shell chipped away from one end with a knife sterilised by heating, and the inoculation made on the exposed white ; the egg is then placed, inoculated end down, in a wine-glass of such a size that it rests on the rim and does not touch the bottom. A few drops of water may with advantage be put at the bottom of the glass to keep the egg-white moist. The preparation is kept in a warm place for twenty-four to forty-eight hours and then examined. Antitoxin itself may be used as a culture medium, *provided it contains no antiseptic* (this is now rarely the case). A test-tube is sterilised by heating, or with boiling water or steam from a kettle, antitoxin to the depth of about an inch is poured in, and is coagulated by holding the tube very obliquely in boiling water or steam. After coagulation and cooling the medium is inoculated. If no incubator is available, the culture may be kept in a warm place, or in an inside pocket.

Many laboratories now undertake the examination of material. Culture outfits are supplied by some, consisting of a sterilised tube containing a sterilised swab. Failing this, a piece of membrane may be forwarded in a tube or bottle which has been sterilised by heating, or with boiling water or steam. If there be no membrane, a swab can be readily extemporised by wrapping a little wool or lint (*non-antiseptic*) round the end of a piece of wire, knitting needle, hair-pin, penholder, or splinter of wood. The wood may be sterilised by moistening with water and then holding in a flame. Membrane or secretion may also be forwarded on pledgets of wool, pieces of lint or calico, and even on paper, but these are not so suitable.

III. *The Schick Test.*—This test enables an estimate to be arrived at as to the presence or absence of specific antibodies in the blood. The principle of the test is that the intra-cutaneous injection of diphtheria toxin gives rise to a local reaction at the site of inoculation in unprotected subjects, while if specific antibodies exist, the toxin is neutralised and no reaction follows.

The diphtheria toxin used for the injection consists of a broth culture of the diphtheria bacillus grown for six days at 37° C. It is then treated with 0·5 per cent. carbolic acid, and the bacteria are allowed to sediment in an ice-box for two to three days. The supernatant fluid is filtered through a Berkefeld filter and standardised. A ripened toxin is usually used of an accurately determined M.L.D. (minimum lethal dose) for a 250 grm. guinea-pig.

A primary dilution is made by diluting with normal saline, so that each c.c. of it will represent 10 M.L.D. The bulk toxin keeps its strength well, the primary dilution also for two weeks in the ice-box, but the final dilution must be made up daily. The final dilution is made by diluting the primary dilution one hundredfold, so that 1 c.c. of it will contain $\frac{1}{10}$ M.L.D., while 0·2 c.c. will contain $\frac{1}{50}$ of a M.L.D. This amount of 0·2 c.c. ($\frac{1}{50}$ of the M.L.D.) is the dose to use.

The injection must be *intra* (not *sub*-) cutaneous and be made with a fine needle.

A *negative* reaction is shown by the skin remaining normal. With a *positive* reaction, a trace of redness appears slowly at the site of injection in from 12 to 24 hours, and usually a distinct reaction in the course of 24 to 28 hours. There is an indurated and reddened area of from 10 to 25 mms. in diameter. This reaches its height on the third or fourth day.

(B) *In milk.*—See section on "Milk."

Vincent's Angina

An infective malady characterised by sore throat, fetor, dysphagia, and ulceration and membrane simulating diphtheria. The diphtheria bacillus, however, is not present, and the affection is caused by an apparent association of a bacillus and a spirochaete. The bacillus (*B. fusiformis*) measures 6–8 μ to 10–12 μ in length, has *pointed* ends and is usually somewhat bent, not straight, often appears feebly motile, and does not stain by Gram. It can be cultivated anaërobically on the ordinary media to which human blood-serum, ascitic or hydrocele fluid has been added. The spirochaete is long and sinuous and very motile, but cannot be cultivated, and is stated to be developed from the fusiform bacillus. Smears may be stained with methylene blue or dilute carbol-fuchsin, and the appearance of the associated organisms is so characteristic that a diagnosis is easily effected (Plate VII, *b*).

Ulcero-membranous inflammatory affections caused by the Vincent's organisms are not confined to the pharynx and tonsils but may be widely distributed on the mucous membrane of the mouth and gums. They sometimes cause a diffuse ulcero-membranous gingivitus, sometimes a more restricted infection of the gums at the junction with the necks of the teeth—fuso-spirillary peridental or marginal gingivitis. These lesions are fairly common and may be confused with pyorrhœa alveolaris. They have been fully described by Taylor and McKinstry.[1]

Fusiform bacilli have been met with in various necrotic processes, *e.g.* noma (see Chapter XX).

The Xerosis Bacillus

The xerosis bacillus was isolated by Neisser from cases of xerosis conjunctivæ, and is met with in follicular conjunctivitis. Lawson and also Griffith isolated it from nearly 50 per cent. of all normal conjunctival sacs. In morphology and staining reactions it resembles the Klebs-Löffler bacillus very closely. It differs from the Klebs-Löffler bacillus in the following particulars: (1) Usually, but not always, in the *primary* cultivations from the eye on blood-serum, colonies do not appear under about thirty hours, while those of the Klebs-Löffler bacillus are visible in sixteen to twenty hours. This does not apply to the *secondary* cultivations, in which the colonies appear as soon as those of the Klebs-

[1] *Roy. Soc. Med.*, Odontological Sect., Nov. 27, 1916.

Löffler bacillus. (2) Upon agar it will seldom or never grow in primary culture, and in secondary cultures it forms only a thin, translucent, *dry* film. (3) Upon gelatin it will never grow in primary culture and seldom in secondary culture. (4) It does not give rise to acid production in milk or glucose broth (5) It is non-pathogenic to guinea-pigs. (6) The Neisser stain is negative. The fermentation reactions will be found in the Table on p. 326.

In all probability the organism is not causative of xerosis conjunctivæ.

To isolate the organism, blood-serum tubes are inoculated with a looped platinum needle from cases of follicular conjunctivitis or xerosis and incubated at 37° C. for forty to forty-eight hours. Half the tubes will usually show a growth. Preparations may be stained with Löffler's blue and by Gram's method.

Bacillus coryzæ (segmentosus)

An organism first described by Cautley, of frequent occurrence in the nasal secretion in cases of "influenza" cold. It bears a striking resemblance morphologically to the *B. diphtheriæ* when stained with methylene blue, and is Gram-positive, but does not show granules either with Löffler blue or with Neisser's stain. On agar it grows more slowly than *B. diphtheriæ*, and in glucose broth and litmus milk acid production is slow and feeble. It is non-pathogenic to guinea-pigs. The fermentation reactions will be found in the Table on p. 326.

Other Diphtheria-like Bacilli

As already mentioned, diphtheria-like bacilli are not infrequent in wounds, pathological discharges and secretions; they are common in the urethra. Some of them may be positive with Neisser's stain. They are always non-virulent. The fermentation reactions of some of these organisms will be found in the Table on p. 326.

Bacillus diphtheriæ columbarum

Pigeon diphtheria is an infectious disease of pigeons, characterised by the formation of diphtheritic-like membranes on the tongue, fauces, and corners of the mouth; occurs in extensive epizootics from time to time. Löffler isolated a bacillus to which he gave this name. It is short, with rounded ends, non-motile,

does not form spores, and does not stain by Gram's method. On gelatin it forms a whitish growth without liquefaction, on agar a creamy growth, and on potato a thin grey film. Milk is not curdled and is unchanged in reaction. It is pathogenic for the mouse and pigeon, but only slightly so for the fowl and guinea-pig. It is possible to prepare a vaccine, and an anti-serum for the disease.[1] Later research has, however, suggested that this disease may be due to a filter-passer.[2]

Diphtheritic roup of poultry is a different disease, and is stated to be due to a protozoan parasite.[3] Macfadyen and the writer [4] found Klebs-Löffler-like organisms to be present in the mouths and throats of healthy pigeons and fowls. These organisms resembled the true Klebs-Löffler bacillus in their cultural reactions, but were quite non-virulent to guinea-pigs (see Table, p. 326).

The so-called diphtheria of calves is produced by an anaërobic streptothrix.

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[1] See *Ann. de l'Inst. Pasteur*, xv, 1901, p. 952.

[2] Dean and Marshall, *Journ. of Path. and Bact.*, vol. xiii, 1908, p. 29.

[3] See also Gordon Sharp, *Lancet*, 1900, vol. ii, p. 18.

[4] *Trans. Path. Soc. Lond.*, vol. li, 1900, p. 13, and *Brit. Med. Journ.*, 1900, vol. i, p. 994.

CHAPTER IX

INFECTIVE GRANULOMATA — GLANDERS — "ACID-FAST" BACILLI—TUBERCULOSIS — LEPROSY — THE SMEGMA BACILLUS

CERTAIN infective diseases are characterized by the formation of cellular nodules in the tissues which have some similarity to granulation tissue. Since these diseases may usually be transmitted by inoculation of material from the nodules they are known as "infective granulomata." The principal infective granulomata are, glanders, tuberculosis, leprosy, actinomycosis and syphilis, and the causative organisms are largely confined to the nodules. Those in which the causative organisms are bacillar in appearance, viz., glanders, tuberculosis and leprosy, will be considered in this chapter.

Glanders [1]

Glanders is a disease which has been known from the earliest times, being recognized by the Greek and Roman writers, by whom it was termed μάλις and *malleus* respectively. It is pre-eminently a disease of the horse, mule, and ass, but is also communicable to man and to certain other animals. It is caused by a small bacillus discovered by Löffler and Schütz in 1882.

In the horse the lungs are always affected, and fre-

[1] See McFadyean, *Journ. of State Med.*, vol. xiii, 1905, pp. 1, 65, and 125.

quently the nasal mucous membrane (Fig. 39). Nodules form which afterwards break down and ulcerate, and a muco-purulent discharge appears; in the older writings the name "glanders" covered only these advanced cases of the disease. In "farcy" the lymphatic vessels and glands are affected, the enlarged glands being known as "farcy buds" (Fig. 40).

In man the disease is rare, an average of four deaths per annum being caused by it in this country. It occurs in

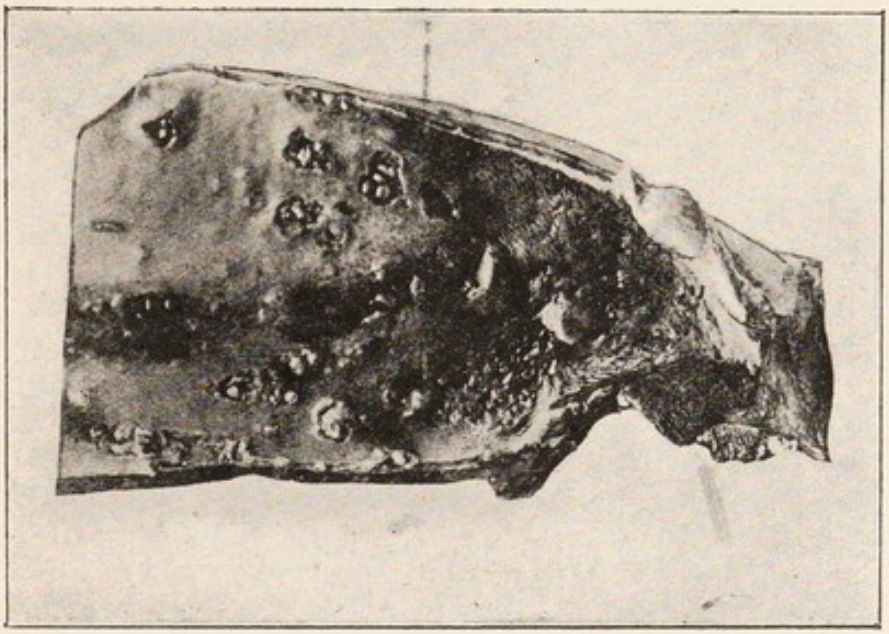


FIG. 39.—Nasal septum of glandered horse, showing ulceration of Schneiderian membrane (McFadyean).

two forms—the acute and the chronic. The former is a very serious affection, accompanied by high fever, prostration, and delirium, and is almost invariably fatal in from two to three weeks. The seat of infection is usually the hand or arm, the nasal mucous membrane being sometimes subsequently involved, and deposits may form, in the lymphatic glands, internal organs, and muscles. In the chronic form intramuscular abscesses are frequent from the breaking down of which indolent ulcers may

result; the disease runs a prolonged course of weeks or even months, and about half the cases end in recovery.

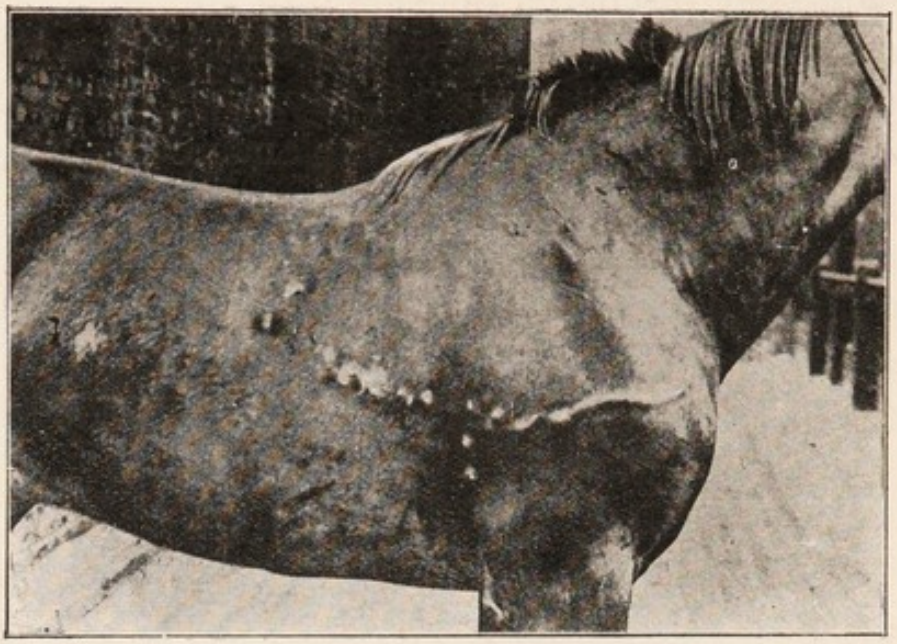


FIG. 40.—Horse affected with farcy (McFadyean).

In the early stage an eruption may develop on the forehead and face simulating very closely that of smallpox.

The Glanders Bacillus

The glanders bacillus (*B. mallei*) is an obligatory parasite with the equine species for its normal host. It hardly grows on artificial media below about 20° C., and probably cannot maintain a saprophytic existence outside the animal body.

Morphology.—The glanders bacillus occurs in the tissues as a cylindrical rod with rounded ends, varying between 2 μ and 5 μ in length, and generally straight, though sometimes slightly curved. The bacilli are usually irregularly scattered, and do not tend to form colonies. In stained preparations they often appear more or less beaded, or may exhibit bipolar staining, but some stain uniformly. The bacilli from young cultures not more

than twenty-four hours old are almost always short rods, a little thicker than those found in the lesions (Plate VIII, *a*). In old broth cultures the surface growth is largely composed of filaments, which do not show any regular segmentation, but may exhibit lateral branching, and may have club-shaped extremities. From these features some have inferred that the glanders organism belongs to the *Streptotricheæ*. The bacillus does not form spores, and is probably non-motile, though in a hanging-drop preparation a very active Brownian movement is present.

Staining reactions.—The bacillus is Gram-negative, is not acid-fast, and from young cultures stains readily with the ordinary anilin dyes. In smears of glanders or farcy material, a simple staining with any of the basic anilin dyes, with subsequent decolorisation with dilute acetic acid, suffices to demonstrate it if it is present in any number, a difficulty in recognising the organism being the presence of deeply staining nuclear detritus. In sections, methylene-blue staining with decolorisation in dilute acetic and mordanting with tannin gives the best results (p. 344). The bacillus shows dark staining dots when treated with osmic acid, suggesting fat-globules (Shattock).

Cultural characters.—The *Bacillus mallei* is an aërobic, and facultatively anaërobic organism. The growth on gelatin at 22° C. is scanty and pale brownish in colour without liquefaction. On glycerin agar it forms a thick cream- or slightly brown-coloured growth, and on blood-serum a somewhat amber-coloured growth, which afterwards becomes brownish. The growth on potato at 37° C. is most characteristic, and practically diagnostic. If the surface of the potato is inoculated with a loopful of farcy pus or material from the centre of a glanders nodule, the resulting growth is usually not distinctly visible until the third day, when raised, translucent,

viscid, amber-yellow coloured growth or colonies appear. With continued incubation the colonies coalesce, the growth becomes thicker and fawn-coloured, then reddish-brown, and finally generally chocolate-brown. The growth is also odourless, limited to the site of implantation, and does not stain the potato. Broth or glycerin broth becomes uniformly turbid, and after a week or so patches of a whitish surface scum form, and after three weeks the broth is nearly covered with this surface growth, which is slimy and easily broken up on shaking. Broth cultures give the indole reaction. Litmus glucose agar becomes pink. Milk is not coagulated.

Resistance to Germicides, etc.—The glanders bacillus is but little resistant, and cultures frequently die out in a month or so. Complete desiccation at 37° C. of nasal discharge, farcy pus, or bacilli from cultures, is frequently fatal in twenty-four to forty-eight hours. Young broth cultures are soon destroyed by bright sunlight, and an exposure of ten minutes to a temperature of 55° C. is fatal to the cultivated bacilli. A 3 per cent. solution of carbolic acid, a 1 per cent. solution of potassium permanganate, and a 1 in 5000 solution of corrosive sublimate are fatal in two to five minutes.

Pathogenicity, etc.—The glanders bacillus varies considerably in virulence, and under continued cultivation may become almost non-pathogenic.

Glanders is met with exclusively among horses, asses, and mules, and man is infected from these animals, nearly all cases of human glanders being among ostlers, grooms, and coachmen, and the usual mode of infection is by farcy pus or nasal discharge coming into contact with a cutaneous wound or abrasion. A remarkable immunity, however, is enjoyed by the slaughterers, who have to deal with the carcases of glandered animals, and who might be supposed to run the greatest risk. But it must be

remembered that Babes frequently found at the post-mortem on persons who had to do with horses, and who died from diseases other than glanders, encapsuled glanders nodules in the lungs and internal organs, suggesting that the disease may often be latent in man, who appears to be relatively insusceptible, and that infection may be possible by inhalation. In the horse glanders is readily transmissible experimentally both by ingestion and by inoculation, and ingestion is probably the common mode of infection naturally, infection by inhalation occasionally occurring. Even when glanders bacilli are administered experimentally by the mouth in the horse, the lesions may be most prominent in, or even be confined to, the lungs. In the horse, the disease has periods of epidemic prevalence, and is particularly frequent in London. In 1903 there were 2499 equine cases in Great Britain, nearly 90 per cent. of which occurred in the Metropolitan area. These, it is to be noted, were cases in which the disease was well developed and manifest, but there are also numerous others in which it is latent. Since 1908, the disease has decreased, and in 1914 there were only 269 equine cases. Guinea-pigs and field mice are highly susceptible to the disease, which may also be contracted by some of the Carnivora, such as the cat, lion, and tiger, by inoculation or by feeding on diseased carcases. The rabbit, sheep, and dog are but slightly susceptible, while cattle, swine, and house mice are stated to be immune. Shattock [1] found that the white mouse is somewhat susceptible, and suggests that probably the house mouse is similarly so.

In the horse the most constant seat of glanders lesions is the lung, and McFadyean states that no case of glanders with lesions elsewhere than in the lungs, and with these organs unaffected, has ever been recorded. In nearly

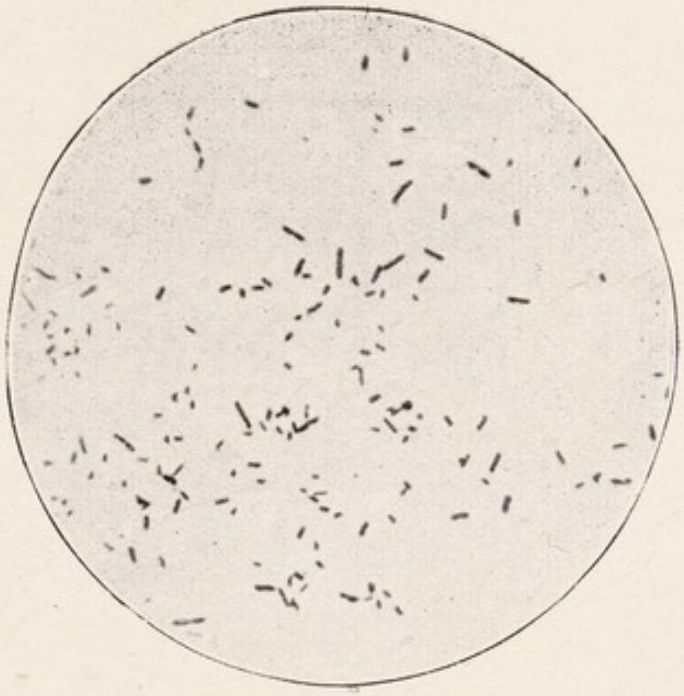
[1] *Trans. Path. Soc. Lond.*, vol. lix, 1898, p. 333.

every case of farcy, also, nodules are present in the lungs. The lung lesions have the form of rounded, firm, or shotty, nodules. The number present is variable, rarely less than a dozen ; exceptionally there are hundreds, fairly evenly distributed throughout the lung tissue. The nodule commences as a collection of polymorphonuclear leucocytes, around which a zone of congestion is present. Later, the alveolar walls undergo necrosis, and the leucocytes necrose and disintegrate, but their chromatin persists as rounded fragments which retain their affinity for nuclear stains (chromatotaxis). The nodule may become surrounded with a layer of thin fibrous tissue, between which and the necrotic central area a zone of endothelioid cells with giant-cells may be present (Plate VIII, *b*).

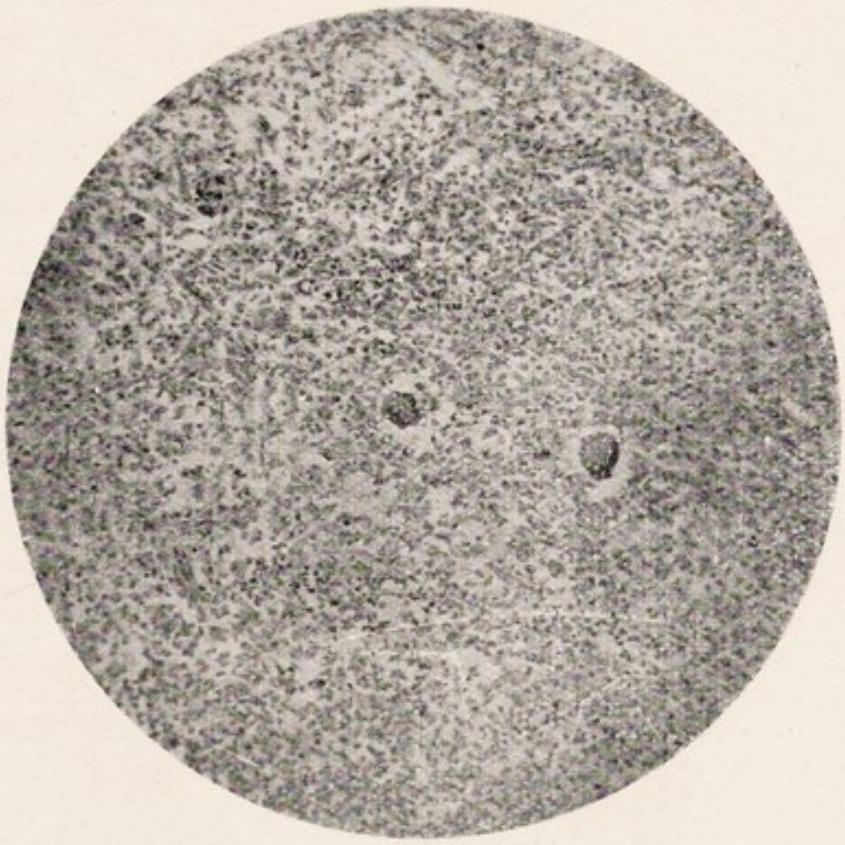
The lesions of farcy are at the onset histologically identical with the glanders nodule, but by the progressive liquefaction of the tissues actual abscesses form.

The lesions set up in an inoculated guinea-pig are very characteristic, and can be used for diagnostic purposes. With a very virulent culture, such as can be obtained by several passages through a susceptible animal, a guinea-pig may die in four or five days, and the post-mortem lesions are slight, consisting of some caseation at the seat of inoculation and slightly enlarged spleen, which contains a few small yellowish nodules resembling miliary tubercles. The material from human cases as a rule seems more virulent than that from the horse, and death of the guinea-pig often ensues a few days after inoculation.

The culture or material from a glandered horse does not usually produce death of a guinea-pig until a lapse of two or three weeks. A male guinea-pig being chosen, the changes observed are caseation followed by ulceration at the seat of inoculation, when this is done subcutaneously, and great enlargement of the testicles ; on cutting into these they are found to be partially or almost entirely



a. The glanders bacillus. Film preparation of a pure culture. × 1000.



b. Section of a glanders nodule, showing giant-cells (after McFadyean).

converted into a pasty caseous material, while the skin covering them is so adherent that it can only be detached by cutting, and the spleen is very much enlarged and studded with small yellowish nodules. In a female guinea-pig the ovaries are attacked. These appearances are of importance in the diagnosis of the disease. The difficulty of finding the bacillus in the discharges by microscopical and staining methods is so great that these cannot be employed with any certainty. Löffler and Straus therefore recommend the inoculation of a male guinea-pig intraperitoneally with the discharge or other material. If the glanders bacillus is present the lesions thus described rapidly ensue, and the diagnosis is established in four or five days (Straus's test [1]). At the present time the inoculation method has been almost entirely superseded by the introduction of mallein, the former being reserved for clinical diagnosis in man.

McFadyean found that the blood of a glandered animal produces agglutination or clumping of the glanders bacillus similar to that obtained in the agglutination (Widal) test for typhoid, and has suggested this reaction as a means of diagnosis. As an aid to the clinical diagnosis of the disease in man it is doubtful if agglutination can be applied, for Foulerton found that sera of typhoid fever and diphtheria also produce agglutination of the glanders bacillus.

Toxins.—Mallein, a preparation analogous to tuberculin, is prepared by growing a virulent glanders bacillus for a month or six weeks in glycerin veal-broth in flat flasks such as are employed for tuberculin (Fig. 42, p. 350), so that there is free access of oxygen. The culture is then autoclaved for fifteen minutes at 115° C., filtered through a Berkefeld filter, concentrated to one fourth of its volume,

[1] See also Nicolle, *Ann. de l'Inst. Pasteur*, xx, 1906.

and mixed with an equal volume of a ½ per cent. solution of carbolic acid. This yields an active mallein, 1 c.c. of which is a dose, and gives a good reaction. Like tuberculin, it possesses feeble curative properties, though a few cases of cure by prolonged use have been reported by Babes and others, but is used for diagnostic purposes; the veterinary authorities are unanimously agreed that it is one of the most certain means we possess for diagnosing glanders in the horse. Injected into an unglandered horse little or no effect is produced, but in a glandered animal, about twelve hours after injection, the temperature rises 1·5° to 3° C. above the normal, a large and painful swelling forms at the seat of inoculation (it may be as large or even larger than half a cocoanut), while any affected lymphatic vessels or farcy buds become swollen. Reaction may, however, be produced in the absence of glanders if the horse is being treated with bacterial products. toxins, etc.[1]

Epizootic lymphangitis has a superficial resemblance to farcy in the horse, and must not be mistaken for the latter (see "Sporotrichosis," Chapter XVI).

The greatest care should be exercised when working with glanders material or cultures, several fatal laboratory accidents having unfortunately happened.

Whitmore [2] describes a glanders-like disease occurring in man in Rangoon; especially among those addicted to the hypodermic injection of morphine. A non-Gram-staining bacillus is present, morphologically like the glanders bacillus, but killing guinea-pigs with septicæmic symptoms and not affecting the testes, growing well and luxuriantly on culture media, liquefying gelatin slowly, growing well on potato with at first a cream-coloured, and subsequently a yellowish growth, curdling milk and not fermenting any sugar.

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[1] See Sudmersen and Glenny, *Journ. of Hygiene*, vol. viii, 1908, p. 14.

[2] *Journ. of Hyg.*, xiii, 1913, p. 1.

Clinical Examination

(1) Prepare and stain film preparations of the pus or discharge in Löffler's blue, with subsequent partial decolorisation in 4 per cent. acetic. The ordinary pyogenic cocci will not be found unless a secondary infection has occurred, and the material may appear sterile, for the glanders bacilli may be very scanty.

(2) Several tubes of glycerin-agar and potato should be inoculated and incubated at 37° C. for seventy-two hours. On the agar, colonies of the glanders bacillus will develop in twenty-four to thirty-six hours, but the potato will not show the characteristic amber-yellow growth under forty-eight to seventy-two hours.

(3) It will usually be necessary (in man, at least) to confirm the diagnosis by an inoculation experiment. A fully developed male guinea-pig is chosen, and a little of the discharge, or an emulsion of the material (0·5 to 1 c.c.) is injected intraperitoneally, if the material be fairly sterile, but if not, subcutaneously. In three to five days the animal should show the characteristic swelling of the testicles if the material be glandered.

(4) An ophthalmo-reaction is stated to be reliable both in man and in animals.

(5) In animals the mallein test may be applied. The dose is injected subcutaneously in the neck over the vertebræ, and midway between the jaw and the shoulder.

(*a*) The temperature of the animal should be taken if possible morning and evening for two or three days previous to inoculation; in any case at the twentieth hour after inoculation, or, better, at frequent intervals from the twelfth to the twentieth hour.

(*b*) A complete reaction comprises (i) a rise of temperature of more than 2·5° F., (ii) an extensive hot and painful swelling at the seat of inoculation. Systemic disturbance, such as prostration, loss of appetite, shivering, etc., may occur.

(*c*) The temperature reaction is unreliable in all cases in which the temperature at the time of inoculation is 2·5° F. above the normal. In such cases, if there be any suspicious clinical signs to assist, reliance may be placed upon the local swelling.

The method of injection of mallein into the eyelid is now practised as it is more convenient. A concentrated mallein is used and 2 minims are injected under the skin of the under-eyelid near the middle. The eyes are inspected twenty-four, thirty-six and forty-eight hours after the injection. A positive reaction consists of a discharge of mucus from the inner canthus and of a

characteristic tender swelling of one or both eyelids, more or less closing the eye.

(6) In animals the agglutination reaction is stated by Moore and Taylor [1] to give accurate results. In man this test might give an inconclusive result (see *ante*).

(7) In the tissues the glanders bacillus is difficult to demonstrate. Sections may be stained for half an hour with carbol methylene-blue, treated with 4 per cent. acetic for a few seconds, washed, and rapidly dehydrated with alcohol, cleared and mounted. McFadyean recommends, after treating with acetic and washing, flooding with a saturated solution of tannic acid in water for fifteen minutes, washing, counter-staining in a 1 per cent. aqueous solution of acid fuchsin for fifteen to thirty seconds, washing, dehydrating, and clearing in cedar oil.

"Acid-fast" Bacilli

An important characteristic of the tubercle, leprosy, smegma, and certain other bacilli is the property they possess when stained with fuchsin of retaining the red colour after treatment with a strong solution of a mineral acid (25 per cent. sulphuric or 30 per cent. nitric). They are therefore termed "acid-fast." Most other organisms are rapidly decolorised even by 1 or 2 per cent. sulphuric acid, but it must be recognised that several apparently saprophytic bacilli are also "acid-fast." The retention of the fuchsin colour in spite of treatment with the acid seems to be due to the presence of substances of a fatty or waxy nature within the organisms with which the fuchsin either combines or is protected from the action of the acid.

Moreover, by cultivating many saprophytic bacilli in media containing butter, Bienstock and Gottstein converted them into "acid-fast" forms.

"Acid-fast" bacilli are also present in Johne's disease, occasionally in rats, in butter (Petri, Rabinowitsch, Rubner), on certain Graminaceæ (the "Timothy-grass bacillus" of Moeller), and in dung (the "Mist bacillus"). It has been suggested that these saprophytic acid-fast bacilli may be derived from the tubercle bacillus, but Panisset's work gives no confirmation of this.

The Streptotricheæ occasionally exhibit "acid-fast" properties. All the acid-fast bacilli seem to be Gram-positive.

[1] *Journ. of Infect. Diseases*, Sup. No. 3, May 1907, p. 85.

Tuberculosis

Tuberculosis is, unfortunately, only too common in the human subject, and most of the domestic animals and wild animals in a state of captivity may be attacked by it.

The conception of tuberculosis was originally a purely anatomical one, the name being given to a condition in which the organs were studded with little yellowish points or nodules, which were termed tubercles. Laennec was the first to indicate the characters of these nodules or tubercles, and traced with considerable accuracy their development from minute lesions, the miliary tubercles, up to the large cheesy masses which may be met with in the glands and lungs.

Microscopically, the structure of a young and typical tubercle is characteristic. At the centre one or more giant-cells are found—large protoplasmic masses, each containing ten to twenty nuclei arranged round the periphery (Plate X, *b*). They are of the nature of plasmodia, similar to the masses of fused cells which surround a foreign body in the lower animals (Adami), and are endothelial in origin. Around the giant-cells are well-defined epithelial-like cells with large and distinct nuclei, which are known as epithelioid, or more properly endothelioid, cells. A zone of smaller cells with scanty protoplasm and small nuclei surrounds the endothelioid cells; they are known as lymphoid cells from their likeness to the cells of lymphoid tissue. This is the structure of a typical tubercle, but one or other of the components may be wanting, and none can be said to be absolutely characteristic of the tubercle. The nodule possesses no blood-vessels, and as its size increases by growth at the periphery the central parts undergo degenerative changes, and may become either structureless or hyaline, or be converted into a soft yellowish material somewhat like cheese and

termed caseous. More or less extensive inflammatory reaction ensues in the tissues surrounding the tubercle, and the cellular elements often become spindle-shaped and ultimately fibrous, so that the tuberculous nodule becomes enclosed by a capsule of fibrous tissue which may contract and convert it into a fibrous nodule. After caseation has occurred calcification may ensue—that is, lime-salts are deposited and the nodule is converted into a calcareous mass.

So far back as 1865 Villemin showed that inoculation of rabbits with human caseous material was followed by a development of nodules similar in all respects to the miliary tubercles in man. Cohnheim, Burdon Sanderson, and Wilson Fox confirmed this observation, but they also showed that the development of tubercles apparently followed the introduction, not only of tuberculous material, but also of setons, pieces of putrid muscle, and gutta-percha. It was pointed out, however, that in all probability these results were due to accidental contamination or inoculation with tuberculous matter, and, by adopting suitable precautions in order to prevent such sources of error, it was conclusively shown that non-tuberculous matter is unable to set up tuberculosis. Tuberculosis is therefore inoculable, and is an infective disease, and as such must be due to a specific infective agent, to the discovery of which observers then directed their attention. In 1882 Koch announced that he had discovered a special bacillus, the tubercle bacillus, in tuberculous tissues, which could be isolated and cultivated, and which reproduced the disease on inoculation.

The Tubercle Bacillus

Morphology.—The tubercle bacillus (*B. tuberculosis*) is a slender rod with rounded ends, often slightly curved, and

PLATE IX.

a. The tubercle bacillus. Film preparation of a pure culture. × 1000.

b. Tubercle bacilli in sputum. × 1500.

averaging 2–3 μ in length, though the length varies in the tissues from 1·25 μ to 6·5 μ; in cultures it tends to be short, on serum being about 1 μ. In stained preparations one or more unstained intervals are often seen in the rods (Plate IX, *a*); these have been considered by some observers to be spores, but there are many objections to this view. Spores are usually single and not multiple, and are regular spherical or ovoid bodies, whereas the unstained spaces in the tubercle rods are irregular. Moreover, in the same specimen of sputum a varying amount of "beading," as it is termed, may be brought out by different staining methods (Plate IX, *b*); in a preparation stained by Gram's method it is usually more pronounced than in one stained with carbol-fuchsin. In class work also it will be found that one student's specimen will show beading much more markedly than another's. These considerations render it probable that the beading is partly due to segmentation of the protoplasm, and partly, perhaps, is an artifact due to the staining process, and is not a spore formation. The tubercle bacillus, however, probably does form spores, though this is a debated point. Some observers have described clear, regular, unstained spaces in bacilli from old cultivations, and consider these to be true spores.

The tubercle bacillus is a non-motile, strictly parasitic organism (it has been described as being both motile and flagellated). It usually occurs singly, occasionally linked in twos or threes so as to form short chains, and under certain conditions, especially in old cultures, filamentous forms develop, and Foulerton[1] and others include it among the *Streptotricheæ*. The bacillus is agglutinated by the blood-serum of a tuberculous animal (see p. 370). There are several varieties of the tubercle bacillus (see pp. 358 and 359).

[1] "Milroy Lectures," *Lancet*, 1910, vol. i, p. 551, *et seq.*

Staining reactions.—The tubercle bacillus stains indifferently with the ordinary watery solutions of dyes, prolonged treatment with, or warming, the solution being required. It stains well by Gram's method. It also stains well and deeply with carbol-fuchsin, particularly on warming, and when so stained is markedly resistant to the decolorising action of 25–30 per cent. mineral acid; that is to say, it is strongly "acid-fast," and this property is made use of for demonstrating its presence in tissues, etc., and for diagnostic purposes. This "acid-fastness" is due to the chemical constitution of the bacillus (see p. 354). In old and particularly healing lesions red-staining granules may take the place of definite bacilli: these are the "splitter" forms of Spengler.

Cultural characters.—The tubercle bacillus is aërobic and facultatively anaërobic, and thrives best at a temperature of 37° C. or thereabouts, but development even then is slow, four weeks at least being required for an appreciable growth. Primary cultivations from the lesions cannot be obtained on ordinary culture media but should be made on (*a*) Dorset's egg medium, (*b*) glycerinated potato in Roux's tubes (Fig. 10, p. 69), the bulb being filled with 5 per cent. glycerin in physiological salt solution, (*c*) glycerin brain agar, or (*d*) glycerinated serum (preferably dogs'). Cultures of the bacillus from the sputum direct may be obtained after treatment of the sputum with antiformin (p. 373). Dorset's egg medium is prepared thus: the contents of four eggs are well beaten up, 25 c.c. of water are added, and the mixture is strained through muslin. The fluid is then tubed, and the tubes are heated in the sloping position to 70° C. for four hours. At the time of inoculation, a drop or two of sterile water should be added. Brain agar is prepared by making a 3 per cent. nutrient agar of + 20 reaction, adding an equal volume of pounded ox-brain, and sufficient glycerin to

make 5 per cent. in the mixture, and sterilising. Egg broth is also a good culture medium.

After culture on these media for some generations, the tubercle bacillus will develop on 5 per cent. glycerin agar (reaction + 15 or 20), and in 5 per cent. glycerin broth (veal is best); it will also grow, though very slowly, on glycerin gelatin at 22° C. Gelatin and blood-serum are not liquefied. On glycerin agar the growth forms a dry, crinkled and wrinkled, cream-coloured or brownish-yellow film, which has been well described as resembling the patches of lichen met with on trees (Fig. 41). The growth, however, varies considerably, both in colour and in the amount of wrinkling, though retaining more or less the characteristics just mentioned. In broth it forms soft, cream-coloured, flaky masses, which increase slowly both in size and number, the broth remaining perfectly bright and clear. Sometimes a dry crinkled film forms on the surface of the broth, and may spread all over it, and tends to creep up the sides of the vessel. This film formation seems to be essential for the preparation of a satisfactory old tuberculin, but it is necessary in order to start it that some of the inoculated particles should float and form nuclei from which the film spreads. The virulent organism from the primary cultivations is difficult to grow on anything but glycerinated potato or serum, or brain agar.

FIG. 41.—Tubercle bacillus. Glycerin-agar culture three months old.

TUBERCULINS.—Extracts of, and suspensions of triturated tubercle bacilli, human or bovine, are employed

in treatment and for the diagnosis of tuberculosis. The preparations are known as tuberculins. Tuberculins may be prepared from (*a*) human, or (*b*) bovine, strains of bacilli. Tuberculin is frequently designated by a capital T, and that prepared from a bovine strain has a capital P prefixed (P=perlsucht). Thus PTR means new tuberculin prepared from a bovine strain.

Old tuberculin (*Syn.* TO or TA=tuberculin—original—alt).—This is prepared by growing the tubercle bacillus in glycerin veal broth in a shallow layer in flat flasks (Fig. 42), so that there is a free supply of oxygen. After some weeks an abundant growth with

Fig 42.—Flask for growing tuberculin.

copious film formation develops; the latter seems to be essential, but it does not appear to matter whether the bacilli be virulent or non-virulent, or whether they be of human or of mammalian origin. The cultures, bacilli and all, are heated at 115° C. in the autoclave for half an hour, then concentrated over a water-bath to about one tenth of their volume, and finally are filtered through porous porcelain; the resulting fluid is thick, owing to the concentration of the glycerin by the evaporation, is of a dark amber colour, and possesses a curious characteristic smell. The large proportion of glycerin preserves the fluid, which keeps indefinitely in a cool dark place.

This old tuberculin possesses remarkable properties. Relatively large amounts (0·1–0·5 c.c.) may be injected into a healthy animal or individual without effect, but in a tuberculous one a minute dose, 0·001 c.c. or less, gives rise to a marked reaction—elevation of temperature with constitutional disturbance more or less severe, and swelling and tumefaction of tuberculous lesions (glands, ulcers, etc.), and this reaction is made use of for diagnostic purposes (see p. 377). By cautiously increasing the amount a toleration is gradually induced, so that considerable doses cause little or no disturbance. Injections of tuberculin tend to produce marked changes in the tuberculous parts, leading to necrosis and exfoliation, with subsequent healthy reaction and repair. This is especially seen in cases of lupus; by continued injections a marvellous improvement results, so much so that a cure is apparently effected; but, unfortunately, when the treatment is discontinued the scar usually breaks down and the disease returns. Nevertheless, a few cases have remained permanently healed.

For treatment, the dose to commence with should not be more than 0·0001 c.c., dilutions being made with 0·5 per cent. carbolic solution, and the dose is repeated when all reaction has passed away and is gradually increased. Tuberculin R, or tuberculin BE (see below), is now more generally employed to commence with, and after a time may be followed by a course of old tuberculin.

Healthy guinea-pigs bear considerable injections of tuberculin without harm; but if they be tuberculous, if the disease is advanced (eight to ten weeks after inoculation), doses of 0·01 c.c. produce death; if less advanced (four to five weeks after inoculation) a larger dose, 0·2 to 0·3 c.c., is required; but 0·5 c.c. always proves fatal. The post-mortem appearances are congestion of the lymphatics and viscera, and dark red spots, from mere

points to the size of a hemp-seed, on the liver and spleen. These are due to enormous engorgement of the capillaries in the immediate neighbourhood of tuberculous deposits, actual extravasations of blood being rarely found. The hæmorrhagic-like spots on the liver are almost pathognomonic of death from tuberculin.

Absolute alcohol precipitates the active principle of tuberculin in the form of a whitish flocculent precipitate which chemically consists of proteoses. This precipitate, re-dissolved, is made use of in the *ophthalmic* reaction (p. 378). Tuberculin applied to the scarified skin also gives a *cutaneous* reaction in tuberculosis (p. 377).

New Tuberculin (*Syn.* TR=tuberculin residual).—This is prepared from young and virulent cultures of the tubercle bacillus. The growth is collected, dried *in vacuo*, and triturated by machinery. Of the triturated material, 1 grm. is treated with 100 c.c. of distilled water, and centrifuged. The supernatant liquid is rejected, and the residue is collected, dried, again triturated and centrifuged. The supernatant liquid is carefully pipetted off and kept, while the residue is again submitted to the same treatment, and the process is repeated until no solid residue remains. The fluids are then mixed, the solid content is estimated gravimetrically, some glycerin is added, and the liquid is diluted to the correct volume, so as to contain 2 mgrm. of solid matter per cubic centimetre (not 10 mgrm. as formerly stated), and for use is diluted with 20 per cent. sterile glycerin solution.

Tuberculin R, according to Koch, possesses distinct immunising properties, and causes neither reaction nor suppuration.

For treatment of tuberculosis in man the initial dose is equivalent to not more than $\frac{1}{100000}$–$\frac{1}{10000}$–$\frac{1}{5000}$ mgrm. of solid matter, according to the nature of the case.

The doses are given subcutaneously at intervals of ten to fourteen days, and the treatment may be controlled in the earlier stages by opsonic determinations. According to Latham, tuberculin may also be given by the mouth. Cases of cutaneous or localised tuberculosis, and those in which the opsonic index to tubercle is moderately reduced, react best.

Tuberculin, bacillary emulsion (BE), is an emulsion of the powdered bodies of tubercle bacilli in 50 per cent. aqueous glycerin. The mixture is allowed to sediment until all heavy particles have deposited, the milky supernatant fluid is pipetted off, and standardised so as to contain 5 mgrm. of solid matter per c.c. The dosage is similar to that of tuberculin R.

Behring prepared another tuberculin, tulase or TC, by treating tubercle bacilli with chloral, which he states has a marked curative action, and is better administered by the mouth than by subcutaneous inoculation. By giving tulase to cows, the milk is said to acquire immunising and curative properties which are transmitted to those consuming it. Rosenbach's tuberculin is prepared by growing the tubercle bacillus with the ringworm organism, Friedmann's is derived from a turtle tubercle bacillus. Other tuberculins are also on the market, and any tuberculin may be prepared with a human or with a bovine strain of bacillus.

Chemical products.—The tubercle bacillus produces no extra-cellular toxin. Crookshank and Herroun obtained from glycerin broth cultures of the tubercle bacillus a proteose and an alkaloidal body. The proteose was also obtained from "perlsucht." Both the alkaloid and the proteose (from both sources) produced a rise of temperature in tuberculous guinea-pigs, while in healthy animals the former caused a slight, and the latter a marked, fall in temperature.

De Schweinitz and Dorset [1] described chemical products isolated from the tubercle bacillus grown in a special glycerin-asparagin mixture. From the bacilli themselves an acid body was isolated, probably teraconic acid, an unsaturated acid of the fatty series. A certain amount of the same body was also obtained from the special culture medium, but only a trace from glycerin broth, in which the bacilli had been cultivated, in the latter case not because it was not formed, but because of the difficulty of isolation. This acid seemed to produce on injection depression of temperature and necrosis of the tissues locally, possessed some immunising power, and may be the substance producing caseation in the tuberculous nodules. The bacilli extracted with hot water yielded an albuminoid, which gave the tuberculin reaction. This they regard as the fever-producing substance.

Bulloch and Macleod [2] state that the acid-fast substance of the tubercle bacillus is an alcohol. Hot xylol will remove this substance from the tubercle bacillus, and ether or 5 per cent. caustic soda that from the smegma bacillus; the organisms after this treatment are no longer "acid-fast."

Maragliano states that toxic bodies are present in the blood and urine of tuberculous individuals. Cellulose also seems to be present in small amount in the bacilli (it has also been found in tuberculous nodules).

Tubercle bacilli, living or dead, are with great difficulty absorbed when in any quantity. The dead bacilli when injected under the skin invariably cause suppuration, and several months later it is still possible to detect in the pus numerous bacilli which stain well; introduced into the circulation of rabbits they give rise to nodules in the lungs

[1] *Med. Journ. N.Y.*, 1897, July 24, p. 195. Also *Fifteenth Annual Rep. Bureau of Animal Industry, U.S.A.*, 1890.

[2] *Journ. of Hygiene*, vol. iv, 1904, p. 1.

similar to the tuberculous nodules produced by living bacilli (Koch).

Action of heat and antiseptics on the tubercle bacillus.—The thermal death-point of the bacillus has been the subject of some controversy. Sternberg found that tuberculous sputum exposed for ten minutes to a temperature of 90°, 80°, and 66° C. failed to infect guinea-pigs in inoculation, while another specimen of the same sputum heated for ten minutes to a temperature of 50° C. produced tuberculosis in a guinea-pig, so that from these experiments the thermal death-point lies between 50° and 66° C.

Yersin in 1888, by culture methods, failed to obtain any growth from bacilli which had been heated to 70° C. for ten minutes, while those heated to 55° C. and 60° C. gave growths in glycerin broth in ten days and twenty-two days respectively. Macfadyen and the writer, in the course of some experiments on the sterilisation of milk, found that milk to which powdered dried sputum had been added was rendered innocuous by a momentary heating to 67°–68° C. These experiments indicate that a temperature of 65° C. and over is probably rapidly fatal to the tubercle bacillus, so that milk which has been pasteurised (*i.e.* heated to 65°–70° C. for twenty to thirty minutes) may be regarded as quite safe. Experiments by the Royal Commission on Tuberculosis with virulent tuberculous milk gave somewhat irregular results; in one instance heating to 65° C. for two and a half minutes rendered the milk innocuous, in another instance after five minutes at 70° C. it was slightly virulent, but twelve minutes at the same temperature rendered it inert (see also section on "Milk"). Foulerton found that emulsified tuberculous material from tuberculous guinea-pigs did not lose its power of infecting unless heated at 70° C. or over for ten minutes.

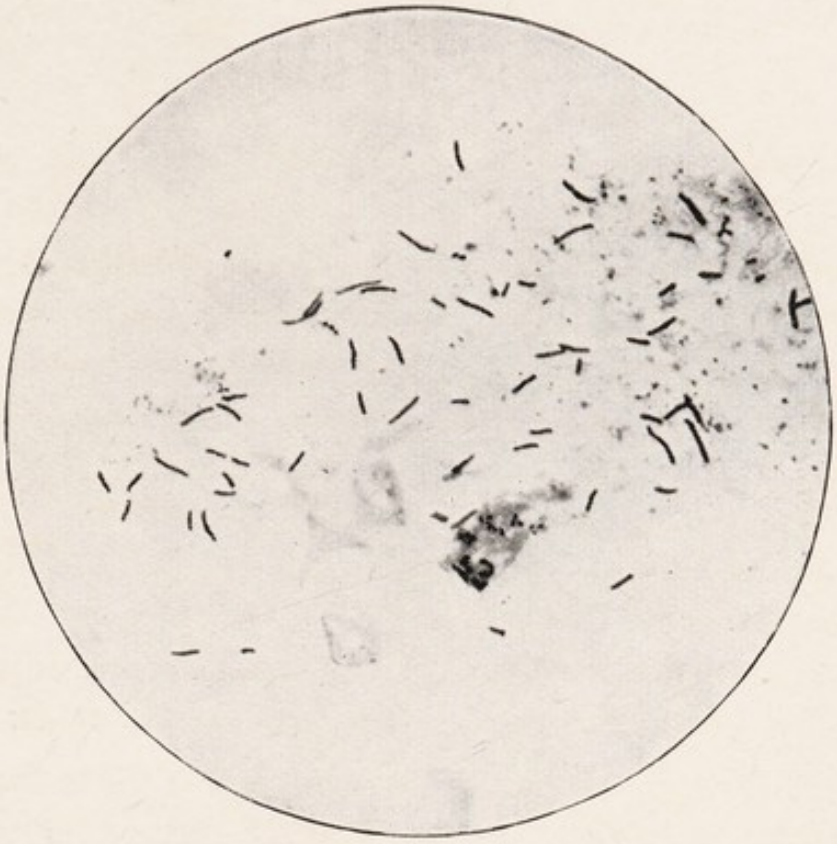
The tubercle bacillus offers considerable resistance to

the action of antiseptics and germicides. Yersin found that it was killed by 5 per cent. carbolic acid in thirty seconds, by 1 per cent. in one minute, by absolute alcohol in five minutes, and by mercuric chloride, 1–1000, in ten minutes. Crookshank found that tuberculous sputum mixed with an equal volume of 5 per cent. carbolic was rendered innocuous in a few minutes, and this without any special precautions as to breaking up the masses. For disinfecting sputum mercuric chloride is unsuitable. (See also Chap. XXI.)

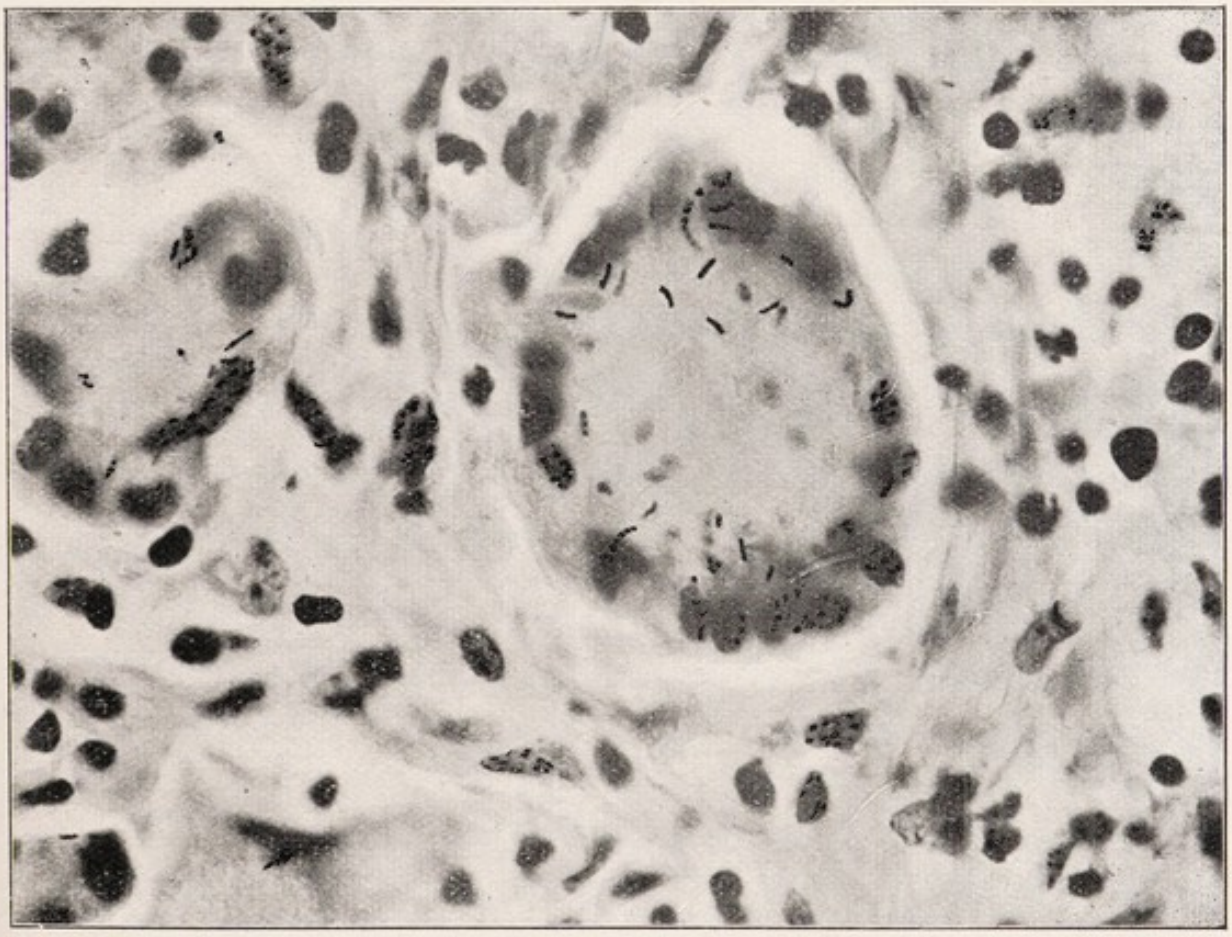
Pathogenesis, etc.—Man is, unfortunately, only too frequently attacked with tuberculosis, the manifestations of which tend to differ somewhat at different age periods. Thus, in the very young, general miliary tuberculosis, tuberculous meningitis, and tuberculous disease of the peritoneum, intestine, and mesenteric glands (tabes mesenterica) are the commonest ; in older children, up to the age of puberty, the lymphatic glands, especially in the neck, joints and bones, and the skin (lupus) are mostly attacked ; young adults suffer from disease of the lung (consumption, phthisis), and older people from chronic disease of the lung and tuberculous disease of the urinary organs and testes, and of the suprarenal capsules (Addison's disease). Scrofula and struma were terms formerly much employed ; both denote a swollen neck, and were applied to cases suffering from chronic tuberculous inflammation with enlargement of lymphatic, especially of the cervical, glands, with which other conditions, such as inflammations of the ear, throat and eye, and implication of bones and joints, are frequently associated.

The distribution of the bacillus in the tissues varies considerably. In young and active tubercles the bacilli are more plentiful and more easily demonstrated than in older and more chronic ones. They tend to be more numerous in some animals than in others—in the ox and

PLATE X.



a. Tubercle bacilli in sputum. × 1000.



b. Giant-cell in a tubercle containing tubercle bacilli. × 1000.

horse than in man, for example. In man the bacillus is frequently difficult to demonstrate (by staining) in enlargedand caseating glands, in pus, in synovial membranes and particularly in lupus. In some animals, especially the ox and horse, bacilli can usually be readily demonstrated, and may be present in large numbers, and frequently have the typical distribution, viz. within and at the periphery of the giant-cells, though they are by no means confined to this locality (Plate X, *b*). The bacilli are comparatively scanty in the lesions in guinea pigs.

It was asserted, particularly by Rosenberger and Forsyth, that tubercle bacilli can be detected in the blood in the majority of cases of pulmonary tuberculosis. Hewat and Sutherland,[1] however, made twenty-two blood examinations on twenty patients in all stages of the disease and in only one detected two acid-fast bacilli. Schroeder and Cotton tested the blood of forty-two cattle in all stages of tuberculosis by inoculation into guinea-pigs with negative results.

Tuberculosis in animals.—The majority of the domestic animals are subject to tuberculosis. It is most common in the ox, pig, and horse, much less so in the sheep and goat, cat and dog. Wild animals, both mammals and birds, in a state of captivity are also specially prone to be attacked, and a large number of the deaths in Zoological Gardens, particularly among the apes, are due to this disease.

In the ox the tu' ·rculous lesions are most frequently met with in the lymphatic glands and serous membranes, particularly the pleura, and in the lungs and liver, while the fat and muscular tissues, which constitute the major part of " meat," are very rarely affected. On the pleura the growths take the form of nodular masses, which from

[1] *Brit. Med. Journ.*, 1909, vol. ii, p. 1119 (References).

their arrangement are popularly termed "grapes" or "angle berries." the "perlsucht" of the Germans.

In carp, tubercle-like nodules are occasionally met with in which a bacillus resembling the tubercle bacillus in morphology and staining reactions is present. It grows, however, much more freely than the true tubercle bacillus, and though inoculable into fish and frogs, is non-inoculable into warm-blooded animals. But it yields a tuberculin which reacts with mammalian tuberculosis, and by feeding carp on the mammalian tubercle bacillus this can apparently be transformed into the piscian variety.[1]

Bird or avian tuberculosis undoubtedly differs in many respects from mammalian tuberculosis. The tuberculous new formations may be very large, but do not show nearly such a disposition to caseation or suppuration as the human lesions. Epithelioid cells form the major part of the growth, and giant-cells are very infrequent. One remarkable feature is the enormous numbers of bacilli which may be present in the tissues ; in places they may be so numerous and closely packed as to form distinct masses or nodules. The bacilli of avian have the same staining reaction as those of mammalian tuberculosis, but on cultivation and inoculation various differences between the two races become evident. Rats, guinea-pigs, and rabbits are practically insusceptible to inoculation with the avian bacillus.

The mammalian bacilli flourish best at about 37° C., and growth ceases at 41° C., whereas the avian bacilli thrive luxuriantly at 43° C., and the growth of the latter on glycerin agar is much moister and more wrinkled, and often more pigmented, than that of the former. Fowls and dogs are with difficulty infected with human bacilli, but dogs are susceptible to infection with avian bacilli. By cultivation on boric-acid agar and on eggs, etc., the

[1] See Himmelberger, *Centr. f. Bakt.*, Abt. I (Orig.), vol. 73, p. 1.

mammalian bacilli are stated to assume the characters of the avian.

Avian tuberculosis is of considerable practical importance as attacking poultry. A few cases have been recorded in which the bacilli cultivated from human cases seemed to be of the avian type, and were therefore probably derived from an avian source of infection. Two types of tuberculosis also occur in the horse—one in which the lesions are chiefly abdominal, in the other the lungs and bronchial glands are most affected. Nocard states that the bacillus obtained from the pulmonary variety is generally of the ordinary mammalian type, while that of the abdominal one belongs to the avian.

Relation of human and bovine tuberculosis.—It was noticed long ago that there are certain differences between the bacilli of human and of bovine tuberculosis, the latter tending to be shorter and thicker and less readily cultivated than the former ; also, whereas human tuberculous material injected into a rabbit generally produces small discrete lesions which tend to retrogress, bovine material induces a progressive disease with large caseating masses.[1] These distinctions were regarded as being due to variations in the bacilli as a result of growing upon a different soil and not to any fundamental difference between the two strains of bacilli. In 1901, however, Koch stated[2] that young cattle and swine cannot be infected with human tuberculous material, and he therefore concluded that human and mammalian tubercle bacilli are essentially different. As a result of his experiments he made the statement that " though the important question whether man is suceptible to bovine tuberculosis at all is not yet absolutely decided, if such a susceptibility really exists,

[1] The bacilli derived from tuberculosis of the sheep, pig, and horse (pulmonary lesions) are also of the bovine type.

[2] See *Brit. Med. Journ.*, 1901, vol. ii, p. 189.

the infection of human beings is but a very rare occurrence."

This view met with considerable opposition, and a second Royal Commission was appointed to investigate the question, and the following summarises the results obtained up to the present, from which it will be gathered that while there is no justification for assuming that man is infected from human sources alone, infection from human sources is probably vastly more frequent than from any other. Thirty different viruses isolated from cases of tuberculosis occurring spontaneously in bovines were studied, and the results of introducing them into a number of different animals by feeding and inoculation are recorded. In calves, inoculation usually results in generalised progressive tuberculosis, but the effect is somewhat dependent on the dose, *i.e.* the number of bacilli, administered. Thus whereas 50 mgrm. of culture always induced a fatal generalised progressive tuberculosis, in two instances much smaller doses—0·01–0·02 mgrm.—produced only limited retrogressive tuberculosis. Feeding, on the other hand, usually produced lesions limited to the neighbourhood of the digestive tract, which generally retrogress and become calcareous. The bovine bacillus, when introduced into *rhesus* monkeys or chimpanzees, either by inoculation (even in so small a dose as 0·001 mgrm.) or by feeding, induces rapid generalised tuberculosis, and, considering the close relation that exists between the anthropoid apes and man, these results are of the highest importance. In pigs, generalised progressive tuberculosis is readily set up both by feeding with, and by the inoculation of, bovine bacilli. Goats, dogs, and cats are relatively less susceptible, but more or less tuberculous infection can similarly be produced in them. On this part of the investigation the Commissioners remark that the bacillus of bovine tuberculosis is not so consti-

tuted as to act on bovine tissues only, and the fact that it can readily infect the anthropoid apes, and, indeed, seems to produce this result more readily than in the bovine body itself, has an importance so obvious that it need not be dwelt on. The viruses isolated from sixty cases of the disease in man were also studied, and the results obtained show that they may be divided into two groups, subsequently referred to as Group I and Group II. The bacilli of Group I comprised fourteen viruses, one obtained from sputum, three from tuberculous cervical glands, and ten from mesenteric glands of primary abdominal tuberculosis in children. The results produced by introducing these viruses into animals are identical with those produced by the bovine bacillus. The bacilli of Group II, comprising forty viruses obtained from various forms of human tuberculosis—cervical glands, mesenteric glands (8), lungs and bronchial glands (10), joint and bone disease (9), testis, kidney, etc.—grow more luxuriantly in culture than those of Group I, and inoculated into calves and rabbits do not produce the generalised and fatal disease caused by the bovine bacillus, but in *rhesus* monkeys and in the chimpanzee set up a general tuberculosis. Certain human viruses, differing in certain respects from those of Groups I and II, were also met with and are classed as Group III, but an opinion on their significance is reserved for a future report.

The Commissioners conclude that the tubercle bacillus in its nutritive and reproductive powers resembles other simple organisms, and that the essential difference between one strain and anothe· depends on variations in these factors, and they classify those bacilli that grow with difficulty on artificial media as *dysgonic*, and those that grow readily on media as *eugonic*. There are no definite morphological differences between the human and the bovine types of bacilli. In the rabbit, 10 mgrm. of serum

culture introduced by subcutaneous inoculation between the shoulder, induces within 100 days after inoculation, for the human type of bacillus little or no infection, for the bovine type of bacillus progressive generalised tuberculosis and usually death.

As regards the histological appearances of the tuberculous process in different animals, Eastwood states that there is an underlying unity of the morbid processes produced experimentally by infection with every variety of bovine and human tubercle bacillus.

In their final Report, the Commissioners conclude that an appreciable amount of human tuberculosis is caused by bacilli of the bovine type, and that tuberculosis may be communicated to man from infected cow's milk, and from tuberculous meat, either beef or pork.

So far, therefore, from any relaxation of the existing supervision of milk-production and meat-production being possible, the Commissioners press upon the Government the enforcement of food regulations, "planned to afford better security against the infection of human beings through the medium of articles of diet derived from tuberculous animals." More particularly they urge such action "in order to avert or minimise the present danger arising from the consumption of infected milk."

Of young children who died of wasting disease of the intestine, the bovine bacillus was present in nearly half the cases. Further, a large proportion of cases of tuberculous cervical glands in both children and adults was due to the same bacillus. The wording of the report is: "Whatever, therefore, may be the animal source of tuberculosis in adolescents and in adult man, there can be no doubt that a considerable proportion of the tuberculosis affecting children is of bovine origin, more particularly that which affects primarily the abdominal organs and the cervical glands. And further, there can be no doubt

that primary abdominal tuberculosis, as well as tuberculosis of the cervical glands, is commonly due to ingestion of tuberculous infective material. The evidence which we have accumulated goes to demonstrate that a considerable amount of the tuberculosis of childhood is to be ascribed to infection with bacilli of the bovine type transmitted to children in meals consisting largely of the milk of the cow.

"We are convinced that measures for securing the prevention of ingestion of living bovine tubercle bacilli with milk would greatly reduce the number of cases of abdominal and cervical gland tuberculosis in children, and that such measures should include the exclusion from the food supply of the milk of the recognisably tuberculous cow, irrespective of the site of the disease, whether in the udder or in the internal organs."

Eber,[1] in an extended investigation, succeeded in infecting calves from three cases of human pulmonary tuberculosis. The bacilli isolated from the human material were of the human type, but after passage through the calf became transformed into the bovine type. He affirms, therefore, the essential identity of the human and bovine types of tubercle bacilli. This is not the general opinion. Fraser has directed attention to the frequency of the bovine type of bacillus in the tuberculous lesions of bone and joints in children. Eastwood and Griffith[2] have investigated the characteristics of the tubercle bacilli in 261 cases of human bone and joint tuberculosis with the following results:

[1] *Centr. f. Bakt.*, Abt. I (Orig.), lix, 1911, p. 193.
[2] *Journ. of Hygiene*, vol. 15, No. 2, 1916, p. 25.

Age period.	Number of cases.	Human.	Bovine.	Atypical.
0–5 years	47	31	14	2
5–10 ,, ...	108	75	31	2
10–16 ,, ...	62	52	7	3
16–25 ,, ...	15	12	3	—
Over 25 years . . .	29	26	—	3
Total	261	196	55	10

The percentages of "bovine" cases are:

All ages (55 out of 261) . . 21·1 per cent.
Under 10 years (45 out of 155) . 29·0 per cent.
Over 10 years (10 out of 106) . 9·4 per cent.

Only three of the above patients yielding the bovine type of bacillus were over 16. In the cervical gland tuberculosis of children under 10 years of age, 72 per cent. yield the bovine bacillus.[1]

With regard to the channel of infection in human tuberculosis opinions differ. Koch insisted that inhalation of air-borne bacilli derived from dried human sputum is the principal source of infection; Von Behring, on the other hand, expressed the opinion that tuberculous milk fed to children is the main source of infection both of children and of adults; in the latter case he suggested that bacilli are ingested in childhood and lie dormant for years before becoming active.

Calmette similarly believes that in the young infection by the digestive tract, especially by tuberculous milk, is the more frequent, and attached little or no importance to dry dust containing tubercle bacilli as a source of infection. Ravenel considers that the alimentary tract, particularly in children, is a frequent portal of entry for the tubercle

[1] Griffith, *Lancet*, 1917, vol. 1, p. 216.

bacillus, which he believes is able to pass through an intact mucous membrane. Of sixty cases of human tuberculosis investigated by the Royal Commission on Tuberculosis, twenty-eight possessed clinical histories indicating that in them the bacillus might have been introduced by the alimentary canal.

Flügge, on the other hand, states that his experiments show that tuberculosis can be communicated to animals by inhalation, and that the dose of bacilli required to infect by the respiratory tract is far less than that required to infect by the alimentary canal. The mode of infection in man doubtless varies, and he believes that children may be infected by the digestive tract, by tuberculous food, particularly milk, but the most extensive source of human infection is the number of droplets of tuberculous expectoration coughed up by consumptives ; these float in the air and serve as sources of infection to others. Ribbert and Schrötter, also, from the evidence of autopsies, considered inhalation as the chief mode of infection in man.

Bulloch,[1] from a careful survey of the literature, concludes that pulmonary tuberculosis is invariably caused by bacilli of the human type, and, therefore, is presumably due to inhalation of human bacilli.

Experiments by the Royal Commission show that infection by feeding is vastly more difficult to attain than by inoculation.

McFadyean,[2] also, from a critical survey of the experimental evidence, concludes that (1) inhalation of tubercle bacilli suspended in the air is a very certain method of infecting susceptible animals ; (2) experimental infection by the digestive tract is comparatively difficult to realise ; (3) inhalation is probably the commonest natural method

[1] "*Horace Dobell Lecture*," 1910.

[2] *Journ. Roy. Inst. Public Health*, vol. xviii, 1910, p. 705.

of infection, both in man and in animals; (4) infection by the digestive tract can be inferred only when the lesions are confined to the abdomen. He finally states that "the whole of the experimental evidence on which the theory of the intestinal origin of pulmonary tuberculosis in man was built up has been swept away."

While the death rate per million living in 1914 from all forms of tuberculosis was 1344, that from phthisis was 1016, so that the greater part of the mortality from tuberculosis must be ascribed to infection with the human bacillus derived from human sources. There still remains the residuum of glandular, abdominal, bone and joint tuberculosis caused by the bovine bacillus which is generally ascribed to infection by ingestion of tuberculous milk.

The writer is doubtful if this be the complete explanation; for instance, cases of bovine bacillus infection with open wounds and discharging sinuses might well infect others.

Spengler and others assert that the bovine bacillus is not virulent to man and Spengler distinguishes two types of human bacillus (*a*) the "*humanus brevis*," the ordinary human type, and (*b*) the "*humanus longus*," more virulent and very like the bovine type.

The occurrence of tuberculosis in the domestic animals raises points of practical importance, especially the occurrence of infection from the consumption of meat and milk from diseased animals. There can be no doubt that the carcase of an animal extensively affected with tuberculosis, especially if wasting has occurred, should be condemned as unfit for food, and likewise all parts in which there are tuberculous deposits. But it becomes an important question for the community, financially as well as from a hygienic point of view, as to the method of procedure with the meat from a beast comparatively slightly affected with tuberculosis—an enlarged gland or two, and a few nodules on the pleura. No doubt the ideal method in such a case is the condemnation and destruction of the whole carcase, be the amount of tubercle ever so little; but from financial

considerations this procedure is hardly practicable on account of the large amount that would have to be paid in compensation. Experiment has demonstrated that the tubercle bacilli are practically confined to the tuberculous areas and are extremely rarely met with in the muscular tissue, and these portions, therefore, it might seem, could be eaten with impunity, especially as they would be cooked before consumption. As regards swine, however, it is generally held that tuberculosis anywhere condemns the whole carcase.

The report of the first Royal Commission on Tuberculosis, however, indicated two dangers. Firstly, in cutting up a carcase the butcher will most likely use the same knife throughout, and in this way may infect the meat with tuberculous matter by smearing with the knife. Secondly, cooking cannot be depended upon to destroy the bacilli unless the joints are under 6 lb. in weight ; when the weight is above this the temperature in the interior may not rise sufficiently high. Evidently one of the first measures to be taken is the abolition of private slaughter-houses and the establishment of municipal abattoirs where the meat would have to be passed by competent inspectors. In this way all badly affected carcases would be condemned, and those only slightly affected could be separately dealt with and special precautions taken to eliminate tuberculous pieces, etc.

Tuberculous milk also raises many important points. Probably some 10–15 per cent. of *all* samples are infective to guinea-pigs, but this does not necessarily indicate that this proportion would be dangerous to man, for the material is introduced into the guinea-pigs by inoculation after concentration by centrifuging (see also section on "Milk"). Tubercle bacilli may gain access to milk not only when the udder is tuberculous, but also when the cows are suffering from tuberculosis elsewhere which is *clinically recognisable*. Thus, when the lungs are affected, bacilli are disseminated from the air-passages and also by the fæces. It is noteworthy that the incidence of abdominal tuberculosis in young children occurs just when cow's milk is the staple article of their diet. At the same time this incidence does not seem to fall on those who consume most milk.

Much might be done by the registration of all dairy premises, the use of selected cows, the elimination of all tuberculous animals, and by enforcing the inspection of dairy cattle by competent inspectors at suitable intervals. The notification of all forms of udder disease is now compulsory. In the absence of inspection

and the use of selected cows, treatment of milk intended for the food of infants and young children by pasteurisation or sterilisation has been recommended, but has disadvantages (see section on "Milk"). The ideal method, and one which commends itself at first sight as being the most satisfactory, is the elimination by slaughter of all animals which are tuberculous. This was adopted in the State of Massachusetts; under an order of the Board of Cattle Commissioners all beasts in the State were tested with tuberculin, and every animal that reacted was slaughtered, and strict quarantine combined with the tuberculin test imposed on all imported cattle. Even in this small area such a plan was found to be unworkable, the expense of compensation becoming formidable. A middle course seems to be the only practicable one, viz. all manifestly tuberculous animals, especially where wasting or a tuberculous udder is present, to be slaughtered; other animals to be tested with tuberculin, and those which react to be separated from the healthy and to be disposed of (for slaughter) as soon as convenient, and in the meanwhile kept as much as possible in pasture.

Tuberculosis is diminishing among the white races; it is, however, spreading among many coloured races. It is to be noted that the decline began long before the germ origin had been demonstrated, and, what is more, the rate of decline was almost as great before any administrative measures were taken as since. Nevertheless, it can hardly be doubted that measures should be adopted by local authorities and others to prevent the spread of tuberculosis. All forms of tuberculosis have now been made notifiable in this country. Patients should be warned of the danger of disseminating their expectoration, and should use pocket-spittoons containing an antiseptic, or handkerchiefs (such as the Japanese paper ones) which can be destroyed. Rooms which have been inhabited by tuberculous patients should be disinfected, for which purpose Delépine recommended spraying with a 1–100 solution of chloride of lime. Although the occurrence of direct infection can rarely be proved, the possibility of this cannot be ignored. Not only should the dissemination of infection be prevented, but the resistance of the individual should be raised by providing a healthy environment and by inculcating the importance of fresh air.

Serum therapeutics and vaccine.—Many sera have been introduced for the treatment of tuberculosis, *e.g.* Mara-

gliano's, Marmorek's, Spengler's, Mehnarto's, etc. Spengler's I.K. serum is of considerable value in many cases: it is prepared by immunising rabbits by intramuscular injections and contains the laked red-corpuscles as well as the serum.[1] Mehnarto's is stated to be a mixture of sheep and snake serums and is reported favourably on by Barcroft.[2]

For *vaccine treatment*, tuberculins R and BE are usually employed (p. 352). Latham has found that tuberculin given *per os* produces its characteristic effects.

Immunity.—Attempts have been made from time to time to produce immunity against the *B. tuberculosis*, particularly in cattle. Thus McFadyean[3] found that heifers which had previously been subjected to repeated doses of tuberculin (old) in some cases resisted infection with virulent bacilli. Behring[4] also employed human tubercle bacilli for the vaccination of cattle with satisfactory results. His tulase likewise confers immunity when given either by the mouth or by the stomach.

Theobald Smith[5] also concludes that vaccination of calves with the human type of bacillus is harmless, and that the procedure leads to a relatively high resistance to fatal doses of the bovine bacillus.

Clinical Examination

I. The "*complement-fixation*" *test* was first used in tuberculosis by Wassermann and Brück. The method has been further elaborated by Emery.[6] He makes use of a standard emulsion of tubercle bacilli in salt solution, containing about 4 per cent. by volume of solid bacillary substance. This is sterilised by inter-

[1] See *Treatment of Tuberculosis by Immune Substances (I.K.) Therapy.* Fearis (John Murray, 1912).

[2] *British Journ of Tuberculosis*, 1913.

[3] *Trans. Path. Soc. Lond.*, vol. liii, 1902, p. 20.

[4] *Brit. Med. Journ.*, 1906, vol. ii, p. 577.

[5] *Journ. Med. Research*, vol. xviii, 1908, p. 451

[6] *Lancet*, 1911, vol. i, p. 485.

mittent sterilisation and keeps for four to six weeks. Bacilli from various sources vary somewhat, so that the emulsion should be standardised so as to give an absorption-time with normal sera of about 20 minutes, *i.e.* the complement of normal serum should be just completely absorbed in about 20 minutes. A water-bath kept at a constant temperature of 38° C. is used to warm all the constituents and mixtures. One part of the serum to be tested is mixed with four parts of the bacillary emulsion in a small tube (*e.g.* a Durham's tube) in the water-bath, the time of mixing being accurately noted. After 2½ minutes' incubation, 4 volumes of the mixture are removed by means of a capillary pipette with teat (Fig. 37, p. 237), into which also a single volume of sensitised corpuscles (*i.e.* a hæmolytic system, p. 237) is taken up and the whole is expelled into a small tube already standing in the water-bath. The process is repeated after 5, 10, 15, and 20 minutes, and longer if necessary. By the occurrence or absence of hæmolysis in the various tubes, the time taken for the absorption of complement is ascertained, the complement used being that contained in the serum itself, which therefore should be fresh. A control with normal serum should always be performed at the same time. With normal serum complete absorption should take place in about 20 minutes; with tuberculous sera it is often complete in 2½ minutes. If, then, absorption of complement is complete in much less than the time necessary for absorption with a normal serum, presumably the serum is derived from a tuberculous individual. (But see Emery's paper for limitations.)

II. *Precipitin reaction.*—Spengler has devised a precipitin reaction for the diagnosis of, and prognosis in, tuberculosis. The reagents are the blood-serum or the laked whole blood, or both, very highly diluted and mixed in different dilutions with tuberculin.[1]

III. *Agglutination reaction.*—The method of agglutination was proposed by Arloing and Courmont for the diagnosis of tuberculosis, but is difficult to carry out and is not much employed. A special method has to be employed to obtain homogeneous cultures of the tubercle bacillus or a powder of pulverised or ground-up bacilli may be used: this powder may be purchased. The reaction may be carried out either microscopically or macroscopically; for the latter small sterile test-tubes may be em-

[1] See Fearis, *Practitioner*, i, 1913.

ployed. For each test three dilutions of the serum are made, a 1 in 5, a 1 in 10, and a 1 in 20, and the tubes filled with these dilutions are allowed to stand in an inclined position (45°) for five to ten hours. In man the serum of normal individuals may agglutinate up to a dilution of 1 in 5, while in animals this is variable—imperceptible in the guinea-pig, rabbit, and calf; feeble in the goat; in the adult ox up to 1 in 5, but in the dog it may be up to 1 in 10 or even 1 in 20.

A positive serum reaction in a suspected subject is a sign of great value in establishing the diagnosis; a negative serum reaction is of less value.

IV. The *examination of sputum, etc.*, for the tubercle bacillus is a routine procedure of the greatest value in forming a diagnosis. Fortunately, owing to the peculiar staining reaction of the tubercle bacillus, the method is comparatively simple.

If it is inconvenient to examine the sputum for a day or two a little 1–20 carbolic should be added. This preserves the sputum, and the tubercle bacilli retain their staining power for months.

1. *Sputum.*—Film specimens are prepared by smearing a little of the sputum on to a slide with a needle so as to form a thin film covering two-thirds of the surface, or by placing a particle of the sputum on one slide, applying another slide, pressing together, and then drawing apart so that a thin film is left on each slide. The thick portion of the sputum should be used, the thin mucoid portion being rejected. If the sputum is thin and watery, the thicker portion can be obtained by covering the bottom of a Petri dish with filter-paper, placing a large drop of the sputum on this, and working it over the paper with a bent steel needle. The paper absorbs the water, leaving the thicker material on the surface. If there are any small yellow caseous particles present these should be chosen, and sufficient material should be used so as to form a distinct but not too thick film; a little experience will soon decide the right amount; too thin a film should be avoided. Preparations may also be made by smearing the sputum on a cover-glass or between two cover-glasses instead of using slides. Whichever plan is adopted, the film is dried and fixed in the usual manner (generally by heat), and then stained by one of the following methods:

(*a*) *Ziehl-Neelsen method.*—Film specimens on slides are most conveniently stained by flooding with filtered, undiluted carbol-fuchsin and warming for 2 to 5 minutes on a piece of asbestos cardboard supported on a tripod, or on a heated penny (p. 122),

or slides or cover-glasses flooded with the stain may be held in the forceps and carefully warmed over a flame, or the preparations may be immersed in a watch-glass or dish of the stain, covered, and placed in the warm incubator for half an hour. In no case must the stain be allowed to boil, or the bacilli may lose their staining power; it should only be warmed sufficiently to steam (50°–60° C.), and with slides or cover-glasses as evaporation takes place more stain (always filtered), or better, 5 per cent. carbolic, should be added. After staining, the preparations are rinsed in water and are then decolorised by treating with 25 per cent. sulphuric or 30 per cent. nitric acid. The preparation may be flooded with the acid, but a better method is to immerse the preparation in a pot (Fig. 22, p. 121) containing the acid. In the acid the colour changes after a few seconds to a yellowish brown, the preparation is then rinsed in water, and some of the pink colour returns. The treatment with acid and with water alternately is repeated until the preparation is nearly colourless when rinsed in water. With sputum this is usually the case after three or four rinses in the acid, but it varies with the thickness of the film and with the number of tubercle bacilli present; when these are absent the film often decolorises more readily than when there are many. The presence of blood renders the decolorisation difficult. After decolorising and washing, the preparations are stained for one minute in Löffler's methylene blue, washed in water, and mounted in water, or, better, dried and mounted in Canada-balsam or cedar oil. When the preparation is made on the slide, after washing and drying, it can be examined directly without a cover-glass with the oil-immersion after applying a drop of cedar oil, unless a permanent specimen is desired, in which case it should be mounted in Canada-balsam.

The tubercle bacilli appear as delicate red rods, often beaded or segmented, on a blue background composed of cells, mucus, and putrefactive or other bacteria. Occasionally here and there a little red colour may be present in addition to the tubercle bacilli. Hair and keratinised material generally, such as horny epithelium, and red blood-corpuscles, retain the red colour after the foregoing treatment, and the spores of bacteria are also liable to retain the red somewhat persistently. These exceptions are not, however, likely to prove a source of error, for the tubercle bacilli should be recognised not only by their red colour, but also by their characteristic size, shape, and general appearance. It is conceivable that acid-fast bacilli not tubercle might be present in sputum, but

such an event is a very unlikely one. For the microscopical examination, a $\frac{1}{6}$-inch with good illumination is sufficient when the tubercle bacilli are present in any number. When they are scanty it is necessary to use a $\frac{1}{12}$-inch oil-immersion, and this is the better lens in any case. (See Plate IX, *b*, and Plate X, *a*.)

If tubercle bacilli are not found, other specimens should be prepared and examined. *It is only by repeated examinations on different occasions that the negative evidence*, the absence of tubercle bacilli, *becomes of any value*.

The tubercle bacillus is occasionally not acid-fast ; [1] probably the bacilli in such cases are degenerate, and, like all degenerate bacteria, fail to stain well. Spengler claims that the following method will stain these and "splitter" forms : (1) Stain with warm carbol-fuchsin by the ordinary method, avoiding overheating ; (2) pour off the stain without washing and treat with picric acid alcohol (equal parts of saturated aqueous picric acid and absolute alcohol) ; (3) after 3 seconds rinse with 60 per cent. alcohol ; (4) treat with 15 per cent. nitric acid until yellow (about 30 seconds) ; (5) rinse again with 60 per cent. alcohol ; (6) counter-stain with the picric acid alcohol until yellow ; (7) wash with distilled water. This is an excellent method, and thick films may be used. In material which has been preserved a long time, *e.g.* sputum with carbolic, or tissue in spirit, the bacilli may be much less acid-fast than in fresh material.

Various methods have been recommended for the solution of the sputum and the examination of the sediment of the bacilli. In one method 5 c.c. of sputum are mixed with 50 c.c. of normal KOH solution ; the mixture is warmed in a water-bath to 60°–65° C. until the sputum is dissolved (about 3 hours) ; 50 c.c. of cold water are next added, the whole is well shaken, and again warmed for $\frac{1}{2}$ hour. Petroleum ether 2 c.c. is next added, the whole is well shaken, and is then kept at 60° C. until the ether has separated. The bacilli will be concentrated in the fluffy layer at the junction of the ether and water ; this is pipetted off and films are made with it and stained. Antiformin (a mixture of sodium hypochlorite and sodium hydrate) has also been recommended. Into a boiling-tube or small flask of 50 c.c. capacity, 5 c.c. of the sputum are introduced. To this are added 25 c.c. of antiformin solution (10–20 per cent. aqueous solution) diluted with 10–20 c.c. of water according to the density of the sputum. The

[1] See *Lancet*, 1908, vol. i, p. 1222.

mixture is well shaken until homogeneous (about 15 minutes), then centrifuged, the deposit is washed three times with salt solution by centrifuging, and films are made with the washed deposit and stained by the Ziehl-Neelsen or Spengler method.

If loopfuls of the antiformin deposit are inoculated on to suitable media, pure cultures of the tubercle bacillus can frequently be obtained.

For obtaining cultures of the tubercle bacillus directly from sputum and post-mortem material Soparkar [1] recommends the use of caustic soda. From 2 c.c. to 5–10 c.c. of the material is used, according to its richness in bacilli. It is mixed with an equal volume of normal sodium hydrate solution and the mixture is kept at 37° C. for about half-an-hour—until the material is quite fluid ; with fluid sputum ten minutes may suffice. At the end of incubation, the mixture is neutralised to litmus with 5 per cent. hydrochloric acid, centrifuged and the deposit used for inoculating the tubes.

If the tubercle bacillus cannot be detected microscopically after repeated examinations, and a certain diagnosis is important, the inoculation method may be employed. A couple of guinea-pigs are inoculated subcutaneously in the thigh or abdomen with 0·5 to 1 c.c. of the sputum. If tubercle bacilli are present the animals will show signs of tuberculosis in three to six weeks (see below, " Urine ").

(*b*) *Other methods* have been devised for staining the tubercle bacillus, but do not seem to be better than the Ziehl-Neelsen or the Spengler. The following may be useful for those who are colour-blind to red :

α. Much's method.—Prepare the following solution : 10 c.c. of a saturated alcoholic solution of methyl violet B.N. in 100 c.c. of 2 per cent. aqueous carbolic ; (1) stain the film with this, warming over the flame, or for 24–48 hours at 37° C. ; (2) treat with Gram's iodine solution, 1–5 minutes ; (3) treat with 5 per cent. nitric acid for 1 minute ; (4) treat with 3 per cent. hydrochloric acid for 10 seconds ; (5) treat with a mixture of equal parts of acetone and absolute alcohol.

β. Herman's method.—Prepare shortly before use the following solution : 3 parts of a 1 per cent. aqueous solution of ammonium carbonate, 1 part of a 3 per cent. solution of krystal violet in 95 per cent. methyl alcohol. (1) Flood the film with this, warm

[1] *Indian Journ. Med. Research,* vol. 4, 1916, p. 28.

until it steams, and stain for 1 minute; (2) decolorise with 10 per cent. nitric acid for a few seconds, and then with 95 per cent. alcohol until the film assumes a pale blue colour, then rinse in tap-water followed by distilled water; (3) counter-stain with 1 per cent. aqueous eosin.

By both these methods the tubercle bacilli appear blue-black.

2. *Tissues.*—The histological appearance of the tubercle is usually sufficient for diagnostic purposes without the demonstration of the tubercle bacilli, which in many instances may be difficult in human material, as they may be very scanty, or practically impossible to find, *e.g.* in lupus. Sections should be prepared either by the freezing or the paraffin method, stained with hæmatoxylin, and counter-stained with eosin, or orange-rubin, or with the Ehrlich-Biondi mixture.

In order to demonstrate the tubercle bacillus in fresh tissue smears may be made and stained like sputum, or sections prepared and stained in warm carbol-fuchsin for about ten minutes. For frozen sections the stain may be contained in a watch-glass or small glass capsule, and is warmed until it steams, but not boiled, on a piece of asbestos cardboard or a sand-bath. Paraffin sections should be fixed to the slides with glycerin albumin, and may be stained by flooding with the carbol-fuchsin and warming on asbestos cardboard, or a heated penny, for ten minutes. After staining, the sections are washed in water and are then decolorised in 25 per cent. sulphuric acid. This is a longer process than with sputum, and the sections after being in the acid for a few seconds are washed in water and then returned to the acid, and this alternate rinsing in acid and in water is repeated until they are nearly colourless when placed in water. It is not necessary to remove the colour absolutely; a faint pink remaining does not matter. After rinsing in fresh water to remove all the acid, the sections are counter-stained in Löffler's methylene blue for two minutes, rinsed in methylated spirit, passed through absolute alcohol somewhat rapidly to avoid removing too much of the blue, cleared in cedar oil or xylol, and mounted in balsam. The sections may also be counter-stained with hæmatoxylin or Bismarck brown.

Instead of using the strong acid solution for decolorising, an acid alcohol solution may be used with advantage, or 2 per cent. aqueous hydrochloride of anilin may be employed.

Gram's method may also be used, but is, of course, not distinctive for the tubercle bacillus.

Sections may also be first stained with Ehrlich's or other hæmatoxylin solution, then stained with warm carbol-fuchsin, washed, treated with 2 per cent. aqueous anilin hydrochloride for a few seconds, decolorised with 75 per cent. alcohol until the red colour is no longer apparent (15–30 minutes), and counter-stained with an aqueous solution of orange.

When a positive diagnosis is important, a small piece of the tissue may be inserted under the skin of the thigh or abdomen of a guinea-pig. If tuberculous, the animal will show signs of tuberculosis in two or three weeks (see below, "Urine").

Films of pure cultivations of the tubercle bacillus may be stained in warm carbol-fuchsin for two to five minutes, rinsed in the sulphuric or nitric acid solution, washed, dried, and mounted. They can also be stained by Gram's method, which usually brings out the beaded appearance very markedly, or by any of the other methods mentioned under *Sputum*. Differentiation from the leprosy bacillus will be found at p. 385, and from the smegma bacillus and other acid-fast organisms at p. 387.

3. *Urine.*—The tubercle bacillus is often very difficult to demonstrate in urine. The urine must be allowed to stand in a conical glass for twenty-four hours or centrifuged, and film specimens are prepared with the sediment and treated by one of the methods for sputum given above. Several specimens should be made and must be very carefully examined. The sediment may also be treated by the antiformin method. It is important to exclude the smegma bacillus, and the urine is preferably drawn off by a catheter. Staining may be carried out by Housell's method, by which the smegma bacillus is decolorised, viz. after staining in warm carbol-fuchsin the specimen is washed and dried. It is then immersed in acid alcohol (alcohol + 3 per cent. hydrochloric) for ten minutes, washed in water, counter-stained for a few seconds in a saturated alcoholic solution of methylene blue, washed, dried, and mounted (see also p. 387). An electrolytic method for the concentration of the tubercle bacilli has been devised by Russ.[1]

If a diagnosis is of importance inoculation should be resorted to. Two guinea-pigs are inoculated subcutaneously in the thigh or abdomen with 0·5 to 1 c.c. of the deposit from the sedimented or centrifuged urine, or one may be inoculated subcutaneously, the

[1] *Proc. Roy. Soc. Lond.*, B. 1909.

other intra-peritoneally. If tubercle bacilli are present the animals may show signs of tuberculosis as early as two to three weeks after inoculation. Sometimes, of course, the animals may die from some intercurrent infection before the tuberculous infection has had time to develop. Delépine [1] recommends the inoculations to be made on the inner aspect of the leg about the level of the knee. The order of infection after inoculation is as follows: the popliteal, superficial and deep inguinal, and sub-lumbar glands, the retrohepatic, mediastinal and bronchial, deep cervical, and subscapular glands, the spleen, liver, and lungs. The inoculated animals are killed in two to three weeks, dissected, and the lesions examined microscopically. Others inoculate two guinea-pigs, one subcutaneously in the abdomen, the other intra-peritoneally. Negative results are nearly as valuable as positive ones.

In *fæces*, if definite yellow caseous particles can be found, these should be picked out, and films made and stained. Antiformin may also be used. About 5–6 c.c. of fæces are mixed with 20 c.c. of 15 per cent. aqueous antiformin in a conical glass, well agitated and broken up, and an equal volume of the dilute antiformin is then added. The mixture is allowed to stand for an hour, and with the white curdy layer which forms, films are prepared, stained, and examined.

4. *Milk.*—See section on milk (Chapter XXI).

V. *The opsonic method.*—The general mode of carrying this out is described at pp. 236–242, the tubercle bacilli being suspended in 1·5 per cent. salt solution.

VI. *Tuberculin reactions.*—The *old* tuberculin is used for diagnostic purposes; it is not perhaps very safe. A dose of 0·0002 c.c. is injected subcutaneously, and the temperature taken four-hourly during the succeeding thirty-six hours. A rise of 2°–3° F. or more ensues a few hours after injection in tuberculous subjects. If no reaction occurs another dose of 0·0005 c.c. may be given after the lapse of some days, followed by a third one of 0·001 c.c. if necessary.

This method has now almost completely been superseded by the cutaneous or by the ophthalmo reaction.

The cutaneous tuberculin reaction.—Von Pirquet [2] discovered

[1] *Brit. Med. Journ.*, 1893, vol. ii, p. 664. The results only apply to ordinary forms of tuberculosis, and not to certain modified forms such as lupus and the avian variety.

[2] *Wien. med. Woch.*, July 6, 1907.

that when tuberculin is introduced into the superficial layers of the skin of tuberculous individuals, as in vaccination, a reaction occurs consisting of the formation of a papule with redness, slight swelling and exudation, and sometimes small vesicles. This reaction is usually at its height twenty-four to forty-eight hours after inoculation. In healthy individuals no reaction follows the inoculation. The method is to scarify a small spot on the forearm through a drop of a dilution of the old tuberculin, and protect the patch with a simple dry dressing. Moro has modified the method by applying the tuberculin to the skin in the form of ointment.

The ophthalmo-tuberculin reaction.—Calmette transferred the site of inoculation from the skin to the conjunctiva. He makes use of material prepared by precipitating the old tuberculin with alcohol, of which a 1–100 solution is prepared in distilled water. One drop of this is instilled into the inner half of the conjunctiva of one eye. In tuberculous individuals a reaction follows, usually in six to sixteen hours after medication, consisting of a conjunctivitis, ranging in intensity from a local redness to a redness extending over the whole eye and having the appearance of an acute conjunctivitis. The reaction soon passes off, generally without leaving any ill effect. On the whole the reaction appears to be fairly constant in tuberculous individuals, but absence of reaction is not certain proof that the case is not tuberculous.[1]

VII. *Tuberculin for veterinary use.*—The dose of the various preparations in the market varies according to their strength; it corresponds to 0·1 c.c. or 0·2 c.c. of Koch's original tuberculin.

The appropriate dose is injected subcutaneously in the neck and the reaction consists of a rise of temperature of from 1·5° to 6° F. above the average normal, commencing 8–12 hours after injection and lasting 12–14 hours, the temperature being taken at the twentieth hour after injection, or, if it can be done, at frequent intervals from the twelfth to the twentieth hour. The temperature should be taken just before inoculation, and, if possible, morning and evening for two or three days previous to inoculation.

A healthy animal is unaffected by the injection, and if an animal be extensively affected with tuberculosis the reaction may not be given, or may be masked by the fever present.

An ophthalmo-reaction may also be employed in cattle.

[1] See *Brit. Med. Journ.* and *Lancet*, 1907, vol. ii, and 1908, vol. i.

Johne's disease,[1] a bovine enteritis, is due to an acid-fast bacillus closely resembling the tubercle bacillus in morphology. It is found in scrapings of the affected mucous membrane of the bowel, and also in sections of the intestinal wall. The Johne bacillus is inoculable into the goat, but not into the guinea-pig or rabbit, and does not grow on any of the ordinary laboratory media. Twort states that it can be cultivated on the medium employed by him for growing the leprosy bacillus (p. 382), and from the cultures a diagnostic vaccine may be prepared.[2]

Pseudo-Tuberculosis

The term "pseudo-tuberculosis" (which is not a good one, and should be discarded) has been applied to a number of different conditions which have as a common character the presence of tubercle-like nodules, but which are not caused by the tubercle bacillus. Such are produced by certain parasitic worms, by *Blastomycetes*, *Streptothrix* and *Aspergillus*, Protozoa, and by several bacteria.

Pfeiffer's *Bacillus pseudo-tuberculosis* produces nodular deposits in the organ, accompanied by wasting, very like true tuberculosis. The disease, however, runs a more rapid course, death ensuing in the guinea-pig two to three weeks after inoculation. Guinea-pigs, rabbits, mice and monkeys can be readily infected. The nodules consist of masses of round cells which undergo necrosis and caseation. The bacillus in the tissues is not readily stained, carbol-methylene blue being the best solution, as it is not acid-fast, nor does it stain by Gram's method. Morphologically it is a small rod 1–2 μ in length, usually non-motile, although, according to Klein, it possesses a single flagellum or two flagella at one end. On gelatin it forms a whitish growth without liquefaction, like that of the colon bacillus, but confined to the needle-track. It pro-

[1] See McFadyean, *Journ. Comp. Path. and Therap.*, vol. xx, 1907, p. 48.

[2] Twort, *Veterinary Record*, Sept. 14, 1912.

duces alkali, forms no gas, and does not curdle milk. Broth remains clear, with a whitish stringy flocculent deposit. The bacillus grows readily and rapidly.

MacConkey has found that the fermentation reactions of this organism and of the plague bacillus are practically identical (see "Plague," p. 448), and sterilised cultures of either will protect against the other.

Ovine caseous lymphadenitis, a disease of sheep simulating tuberculosis, is due to a short plump bacillus with rounded ends which stains well by Gram's method, and grows best on blood serum, on which it forms greyish colonies.[1]

Much finds in the glands in Hodgkin's disease antiformin-resistant bodies, non-acid-fast, and similar to the non-acid-fast tubercle bacilli which he has described (see p. 374).

Leprosy

Leprosy, elephantiasis Græcorum or true elephantiasis, is a disease of which we have records from the earliest times. It was undoubtedly somewhat prevalent in the British Isles from the twelfth to the fifteenth centuries, as the many leper houses and enactments against lepers testify, though no doubt other skin diseases, psoriasis, lupus, etc., were at that early period of medical diagnosis confounded with it. At the present day leprosy, although extinct in the British Isles, may be said to have a world-wide distribution, for it is met with in Iceland and Scandinavia, Russia and the Mediterranean coasts; in Persia, India, China, Siberia, and Japan; in Africa from north to south; in many districts of the American continent; and in the Pacific Islands. Three varieties of leprosy are described—the tuberculated or nodular, the anæsthetic, and the mixed.

[1] *Sixteenth Ann. Rep. Bureau of Animal Indust. U.S.A.*, p. 638.

The mode of spread is probably by personal contact (though possibly insects play some part), and throughout ancient and mediæval times leprosy was considered to be a contagious and communicable disease, as witness the stringent regulations in the Mosaic and other laws for the segregation of lepers. J. Hutchinson supposed that fish in the diet, particularly if stale, decomposed, or badly cured, in some way is a causative factor; but this view is now discredited.

A bacillus, the *Bacillus lepræ*, is abundant in the tissues and was discovered by Hansen in 1879. In form it resembles the tubercle bacillus, but is slightly more slender; it probably does not form spores, though in stained preparations the same irregularity in staining—namely, the occurrence of unstained intervals, the so-called "beading"—is met with as in the tubercle bacillus, and is assumed by some to be due to the presence of spores. The organism as obtained from the tissues is non-motile, stains readily with the ordinary anilin dyes, and by Gram's method, which brings out the beaded appearance very well, and is markedly acid-fast, thus closely resembling the tubercle bacillus, and the methods used to demonstrate it are the same as for the latter organism.

The *Bacillus lepræ* is found in enormous numbers, usually crowded together in bundles or masses, in the leprous nodules in the skin (Plate XI, *a*), liver, spleen, and testicles, in the affected nerves in the anæsthetic form and even in the ganglion cells of the central nervous system—in fact, any viscus may be affected; it has also been found in the blood, but only in the febrile paroxysms which set in when the disease is approaching a fatal termination. The exact situation of the leprosy bacilli in the tissues has been a matter of controversy. By some it has been held that they are contained within certain round cells, the so-called leprous cells, and this may be

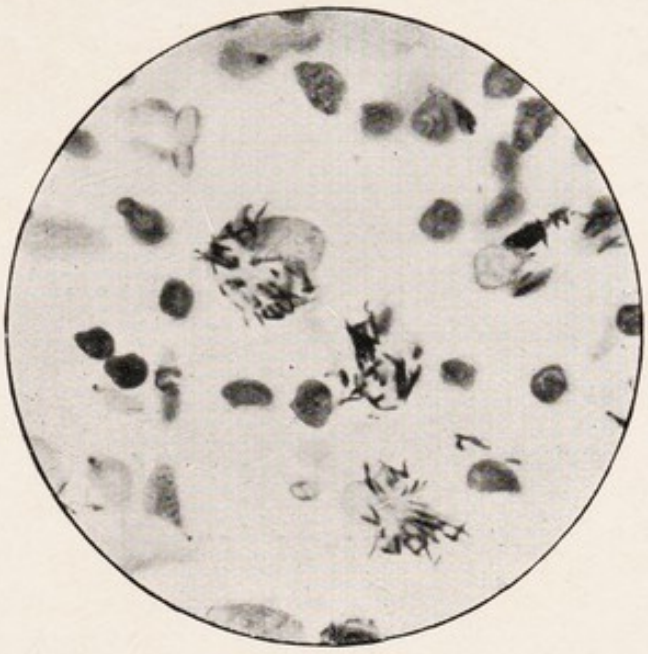
the case, but to an inconsiderable extent. Unna always regarded these leprous cells as really being transverse sections of lymphatic vessels containing bacillary thrombi, and this seems to be usually the case. Giant-cells are occasionally present in the leprous nodules. One of the most constant and earliest situations in which the *B. lepræ* is found is the nasal mucous membrane.

Although the organism is present in such enormous numbers and is so readily demonstrable, to cultivate it on artificial media and to infect animals with it are both difficult matters. Babes, Bordoni-Uffreduzzi, Czaplewski, are some of those who in the past believe that they have cultivated the leprosy bacillus. Van Houten [1] claimed to have succeeded by growing it in glycerin fish broth. The bacillus cultivated was acid-fast, and agglutinated with, and was sensitised by, lepers' serum.

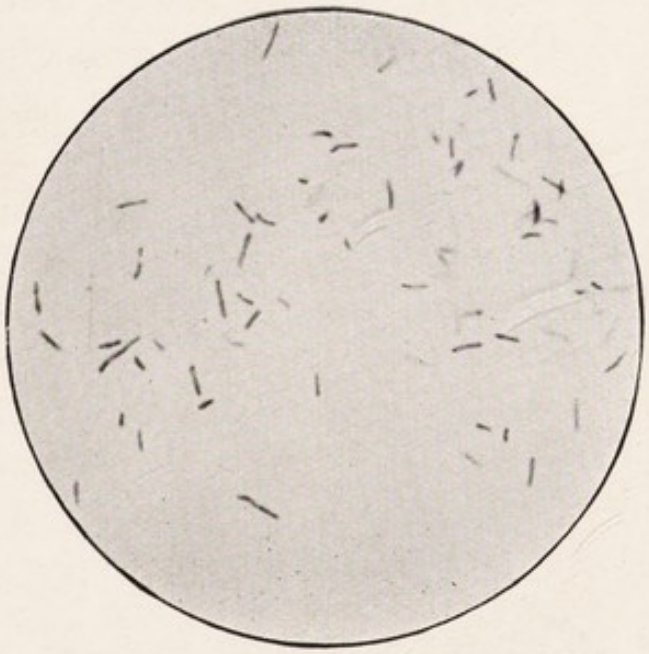
Deycke,[2] by taking fragments of leprosy tissue and incubating for several weeks in physiological salt solution at 37° C., obtained a growth of a semi-acid-fast streptothrix, *S. leproides*. He is uncertain if this is a true growth of the leprosy bacillus. Twort [3] claimed to have cultivated the *B. lepræ* on a medium consisting of eggs, glycerin, and ground-up tubercle bacilli. Clegg states that the leprosy bacillus will grow in symbiosis with amœbæ, and Duval that it grows in 1 per cent. human serum in symbiosis with some bacteria. Kedrowsky and Bayon claim to have grown the organism on a placental-juice agar, and Bayon obtained complement fixation with his cultures with leper serum. Kedrowsky's organism is a non-acid-fast diphtheroid, Clegg's an acid-fast chromogenic bacillus, Duval's and Bayon's are acid-fast leproid bacilli.

[1] *Journ. Path. and Bact.*, vol. viii, 1903, p. 260.
[2] *Brit. Med. Journ.*, 1908, vol. i, p. 802.
[3] *Proc. Roy. Soc. Lond., B.*, 1911.

PLATE XI.



a. Leprosy. Section of skin. × 1500.



b. The smegma bacillus. Smear preparation of smegma, × 1500.

The serum of few lepers agglutinates the acid-fast organisms isolated from leprosy, and then only with a small proportion of the cultures.

In 1904 Rost announced that he had obtained cultures in the leprosy bacillus in a chlorine-free medium, but this was not confirmed. In 1909 he again claimed success by cultivating in a medium consisting of the fluid obtained by the steam distillation of rotten fish to which is added a little Lemco broth and milk, and Bannermann believes that he is correct.[1] Williams has grown a non-acid-fast streptothrix in ordinary broth, and has also cultivated acid-fast bacilli in a modified Rost medium (substituting distilled water for the fish distillate). The writer has also grown a non-acid-fast streptothrix from a case of leprosy on brain agar containing the juice from disintegrated *B. megaterium*. As a result of these alleged positive cultural results, it has been surmised that the *B. leprœ* is really a streptothrix, that it is acid-fast only under certain conditions, viz. in the body or in media containing fat, and that under cultivation the streptothrix may break up into non-acid-fast diphtheroid bacilli or into acid-fast leproid bacilli. On the other hand, Fraser and Fletcher [2] have made 373 inoculations from 33 non-ulcerating cases of leprosy on a variety of culture media with entirely negative results. More work is therefore required before it can be definitely stated that the leprosy bacillus has been cultivated.

A certain number of positive results of the inoculation of leprous material into the lower animals have been reported by Ortmann and others. Nicolle [3] has reported the successful inoculation of a macaque monkey, but most of the attempts have ended in failure; positive

[1] See *Sc. Mem. Gov. of India*, No. 42, 1911.
[2] *Lancet*, Sept. 27, 1913.
[3] *Comp. Rend. Acad. Sc.*, 1905.

results are open to criticism and may be fallacious, for lepers not infrequently suffer from coincident tuberculosis, and the animals therefore may have been infected with tuberculosis. Japanese dancing mice are also stated to be slightly susceptible. The local lesion induced in animals may be simply inflammatory, produced by the leprous material acting as a foreign body, and the bacilli may be diffused without proliferating. Human beings have also been inoculated, but the positive results obtained are all open to objection.

The differentiation of leprosy from tuberculosis, although the bacilli are so similar, does not in the majority of cases present much difficulty. The large number of bacilli present in the lesions, and particularly in the skin, forms a marked distinction from tuberculosis. The *Bacillus lepræ* also stains more readily, and with watery solutions in a shorter time, than does the *Bacillus tuberculosis*, though this distinction is hardly marked enough for diagnostic purposes. About 50 per cent. of lepers give a positive Wassermann reaction.

Cases of leprosy, both of the nodular and anæsthetic varieties, have been treated with injections of Koch's old tuberculin, which produces a reaction, sometimes marked, followed by some amelioration in their condition. Rost and Williams with their cultures have prepared vaccines with which treatment is being pursued. Nicholls and others have used extracts of leprous tissue as a vaccine, and Bayon states that a filtered extract of the Kedrowsky culture is of service for treatment.

Deycke injected a vaccine prepared with his streptothrix into lepers with apparently a beneficial effect. The acid-fast property of the streptothrix resides in a fatty substance which can be extracted with solvents, particularly benzoyl chloride. The fatty substance Deycke terms " nastin " ; it is a neutral fat, the glycerin ester of

a fatty acid of high molecular weight. Injected into leprosy patients it sometimes produces marked reaction. In solution in benzoyl chloride it is much more active, and Deycke introduced it as a curative vaccine in leprosy. In India the results of nastin treatment have been favourable on the whole, in Guiana and South Africa they have been unfavourable.[1]

Dean [2] and others have met with a leprosy-like disease in the rat. Marchoux found about 5 per cent. of the sewer rats in Paris infected with it. Nodules are found in the tissues which contain large numbers of an acid-fast bacillus closely resembling the *B. lepræ*. Material from infected rats inoculated into healthy rats reproduces the disease after some months, but has no effects on guinea-pigs. The disease is probably conveyed by contact.

Dean cultivated a diphtheroid non-acid-fast bacillus from this disease ; Bayon an acid-fast leproid bacillus which he finds to be very similar to that obtained by him from human leprosy.

Clinical Examination

(1) If cutaneous nodules be present, one is clamped, pricked, and films are prepared with the juice that exudes and stained as for tubercle. The occurrence of large numbers of bacilli, having the same staining reactions as the tubercle bacillus and obtained from the cutaneous structures, is diagnostic of leprosy (the smegma bacillus may be present *on*, but not *in*, the skin).

(2) In the tissues, sections of which are stained in the same manner as tuberculous material, the diagnosis must be based on the presence of the bacilli in large numbers in the so-called leprosy-cells.

(3) Leprosy is not inoculable in guinea-pigs.

N.B.—It must be remembered that lepers not infrequently suffer from coincident tuberculosis.

(4) The differentiation of the leprosy from the tubercle bacillus by staining methods cannot be said to be satisfactory. By staining in a saturated aqueous solution of fuchsin in the cold

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[1] Scott, *Indian Journ. Med. Research*, vol. i, No. 2, 1913, p. 352.

[2] *Journ. of Hyg.*, vol. v, 1905, p. 99 ; Marchoux and Sorel, *Ann. de l'Inst. Pasteur*, xxvi, 1912, p. 778.

for five to seven minutes, and subsequently decolorising with acid alcohol (nitric acid 1 part, alcohol 10 parts), it is stated that the leprosy bacillus is stained, the tubercle bacillus not.

The Smegma Bacillus [1]

The smegma bacillus is an organism found in the smegma præputii, between the scrotum and thigh, and between the labia. It also occurs in the cerumen, occasionally on the skin, and possibly in the sputum.

It is a small bacillus resembling the tubercle bacillus in size and appearance, and, like the latter, is difficult to stain, but when stained with carbol-fuchsin, retains the colour after treatment with a 25 per cent. mineral acid (Plate XI, *b*); it is also Gram-positive. It has, therefore, to be distinguished from the tubercle bacillus in certain localities, viz. in urine and about the external genitals. It is non-inoculable on animals, and does not usually grow in primary cultures on ordinary media, but can be isolated by the use of blood-serum or nutrose-agar, on which it forms delicate, ropy colonies. After isolation it grows freely on agar as a thin, slightly brownish, creamy layer, in which the bacilli may be very short but retain their acid-fast properties; on potato it forms minute (0·5–1 mm.) greyish colonies. It has been suggested that the syphilis bacillus of Lustgarten is identical with the smegma bacillus; neither is decolorised by Lustgarten's permanganate method, but while the smegma bacillus after staining is with difficulty decolorised by acid, and is easily decolorised by alcohol, the reverse is the case with Lustgarten's bacillus.

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[1] See Neufeld, *Arch. f. Hygiene*, xxxix, p. 184; *Zeitschr. f. Hyg.*, xxxix, 1901; and Moeller, *Centr. f. Bakt.*, xxxi, 1902 (Originale), p. 278.

Staining and Differentiation

Film preparations of smegma may be stained in exactly the same manner as for tubercle, after treating the preparations with ether to get rid of fatty material.

The urine should be drawn off with a catheter when it is to be examined for the tubercle bacillus ; this will generally exclude the smegma bacillus. Young and Churchman [1] conclude that the smegma bacillus is a scant invader of the male urethra, and that by washing the glans and irrigation of the urethra it may be eliminated from the urine.

If there is reason to suspect the presence of the smegma bacillus when staining for tubercle, Bunge and Tranteroth [2] recommend that the film specimens should be treated as follows :

(1) Immerse in absolute alcohol for three hours.

(2) Immerse in 5 per cent. chromic acid for fifteen minutes.

(3) Stain in warm carbol-fuchsin.

(4) Decolorise in 25 per cent. sulphuric acid for two to three minutes.

(5) Counter-stain in a concentrated alcoholic solution of methylene-blue for five minutes.

The smegma bacillus will be decolorised by this method (see also p. 376).

Coles recommends (*Journal of State Medicine*, vol. xii, 1904, p. 225) the following staining method:

(1) Spread thin and even films on slides, and fix by heat, in the ordinary way.

(2) While still warm from the heat fixation flood with filtered carbol-fuchsin for half a minute. Again warm for a few seconds over the flame without actual boiling. Allow it to stand and stain for seven minutes.

(3) Wash thoroughly in running water, and then decolorise in either of the following solutions :

(*a*) *In Pappenheim's solution.*[3]—Place the preparation in a

[1] *Johns Hopkins Hospital Rep.*, vol. xiii, 1906, p. 15.

[2] *Fortschrit. der Med.*, xiv, 1896, Nos. 23 and 24. See also *ibid.* No. 9.

[3] *Pappenheim's solution* consists of one part of corallin (rosolic acid) in 100 parts of absolute alcohol, to which methylene-blue is added to saturation ; 20 parts of glycerin are then added.

wide-mouthed bottle containing the solution for not less than four, and not longer than twelve, hours. Wash, dry, and mount. Tubercle bacilli are the only organisms stained red.

(*b*) *In Pappenheim's solution without methylene-blue.*—Proceed as in (*a*); wash in water and counter-stain for a minute in weak aqueous methylene-blue solution. The tubercle bacilli are brilliantly red.

(*c*) *In 25 per cent. sulphuric acid.*—Pour on a few drops of the acid and allow it to act for half a minute. Pour off, and then place the preparation in a wide-mouthed bottle containing the acid for not less than sixteen hours and not more than twenty-four hours. Wash thoroughly, counter-stain with weak aqueous methylene-blue. Tubercle bacilli are the only bacilli which retain the red.

Acid-fast bacilli in milk and butter.—Numerous acid-fast bacilli have been obtained from milk and butter. They usually grow freely and quickly on agar and on gelatin without liquefaction, sometimes as a creamy layer, sometimes as a dry, crinkled film, which may be pigmented (yellow, orange, pale brown or brick red). Some are pathogenic to guinea-pigs by massive intra-peritoneal inoculation only, producing a plastic peritonitis, but not nodules in the organs. In culture, the bacilli are acid-fast and occasionally resemble *B. tuberculosis*, but are generally thicker. (See Petri, *Arb. a. d. Kais. Gesundheitsamte*, xiv, 1897; Rabinowitsch, *Zeitschr. f. Hyg.*, xxvi, 1897; Grassberger, *Münch. med. Woch.*, 1899, Nos. 11 and 12; Tobler, *ibid.* xxxvi; Swithinbank and Newman, *Bacteriology of Milk* [Murray, 1903]).

Grass bacilli and mist bacillus.—Moeller isolated from a grass (*Phleum arvense*) an acid-fast bacillus which he termed the Timothy-grass bacillus; other grasses also yield acid-fast bacilli (Grass Bacillus II). They grow readily on culture media, and are not so acid-fast as the tubercle bacillus. The Mist bacillus was isolated from dung, and is considered by Pettersson to be identical with the Timothy-grass bacillus. (See Moeller, *Deutsch. med. Woch.*, 1898, p. 376; Herr, *Zeitschr. f. Hyg.*, xxxviii, 1901; Pettersson, *Berl. klin. Woch.*, 1899, p. 562.)

CHAPTER X

TYPHOID FEVER — PARA-TYPHOID FEVER — BACILLUS ENTERITIDIS AND THE GÄRTNER GROUP—SWINE FEVER —BACILLUS DYSENTERIÆ—BACILLUS COLI

The organisms considered in this chapter form a natural group or family, the "Typhoid-Colon" group, and pass as it were by gradations in cultural characters from the typhoid bacillus to the colon bacillus. Löffler classes them together in a family, the Typhaceæ, which is divided into sub-families: (*a*) Typheæ, which includes the *B. typhosus* and *B. dysenteriæ*; (*b*) Iosarceæ,[1] which includes the Gärtner group of organisms; and (*c*) Coleæ, the *B. coli* group of organisms.

The group can be divided into lactose fermenters and non-lactose fermenters. The former includes *B. coli* and its variants. There is also a group of *late* lactose fermenters (after six days) which occur in the intestine, *e.g. B. coli mutabilis*. The non-lactose fermenters are classified by Henderson-Smith [2] as follows:

I. Certain groups of no known pathogenic importance. Frequent in the intestine.

II. The Typhoid group, *B. typhosus*.

III. Paratyphoid-Enteritidis (Gärtner) group.

1. Atypical members.
   - *a*. Saccharose fermenters. Not agglutinated with Gärtner or paratyphoid serum.
   - *b*. Dulcitol non-fermenters.
   - *c*. *B. paratyphosus* A.
   - *d*. Salicin fermenters. Frequent in animals.

2. Typical members.
   - *a*. *B. enteritidis* of Gärtner.
   - *b*. *B. paratyphosus* B.
   - *c*. *B. suipestifer*.

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[1] From ἰός, poison, and σάρξ, flesh.

[2] *Centr. f. Bakt.* Abt. I (Orig.), 68, 1913, p. 151 (Bibliog.).

IV. Dysentery group.
1. Mannitol non-fermenters. *B. dysenteriæ*, Shiga.
2. Mannitol fermenters.
   a. *B. dysenteriæ*, Strong.
   b. Sorbite fermenters.
      (a) Dextrin non-fermenters.
      (b) Dextrin fermenters.
   c. Sorbite non-fermenters.
      (a) Dextrin non-fermenters.
      (b) Dextrin fermenters.
         α. Maltose fermenters. *B. dysenteriæ*, Fexner.
         β. Maltose non-fermenters. *B. dysenteriæ* Y.

The typhoid bacillus is a remarkably stable and well-defined organism showing little or no variation, unlike most other members of the group.

All the foregoing are non-liquefiers; for convenience certain liquefying forms, *e.g. B. cloacæ*, may be placed in this group.

Chalmers and Macdonald [1] also give some useful tables of differentiation of the members of this group:—

Non-fermenters.

A. Glucose, lactose, raffinose and mannitol not fermented.
   I. *Alkaligenes Sub-group.* *B. (fœcalis) alkaligenes.*

Partial Fermenters.

B. Glucose partially fermented with production of acid only, no gas. Lactose, raffinose and mannitol may or may not be partially fermented:
   1. Motile. II. *Typhoid Sub-group.*
   2. Non-motile. III. *Dysentery Sub-group.*

Complete Monosaccharide Fermenters.

C. Glucose completely fermented with the production of acid and gas:
   a. Lactose not fermented:
      1. Mannitol and raffinose not fermented.
         IV. *Morgan I. Sub-group.*
      2. Mannitol and sometimes raffinose fermented with production of acid and gas. V. *Paratyphoid —Gärtner Sub-group.*

[1] *Lancet*, 1916, vol. ii, p. 139.

Disaccharide Fermenters.

b. Lactose partially fermented with production of acid only, no gas; mannitol partially or completely fermented; raffinose usually not fermented.

VI. *Wesenberg Sub-group.*

c. Lactose completely fermented with the production of acid and gas:

1. Bacilli encapsuled. VII. *Encapsuled Sub-group.*

2. Bacilli not encapsuled:

a. Raffinose not fermented. VIII. *Entericus Sub-group.*

Trisaccharide Fermenters.

β. Raffinose completely fermented with the production of acid and gas. IX. *Colon Sub-group.*

The *Morgan Bacillus Sub-group* IV is met with in diarrhœa (p. 417), the *Wesenberg Sub-group* VI is also met with in diarrhœa. The *Encapsuled Sub-group* VII includes the *B. pneumoniæ* (p. 467). The *Entericus Sub-group* VIII includes the *B. entericus* and *B. giumai* of Castellani and *B. khartoumensis* of Chalmers and Macdonald.

Typhoid Fever

The specific organism of typhoid or enteric [1] fever is a bacillus originally isolated by Eberth in 1880, and more closely studied by Gaffky in 1884.

The Eberth-Gaffky bacillus, or *Bacillus typhosus*, is best observed in sections of the spleen, in which it occurs in groups or colonies consisting of short rods with rounded ends, each measuring about 3 μ in length. It has also been demonstrated in the mesenteric glands (Plate XIII, *b*),

[1] It has recently been suggested to include both typhoid fever and the paratyphoid fevers under the term "enteric fevers."

and liver, in the swollen Peyer's patches before ulceration, and in other situations.

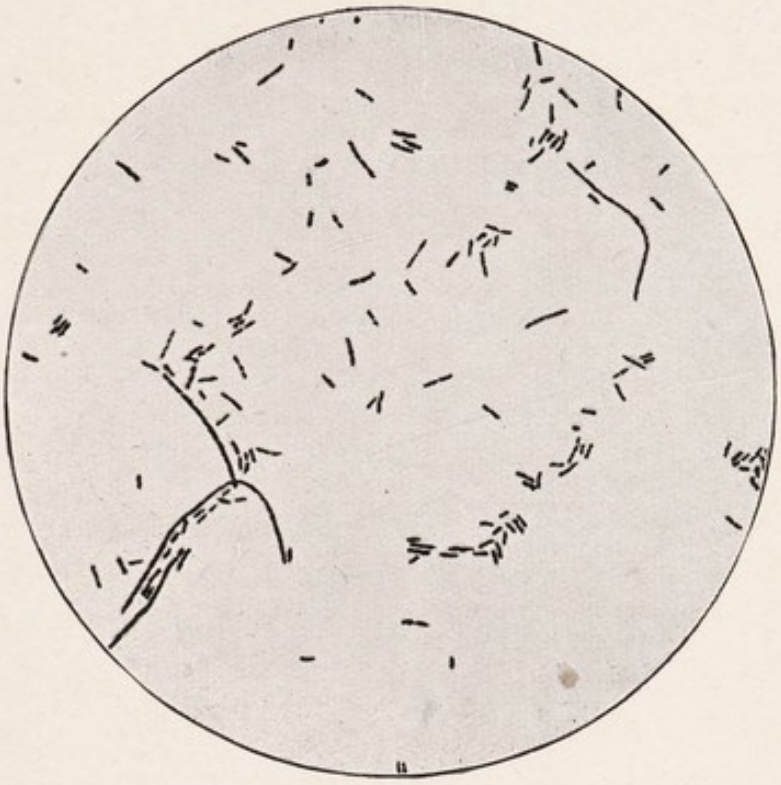
Pure cultivations may be obtained from the spleen during life by puncture (p. 414), from the blood (p. 409), sometimes from the urine and fæces, or from the spleen of a cadaver. In the latter case the organ is washed, and then cauterised lineally with a red-hot iron, in order to destroy the saprophytic bacteria on and near the surface. An incision is made with a sterilised knife through this cauterised area, and a little of the splenic pulp is taken with a sterilised platinum needle and inoculated on to tubes or plates, preferably of litmus lactose, Conradi-Drigalski, or malachite-green, agar. These are incubated at 37° C. for twenty-four to forty-eight hours, and the growths which develop are examined microscopically and are tested by agglutination and by cultural methods. The *Bacillus typhosus* has the following characters :

Morphology.—Bacilli with rounded ends averaging 3 μ in length, and 0·6 μ broad. It is, however, in cultivation a markedly pleomorphic organism, and very short rods, long rods, and thick filaments 10 to 30 μ in length occur ; the latter are known as involution forms (Plate XII, *a*). It does not form spores, but granulation and vacuolation may be observed in the protoplasm, particularly in old cultures.

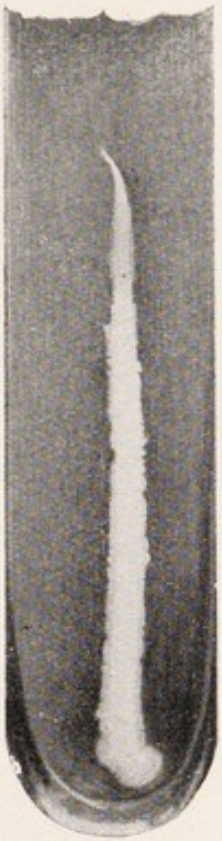
It is actively motile, and possesses a number of flagella, arranged peritrichically both at the poles and sides (Plate XII, *c*). The flagella are long and wavy, and average eight to twelve in number, a point of differentiation from the *Bacillus coli*, which usually has only three or four. It stains by the ordinary anilin dyes, but not by Gram's method.

Cultural characters.—The *B. typhosus* is aërobic and facultatively anaërobic, and grows well on the ordinary culture media. On agar it forms a thick, moist, greyish

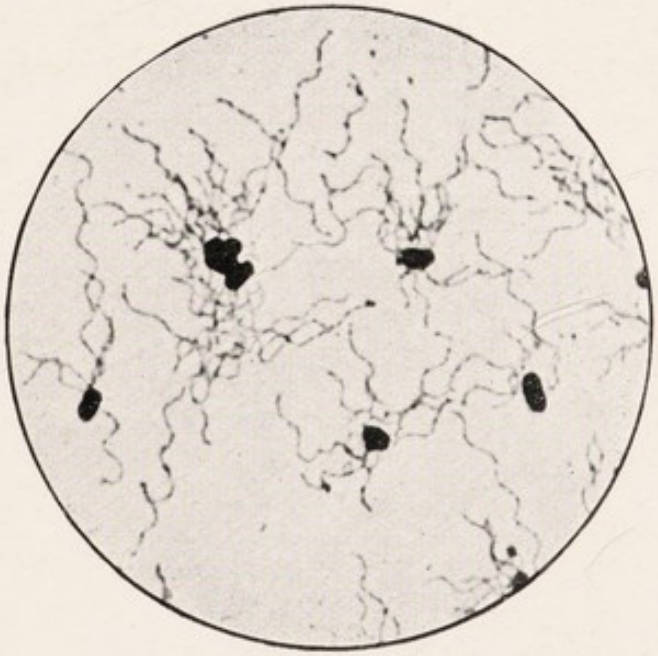
PLATE XII.



a. Bacillus typhosus. Film preparation of a pure culture. × 1500.



b. Gelatin culture of *B. typhosus*, six days old.



c Bacillus typhosus. Film preparation showing flagella. × 1500.

layer. On gelatin it grows slowly, and the growth, which is usually scanty and confined to the needle-track, is white and shining, and somewhat irregular (Plate XII, *b*). The colonies in gelatin are visible in about forty-eight hours, and form small roundish-white points, which are granular and brownish in colour by transmitted light. In broth it produces a general turbidity, without film formation. The growth on potato acid in reaction is somewhat characteristic ; it forms a moist, grey, shining layer, which is almost invisible. If, however, the reaction of the potato is neutral or alkaline, the growth may be yellowish. The *B. typhosus* grows well in milk, with slight permanent acidity, but without coagulation. A lead acetate medium is blackened.

Acid is formed in small quantity during its growth in many media (volatile fatty acids, and lactic acid), and the organism will grow in slightly acid media. Neither gas nor indole [1] is formed in cultures ; acid is produced from glucose, but no gas ; lactose is unacted upon. The fermentation reactions on various media are given in the Table on p. 432, and are there contrasted with those of the *B. coli* and other organisms (see also p. 438). Chatterjee [2] finds that agar on which the typhoid bacillus has been grown contains substances which inhibit further development of the organism if it be inoculated on to an agar culture which has been scraped so as to remove all growth.

Pathogenicity.—In cases of typhoid fever in man the *Bacillus typhosus* is widely distributed in the body, in the various tissues, and in the blood, from which it may be obtained by cultivations made from at least 0·5 c.c. (see "Clinical Diagnosis," p. 409). The bacillus is constantly

[1] Occasionally a feeble indole reaction may be obtained by careful testing.

[2] *Trans. XIVth Internat. Cong. of Hygiene* (Berlin, 1907), Bd. iv, p. 34.

present in the blood from the commencement of the disease, though not in large numbers, and cultures from the blood in competent hands result in the recovery of the organism in approximately 100 per cent. of the cases; in the later stages of the disease it is less frequently recovered. In addition to being present in the Peyer's patches, mesenteric glands, and spleen, the *B. typhosus* has been found in the rose-spots of the eruption, in the sweat, in the sputum and lungs in the pulmonary complications, and in the urine. In the urine it is so frequently present that special disinfection should be practised, more particularly during convalescence, and in some cases it may be so abundant as to produce a turbidity (typhoid bacilluria) and cystitis. It is also pyogenic, and occurs (usually in pure culture) in concurrent or post-typhoidal complications, *e.g.* empyema, abscesses, osteomyelitis, suppurating ovarian cysts, etc. Clumps of bacilli in the gall-bladder have been suggested as the nuclei of gall-stones, and the bacilli may be so numerous in the gall-bladder and bile-ducts as to cause cholecystitis and cholangitis. It is not easy to isolate the organism from the stools, and plate cultivations on special media must be employed, *e.g.* bile-salt, Conradi-Drigalski, malachite-green, or brilliant-green, agar (see p. 420).

Injected intraperitoneally into mice and guinea-pigs the *B. typhosus* usually produces death, and the same result follows from intravenous injections in rabbits, but the pathogenic effects so obtained are not specific. By continuous cultivation it loses its pathogenic properties. Given by the mouth no result follows, and the same is the experience of most observers who have fed animals on typhoid stools; a disease analogous to typhoid fever in man has rarely been induced experimentally. Remlinger [1] states that by feeding young rabbits on vege-

[1] *Ann. de l'Inst. Pasteur*, xi, 1897, p. 829.

tables, cabbage, etc., soaked in water, to which had been added some culture of the typhoid bacillus, he has succeeded in inducing a condition resembling typhoid fever in man. The charts which accompany the paper show a typical rise of temperature, a period of pyrexia with morning remission, followed by a typical fall of temperature. The animals suffered from diarrhœa, and their blood gave the agglutination reaction. Post mortem, the intestine was congested and filled with yellow diarrhœic matter, the Peyer's patches were swollen and in some places commencing to ulcerate. The spleen was increased to two or three times its normal size, and cultures of the typhoid bacillus were obtained from it. Metchnikoff [1] has infected the chimpanzee *per os* with typhoid fæces.

The proof of the causal relation of the *Bacillus typhosus* to typhoid fever is based on the following facts. It is met with in the tissues in cases of typhoid fever, can be obtained from the spleen during life by puncturing with a hollow needle, and may be isolated from the urine, fæces and blood during the course of the disease, and is not met with in other diseases. Cases of the disease have occurred in institutions which could not be traced to any source of infection other than the presence of a well "carrier" having the organism in the intestine. The writer has had under his care three cases, and knows of several others, in which the disease was almost certainly contracted in the laboratory from working with pure cultures. Agglutinins specific for the *B. typhosus* are present in the blood of the patient and in that of an animal inoculated with the organism, but are practically absent from the blood of those who have not suffered from an attack and in other diseases (Plate XIII, *a*). This indicates that in the body of an individual suffering from typhoid fever the same substances are formed as in an animal artifi-

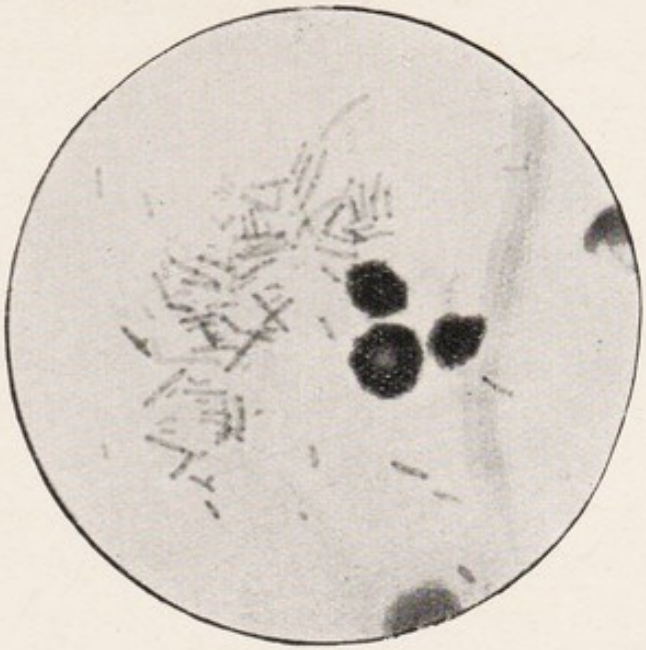
[1] See *Ann. de l'Inst. Pasteur*, xxv, 1911, p. 193.

cially immunised by cultures of the *B. typhosus*. This reaction is now recognised as a valuable clinical test in doubtful cases of enteric fever (the " Widal " or agglutination reaction [1]). Finally, inoculation with a vaccine consisting of killed culture of the *B. typhosus* confers considerable protection against acquiring the disease.

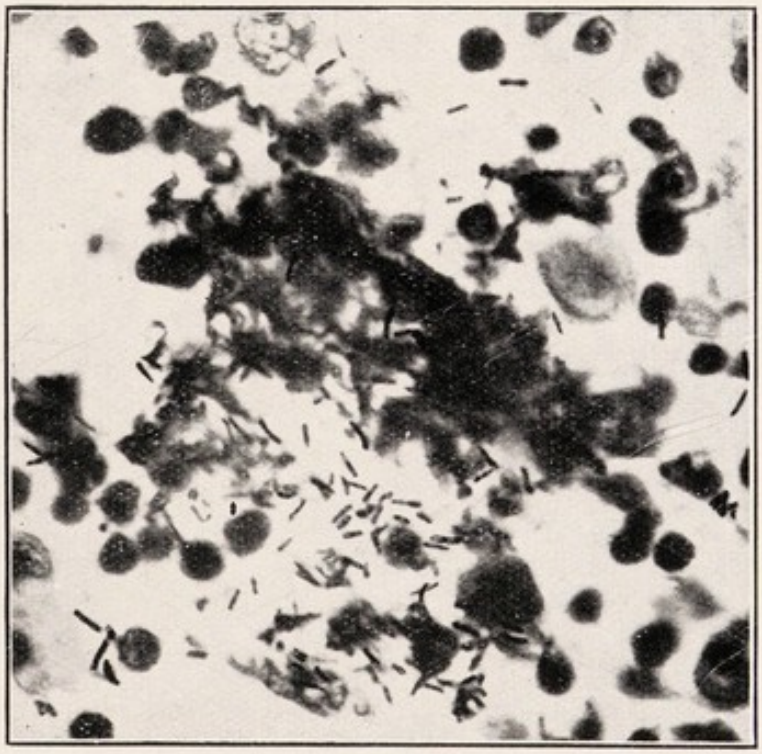
The agglutination reaction.—For the method of carrying out the agglutination reaction see p. 410. *Normal* serum will generally agglutinate the typhoid bacillus in a dilution up to 1 in 3 or 4, but occasionally is more active. Dead bacilli may be used. The reaction is not obtained before the sixth or seventh day of fever, occasionally not until much later. Very rarely the reaction seems to be intermittent. The blood may retain its agglutinating power for years after an attack, and inoculation with anti-typhoid vaccine also confers agglutinative properties. Cases do occur in which agglutination is absent throughout, but they are rare and often tend to be severe and to terminate fatally. Usually, if the blood during the course of an attack fails to give a reaction when tested on three occasions at intervals of three to four days, it is improbable that the case is one of typhoid fever ; it may be a case of paratyphoid fever due to infection with one of the paratyphoid bacilli which belong to the Gärtner group of organisms (see p. 418). If a positive reaction be obtained, yet the case does not seem to be one of typhoid, a previous attack or inoculation with typhoid vaccine must be excluded. Agglutination may be obtained up to two or three years, after an attack of typhoid fever, but the reaction has generally practically disappeared nine months after anti-typhoid inoculation. The previous injection of a typhoid anti-serum into the patient might induce a non-typhoid infection to give the reaction.

[1] Some controversy has arisen as to the discoverer of this reaction. Grünbaum claims to have first observed it.

PLATE XIII.



a. The agglutination reaction. A clump of typhoid bacilli. × 1500.



b. *Bacillus typhosus* in mesenteric gland. Thionine blue. × 1000.

Gwyn [1] found that out of 265 cases diagnosed as typhoid and accurately studied, only one persistently failed to give the reaction. The blood of this case, however, reacted typically with a Gärtner-like organism obtained from the blood (a case, therefore, of paratyphoid infection). Cases of mixed infection with typhoid and paratyphoid bacilli occasionally occur.

Johnson and McTaggart [2] found that typhoid blood dried for sixty days still gave a typical agglutination reaction. An incomplete reaction was occasionally obtained as early as the end of the second day, and the complete reaction was rarely delayed beyond the fifth day. They also noticed that the blood of the horse often produced clumping, etc., of typhoid bacilli, indistinguishable from an agglutination reaction with typhoid blood; but the same agglutinating effect was also produced on the colon bacillus. Many chemical substances also produce agglutination of typhoid bacilli, so that it is necessary to exclude them in making a diagnosis. For example, corrosive sublimate (0·7 : 1000), alcohol, salicylic acid, vesuvin, and safranin (1 : 1000) agglutinate, while carbolic and lactic acids, chloroform, caustic soda, and ammonia do not, the two last only provided the test typhoid emulsion be made with distilled water. Safranin has a powerful agglutinating action on the typhoid bacillus, but not on the colon bacillus.

While there is no constant connection between the activity of agglutination and the severity of the disease, active agglutination tends to go with cases which recover, and cases in which agglutination is feeble or absent tend to be severe.

Toxins.—From cultures of the typhoid bacillus Brieger isolated a base which he termed typhotoxin, and which is

[1] *Johns Hopkins Hosp. Bull.*, vol. viii, 1900, p. 387.
[2] *Brit. Med. Journ.*, 1896, vol. ii, p. 629.

isomeric with gadinine. In animals it produced salivation, profuse diarrhœa, paralysis, and death. Brieger and Fränkel isolated from cultures a toxic protein body. Fenwick and Bokenham [1] extracted from spleens of typhoid fever patients a proteose, an alkaloid, and a fatty residue. The proteose produced fever, anorexia, and loss of weight in guinea-pigs and rabbits, but the alkaloid and fatty matter were without effect.

The toxins of the typhoid bacillus, however, seem to be largely intra-cellular, and filtered broth cultures are usually almost non-toxic. Sidney Martin [2] by cultivating in a protein medium was able sometimes to obtain a toxic filtrate, a few c.c.'s of which produced lowered temperature, diarrhœa and death. Macfadyen and Rowland,[3] by disintegrating large quantities of typhoid bacilli, filtering, and so obtaining the intracellular constituents in the filtrate, found that small doses of the latter produced a transient rise of temperature in guinea-pigs and a loss of weight which was soon recovered from. Animals so treated were protected against a certain lethal dose of typhoid bacilli, and their blood exhibited agglutinative and bacteriolytic properties towards the typhoid bacillus. Macfadyen [4] later obtained the intra-cellular juice of typhoid bacilli by disintegration after freezing with liquid air, and found it to be very toxic to guinea-pigs by intra-peritoneal, and to rabbits by intra-venous, inoculation. The writer found that cultures of the *Bacillus typhosus* do not give the "diazo" reaction.

Survival of the typhoid bacillus in the body.—Bacilli may persist in the spleen for weeks, in the gall-bladder for years, and in suppurative lesions for six years or more.

[1] *Brit. Med. Journ.*, 1895, vol. i, p. 801.
[2] *Ibid.* 1898, vol. ii, pp. 11 and 73.
[3] *Centr. f. Bakt.*, xxx, p. 753.
[4] *Proc. Roy. Soc. Lond.*, B. lxxi, 1902, p. 77.

Foster and Kayser obtained pure cultures from the gall-bladders of seven out of eight cases, and in 2 per cent. of the cases this "cholecystitis typhosa" becomes a chronic process, and typhoid bacilli may be discharged into the bowel for long periods. Dean [1] found this to be the case in a patient who had had enteric fever twenty-nine years previously. Such "typhoid carriers" have been the subject of much investigation recently.[2] A. and J. Ledingham record three instances met with in an asylum in which mysterious cases of typhoid had occurred—31 cases during fourteen years. Davies and Walker Hall [3] relate similar outbreaks, the carrier in this case being a woman who had suffered from enteric fever in 1901, milk serving as the vehicle of transmission, and a number of other instances have been recorded. Three-fourths of the cases are women (and three-fourths of the cases of gall-stones occur in women), and usually the serum of the carriers gives a marked agglutination reaction, and their stools frequently contain such large numbers of typhoid bacilli that these largely replace the natural bacterial flora of the intestine and may often be recovered from the stools by simple plating. Firth's statistics give an idea of the frequency of the development of the carrier state. Of 1229 cases of enteric fever among the British troops in India bacteriologically examined, 13 cases of chronic carriers and 13 cases of temporary carriers were detected. Obviously the typhoid carrier is a source of serious risk to the community, and mysterious outbreaks of enteric fever, ascribed by some in the past to a "*de novo*" origin of the specific organism, become explicable. Typhoid convalescents should be bacterio-

[1] *Brit. Med. Journ.*, 1908, vol. i. p. 562.

[2] See Ledingham, *Rep. Med. Off. Loc. Gov. Board* for 1909–10 (Bibliog.); *ibid.* for 1912–13, p. 336.

[3] *Proc. Roy. Soc. Med.*, vol. i, 1908, Epidemiolog. Sect., p. 175.

logically examined three or four times at weekly intervals before discharge from hospital, and the negative cases may with reasonable safety be allowed to resume their civil life (Ledingham). The typhoid bacillus may occur in the contents of ovarian cysts, usually causing suppuration, and may survive for months—twelve in a case recorded by Taylor [1]—after the attack of typhoid.

Survival of the typhoid bacillus outside the body.—The *Bacillus typhosus* has been isolated in a few instances from WATER SUPPLIES which have become infected, and have given rise to epidemics, as in the case of the Lincoln epidemic in 1905.[2] This is the exception, however, and the isolation of the typhoid bacillus from an infected water is a very difficult matter on account of the fact that the bacillus may have died out before the investigation is commenced, that it is generally in a small minority and admixed with numbers of coliform organisms, and that until recently no medium was available which inhibited the growth of the coliform organisms without at the same time inhibiting the growth of the *B. typhosus*. By the use of malachite or brilliant green media, the last-named difficulty seems to have been overcome (see section on "Water").

In sterilised waters, including distilled water, the *Bacillus typhosus* maintains its vitality for upwards of a month, and in some cases for much longer. The survival is not necessarily longer in an organically polluted water than in a pure water. Infecting sterilised Thames water (from the Temple Embankment) and sterilised tap-water of the Chelsea Water-works with typhoid cultures, the writer found that, examining small quantities (1 c.c.) of the water, the bacillus appeared to die out in the former in two to three weeks, in the latter in four to five weeks.

[1] *Journ. Obstet. and Gynæcol. Brit. Empire*, November. 1907.

[2] *Rep. Med. Off. Loc. Gov. Board* for 1905–06.

The survival of the typhoid bacillus in natural waters must be influenced by many circumstances—temperature, chemical composition, struggle for existence with the natural bacterial flora, etc., of the water. Experiments by Russell and Fuller,[1] in which the organism, suspended in collodion sacs, was subjected to the action of lake water, indicated that the maximum was eight to ten days. Houston,[2] using raw Thames, Lee, and New River waters artificially infected with varying quantities of ordinary laboratory typhoid cultures, and examining quantities of 100 c.c. of the water, found that in none of eighteen experiments was a negative result obtained in four weeks, and it was only after nine weeks that the typhoid bacillus could not be isolated from this quantity in all the experiments. But in subsequent experiments,[3] in which typhoid bacilli, obtained directly from the urine of a carrier case by centrifuging and without culturing, were added to the water, the number of bacilli was reduced by 99·99 per cent. after a week, and after ten days the organism could not be isolated from 100 c.c. of the infected water, indicating that the uncultured bacillus rapidly dies in a natural water, and that even a week's storage of water affords enormous protection against water-borne typhoid. In aërated (CO_2) waters the *B. typhosus* does not survive a fortnight. The methods of isolation from water are given in Chapter XXI.

The *Bacillus typhosus* may gain access to shell-fish,[4] oysters, mussels, cockles, etc., particularly if obtained

[1] *Journ. Infect. Diseases*, Sup. No. 2, February 1902, p. 40.

[2] *First Rep. on Research Work*, Metropolitan Water Board, 1908.

[3] *Sixth Research Report*, Metropolitan Water Board, 1911.

[4] On pathogenic organisms in shell-fish see Reports by Bulstrode to the Local Government Board, 1894 and 1911 ; *Rep. Med. Off. Loc. Gov. Board* for 1899–1900, p. 574 ; Houston, *Fourth Report of the Sewage Commission*, vol. iii, 1904 ; McWeeney, *Loc. Gov. Board, Ireland*, 1904 ; Buchan, *Journ. of Hygiene*, vol. x, 1910, p. 569.

from sewage-polluted laying. Such polluted shell-fish may give rise to typhoid epidemics—as at Winchester and Southampton in the case of oysters, and in the case of cockles, derived from the Thames Estuary and imperfectly cooked, to typhoid cases. Buchan found that out of 855 primary cases of typhoid fever occurring in households in Birmingham, 124, or 14·5 per cent., had a history of mussel eating, and in seventeen instances the histories were conclusive of mussel infection. Mussels, under certain conditions (which are not well understood), are liable to develop mytilotoxin, etc. (p. 40), which gives rise to gastro-enteritis. Shell-fish from sewage-polluted layings contain *B. coli* in varying numbers, but from uncontaminated layings are free from this organism, which may therefore serve as an index of pollution (see "Examination of Shell-Fish," Chapter XXI). Contaminated shell-fish, removed to pure water, gradually cleanse themselves—probably after two to three weeks' sojourn. Klein obtained the typhoid bacillus from artificially infected oysters, kept in tanks of sea-water, after nine, sixteen, and even eighteen days from the commencement of the experiment, the oysters showing no abnormal condition.

As regards the vitality of the *Bacillus typhosus* in sewage we have little certain information; probably it tends to die out within a few days. In sterilised sewage inoculated with it the *B. typhosus* hardly multiplies at all, and at the end of ten days has died out. Certain organisms in sewage seemed to have a deleterious action on the *B. typhosus*, hastening its extinction, viz. the *B. fluorescens liquefaciens* and *B. fluorescens stercoralis*. Russell and Fuller, subjecting the bacillus to the direct action of sewage, found the survival to range from three to five days.

In *dry* garden earth, according to Dempster,[1] the *Bacil-*

[1] *Med.-Chir. Trans.*, vol. lxxvii, 1894, p. 263.

lus typhosus does not live longer than eighteen days (Firth and Horrocks recovered it up to twenty-five days), and in peat it dies within twenty-four hours. In *moist* soil, however, the bacillus still survived on the forty-second day. In an *artificially* dried soil it was not found alive after the seventh day.

Sidney Martin found that in moist *sterilised* soil kept at temperatures from 3° to 37° C., the *B. typhosus* maintains its vitality for upwards of fifteen months, but that in unsterilised soil it rapidly dies.[1]

Mair [2] concludes that the typhoid bacillus can survive in natural soil in large numbers for about twenty days, and is still present in a living condition after seventy to eighty days, but that there is no evidence that it is capable of multiplying and leading a saprophytic existence in ordinary soil. He suggests that Martin's result (the rapid extinction of the bacillus in unsterilised soil) may be explained by the use of *broth* cultures for infection, the broth added causing a multiplication of the saprophytes. Firth and Horrocks [3] similarly conclude that the typhoid bacillus displays no tendency to increase in numbers, nor to grow upwards or downwards in soil, though it may be washed by water through a thickness of 18 inches. Neither virgin nor sewage-polluted soils differed much in these respects.

Vitality of B. typhosus in dust, fomites, etc.—Firth and Horrocks found the *B. typhosus* to be alive in soil dry enough to form dust for as long as twenty-five days, and consider that infective material can be readily transmitted from dried soil and sand by means of winds and air-currents. Doubtless much depends on the degree of dryness of the substratum. From khaki drill and serge

[1] *Reps. Med. Off. Loc. Gov. Board* for 1896–1901.
[2] *Journ. of Hygiene*, vol. viii, 1908, p. 37.
[3] *Brit. Med. Journ.*, 1902, vol. ii, p. 936.

inoculated with cultures the bacillus was recoverable for from ten to twelve weeks, and for from ten to seventeen days from the same materials fouled with enteric fæces.

Semple and Grieg,[1] with cloth and blanket infected with typhoid urine, failed to obtain the bacillus after seventeen days. This, however, was in India, and the survival of the typhoid bacillus on fomites probably greatly depends on the degree of drying of the material. A striking instance of the conveyance of infection by fomites was that of the blankets used in the South African War and brought to this country, which gave rise to many cases of typhoid fever.

Firth and Horrocks demonstrated that house-flies can convey enteric infective material from specific excreta or other polluted material to objects on which they settle or feed, and the Commission which investigated the prevalence of enteric fever in the Spanish-American War ascribed to flies the principal part in the dissemination of the disease (see also p. 443).

There has always been considerable discussion on the exact relation of "sewer-gas" to disease. It is generally held that sewer-gas is at least a predisposing cause to enteric fever, diphtheria and tonsillitis. Some have considered that the specific organisms are present in the emanations from sewers, and this may occasionally be the case. Thus Horrocks,[2] in some experiments performed at Gibraltar, by pouring sewage artificially infected with typhoid culture down drains, showed that specific bacteria present in sewage may be ejected into the air of ventilating pipes, inspection chambers, drains and sewers by (*a*) the bursting of bubbles at the surface of the sewage, (*b*) the separation of dried particles from the walls of pipes, chambers and sewers, and probably by (*c*) the ejection of

[1] *Sc. Mem. Gov. of India*, No. 32, 1908.

[2] *Journ. Roy. San. Inst.*, May 1907, p. 176.

minute droplets from flowing sewage. "Sewer-gas" may also lower vitality and increase susceptibility. Thus Alessi found that animals exposed to drain emanations are at first more susceptible to infection, but after a month or so acquire tolerance and are no more susceptible than animals kept under ordinary conditions. Exposure to the gaseous emanations from putrefying matter is stated by Trillat to increase the virulence of pathogenic bacteria. There is no evidence that sewer-men or those employed at sewage-works suffer from ill-health.

Action of heat, germicides, etc.—The *B. typhosus* in broth culture is killed by a temperature of 53°–54° C. in half an hour, and of 56°–60° C. in ten minutes. It is readily destroyed by antiseptics. (See Table, Chap. XXII.)

Semple and Grieg (*loc. cit.*) found bright sunlight to be germicidal in from two to six hours.

Wines and spirits have some germicidal action on the typhoid bacillus. Champagne destroys the bacillus in ten minutes, white wines in fifteen to twenty minutes, red wines in thirty minutes or thereabouts. If diluted with water the germicidal action takes much longer to accomplish, and the acidity, not the alcohol content, seems to be the determining factor.[1] Spirits, such as whisky or brandy, if diluted with not more than one to two times the volume of water, kill in ten to twenty minutes.

Anti-typhoid serum.—Attempts have been made to prepare an anti-typhoid serum by inoculating horses with increasing doses of typhoid bacilli, first killed (by heat, chloroform, etc.) and then living, but such sera have proved quite useless.

Macfadyen [2] prepared an endotoxic serum by treating horses with the endotoxin obtained by triturating the

[1] Sabrazès and Marcandier, *Ann. de l'Inst. Pasteur*, 1907.

[2] *Proc. Roy. Soc. Lond.*, B, vol. lxxi, 1903, pp. 76 and 351; *Brit. Med. Journ.*, 1906, vol. i, p. 905.

bacilli in the presence of liquid air. The writer continued the work, and obtained a serum which gave promising results.[1]

Chantemesse,[2] by cultivating a virulent strain of the typhoid bacillus in a special broth made with ox spleen, heating the culture to 55° C., centrifuging and injecting horses with the fluid, obtains a serum which he claims has marked curative properties, the mortality being 4·3 per cent., as against 17 per cent. for those subjected to ordinary treatment. The patients receive very small doses of the serum—five or six drops—and the dose is repeated only two or three times. This dosage is quite different from that of an ordinary antitoxic or antimicrobic serum, and Wright suggested that *toxins* (and not anti-bodies) in the serum may be the active agents. Chantemesse has accepted this view, and the treatment, therefore, seems to be a vaccine one.

The disease has also been treated with a *vaccine* (consisting of a killed culture) with promising results by Semple, Smallman, Leishman, and others. The initial dose is 40–100 millions, and the amount is cautiously increased up to 300–400 millions.

Anti-typhoid vaccine.—Wright first prepared an anti-typhoid vaccine by the following method.[3] A typhoid culture of moderate virulence (the virulence being kept up by intraperitoneal passage through guinea-pigs) is grown in peptone beef broth in flasks at 37° C. for from fourteen to twenty-one days. The flasks are then so heated that their contents attain, and remain at for a few minutes, a temperature of 60° C. To obtain uniform

[1] See Hewlett, Goodall and Bruce, *Proc. Roy. Soc. Med.*, vol. ii, 1907–08 (Medical Section), p. 245 *et seq.*; and Hewlett's *Serum Therapy*, p. 220.

[2] *Trans. Fourteenth Internat. Cong. Hygiene and Demography*, 1907.

[3] Wright and Semple, *Brit. Med. Journ.*, 1897, vol. i, p. 256.

toxicity, the contents of several flasks should be mixed, and to safeguard the vaccine from contamination one twentieth of its volume of 10 per cent. lysol is added. Various ingenious devices have been adopted by Wright and Leishman to prevent contamination and for standardisation.

The immunising power of a typhoid vaccine depends upon the number of bacilli it contains, and on the particular strain of bacillus used. The vaccine is standardised by counting the number of bacilli it contains by Wright's method (p. 242). Leishman [1] now cultivates for about forty-two hours, and the bacteria are killed by heating to 53° C. for one hour, the higher temperature having proved to be deleterious, and after cooling 0·25 per cent. of lysol is added ; it is not necessary to employ a virulent bacillus. In the early days the symptoms produced by the inoculation were often severe, but with more moderate doses are now hardly appreciable. Two doses of the vaccine should be given, with an interval of about ten days between the two, the doses being 500 and 1000 millions respectively. The vaccine deteriorates on keeping. Emulsions of agar cultures and autolysed cultures have also been used for preparing vaccines. Polyvalent vaccines have been introduced by Castellani, and are now being used, *e.g.* Typhoid and Paratyphoid A and B. Cholera may also be introduced into it.

Inoculation is now being extensively practised, and Leishman (*loc. cit.*) gives the following statistics of its value : total number under observation, 18,483–19,314 ; average period under observation, twenty months ; number inoculated, 10,378 ; number uninoculated, 8,936 ; case-incidence of enteric per 1000, inoculated 5·39 $\pm$0·48, uninoculated 30·4 $\pm$1·23 ; case-mortality per 100, inoculated 8·9, uninoculated 16·9. In the French navy Chante-

[1] See *Journ. Roy. Inst. Pub. Health*, vol. xviii, 1910, pp. 385, 449, 513.

messe states that during nine months in 1912, among 67,843 unvaccinated persons 542 cases of typhoid fever occurred, while among 3,107 vaccinated ones not a single case of typhoid occurred.

Variation of the B. typhosus.—Allusion has already been made to Twort's work on the "education" of *B. typhosus* to ferment lactose, and on the apparent conversion of *B. typhosus* into *B. alkaligines* by Horrocks (p. 6). Penfold also records variations in the fermentive powers of *B. typhosus* (*Journal of Hygiene*, vol. xi, 1911, p. 30).

Other Continued Fevers

Besides typhoid and paratyphoid fevers, other continued, enteric-like fevers occasionally occur caused by members of the typhoid-colon group. For instance, Castellani has recorded cases in Ceylon and the Balkans caused by *B. columbensis.*

Relapses

Various hypotheses have been advanced to account for the relapses which occur in typhoid and other diseases (*e.g.* Malta and relapsing fevers). Chantemesse and Widal [1] showed that if the *B. typhosus* is injected into an animal together with toxins of the streptococcus, *B. coli*, or *Proteus*, its virulence is enhanced, or the animal's resistance may be lowered. If, then, immunising and bactericidal properties of the blood and tissues are but slightly acquired during the attack, an absorption of toxic substances from the alimentary tract may be sufficient to give the typhoid bacilli still present a fresh start, and so produce a relapse. This Sanarelli [2] was able to do experimentally. This "sensitisation" may be the cause of relapses in rheumatic fever. Wright and Lamb formulated another hypothesis.[3] The organisms in typhoid, Malta, and relapsing fevers, are deposited in the spleen and internal organs, multiply and form colonies there, which become

[1] *Ann. de l'Inst. Pasteur*, vi, 1892, p. 755.

[2] *Ibid.* vi, 1892, p. 721 ; and *ibid.* viii, 1894, p. 193.

[3] *Lancet*, 1899, vol. ii, p. 1727 ; *Sc. Mem. Med. Officers of Ind. Army*, pt. xii.

protected from the bactericidal substances by the formation of a non-anti-bacterial envelope. When the anti-bacterial substances in the blood and lymph have increased to such an extent as to penetrate and abolish the non-anti-bacterial envelopes which surround these colonies, the production of toxins will be so diminished that the temperature will fall. If, however, for some reason or other, even a single colony escapes the full anti-bacterial power of the lymph, owing, it may be, to being shut off in a capillary which has become blocked, or in some other part not freely infiltrated by the blood- or lymph-streams, the bacteria of this colony will go on multiplying until the blood has become modified in such a manner as to bring about a diminution of the anti-bacterial substances, and thus render a relapse possible.

A third theory was suggested by Durham.[1] He regards a given infection as due to the "result of the action of a sum of a number of infecting agents, each of which is similar but not identical in its nature," the apparently simple infection being "in reality a complex phenomenon brought about by a number of varieties and sub-varieties of the given microbe." He suggests, therefore, that in a typhoid infection a particular race of typhoid bacilli is in excess, and when the anti-bodies for this particular race have been formed in sufficient quantity, the disease process comes to an end. There may, however, be present at the same time other races which have produced little of their specific anti-bodies; these then begin to grow and multiply, and a relapse ensues.

The acquisition of the state of "fastness" by bacteria and protozoa under the influence of specific serums and drugs has already been alluded to (p 220). It is quite likely that in typhoid fever and some other diseases, the relapse may be due to the infecting organism becoming "fast" or resistant to the anti-bacterial substances, so that it again grows and multiplies and produces a recrudescence of the disease.

In the case of relapsing fever the organism is probably a protozoon, and in protozoal diseases relapses coincide with developmental cycles of the parasite, *e.g.* in malaria.

Clinical Diagnosis

(I) *Blood cultures.*—Five to 10 c.c. of blood are withdrawn from a superficial vein with a syringe with aseptic precautions,

[1] *Journ. Path. and Bact.*, vol. vii, 1901, No. 2, p. 240.

and sown into one or two tubes containing 15 to 20 c.c. of broth, or trypsin broth. The tubes are incubated at 37° C., and if organisms develop these are isolated and examined culturally and by agglutination for the typhoid bacillus. Coleman and Buxton recommend the following culture medium: Ox-bile 90 c.c., glycerin 10 c.c., and peptone 2 grm. Distribute in small flasks, 20 c.c. in each, and sterilise. Each flask is inoculated with 2 to 3 c.c. of blood, incubated for eighteen to twenty-four hours, then streaks from each are made on to litmus lactose agar plates, which are incubated for a few hours. If the growth does *not* redden the medium and a typhoid-like bacillus is present, it is tested for agglutination with typhoid-immune serum.

(II) *Agglutination reaction.*—This may be carried out by the microscopic or the macroscopic (sedimentation) method described at p. 210. Dilutions of 1 : 30, 1 : 50, and 1 : 100 should be made. The microscopic method is the more rapid. Various apparatus (agglutinometers) can be obtained, consisting of measuring devices and a supply of dead culture, with which the sedimentation test can be carried out by any one, but are unsatisfactory in the tropics.

Agglutination is now commonly carried out by Dreyer's "Standard Method" (p. 209), the blood serum being tested for typhoid and paratyphoid A and B agglutinations at the same time. The apparatus (supplied by Messrs. Baird and Tattock, 14 Cross Street, Hatton Garden, E.C.) consists of a small metal stand to hold 16 tubes—one larger dilution tube on the left-hand side and 15 smaller agglutination tubes in three rows of 5 each—a dropping pipette with teat and a supply of standard agglutinable cultures of typhoid and paratyphoid A and B (obtainable from the Department of Pathology, University of Oxford). The following are the directions issued:—

1. Technique.

Take a stand containing 15 agglutination tubes in 3 rows of 5 each, and a dilution tube.

With the proper dropping pipette measure out into the dilution tube 54 drops of normal saline solution, 0·85 per cent. sodium chloride, in distilled water (where the water supply is pure, tap-water can be used instead of saline solution) by means of gentle pressure on the teat.

Wash the pipette with distilled water.

Dry out the pipette with successive quantities of absolute

alcohol, followed by successive quantities of ether, and get rid of the ether.

Take up the serum to be tested into the dried pipette. Measure out 6 drops of the serum into the dilution tube already containing the 54 drops of saline solution, thus obtaining a dilution of 1 in 10. Mix thoroughly.

Carefully wash out the pipette.

With the pipette measure out into each row of tubes as follows :

Number of tube.	Drops of Normal Saline Solution.	Drops of Serum Dilution 1 in 10.	
1	0	10	to each tube in row 1 add 15 drops of *B. Typhosus* Standard Agglutinable Culture.
2	5	5	to each tube in row 2 add 15 drops of *B. Paratyphosus A.* Standard Agglutinable Culture.
3	8	2	
4	9	1	to each tube in row 3 add 15 drops of *B. Paratyphosus B.* Standard Agglutinable Culture.
5	10	0	

At each stage of the procedure the pipette is carefully washed and dried out as before described.

Shake each tube thoroughly in order from right to left, *i.e.* beginning each row with the highest dilution.

Place the stand for 2 hours in a water-bath at 50°–55° C. (*not* in dry air).

In Tube 1 of each row the serum acts in a dilution of 1 in 25.
„ 2 „ „ „ 1 in 50.
„ 3 „ „ „ 1 in 125.
„ 4 „ „ „ 1 in 250.
Tube 5 containing no serum is control against spontaneous agglutination.

If the limit of agglutination is not reached within this series higher dilutions are followed out in a similar manner.

The tubes are examined after 2 hours at 50°–55 C. followed by 15 minutes' standing at room temperature. The reading is taken by comparing each tube in succession with the control tube, and is preferably made by means of artificial light against a black background. If daylight is used, the tubes inspected should be partly shadowed by passing a finger up and down behind them.

The highest dilution in which marked agglutination (without

sedimentation) can be detected by the naked eye is *Standard Agglutination.* But owing to the rate at which the dilution increases in the series of tubes employed it will commonly happen that no tube in the series exhibits *Standard Agglutination.* If this be so it will be found in looking along the series that while one tube shows strong agglutination with sedimentation the next succeeding tube shows no agglutination at all or only a trace. In such cases Standard Agglutination lies approximately midway between the two dilutions.

(If the stand is left at the room temperature, 16 to 24 hours must be allowed before the reading is taken, but the reaction is not then so sharply defined. In this case the highest dilution in which a definite flocculent sedimentation appears corresponds approximately to Standard Agglutination.)

When the standard degree of agglutination ("*Standard Agglutination*") occurs with Standard Agglutinable Culture in a serum dilution of 1 in x, then x divided by the figure given on the label of the standard Agglutinable Culture employed gives the number of "*Standard Agglutinin Units*" [1] contained in 1 c.c. of the serum examined.

Thus if standard agglutination occurs in a dilution of 1 in 1000 and the number on the label is 2·5, then $\frac{1000}{2 \cdot 5}$, *i.e.* 400, is the number of Standard Agglutinin Units contained in 1 c.c. of the serum examined.

For uniformity and simplicity in recording results they should be expressed in *Standard Agglutinin Units.*

2. Diagnosis.

A. In non-inoculated persons who have not had typhoid (or paratyphoid) fever, agglutination in a dilution of 1 in 25 justifies a strong suspicion of typhoid (or paratyphoid) infection. But the test must be applied again in the course of a few days to ascertain whether there is any change in the titre of agglutination. Marked agglutination in a dilution of 1 in 50 or more is nearly always diagnostic of active typhoid (or paratyphoid) infection.

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[1] *Note.*—The *Standard Agglutinin Unit* is that amount of agglutinating serum which when made up to 1 c.c volume with normal saline solution causes Standard Agglutination on being mixed with 1·5 c.c. of a particular Standard Agglutinable Culture and maintained at 55° C. for 2 hours in a water-bath followed by 15 minutes at the room temperature.

A *non-inoculated* "carrier" will normally show no important change in the titre of this serum on repeated examination at short intervals.

B. Inoculated persons if quite recently inoculated will usually show a high titre of specific agglutination. A rapid rise in titre sets in within two to four days of inoculation. This is followed by a fall at first rapid, but subsequently becoming very slow, so that a relatively high titre is maintained for a long period (even for years). During this period examinations made at intervals of a few days give practically identical readings.

It follows that in the case of inoculated persons the diagnosis of active typhoid (or paratyphoid) infection will require two or more successive examinations of the serum.

(*a*) If the individual is suffering from active *typhoid* infection his titre of typhoid agglutination will exhibit the usual rise and subsequent regular fall seen in non-inoculated subjects, but starting from and returning towards the higher base line of inoculated persons.

(*b*) If the individual is suffering from active *paratyphoid* infection one of three things may occur as regards his *typhoid* agglutination titre, namely :

1. No appreciable change may occur in the titre of typhoid agglutination.
2. A relatively slight rise may occur, followed by a fall towards the former level.
3. A marked rise may occur synchronous with the rise in paratyphoid agglutination titre, and subsequently followed by the usual fall towards the former level.

Meanwhile the titre of *paratyphoid* agglutination runs the normal course of rapid rise to a maximum (usually exceeding the maximum typhoid titre) followed by a fall, at first rapid and then slower as already described for typhoid subjects, and falling *below* the persistent base line of typhoid agglutination of inoculated persons.

C. In the case of **mixed infections** whether in inoculated or non-inoculated persons the agglutinin curves for the different infecting organisms are usually not synchronous, and they pursue their ordinary course independently of each other.

(III) *Ophthalmo-diagnosis.*—Chantemesse[1] (*loc. cit.*) has devised a method analogous to the ophthalmo-diagnosis for tuberculosis (p. 378). The material is prepared from agar cultures of typhoid

which are emulsified, dried, triturated, and extracted, and the extract is precipitated with absolute alcohol and dried (for details see Hewlett's *Serum Therapy*, p. 382). The dry substance is powdered in an agate mortar, and for use 8 to 10 mgrm. are dissolved in 1 c.c. of sterile water. Of this solution a drop is instilled into the conjunctival sac ; in a case of typhoid, after a lapse of two to three hours the conjunctiva becomes red and there is a sensation of heat, after six to ten hours there is a marked conjunctivitis, which may persist for one to three days and then passes off. In healthy persons and in other diseases no conjunctivitis ensues. A cutaneous reaction has also been devised.

(IV) *Puncture of the spleen with a sterilised hypodermic needle and syringe.*—A little of the blood and pulp is withdrawn with the syringe, and cultivations are made as in (I). This method seems hardly justifiable, and now that the blood-culture method and agglutination reaction have been introduced should be discarded.

(V) *Examination of pus.*—Cultivations may be made as in (I) if the bacillus is present, apparently in pure culture. If not, plate cultivations, preferably on litmus lactose agar, Conradi-Drigalski, malachite- or brilliant-green, agar, may be prepared (see " Water ").

(VI) *Examination of the fæces and urine.*—See p. 376, 377.

The Gärtner or Enteritidis Group of Bacilli

The Gärtner group of bacilli, of which the type is the *B. enteritidis* of Gärtner, are bacilli morphologically resembling the *B. typhosus*, *i.e.* they are pleomorphic, actively motile, multiflagellate, non-sporing, and non-Gram-staining, but culturally are intermediate between *B. typhosus* and *B. coli*. Thus, like *B. coli*, they ferment glucose with the production of gas and acid and change neutral red ; like *B. typhosus* they do not attack lactose and do not curdle milk. In litmus milk they usually first produce slight acidity, followed after three to four days by a change to alkalinity, and the milk ultimately becomes limpid. The fermentation reactions of some members of the Gärtner group are given in the Table on p. 428 *et seq.* The organisms of the Gärtner group may be divided into four sub-groups :

1. *Enteritidis group.*—Produce acute gastro-intestinal disturbance in man. The cause of epidemic meat-poisoning, *e.g.* the *B. enteritidis* of Gärtner.

2. *Pneumonic group.*—Produce pneumonic symptoms in man. The cause of some outbreaks of epidemic pneumonia, *e.g. B. psittacosis.*

3. *Paratyphoid group.*—Produce a disease resembling typhoid fever in man. Subdivisions A and B.

4. *Group non-pathogenic to man, e.g. B. typhi murium.*

The Bacillus enteritidis

A number of outbreaks of what has been termed "epidemic meat poisoning" have been traced to infection with the *B. enteritidis.* (See also "Food Poisoning," Chap. XXI.) The disease takes the form of an acute gastro-enteritis—urticaria, abdominal pain, vomiting, diarrhœa, nervous symptoms and collapse—occurring from eight to thirty-six hours after partaking of a meat meal, usually pork (sausage, pork-pie, ham), occasionally beef and tinned meat. The principal outbreaks of this nature have been those at Jena, in 1888, investigated by Gärtner, and from which he isolated the type form of the *B. enteritidis*; Welbeck in 1880; Middlesbrough in 1888; Mansfield in 1896; and Derby in 1902. A small outbreak occurred at Bedford in 1907.[1] These outbreaks are usually caused by varieties of the *B. enteritidis* having the general characters of the group, which usually do not ferment lactose, and are distinguishable by agglutination reactions and fixation tests, the organism isolated as a rule agglutinating well with the patient's serum.

The *B. enteritidis* in morphology, motility, and staining reactions resembles the *B. typhosus*, forms no, or only traces of, indole, and changes neutral red to a fluorescent yellowish colour. Litmus milk after a faint acidity becomes alkaline, and is converted into a thin watery translucent fluid, without coagulation. It does not attack either salicin or glycerin. The fermentation reactions are

[1] *Public Health*, vol. xx, 1907–8, p. 310.

given in the Table on p. 430. Savage [1] obtained this organism from only one out of fifty-three specimens of human excreta examined. A number of variants were isolated from various materials, some fermenting salicin, some glycerin, and some both these substances (see also "Meat," Chap. XXXI).

Clinical Examination.—See p. 420.

Swine Fever or Hog Cholera [2]

Swine fever, or hog cholera (to be distinguished from swine erysipelas, which see), is an infective disease of pigs, highly contagious, and causing considerable mortality. The duration of the affection is usually three to four weeks; the animals lie about, their temperature is raised, and they may suffer from cough and frequent respiration, and some lameness in the hind legs. Towards the end mucous diarrhœa is a prominent symptom. Post mortem, the large intestine is found to be ulcerated, the ulcers much resembling the typhoid ulcers of man, and according to Klein, pneumonia is commonly present, whence he termed the disease "pneumo-enteritis." McFadyean, however, from his own experience and that of the Board of Agriculture, considers pneumonia very infrequent. The ulcers occur mainly in the cæcum and colon, and are due to a well-defined circular necrosis involving the whole thickness of the mucous membrane and occasionally extending to the wall of the bowel. A diffuse diphtheroid lesion also occurs, due to a superficial necrosis with deposition of a thin layer of fibrinous exudate on the surface of the mucous membrane. All gradations are found between the well-defined circular necrosis and the diffuse diphtheroid lesion.

An organism constantly present is a member of the paratyphoid sub-group of the Gärtner group (*B. suipestifer* or *suicholeræ*, apparently identical with *B. aertryck*), but it seems to be a terminal infection and not the true etiological agent, as the blood and tissues filtered through a porcelain filter are still infective—*i.e.* the organism is probably ultra-microscopic. Some confusion exists in the nomenclature of the disease. Swine fever is the

[1] *Rep. Med. Off. Loc. Gov. Board* for 1909–10, p. 446.

[2] See Uhlenhuth, *Trans. Fourteenth Internat. Cong. of Hygiene* (Berlin, 1907), Bd. iv, p. 50; *Journ. Roy. Inst. Pub. Health*, 1911.

British, and hog cholera the American, name. In addition, a disease of swine was formally described under the designation "swine plague" ("Schweineseuche," Schütz). This clinically much resembles swine fever, but pneumonia is a prominent lesion, and a non-motile, stumpy, bi-polar staining bacillus belonging to the group of the hæmorrhagic septicæmic bacilli is present (see under "Chicken Cholera"). This is now regarded as a secondary infection and the disease as being really swine fever. The *B. suipestifer* is apparently identical with the *B. icteroïdes* of Sanarelli. (See also Chap. XIX.)

Although the lesions are very similar, swine fever has nothing to do with typhoid fever of man, nor with ulcerative colitis.

Other organisms belonging to the Gärtner group are:

1. The Danysz bacillus, used as a virus for exterminating rats (the Danysz virus).

2. The *B. icteroides* of Sanarelli, supposed by him to be the cause of yellow fever, but apparently identical with the *B. suipestifer* (see table, p. 430 and "Yellow Fever," Chap. XIX).

3. The *B. typhi murium* of Löffler, used as a virus for exterminating mice.

4. The *B. psittacosis* of Nocard, causing an infective disease of parrots and transmissible to man (bird-fanciers, etc.), in whom it produces a severe and often fatal broncho-pneumonia.

5. *Summer diarrhœa.*—Morgan [1] concluded that the summer or epidemic diarrhœa of infants is not caused by the dysentery bacillus (see p. 427). In 50 per cent. of the cases he isolated a motile bacillus producing acid and gas from glucose which appears to be most closely allied to the hog-cholera bacillus, differing from the latter by producing alkalinity in litmus milk (without previous acidity) and much indole, and by failing to produce acid and gas from mannitol, arabinose, maltose, and dextrin. It does not ferment dulcitol, saccharose, salicin and sorbite. There are two variants, designated as No. 1 and No. 2. Eyre and Minett [2] examined the normal fæces of sixty young children, and in four only isolated a bacillus allied to the Morgan bacillus. The method of isolation was by means of plates of bile-salt agar containing 1 per cent. of mannitol and coloured with neutral red. (See also Chap. XX.)

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[1] *Brit. Med. Journ.*, 1906, vol. i, pp. 908 and 1131; *ibid.* 1907, vol. i, p. 16.

[2] *Brit. Med. Journ.*, 1909, vol. i, p. 1227.

Paratyphoid Fever [1]

The name "para-colon" bacillus was given by Gilbert in 1895 to races of bacilli intermediate in type between the typhoid bacillus and the colon bacillus, and this designation was also applied by Widal and Nobécourt to a bacillus isolated by them from an abscess in the neighbourhood of the thyroid. The name "paratyphoid" bacillus appears first to have been used by Archard and Bensaude in 1896, and was reintroduced by Schottmüller in 1901, and would seem to be the preferable designation for those micro-organisms that produce typhoidal symptoms.

Paratyphoid fever may be defined as a disease much resembling typhoid fever in its clinical aspect, which is, however, caused, not by the typhoid bacillus, but by organisms belonging to the paratyphoid sub-group of the Gärtner group of bacilli. The disease is generally milder than typhoid fever and the mortality is only 1–4 per cent. Some 3–6 per cent. of the cases notified as "typhoid fever" are probably cases of paratyphoid infection. Paratyphoid infections may occur in epidemics, may be spread by drinking-water, by "carriers," and in other ways, like typhoid fever, and occur in all parts of the world.

Paratyphoid fever is caused by one of two paratyphoid bacilli, known respectively as para A and para B. Both these bacilli are culturally and morphologically similar to the typhoid bacillus and are actively motile, but they ferment glucose with the production both of acid and of gas. In their behaviour with specific immune serums the paratyphoid bacilli also differ markedly from the typhoid bacillus and from one another, and this is the most reliable method for distinguishing them from one another and from other somewhat similar organisms.

[1] See Torrens and Whittington, *Brit. Med. Journ.* 1915, vol. ii, p. 697. Bainbridge and O'Brien, *Journ. of Hygiene*, vol. xi, 1911, p. 68 (Bibliog.).

Bacillus paratyphosus A produces less gas in glucose media than *B. paratyphosus* B (with some strains very little gas is produced); with para A milk remains permanently acid; with para B it becomes alkaline after a transient acidity; and though para A changes neutral red to yellow, the red colour tends to return after three weeks or so, while with para B the yellow colour is permanent. That is to say, in its reactions para A is more closely allied to the typhoid bacillus than is para B. Para B, however, blackens a lead acetate medium, while para A does not.

B. paratyphosus A infection is relatively common in the East, but paratyphoid fever in Great Britain and Western Europe is commonly caused by para B. The fermentation reactions of the paratyphoid bacilli are given in the Table on p. 432.

As regards the agglutination reaction, the blood of the paratyphoid fever patient either does not agglutinate the typhoid bacillus or agglutinates it only in low dilution —*e.g.* 1 in 10 to 40, while it agglutinates the corresponding paratyphoid bacillus of the infection in higher dilution —*e.g.* 1 in 100 or 200, or even higher. The agglutination titre of the serum in cases of paratyphoid A infection is sometimes very low—1 in 25 or even 1 in 10. In cases that have been inoculated with typhoid vaccine, the agglutination titre of the serum for the typhoid bacillus may be somewhat increased as a result of paratyphoid infection.

Cases of mixed infection with typhoid and paratyphoid bacilli occasionally occur.

The diagnosis of paratyphoid fever is determined by (*a*) the agglutination reaction, as for typhoid fever, p. 410; (*b*) the isolation of a paratyphoid bacillus by blood-culture, as for the typhoid bacillus (p. 409). The recovery of the bacillus from the blood is sometimes not practicable as the bacillæmia of paratyphoid fever is often very

transient and cases are frequently not seen until too late. The paratyphoid bacilli are present in the fæces and urine, but frequently only during the height of the disease, unless the carrier state is being established; the method of isolation and differentiation is given below. Prophylactic vaccines for paratyphoid fever may be prepared with paratyphoid bacilli in the same manner as for typhoid fever, and Castellani has made use of a mixed typhoid-paratyphoid vaccine.

Clinical Examination of the Fæces and Urine for Typhoid and Paratyphoid Bacilli, etc.

The following is Ledingham and Penfold's method (*Brit. Med. Journ.*, 1915, vol. II, p. 704):—

Essentials.

1. MacConkey's bile-salt-lactose-neutral-red-agar.
2. Mannite peptone water contained in Durham's tubes.
3. Thoroughly tested specific agglutinating serums of high potency for *B. typhosus*, *B. paratyphosus* A., *B. paratyphosus* B., *B. enteritidis* Gaertner, *B. dysenteriæ* Shiga, and *B. dysenteriæ* Y. These serums we obtain from the Lister Institute.

The following dilutions of these serums are made with carbolised saline (0·5 per cent. carbolic in normal saline) and kept in bulk preferably but not necessarily in the cold chest.

1. *P. G. mixture* containing equal parts of *Paratyphosus* A serum (1 in 66), *Paratyphosus* B. serum (1 in 66), and *Gaertner* serum (1 in 66). Concentration of each in final mixture = 1 in 200.

2. *P. T. mixture* containing equal parts of *Paratyphosus* A serum (1 in 66), *Paratyphosus* B serum (1 in 66), and typhoid serum (1 in 66). Concentration of each in final mixture = 1 in 200.

3. *Y serum* diluted 1 in 100, or 1 in 200.
4. *Shiga serum* diluted 1 in 100, or 1 in 200.
5. *Paratyphosus A serum* diluted 1 in 200.
6. *Paratyphosus B serum* diluted 1 in 200.
7. *Typhoid serum* diluted 1 in 200.
8. *Gaertner serum* diluted 1 in 200.

Steps.

Portion of fæces emulsified in broth and allowed to stand on bench for an hour. One or two drops from the upper layers of the fluid are spread on the bile-salt-agar plates; incubate.

Next Morning.—Pick off several non-lactose colonies (discrete colonies wherever possible) and inoculate *each colony* into mannite and into ordinary broth. The number of colonies taken off will depend on many factors—for example, (1) the number of white colonies present, (2) the appearance of the colonies, (3) the clinical symptoms of the case if known, (4) the appearances of the stool.

Evening of Same Day.—After at least five hours' growth, examine the broth tubes, preferably with the dark ground microscope, and record motility or non-motility. At the same time inoculate an agar slope from each broth tube.

Second Morning.—The mannite tubes are examined and the results (acid and gas, acid only, or no change) recorded on the corresponding agar slopes.

1. If *acid and gas and motile*, test the corresponding slope with P. G. mixture. A loop of culture is emulsified in 4 or 5 c.cm. of saline. Equal parts of this emulsion and the P. G. mixture are placed in a small "agglutination" test tube and kept at 37° C. Examine the tube every hour and note if agglutination is taking place. If so, test the corresponding emulsion with *Paratyphosus* A serum, *Paratyphosus* B serum, and Gaertner serum separately (one dilution of each giving 1 in 400 in the final mixture). If agglutination takes place with A serum but not with B or Gaertner serum the emulsion may then be tested up to the full titre of the serum.

2. *If acid and gas and non-motile*, no further steps need be taken, but it must be remembered that a positive motility is final, while a negative result may leave a certain element of doubt, in which case it is wise to treat the culture as in 1. The extra labour is small.

3. *Acid only and motile.* Test with the P. T. mixture. (Many paratyphoid strains give little or no gas in the sugars they normally ferment, and for this reason it is necessary to treat all cultures giving acid only in mannite (and motile) as possible paratyphoids as well as typhoids.) If agglutination occurs, test further with A, B, and T serums separately.

4. *Acid only and non-motile.* Test with "Y" serum.

5. *No change on mannite and motile.* Discard for the time being,

or, if time permits, test further to determine whether the culture is Morgan's *No.* 1 *Bacillus* or a member of the *Proteus* group.

6. *No change on mannite and non-motile.* Test with Shiga's serum.

The emulsions are arranged in a test rack according as they have been tested with P. G. mixture, P. T. mixture, Y serum, etc. Equal volumes of serum (say from the stock P. G. mixture) are placed in small "agglutination" tubes by aid of a large-bored capillary pipette with rubber teat and fiducial mark, delivering about 0·4 c.cm.

Similar volumes of the corresponding emulsions are added by means of the same pipette, which is cleansed (between each operation) by boiling water from a beaker. As mentioned above, the tubes, after being placed in the incubator, should be examined every hour or so, and any that have reacted should be further tested if necessary. No *negative* results should be recorded, however, until the following morning, when the final readings are made.

Browning, Gilmour and Mackie's Method for Typhoid.—Peptone water (2 per cent. Witte's peptone and ½ per cent. sodium chloride in distilled water) is prepared, steamed for ¾ hour and filtered through paper. It is then distributed in test tubes (6 in. by ¾ in.) 5 c.c. in each and autoclaved at 120° C. for fifteen minutes. Six tubes of the peptone water are taken and to these are added respectively 0·04, 0·08, 0·12, 0·16, 0·22, 0·3 c.c. of a 1–10,000 solution of brilliant green. A large loopful (up to 0·4 c.m. diameter) of fæces is then added to each tube and well emulsified and mixed. The inoculated tubes are incubated at 37° C. for 20–24 hours and then a loopful of the contents is taken from each tube and three successive streaks are made on to a plate of McConkey's medium. The plates are then incubated and examined for the presence of the typhoid (or paratyphoid) bacillus. The brilliant green (Bayer's brillant green extra cryst.—the sulphuric acid salt, free from zinc) may be made up as a 1 per cent. solution in distilled water and keeps for 2–3 weeks. For use, the 1–10,000 solution is freshly prepared by adding 0·1 c.c. of the stock solution to 9·9 c.c. of distilled water.

(See also Rajchman and Western's method, p. 427.)

Bacillus dysenteriæ [1]

In one type of dysentery, the so-called epidemic or bacillary form (see "Dysentery," Chap. XX), a bacillus *B. dysenteriæ*, is the causative agent. The *B. dysenteriæ* includes a group of closely allied organisms.

The dysentery bacillus was first isolated in 1897 by Shiga in Japan. Somewhat later Kruse isolated an almost identical bacillus in Germany, and this type is known as the Shiga-Kruse type. Later, Flexner and Strong isolated another type of the dysentery bacillus, and during the last few years similar organisms, but differing from the Shiga-Kruse and Flexner types in some of their fermentation and other reactions, have been isolated; these are sometimes termed "pseudo-dysentery" bacilli.

The Shiga-Kruse and other types of dysentery bacilli have been isolated by Flexner and Strong in the Philippines, Park, Duval, Bassett, Martini, Hiss, Russell and others in the United States, Castellani in Ceylon, Rogers and others in India, Ruffer and Willmore in Egypt (El Tor), and Eyre, McWeeney and others in the British Isles. The dysentery bacilli have also been frequently met with during the present war, in Gallipoli, in Serbia and on the Eastern and Western fronts. The predominant infection in the East was the Shiga strain, in the West the Flexner strain.

Morphology.—The *B. dysenteriæ* are small slender bacilli much resembling the colon bacillus. They are non-motile, but Brownian movement is often active, Gram-negative, and non-sporing, and are readily destroyed by heat (58°–60° C.) and antiseptics.

Cultural characters.—The dysentery bacilli are aërobic

[1] See P H. Bahr, *Dysentery in Fiji* (Witherby & Co., London, 1912) and Rajchman and Western, Report to the Medical Research Committee, *Special Rep. Series*, No. 5, 1917.

and facultatively anaërobic. On agar a thinnish creamy growth develops ; on gelatin a white growth nearly limited to the inoculation track, and without liquefaction. The colonies on a gelatin plate closely resemble those of the typhoid bacillus. On potato the growth is either thin, grey and slightly visible, or thicker and yellowish or brownish. The colour of neutral red media is unaltered. Litmus milk first becomes faintly acid, then markedly alkaline ; no clotting. Indole is generally not formed (never by the Shiga type) ; occasionally a trace may be detected. All strains ferment glucose with the formation of acid only, no gas ; none ferments lactose. Some strains (the Flexner type) ferment mannitol with the formation of acid only, no gas ; other strains (the Shiga-Kruse type) have no action on this alcohol. The principal fermentation and other reactions are given in the Table on p. 430. These reactions are somewhat variable with different stains, but differentiation may be accomplished by agglutination, saturation, and complement fixation, tests. Shiga [1] distinguished five groups of dysentery bacilli as follows :

1. Fermenting dextrose alone [Shiga, Kruse, Flexner (Newhaven)]. The *Shiga-Kruse Bacillus.*
2. Fermenting dextrose and mannitol (Hiss and Russell's Y bacillus, Ferran, Seal Harbour bacillus). The *Y-Bacillus.*
3. Fermenting dextrose, mannitol and saccharose [Strong (Manila)]. The *Strong Bacillus.*
4. Fermenting dextrose, mannitol, maltose and saccharose (Flexner, Harris, Gay, Woolstein). The *Flexner Bacillus.*
5. Fermenting dextrose and maltose, and giving a feeble acid reaction with mannitol (Shiga).

[1] *Zeitsch. f. Hyg.*, lx, 1908, pp. 75, 120.

The dysentery bacilli are therefore *culturally* divisible into four types; (1) the Shiga-Kruse, (2) the Flexner, (3) the Strong, and (4) the Y. By agglutination, however, the *four* types form *three* groups, for the Shiga strain is agglutinated by Shiga serum only, the Flexner and Y strains by either of the two sera, but not by Shiga serum. and the Strong by its own serum only. The Strong, therefore, remains separate both culturally and by agglutination, but as it has not been isolated anywhere since its discovery in the Philippines, the dysentery bacilli fall into two principal groups, the Shiga and the Flexner.

Bahr found occasional variations in fermentive power after sub-culturing and after a sojourn in flies.

Agglutination reaction.—The agglutination reaction is given by the blood of patients suffering from the bacillary form of dysentery, but not by the amœbic form (unless a double infection be present, which occasionally is the case). Agglutination in Shiga infections commences between the fifth and twelfth day of the disease. A non-dysentery serum may agglutinate the Shiga bacillus up to a dilution of 1 in 50, but agglutination in a dilution of 1 in 64 or over may be considered to be diagnostic. In Flexner infections, no agglutination should be considered to be diagnostic in dilutions lower than 1 in 256. Not every case of dysentery gives agglutination, which may occur only with the particular strain causing the infection.

Pathogenic action.—The organism generally seems limited to the bowel and its mucous membrane and rarely gains access to the blood. No characteristic lesions are produced in animals by administration of the dysentery bacillus *per os*. In man, cultures given by the mouth are stated to have induced a typical dysentery. Animals such as rabbits, guinea-pigs and mice are very sensitive to injections of living and killed cultures; in fact, it is very difficult to immunise animals against the organism.

Amounts of 0·1–0·2 mgrm. of an agar culture given intravenously or intraperitoneally are fatal to these animals. The Shiga strain seems generally to be the more virulent one.

In man the organism is abundant in the bloody mucoid discharge from the bowel, and at an early stage may be isolated by means of litmus lactose agar plates, on which it forms small transparent blue colonies ; at a later stage (after two to three days) the other organisms in the bowel multiply to such an extent that isolation may become difficult. Anilin-dye media are better avoided. "Carriers" occur and help to spread the disease, which may be conveyed by infected water and food and by flies.

Toxins.—The filtrate of dysentery cultures (four to six weeks old), particularly the Shiga strain, in a somewhat highly alkaline broth (broth just alkaline to litmus + 7 c.c. normal NaOH per litre) is markedly toxic, 0·1 c.c. being a fatal dose for a large rabbit.[1]

Anti-serum and vaccine.—The serum of horses immunised with the toxin, or with dead and then with living cultures, possesses marked antitoxic properties, and the use of this antitoxic serum has been successful in cases of acute bacillary dysentery. Shiga obtained a reduction in mortality from 22 to 7 per cent. by the use of serum in a severe epidemic, and striking results were obtained by Ruffer and Willmore [2] in Egypt and by Bahr in Fiji. It is necessary, however, to employ a serum prepared with the particular strain of the disease.

When the disease has become chronic the use of a *vaccine*, consisting of a culture sterilised by heat, is sometimes beneficial. Dysentery vaccines for *prophylaxis* are now being tried.

Para-dysentery bacilli.—In the dysenteries of Ceylon,

[1] Todd, *Journ. of Hygiene*, vol. iv, 1904, p. 480 (Bibliog.).

[2] *Brit. Med. Journ.*, 1909, vol. ii, p. 862, and 1910, vol. ii, p. 1519.

Castellani [1] has sometimes isolated dysentery bacilli nearly related to the Shiga-Kruse type, but showing differences from it in agglutination, persistence of acid reaction in litmus milk, and virulence; these he has termed "para-dysentery" bacilli.

Asylums dysentery and summer diarrhœa of infants.—Both in America and in England some cases of summer diarhœa of infants are found to be associated with the *B. dysenteriæ* (see above, p. 417). The asylums or institutional dysentery, or ulcerative colitis, is also due to this organism, and in this country is almost invariably due to the Flexner type.

Clinical Diagnosis

1. *Agglutination.*—The patient's serum may be examined for agglutination against the Shiga and the Flexner strains according to the details given on p. 410. "Standard" cultures are also supplied by the Oxford laboratory.

2. *Examination of the Fæces.*—This may be carried out by the method given on p. 420. Rajchman and Western's method (*loc. cit.*) is as follows:—

A loopful or so of the fæces is emulsified in 10 c.c. of sterile saline or tap-water. A glass rod spreader of L-form is dipped into the emulsion and two Petri plates of litmus lactose agar are inoculated successively over the whole surface. These are incubated overnight at 37° C. and suspicious blue colonies are then picked out and sub-cultured on sloped agar tubes; not less than three colonies should be sub-cultured. Experience will decide which blue colonies are likely to be the right ones, for some blue colonies may be alkali-producers and not simply non-lactose fermenters which often give at this stage colourless or pale colonies. The general appearance, structure and consistency of the colonies are important characters, as well as the colour.

The sub-cultures on agar are incubated overnight and then some of the cultures may be excluded on account of the macroscopic appearance of the growth. Those finally selected are then sub-cultured into Durham's tubes of the following sugars,

[1] *Journ. of Hygiene*, vol. iv, 1904, p. 495.

Table showing Cultural Reactions of Certain Aërobic Non-spore Producing

Name of Micro-organism.	Motility.	Gram.	Gelatin.	Serum.	Litmus Milk.	Lactose.	Saccharose.	Dulcite.	Mannite.	Glucose.	Maltose.	Dextrin.	Raffinose	Arabinose.	Adonite.
B. acidi lactici, Hüppe . .	O	O	O	O	AC	AG	O	O	AG	AG	AG	AG	AG	AG	AG
B. aertryck, De Nobele . .	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	O	O	AGs	O
B. archibald	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	..	O	..	O
B. asiaticus, Castellani . .	O	O	O	O	A, Alk	O	AG	O	AG	AG	AG	AG	AG	AG	O
B. asiaticus mobilis, Cast. .	+	O	O	O	A, Alk	O	AG	O	AG	AG	AG	AG	AG	AG	O
B. bentotensis, Cast. . . .	+,	O	O	O	A	A	A	As	O	A	A	O	As	O	O
B. capsulatus, Pfeiffer . .	O	O	O	O	AC	AG	AG	O	AG	AG	AG	AG	AG	AG	AG
B. caralinus, Cast.. . . .	+	O	O	O	A, Alk	O	O	O	A or AG	AG	AG	..	AG	AG	..
B. cavicida, Brieger . . .	+	O	O	O	AC	AG	O	AG	AG	AG	O	AG	AG	AG	O
B. ceylonensis A, Cast. . .	O	O	O	O	AC	O	O	O	O	A	O	O	O	O	O
B. ceylonensis B, Cast. . .	O	O	O	O	AC	A	A	A	A	A	A	A	A	A	O
B. cloacæ, Jordan	+	O	+	+	AC	AG	AG	O	AG	AG	AG	AG	AG	AG	O
B. coli, Escherich	+	O	O	O	AC	AG	O	AG	AG	AG	AG	AG	AG	AG	O
B. coli mutabilis, Massini .	O	O	O	O	AC	AG	O	O	..	..	..	..	..	..	O
B. coli-tropicalis, Cast. . .	O	O	O	O	AC	AG	O	O	AG	AG	AG	AG	AG	AG	O
B. columbensis, Cast. . . .	+	O	O	O	A vs, Alk, D or A	O or Gvs	O	AG	AG	AG	AG	As Gs	O	AG	O
B. coscoroba	O	O	O	O	AC	AG	AG	O	AG	AG	AG	AG	AG	AG	O

Abbreviations used in the table.—A = acid; G = gas; C = clot; D = decolorised; Alk = alkaline; O = negative result; viz., neither acid nor clot in milk, neither acid nor gas in sugar media, non-production of positive, sometimes negative.

Intestinal Bacilli, with Names arranged in Alphabetical Order.

Inulin.	Sorbite.	Galactose.	Lævulose.	Inosite.	Salicin.	Amygdalin.	Isodulcite.	Erythrite.	Glycerine.	Indol.	Voges-Prosk.	Broth.	Remarks.
O	AG	AG	AG	O	O	..	..	O	..	+	O	Gt	Belongs to capsulated bacilli; differs from *B. lactis aerogenes* in not fermenting inosite; differs from *B. coli tropicalis* in being capsulated and in fermenting adonite and not fermenting salicin.
O	AG	A G	AG	AG	O	O	AG	O	A	O or +s	O	Gt	Identical culturally and serologically with *B. suipestifer*; identical culturally with *B. enteritidis*, Gaertner (differentiation by agglutination tests), and *B. paratyphosus* B (differentiation by absorption tests, agglutination not sufficient).
O	..	AG	..	..	..	..	..	..	..	+	+	Gt	
O	AG	AG	AG	O	O	O	AG	O	AG	+s	O	Gt	
O	AG	AG	AG	O	O	O	AG	O	AG	+s	O	Gt	Differs from *B. asiaticus* only in being motile.
O	O	A	A	A	As	O	O	O	A	+	O	Gt	
O	AG	AG	AG	AG	..	..	..	..	..	+	+	Gt	Capsulated, generally considered to be identical with *B. lactis aerogenes*.
O	A	AG	AG	..	..	..	..	..	..	+	O	Gt or + P	
O	..	AG	AG	O	A	..	..	..	..	+	O	..	Breiger described it at first as non-motile; differs from *B. coli* in not fermenting maltose.
O	O	O	O	O	O	O	O	O	O	O	O	Gt	
O	A	A	A	O	O	O	A	O	A	+	O	Gt	
O	AG	AG	AG	A or O	O	..	..	O	..	+	+	Gt	Liquefaction of gelatine very slow. The more important intestinal liquefying bacilli may be grouped as follows: (1) lactose fermenters (*B. cloacæ*); (2) lactose non-fermenters, Gram + (*B. proteus vulgaris*); (3) lactose not fermenters, Gram O (*B. diffluens*).
O	AG	AG	AG	O	AG	O	AG	O	AG	+	O	Gt	
O	..	..	..	..	..	..	..	..	..	O	..	..	Incompletely described; late lactose fermenter (after six days); said not to produce indol.
O	AG	AG	AG	O	AG	O	AG	O	AG	+	..	..	Differs from *B. coli* in being non-motile and in non-fermenting dulcite; from *B. neapolitanus* in not fermenting saccharose and dulcite.
O	AG	AG	AG	O	AG	O	AG	O	AG	+	O	Gt	
..	AG	AG	..	A	..	..	..	..	..	O	..	..	Differs from *B. coli-tropicalis* in ferment-saccharose. Certain Authors use the term *B. cosoraba* to indicate a totally different germ, with all the characters of the fowl cholera bacillus (Pasteurella).

s = slight; A, Alk = acid, then alkaline; Gt = general turbidity; P = pellicle; vs = very slight; indol, non-liquefaction of gelatin or serum as the case may be. + = positive result; ± = sometimes

Table showing Cultural Reactions of Certain Aërobic Non-spore Producing

Name of Micro-organism.	Motility.	Gram.	Gelatin.	Serum.	Litmus Milk.	Lactose.	Saccharose.	Dulcite.	Mannite.	Glucose.	Maltose.	Dextrin.	Raffinose.	Arabinose.	Adonite.
B. danysz	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
B. diffluens, Cast.	+	O	+	+	Alk, DP	O	O	O	O or A	A or AG	O	O	O	O	O
B. dysenteriæ, Shiga-Kruse .	O	O	O	O	A, Alk	O	O	O	O	A	O	O or As	O	O	..
B. dysenteriæ, Flexner . .	O	O	O	O	A, Alk	O	O	O	A	A	A	A	A	A	O
B. dysenteriæ, Hiss and Russell	O	O	O	O	A, Alk	O	O	O	A	A	O	A	A	A	O
B. dysenteriæ, Strong . . .	O	O	O	O	AC	O	A	A	A	A	A	O	A	A	O
B. entericus, Cast.	O	O	O	O	O	AG	O	AG	AG	AG	AG	AGs	OD	AG	O
B. enteritidis, Gärtner . .	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	AG	O	AG	O
B. fæcalis alkaligenes, Petruschky	+	O	O	O	Alk	O	O	O	O	O	O	O	O	O	O
B. gasoformans nonliquefaciens	O	O	O	..	AC	AG	AG	O	..	..	..	..	..	..	AG
B. giutotensis, Cast. . . .	O	O	O	O	D, AC	O	O	O	O	A	O	O	O	A	O
B. giumai, Cast.	O	O	O	O	A, Alks	A	O	O	O	AG	AG	AGs	O	AG	O
B. grunthali, Cast. . . .	+	O	O	O	AC	AG	O	O	AG	AG	..	AG	AG	AG	O
B. icteroides, Sanarelli. . .	+	O	O	O	A, Alk	O	O	A or MG	AG	AG	AG	AG	O or A	A or AG	..
B. kandiensis, Cast. . . .	+	O	O	O	As, D, Alk	O	As	O	A	A	O	O	O	O	A
B. khartoumensis, Chalmers and Macdonald	O	O	O	O	A	AG	O	AG	AG	AG	AG	O	O	AG	..
B. lactis aerogenes, Escherich.	O	O	O	O	AC	AG	AG	O	AG	AG	AG	AG	AG	AG	AG
B. levans, Wolffln	+	O	+	..	AC	AG	O	O	AG	AG	..	AG	AG	AG	O
B. lunavensis, Cast. . . .	O	O	O	O	As, Alk	O	A	O	O	A	A	A	O	A	O
B. madampensis, Cast. . .	O	O	O	O	AC	A	A	O	A	A	A	A	AS	A	O
B. morgan	O	O	O	O	O, Alk or As, Alk	O	O	O	O	AG	O or A	O or A	O	O or A	O
B. neapolitanus, Emmerich .	O	O	O	O	AC	AG	AG	AG	AG	AG	AG	AG	AG	AG	O
B. negombensis, Cast.. . .	O	O	O	O	O, Alk	O	O	O	O	A	O	O	O	O	O
B. oxytocus perniciosus, Wysokowitsch	O	O	O	O	AC	AG	AG	AG	AG	AG	..	AG	AG	AG	AG

Abbreviations used in the table.—A = acid ; G = gas ; C = clot ; D = decolorised ; Alk = alkaline ; s = slight ; viz., neither acid nor clot in milk, neither acid nor gas in sugar media, non-production of indol, non-liquefaction

Inulin.	Sorbite.	Galactose.	Lævulose.	Inosite.	Salicin.	Amygdalin.	Isodulcite.	Erythrite.	Glycerine.	Indole.	Voges-Prosk.	Broth.	Remarks.
..	..	..	..	..	..	..	..	..	..	..	..	..	Culturally and serologically identical with *B. enteritidis* Gärtner (Bainbridge).
O	..	AG	A or A Gs	..	O	..	..	..	A	O	..	Gt	See remarks on *B. cloacæ*. On agar growth spreads very quickly all over the surface.
O	O	A	A	..	O	..	..	As	..	O	..	..	
O	O	A	A	..	O	O	O	O	O	+	..	..	
O	O	A	A	..	O	O	O	O	O	+or ±	..	..	
O	A	A	A	..	O	O	A	O	O	+	..	..	
O	AG	AG	AG	..	..	..	..	..	..	+	O	Gt, PS	
O	AG	AG	AG	O	O	O	..	..	O	O	..	Gt	Identical culturally with *B. suipestifer* (= *B. aertryck*) and *B. paratyphosus* B.; differs serologically.
O	O	O	O	O	O	O	O	O	O	O	O	Gt	The typical *B. fæcalis alkaligenes* produces strong alkalinity in all sugar broth, but certain strains may produce slight acidity in glucose, maltose, dextrin, raffinose, sorbite, galactose, lesulose. Certain strains peptonise milk.
O	..	..	..	A	..	..	..	..	..	O	+	..	Incompletely described. It is probably very similar to *B. colitropicalis*, but indol O.
O	O	A	O	O	O	O	O	O	O	O	O	GtP	
O	AG	AG	AG	O	AG	O	AG	O	AS	+	O	Gt	
O	AG	AG	AG	O	..	..	..	O	..	+	O	Gt	
O	AG	AG	AG	..	..	..	..	..	..	±	O	Gt or GtP	Considered to be identical with *B. suipestifer*, but complete serological tests have not been carried out.
O	O	A	A	A	O	O	A	A	A	O	O	Gt	Growth on agar somewhat resembles *S. choleræ*. Found in certain areas of diarrhœa in the tropics.
O	AG	AG	AG	O	AG	O	..	..	AG	+	O	..	
O	AG	AG	AG	AG	AG	..	..	..	..	O	+	Gt	Differs from *B. acidi lactici* in fermenting inosite.
AG	AG	AG	AG	O	AG	..	..	O	..	O	+	..	
O	O	A	A	O	O	O	O	O	Avs	+	O	Gt	
O	A	A	A	O	O	O	A	O	A	+	O	Gt	
O	O	A or AGs	A or AGs	O	O	O	O	O	O	+ +	O	Gt	
O	AG	AG	AG	O	AGs	O	AGs	O	AGs	+	O	..	Differs from *B. coli* in being non-motile and in fermenting saccharose; from *B. pseudocoli* in being non-motile; from *B. colitropicalis* in fermenting dulcite and saccharose.
O	O	A	AS	O	O	O	O	O	O	O	O	Gt	
AG	AG	AG	AG	AG	AG	O	AG	O	AG	+	+	..	

A, Alk = acid, then alkaline; Gt = general turbidity; P = pellicle; vs = very slight; O = negative result, gelatin or serum as the case may be. + = positive result; ± = sometimes positive sometimes negative.

Table showing Cultural Reactions of Certain Aërobic Non-spore Producing

Name of Micro-organism.	Motility.	Gram.	Gelatin.	Serum.	Litmus Milk.	Lactose.	Saccharose.	Dulcite.	Mannite.	Glucose.	Maltose.	Dextrin.	Raffinose.	Arabinose.	Adonite.
B. para-colon, Day . . .	+	O	O	O	A, Alk	O	O	A	AG	AG	AG	AG	AG	AG	..
B. para-eutericus, Cast. . .	+	O	O	O	A	AG	AG	AG	AG	AG	AG	AGs	AG	AG	O
B. para-aertryck, Cast. . .	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	O	O	AG	O
B. paratyphosus A, Schotmüller	+	O	O	O	A	O	O	AG	AG	AG	AG	AG	O	AG	O
B. paratyphosus B, Schotmüller	+	O	O	O	A Alk	O	O	AG	AG	AG	AG	O	O	AG	O
B. paradysentericus, Cast. .	O	O	O	O	A	O	O	O	O	O or A	O	O	O	O	O
B. pneumoniæ, Friedländer .	O	O	O	..	AC	A	AG	AG	AG	AG	AG	AG	AG	AG	AG
B. pritnitzi, Cast.	+	O	O	O	A	O	O	O	O	A	A	A	O	O	O
B. proteus vulgaris, Hauser .	+	+	+	+	AC	O	AG	O	O	AG	AG	O	O	O	O
B. pseudo-asiaticus, Cast. .	O	O	O	O	A, Alk	O	AG	AGs	AG	AG	AG	AG	AG	AG	O
B. pseudo-coli, Cast. . . .	+	O	C	O	AC	AG	AG	AG	AG	AG	AG	AGs	AG	AG	O
B. psittacosis, Nocard . .	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	AG	AG	AG	O
B. pyogenes fœtidus, Passet .	+	O	O	O	AC	A	A	A	A	A	A	A	A	A	..
B. Schæfferi, Freudenreich .	O	O	O	..	AC	AG	O	AG	..	..	..	..	..	..	O
B. suipestifer, Kruse . . .	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	O	O	AGs	O
B. talavensis, Cast. . . .	+	O	O	O	Alk, D	O	A	O	O	A	O	O	O	O	O
B. tangallensis, Cast. . . .	O	O	O	O	As, Alk	O	A	A	A	A	A	A	A	A	O
B. typhi murium, Löffler .	+	O	O	O	A, Alk	O	O	AG	AG	AG	AG	AG	O	AG	O
B. typhosus, Eberth . . .	+	O	O	O	A	O	O	O	A	A	A	A	As	O	O
B. vekanda, Cast.	+	O	O	O	A	AG	O	AG	AG	AG	AG	O	O	AG	AG
B. veboda, Cast.	+	O	O	O	A, Alk	O	O	AG	A	AG	AG	AG	AG	AG	O
B. vesiculosus, Henrici . .	O	O	O	O	AC	AG	O	O	..	..	..	..	..	..	O
B. Watareka, Cast. . . .	+	O	O	O	A	O	O	AG	AG	AG	O	AG	AG	AG	O
B. Werahensis, Cast. . . .	+	O	O	O	A	O	O	A	A	A	AG	AG	AG	AG	..
B. Wesenberg	+	..	O	O	A	A	AG	A	A	AG	..	..	..	..	..
B. Willegodai, Cast. . . .	+	O	O	O	A, Alk	O	O	A	A	AG	AG	AG	AG	AG	O

Abbreviations used in the table.—A = acid ; G = gas ; C = clot ; D = decolorised ; Alk = alkaline ; s = slight
viz., neither acid nor clot in milk, neither acid nor gas in sugar media, non-production of indol, non-liquefactio

Inulin.	Sorbite.	Galactose.	Lævulose.	Inosite.	Salicin.	Amygdalin.	Isodulcite.	Erythrite.	Glycerine.	Indole.	Voges-Prosk.	Broth.	Remarks.
..	AG	AG	AG	..	..	..	..	..	..	+	O	Gt	
O	AG	AG	AG	..	..	..	..	..	..	+	O	Gt, Ps	
O	AG	AG	AG	AG	O	O	AG	O	AG	O	O	Gt	
O	AG	AG	AG	O	O	O	AG	O	O or As	O	O	Gt	
O	AG	AG	AG	AG	O	O	AG	O	O	O	O	Gt	Certain strains typical serologically, may produce at times only A instead of AG; some strains do not ferment inosite (Weiss and Rice).
O	O	O or As	O or As	..	O	..	..	O	..	O	..	Gt	
O	AG	AG	AG	AG	AG	..	..	O	..	O	O	Gt	
A	O	A	A	O	A	O	O	O	O	O	..	..	
O	O	AG	A or A G	O	O	..	..	..	O	+	..	Gt	Cultures emit a disagreeable odour. Hauser distinguished at first three varieties of proteus: *P. vulgaris* (rapid liquefaction of gelatine), *P. mirabilis* (slow liquefaction), *P. Zenkeri* (no liquefaction); later abandoned this differentiation.
O	AG	AG	AG	O	AG	O	AG	O	AG	+	O	Gt	Differs from *B. asiaticus* in fermenting dulcite.
O	AG	AG	AG	O	AG	O	AG	O	AGs	+	O	Gt, Ps	Differs from *B. coli* in fermenting saccharose, belonging to the group *communior* of coliform bacilli.
O	AG	AG	AG	..	O	..	..	..	..	O	O	Gt	Identical with *B. aertryck*, according to Bainbridge.
..	..	A	A	..	..	..	..	..	..	+	O	Gt	
O	O	..	..	O	..	..	..	..	..	+	O	Gt	Incompletely described.
O	AG	AG	AG	AG	O	O	AG	O	AS	+s	O	Gt	Identical culturally and serologically with *B. aertryck*. Other synonyms for *B. suipestifer* are *B. choleræ suis*, *B. of hog-cholera*, Salmon and T. Smith (1885).
O	O	A	A	A	A	O	O	O	A	+	O	Gt	
O	A	A	A	O	A	O	A	O	A	+	O	Gt	
O	AG	AG	AG	..	O	O	..	..	O	O	O	Gt	Bainbridge has found that the name is applied to different organisms, some strains being serologically identical with *B. aertryck*, others with *B. enteritidis*, Gaertner, others with *B. paratyphosus* B.
O	A	A	A	O	O	O	O	O	As	O	O	Gt	Certain strains milk A, Alk.
O	AG	AG	AG	O	O	O	AG	O	AG	O	..	Gt	
O	AG	AG	AG	AG	O	O	A	O	..	O	..	Gt	
O	..	..	..	O	..	..	..	..	..	+	O	..	
O	AG	AG	AG	AG	O	O	AG	O	A	+	..	Gt	
..	..	O	O	..	A	O	AG	..	O	+s	..	Gt	
..	..	..	..	..	..	..	..	..	..	+	..	Gt	
..	..	AG	A	..	AG	O	AG	..	O	+s	..	Gt	

, Alk = acid, then alkaline; Gt = general turbidity; P = pellicle; vs = very slight; O = negative result, gelatin or serum as the case may be. + = positive result; ± = sometimes positive, sometimes negative.

etc. : 1 per cent. glucose, lactose, maltose, saccharose, mannitol and dulcitol in litmus peptone water. These are then incubated overnight and some may again be rejected owing to fermentation of lactose. The likely strains are then agglutinated by specific sera according to the indications given by the sugar reactions. Any sugar tubes which show no change should be incubated for a further period.

Bacillus coli

The *Bacillus coli*, or colon bacillus (*B. coli communis*), is an organism of considerable importance, both in connection with the *Bacillus typhosus*, in pathological processes, and in water supplies as an indication of pollution. As its name implies it is a constant inhabitant of the intestinal tract in man and animals (except perhaps in certain arctic animals), and is one of the most widely distributed organisms in nature. While the term " colon bacillus " is applied to a fairly well-defined organism (the " typical *B. coli* "), there are a number of allied organisms differing from the type in one or more characters—*e.g.* motility, indole production, fermentation reactions, rate and extent of milk curdling, etc.—and these varieties are said to belong to the " colon group," or are termed " coliform."

The *B. coli* may be readily isolated by inoculating litmus lactose bile-salt peptone-water tubes with a trace of a suspension of fresh fæces, growing for from twenty-four to forty-eight hours at 42° C., and plating the culture on litmus lactose agar, on gelatin, or on Conradi-Drigalski agar, or by direct plating of the fæces suspension on the last-named medium (see also " Water ").

Morphology.—The *B. coli* is a short rod with rounded ends, 2 or 3 μ long and 0·5 μ broad, frequently linked in pairs or more. It is often so short that it is merely ovoid in shape ; and, on the other hand, longer individuals and involution forms occur 10 μ or more in length (Plate

XIV, *a*). It is feebly motile, and possesses lateral flagella to the number of three or four on an average, which are shorter and straighter than those of the typhoid bacillus. It is sometimes met with in diplococcoid form, which by cultivation in ascitic fluid may become fixed. Capsulated forms have been described.

Spore-formation does not occur, but vacuolation may

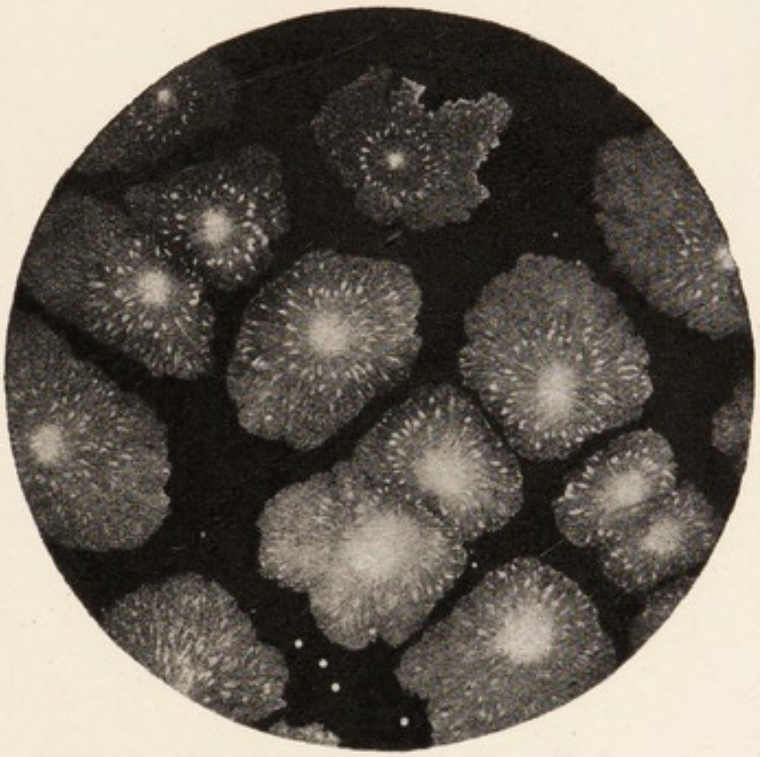


FIG. 43.—Colonies of the colon bacillus, superficial and deep.

sometimes be observed. The organism stains well by the ordinary anilin dyes, but is Gram-negative.

Cultural characters.—The *B. coli* is aërobic and facultatively anaërobic, and grows readily on the ordinary culture media from 20° to 42° C. In gelatin plates the colonies are visible in twenty-four to forty-eight hours. The deep colonies are spherical, granular, and of a pale brownish colour, darker at the centre than at the periphery. The superficial colonies are at first punctate, round and almost transparent, but subsequently spread on the surface and

may attain a diameter of 3 mm., their margins become irregular, the surface is smooth, they are finely granular, opalescent in appearance, and are thicker at the centre than at the periphery (Fig. 43). On a gelatin streak a copious white, shining, smooth growth develops, the margins of which are irregular and crenated (Plate XIV, *b*), and in old cultures the medium becomes opalescent. In a gelatin stab-culture a white growth develops along the

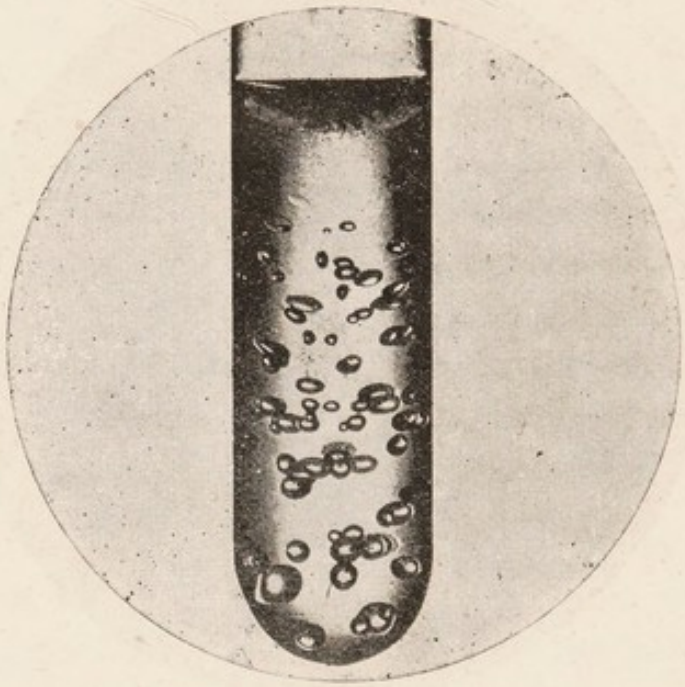
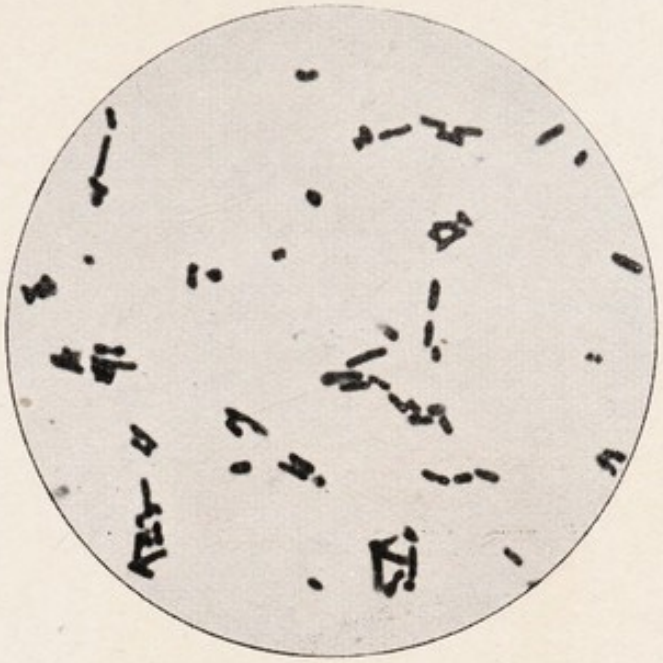


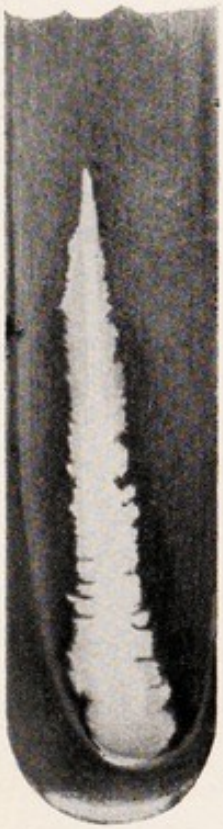
FIG. 44.—Colon bacillus. Gelatin shake culture showing gas production.

line of inoculation with one or more gas-bubbles. The gelatin is not liquefied. On agar and on blood-serum a thick, moist, shining, greyish layer forms. There is abundant formation of gas in a stab-culture in glucose-agar and in gelatin shake cultures (Fig. 44), provided the latter medium be made with meat, for "lemco" gelatin generally fails to give gas. On acid potato it forms a straw-yellow or brownish-yellow, moist, thick growth, but if the potato is not fresh and acid in reaction the growth may be colourless. Milk is a good culture medium, and

PLATE XIV.



a. Bacillus coli. Film preparation from a pure culture. × 1000.



b. Gelatin culture of *B. coli*, six days old.

c. Bacillus pestis. Smear preparation from a bubo. × 1000.

is curdled in twenty-four to seventy-two hours. This curdling is principally due, not to an enzyme, but to the formation of a considerable amount of lactic acid, though a milk-curdling enzyme has been described by Savage [1] as being formed under certain conditions. The gas which is produced in culture media under anaërobic conditions consists of hydrogen and carbon dioxide. Under aërobic conditions marsh gas is stated to be also formed. The ratio of H to CO_2 is about 2 : 1 for dextrose and lactose. In broth it produces a general turbidity without film formation, and the culture gives the indole reaction on the addition of a nitrite in twenty-four to forty-eight hours.

The table, p. 428 *et seq.* gives the fermentation reactions of *B. coli* and of a number of organisms belonging to the typhoid-coli group, for which the writer is indebted to Professor Castellani.

B. coli is an active fermenter of many carbohydrates, alcohols, and glucosides, [2] *e.g.* glucose, lactose, galactose, mannitol and dulcitol, but not of adonitol. Cane-sugar may or may not be fermented; sometimes only acid is formed, sometimes both acid and gas are produced. To the variety producing both acid and gas from cane-sugar Durham gave the name *B. coli communior*. Prescott and Winslow consider that the term *B. coli* should be applied only to an organism that does not attack ketonic sugars. Neutral red in glucose broth is changed to a fluorescent yellow, and Houston describes a typical *B. coli* as "flaginac," *i.e.* producing fluorescence in neutral red glucose peptone-water (fl), acid and gas from glucose (ag), indole in peptone-water (in), and acid and

[1] *Journ. Pathol. and Bact.*, November 1904.

[2] See Twort, *Proc. Roy. Soc. Lond.*, B, vol. lxxviii, page 329; MacConkey, *Journ. of Hygiene*, vol. v, 1905, p. 333, and vol. vi, 1906, p. 385.

curd in milk (ac). The colonies on Conradi-Drigalski agar are large and red (see "Water"). The *B. coli* does not give the Voges-Proskauer reaction (p. 442).

The differentiation of the *B. coli* from the *B. typhosus* should present no difficulty if the morphology and motility of the organisms and their fermentation and agglutination reactions be compared. Bacteriologists usually make use of the following tests for the differentiation of *B. coli*: (1) Morphology, (2) motility, (3) Gram staining, (4) characters of growth and of colonies on gelatin, (5) non-liquefaction of gelatin, (6) action on milk, (7) indole formation, (8) fermentation of glucose, (9) fermentation of lactose and saccharose, (10) action on neutral red. MacConkey suggests that instead of tests Nos. 4, 6, 7, 8, and 10, the following should be substituted: (*a*) fermentation of dulcitol, but not of adonitol and inulin; (*b*) the Voges-Proskauer reaction.

Other media which have been recommended for the differentiation of *B. coli* from *B. typhosus* are the Proskauer-Capaldi media, and Petruschky's litmus whey, but are not now much used.

The Proskauer-Capaldi medium No. 1 is an asparagin-mannitol solution with certain salts; medium No. 2 is a peptone-water-mannitol solution. Both solutions are carefully neutralised and tinged with litmus.

If these media be inoculated with *B. typhosus* and *B. coli* respectively and incubated at 37° C for twenty-four hours, the following changes will be noted:

	Medium No. 1.	*Medium No.* 2.
B. typhosus	No growth or change in reaction.	Growth with strongly acid reaction.
B. coli	Growth with acid reaction.	Growth with neutral or faintly alkaline reaction.

Petruschky's litmus whey is prepared as follows: Fresh milk is warmed and the casein precipitated by the addition of a minimal amount of hydrochloric acid. It is filtered, and the filtrate of

clear whey is carefully neutralised with dilute caustic soda solution. The fluid is then steamed for two hours and filtered; the filtrate should be clear, colourless, and neutral in reaction. Enough neutral litmus solution is then added to render it well coloured, and the mixture is distributed into test-tubes and sterilised. This medium is rendered slightly acid (represented by 6–10 c.c. N/10 caustic soda per cent.) by *B. typhosus*, very acid (40–50 c.c. ditto) by *B. coli*.

The thermal death-point of the organism, according to Weisser and Sternberg, is 60° C. with an exposure of ten minutes. The *B. coli* will grow freely in a slightly acid medium, and in media containing as much as 0·15 per cent. of carbolic acid. In this respect it is a more resistant organism than the *B. typhosus*.

Chemical products.—The acids produced are mainly lævo-lactic acid with some dextro-lactic acid from glucose, lævo-lactic acid only from mannitol; also acetic, formic and succinic acids, and alcohol. According to Harden, *B. coli* attacks glucose in a characteristic manner, each molecular proportion of sugar yielding half a molecular proportion of acetic acid and of alcohol, and one molecular proportion of lactic acid, together with a small amount of succinic acid, and gaseous carbonic acid and hydrogen.[1] Nitrates are reduced to nitrites.

No toxin, or a trace only, is formed in cultures, but the dead bacilli are toxic and pyogenic, and a toxin is obtained by autolysis of cultures or by triturating the bacilli with liquid air (Macfadyen).

Vaughan,[2] by washing large quantities of colon and typhoid bacilli, extracting the bacterial cells first with alcohol, then with ether, and then digesting the ground residue with alcohol containing 2 per cent. NaOH, states that two constituents are obtained, one soluble in alcohol and toxic, the other insoluble in alcohol and non-toxic.

[1] See also Revis, *Centr. f. Bakt.* (2te Abt.), xxvi, 1910, p. 161.

[2] *Trans. XIV Internat. Cong. Hygiene* (Berlin, 1907), Bd. iv, p. 28.

The latter confers a certain degree of immunity on animals injected with it.

Pathogenicity.—The pathogenic action and pathogenicity of the *B. coli* are very varied. Introduced into the circulation or into the peritoneal cavity in guinea-pigs or rabbits it usually causes death in from one to three days with a general septicæmia. Some varieties are, however, non-virulent to animals.

In man the colon bacillus is associated with a number of important pathological processes. It is usually the organism causing the peritonitis which is due to infection from the intestine, as in hernia with obstruction or perforation, in ulceration of the bowel and enteritis, in cancerous growths, and affections of the appendix, biliary canals, and gall-bladder. The exudation in these cases is often characteristic; at first it is clear and greenish, it then becomes greenish-yellow, thin, semi-opaque and foul-smelling, and finally purulent. The colon bacillus may pass through the intestinal wall where it has been damaged, but not yet perforated, as in strangulation.

The *B. coli* is a pyogenic organism, and has been met with in ischio-rectal abscesses (probably the *B. pyogenes fetidus* of Passet). Possibly it causes in some instances the pneumonia and pleurisy occurring after peritonitis for it has been obtained from the lung and pleura in these conditions, but it must be recognised that the *B. coli* is a common secondary or terminal infection. *B. coli* sometimes induces puerperal fever and other forms of septicæmia and it is a common cause of cystitis and other infections of the urinary tract. Coliform organisms are also frequent in dirty septic injuries and gunshot wounds, particularly in the early stages.

In the Pictou cattle disease, characterised by extensive hepatic cirrhosis, Adami found a minute diplococcus or short bacillus. A similar form was afterwards isolated

by him in hepatic cirrhosis in man. Miss Abbot,[1] from a study of several such cases, came to the conclusion that this organism is a variety of the *B. coli*. It has been suggested that hepatic cirrhosis is produced by poisons or toxins, *e.g.* of the *B. coli*, and that alcoholism, the usual cause assigned, is but an exciting or secondary agent.

Anti-serum and vaccine.—Attempts have been made to prepare an anti-serum for *B. coli* infections, but they have met with little or no success.

A *vaccine* consisting of a killed culture has been used successfully in the treatment of chronic *B. coli* infections, *e.g.* cholangitis, cholecystitis, pyelitis, and cystitis; it should always be an autogenous one. The *B. coli* vaccine is more toxic than most vaccines, and small doses must therefore be given (see p. 244).

Clinical Examination

(1) The appearance and odour of the pus are often characteristic. Smears of the pus show small bacilli, which are decolorised by Gram's method.

(2) The organism may be isolated by plating on gelatin, agar, litmus lactose agar, Conradi-Drigalski agar, or by the use of neutral red or bile-salt media (see "Water"). The isolated organism must be tested as to its morphology, motility, non-Gram staining, non-liquefaction of gelatin, indole production, curdling of milk, and fermentation of glucose, lactose, dulcitol, mannitol, etc.

(3) An agglutination reaction may likewise be tried, but if negative is of little value, as there are so many varieties of the colon bacillus, and one variety may not be agglutinated by the specific serum obtained with another variety. A positive reaction must also be carefully controlled, as the colon bacillus is much more readily agglutinated by normal serum than is the typhoid bacillus.

'Coliform' Organisms

Organisms are frequently met with in fæces, manure, sewage and polluted water which resemble the typical *B. coli* in many of their

[1] *Journ. Path. and Bact.*, vol. vi, 1900, No. 3, p. 315 (Bibliog.).

characters, but which differ from it in certain particulars. Thus the colonies on gelatin, instead of being smooth, may be wrinkled ; milk may be but slowly curdled (three to eight days) ; acid or gas production, or both, in sugars may be less marked than usual. These forms, termed 'coliform' organisms, are generally regarded as varieties of the *B. coli*, and are perhaps derived from typical *B. coli*. There is, however, little evidence that *B. coli* can be transformed into such varieties, or that these varieties can be reconverted into typical *B. coli* ; Revis (*loc. cit.*) has produced considerable alterations of fermentive power, and in the characters of the colonies of certain coliform organisms.

A number of other organisms, which have been given distinctive names, are allied to *B. coli* (consult Table of fermentation reactions, p. 428 *et seq.*).

The following may be referred to :

(1) *Bacillus cavicida* (Brieger).—This resembles *B. coli* in most of its characters, but was stated to be non-motile. MacConkey says it is motile. Does not ferment maltose.

(2) *Bacillus neapolitanus* (Emmerich).—Isolated from the bowel in cases of cholera. It differs from *B. coli* in not being motile, and in fermenting cane sugar.

(3) Gas-forming bacilli of Laser and Gärtner.[1]

(4) Aërobic bacillus of malignant œdema (Klein).

(5) *Bacillus lactis aërogenes* of Escherich.—Found in the intestine of nurslings and in milk. Much like *B. coli*, but is non-motile. It differs from *B. coli* by not fermenting dulcitol, by fermenting saccharose and adonitol, and by giving the Voges-Proskauer reaction (see Table, p. 430). According to Harden and Walpole,[2] its action on glucose differs from that of *B coli*, more alcohol being produced and formed at the expense of that part of the molecule of the sugar which in the *B. coli* fermentation yields acetic and lactic acids.

The Voges-Proskauer reaction is obtained by growing the organism in 2 per cent. glucose broth in a fermentation tube (Fig. 12, p. 73) for three days and adding some strong caustic potash solution ; on standing exposed to the air a pink colour develops. According to Harden and Walpole [3] the reaction is probably due to acetylmethyl-carbinol, which in the presence of

[1] *Centr. f. Bakt.* (I^te^ Abt.), xiii, 1893, p. 217 ; xv, 1894, pp. 1, 276.

[2] *Journ. of Hygiene*, vol. v, 1905, p. 488 ; *Proc. Roy. Soc. Lond.*, B, vol. lxxvii, 1906, p. 399.

[3] *Proc. Roy. Soc. Lond.*, B, vol. lxxvii, 1906, p. 399.

air and potash is oxidised into diacetyl, which then reacts with some constituent of the peptone in the medium, giving the pink colour.

The *B. lactis aërogenes* (which may be classed among the capsulated bacilli, see p. 291) is occasionally pathogenic, causing peritonitis.[1] In these circumstances, it is capsulated, but the capsule is difficult to stain. It seems probable that the *B. capsulatus* of Pfeiffer is identical with this organism.

(6) *B. cloacæ* (Jordan).—Met with in sewage. In general characters it much resembles *B. coli*, but produces more gas (75 per cent.) from glucose and liquefies gelatin in four or five to thirty days. Like *B. lactis aërogenes*, saccharose is always fermented and the Voges-Proskauer reaction is positive, but neither dulcitol nor adonitol is fermented. (See Table, p. 428.)

Flies as Carriers of Infection

Flies and other "insects" may convey infection (1) by acting as "porters" and infecting food, etc., (2) by direct inoculation, (3) by inoculation after a cycle of development—in which case the carrier is more or less specific; *e.g.* anopheline mosquitoes in malaria. In the first method the organisms are generally bacteria, occasionally ova of worms; in the second, bacteria or protozoa; in the third, invariably protozoa, filaria, etc., *i.e. animal organisms.*

The ordinary domestic fly, the blue-bottle and other similar flies (of which there are many) have no biting proboscis, but undoubtedly directly convey infection to food, etc., by carrying organisms upon various parts of their body, or by the organisms passing through the digestive tract and infecting the food with the fæces. In this way, typhoid and paratyphoid fevers, bacillary dysentery, *B. enteritidis*, summer diarrhœa, cholera, and possibly anthrax, and also the ova of certain worms, may be conveyed.

The ordinary house-fly breeds in dung and garbage containing dung, and it has a possible range of flight of about a mile. The house-fly experimentally infected remains grossly infected for at least three days, and a smaller degree of infection persists for ten days or even longer.[2]

[1] See Churchman, *Johns Hopkins Hosp. Bull.*, vol. xxii, 1911, p. 116.

[2] See *Reports to the Loc. Gov. Board. on Flies as Carriers of Infection*, Nos. 1–4, 1910 and 1911. Martin, *Brit. Med. Journ.*, 1913, I., p. 1.

CHAPTER XI

BUBONIC PLAGUE—CHICKEN CHOLERA—MOUSE SEPTICÆMIA

Bubonic Plague

Plague was epidemic throughout Europe during the Middle Ages; in England in the fourteenth century it appeared as the Black Death, and in the seventeenth century as the Great Plague of London, while numerous lesser visitations have been recorded. For some years plague has been practically pandemic. The disease seems always to have been endemic in certain centres, *e.g.* in Asia Minor, on the Persian Gulf, in Yunnan, in Uganda, etc. A characteristic of plague is the manner in which it appears and remains prevalent for a time in a district and then disappears, to reappear again after a considerable interval; this has happened not only in Europe, but also in Persia, Syria, India, and China.

Three principal types of the disease are recognised, the bubonic in which the femoral (rarely the inguinal), axillary and other glands become enlarged (whence the disease derives its name), the septicæmic, and the pneumonic. In India the disease has been mainly bubonic (70 per cent. of the cases). Occasionally the majority of the cases are pneumonic, as for instance in Accra, in China in 1910–11, and in the small outbreak in Suffolk in 1910. Septicæmic cases are the exception, but any form tends to become septicæmic on the approach of death.

At the commencement and at the end of an epidemic the disease may assume an extremely mild type, the so-called "pestis minor."

Bacilli were first observed in this disease in the blood, buboes, and organs by Kitasato in 1894. In the same year (1894) Yersin investigated the outbreak of bubonic plague at Hong Kong, and described the bacillus met

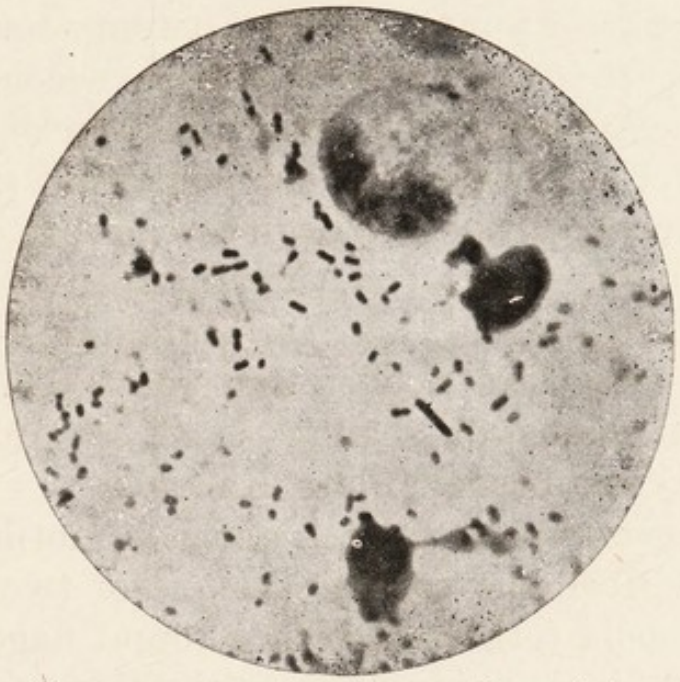


FIG. 45.—Smear preparation from spleen of inoculated guinea-pig. × 1000.

with in the buboes and its cultural and pathogenic properties very fully. This organism is known as the *Bacillus pestis*.

Morphology.—The *B. pestis* belongs to the group of hæmorrhagic septicæmic bacilli (chicken cholera, rabbit and ferret septicæmia, swine plague, etc., see p. 458), and is a markedly pleomorphic organism. *In the animal body* it occurs for the most part as a short, plump, non-sporing rod, measuring 2–3 μ by 1–2 μ, but longer forms may be seen here and there measuring as much as 5 μ (Fig. 45). Polar staining is a marked feature (Plates XIV, *c*, and

XV, *a*), and swollen involution forms occasionally occur. The typical form of the organism, the bi-polar staining, short, stumpy bacillus, is met with in smears from the buboes, in the sputum in the pneumonic form, and in the blood in the septicæmic variety, but only in the earlier stages of the disease. Later the typical forms tend to disappear, their place being taken by a few large, rounded, ovoid, or pear-shaped involution forms. *Under cultivation* the bacilli in young cultures (twenty-four to forty-eight hours) are so short as to be almost coccoid or slightly ovoid ; on agar their size is about the same as that in the animal body, on gelatin they are somewhat smaller, but a few well-marked rods and even threads are always present. In older cultures, rod, thread and involution forms occur more numerously ; on agar containing 2–3 per cent. of salt the latter are swollen and yeast-like.

In broth chains of slightly ovoid organisms occur resembling streptococci (Plate XV, *b*).

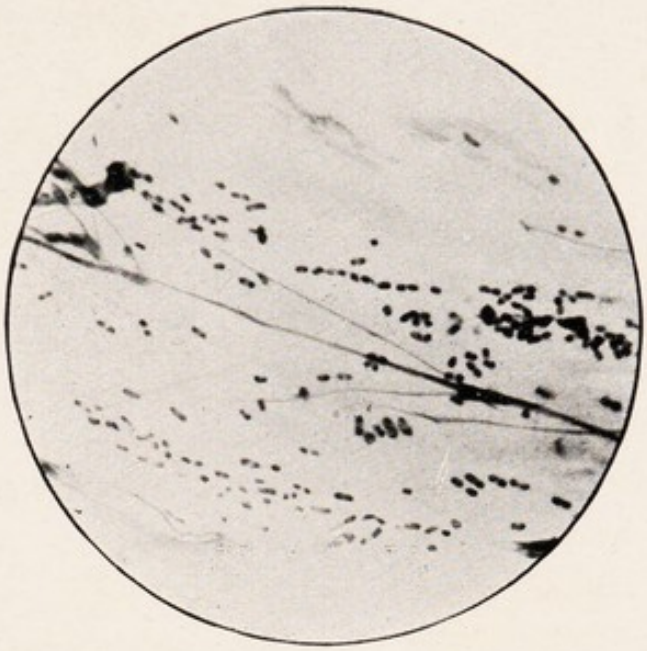
The organism is non-sporing and non-motile, although Gordon described the presence of one or two fine spiral terminal flagella (others have not found flagella).

Sometimes in hanging-drop cultivations a capsule is apparently present, but the writer has failed to verify this by staining methods.

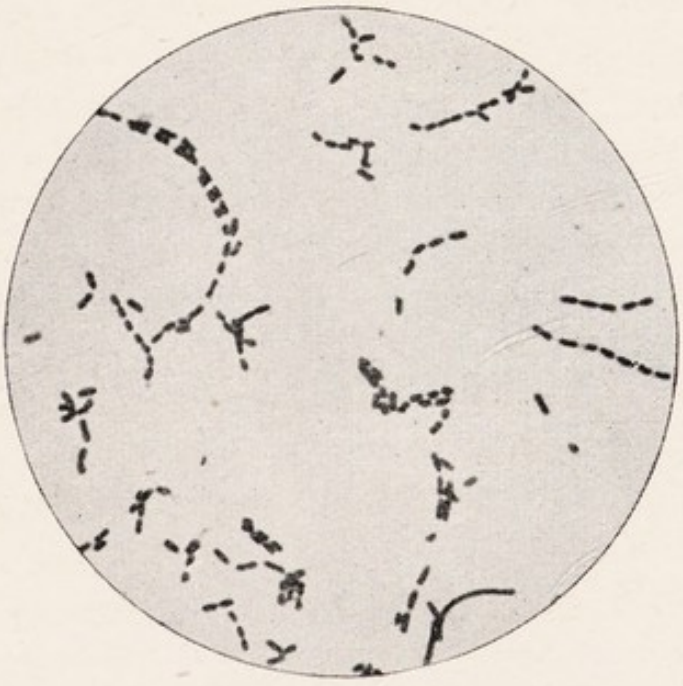
The *B. pestis* stains well with Löffler's blue and anilin-gentian violet, polar-staining being a marked feature, especially in smear preparations. It does not stain by Gram's method. With old laboratory strains polar staining may be completely absent, but in such cases may sometimes be obtained by first treating the preparations with alcohol or by the Gram method, and subsequently staining with Löffler's blue or weak gentian violet. Sections are best stained with carbol methylene or thionine blue.

Cultural characters.—The *B. pestis* is aërobic and facul-

PLATE XV.



a. Bacillus pestis. Smear preparation of sputum. × 1000.



b. Bacillus pestis. Film preparation from a 72-hours' broth culture. × 1000.

tatively anaërobic. On blood-serum it forms moist, smooth, shining, cream-coloured colonies or growths, slightly raised above the surrounding medium. The blood-serum is not liquefied.

On agar the colonies are raised, round and cream-coloured, finely granular, denser at the centre than at the margins, which are regular. Size 0·25 to 0·5 mm. in two days at 37° C.

On surface agar the *B. pestis* forms a thick, opaque, moist, smooth, cream-coloured growth, the margins of which are usually markedly crenated; the growth is very sticky and tenacious. Haffkine states that when grown on *dry* agar (agar which has been kept in the warm incubator for two to three weeks) and viewed from behind the growth has an appearance like that given by the back of a mirror—*i.e.* a dull, silvery appearance.

On a salt agar (2·5–3·5 per cent. of sodium chloride) Hankin describes the development of remarkable spherical or pear-shaped involution forms.

On gelatin the colonies are whitish, filmy, finely granular with regular margins. Size, 0·1 to 0·25 mm. in five days at 22° C.

On surface gelatin the organism forms a thin, white, granular growth, with slightly irregular surface and margins, and nearly confined to the inoculation track (Fig. 46). The growth does not penetrate into the medium, nor does it render it cloudy. The growth is very adherent.

In a stab gelatin culture a delicate whitish, finely granular growth develops to the end of the stab, with little tendency to spread from the needle track. The gelatin is not liquefied. Both in agar and gelatin cultures fresh punctate growths sometimes develop in the original growth, simulating a contamination. No growth occurs on ordinary potato, and milk is not coagulated.

In broth the growth is somewhat characteristic. For

two or three days the broth remains perfectly clear, but a flocculent growth forms and gradually increases in amount on the bottom and sometimes upon the sides of the tube. After some days the broth may become a little cloudy. A delicate flocculent film develops if the tube be kept absolutely at rest. In broth to which a little butter-fat or ghee has been added little islands of growth appear on the surface, and from these flocculent tapering dependent growths form in about a week, provided the tubes or flasks be kept absolutely at rest, the bulk of the broth remaining clear. This is the stalactite growth of Haffkine, and is very characteristic (*B. pseudo-tuberculosis* also gives it). Broth cultures reduce a weak solution of methylene blue.

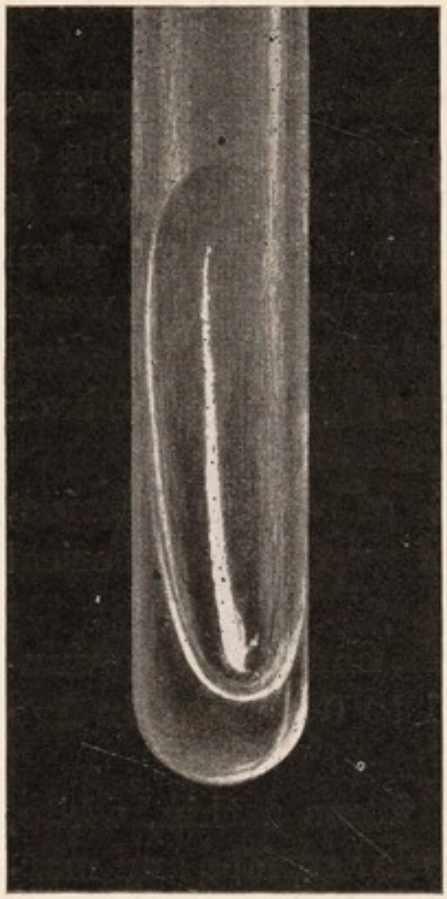


FIG. 46.—Plague, surface culture on gelatin four days old.

With sulphuric acid alone a feeble indole reaction can be obtained with week-old broth cultures. With sulphuric acid and a nitrite a well-marked indole reaction can be obtained under the same conditions.

The fermentation reactions of the *B. pestis*, which MacConkey has pointed out are practically identical with those by the *B. pseudo-tuberculosis*, are as follows : Acid production, but no gas, in glucose, lævulose, galactose, maltose, mannitol, and dextrin, no change in lactose, cane-sugar, and dulcitol.

Action of antiseptics, etc.—The plague bacillus is readily destroyed by antiseptics ; a 1 : 1000 corrosive sublimate

or 1 : 100 chloride of lime solution being efficient. An *acid* solution of corrosive sublimate is preferable, and for the practical disinfection of native houses a 1 : 250 solution of sulphuric acid may be employed. A temperature of 65° C. kills the organism in about fifteen minutes. Desiccation over sulphuric acid at 30° C. is also rapidly fatal.

Vitality and virulence of cultures.—Cultures retain their vitality for at least a month. As regards virulence, the organism varies much according to the source from which it is obtained. Under cultivation it gradually loses its virulence unless subcultured in the following manner: The cultures are made every week on surface agar, are placed in the blood-heat incubator for twenty-four hours, and are then removed and kept at room temperature. If inoculated into animals the virulence may be heightened for a particular species by successive passages, but in so doing is diminished for other species.

Pathogenic action.—In addition to man, the following animals are liable to contract plague under natural conditions—the monkey, cat, rat, mouse, squirrel, hare, ground squirrel, ferret, bandicoot, and marmot. The guinea-pig and rabbit are also susceptible to inoculation. The horse, cattle, sheep and goat are relatively insusceptible, though Simpson stated that calves and poultry may be infected by feeding, and suffer from a chronic form of the disease (this observation of Simpson's has not been confirmed by other workers). Birds are not easily susceptible, and vultures feeding on the corpses of the plague-stricken do not seem to contract the disease. The mouse, rat, and guinea-pig are the animals chiefly used for experimental purposes in the laboratory; the first two are highly susceptible.

A guinea-pig inoculated with plague material or with a pure cultivation usually dies in from two to seven days,

the symptoms being sluggishness and loss of appetite, sometimes a discharge from the eyes, and towards the end, staring coat and perhaps convulsive and paralytic attacks. The post-mortem appearances are extensive hæmorrhagic œdema at the seat of inoculation, enlargement and congestion of the spleen, and enlargement of, and hæmorrhages into, the inguinal and axillary lymphatic glands. If the animal live six or seven days, the glands may be as large as small nuts (see some admirable preparations in the College of Surgeons Museum). The spleen

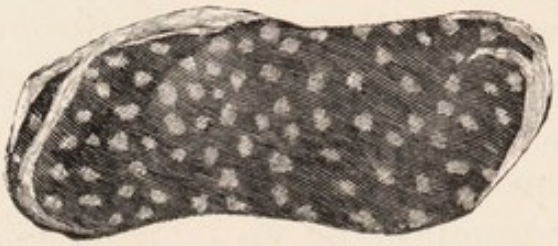


FIG. 47.—Spleen of guinea-pig inoculated with plague. (Nat. size.)

may be enormous, six times its natural size, and studded with small yellowish nodules resembling miliary tubercles, consisting of necrotic areas with masses of bacilli (Fig. 47); the lungs also may be more or less inflamed, and contain small and large necrotic foci. The bacilli are extremely numerous at the seat of inoculation, in the glands, and in the spleen, less so in the peritoneal fluid, liver, and blood; if the death of the animal is delayed the exudation in the bronchi may contain considerable numbers. Some bacilli may generally be found in the duodenum, trachea, and larynx. Mice usually die in from two to three days, and rats in from three to seven days after inoculation. In rats and mice the post-mortem appearances are similar to those in the guinea-pig. Rabbits are much less susceptible to plague than guinea-pigs, but much depends upon the virulence of the strain. A very small dose of

a pure culture may fail to kill an inoculated animal. Rats can be infected by feeding on the corpses or carcases of men or animals dead from the disease.

In man the bacilli are found in large numbers in the fluid in the buboes, either alone or mixed with streptococci or micrococci, and in the sputum in the pneumonic form. They are not usually found in any number in the blood except in the septicæmic variety, or shortly before death, and in stained preparations appear as short plump bacilli, often in pairs, with polar staining and unstained centres (Plates XIV,*c* and XV,*a*). If theorganisms are found to be free and numerous in the buboes the prognosis tends to be grave, but if they are largely present within the phagocytic polymorphonuclear leucocytes the prognosis is better and the disease will probably remain localised.

Toxins.—The plague bacillus forms but little toxin, the minimal fatal dose of the most active filtered broth culture for a mouse being about 0·02 c.c. In order to prepare a vaccine or an anti-serum it is necessary, therefore, to employ unfiltered cultures—*i.e.* the microbes themselves.

Macfadyen obtained an endotoxin by triturating the bacilli frozen with liquid air.

Vaccines and immunity.—Of the plague vaccines, that of Haffkine, the Haffkine prophylactic, is the best known, and has been extensively employed. It consists essentially of a four to six weeks old butter-fat broth culture of the plague bacillus, killed by heating to 65° C. for an hour, with a small addition of antiseptic. As to the value of Haffkine's prophylactic a mass of figures is available. By its use both the incidence of, and mortality from, plague are markedly diminished. Wilkinson collected the following data of the efficiency of the vaccine: Among the inoculated the case incidence was 1·8 and the case mortality 23·9 per cent.; among the uninoculated the figures were 7·7 and 60·1 respectively. The immunis-

ing products seem to be mainly intracellular, but the broth itself is not without action.

Other vaccines have also been devised. Lustig and Galeotti prepared one by digesting the growth from agar cultures with 1 per cent. caustic soda solution, filtering through paper, and precipitating with very dilute acetic or hydrochloric acid, or by saturation with ammonium sulphate. The precipitate is dissolved in a 0·5 per cent. solution of sodium carbonate, and filtered through a Chamberland filter; this forms the vaccine fluid. Calmette prepared a vaccine by emulsifying an agar growth in water, well washing the organisms with sterile water to remove adherent toxin, emulsifying again in sterile water, heating to 70° C. for an hour, and finally drying *in vacuo*. The dry substance can be kept for a considerable time without change. For use 1–2 mgrm. are emulsified in 2–3 c.c. of sterile salt solution and injected.

Yersin proposed vaccinating with living culture of feeble virulence, which has been done by Strong in Manila. Though such a method might be used in a plague-stricken district, it is obviously one that can be used only with the greatest caution.

Klein [1] prepared a prophylactic by drying the organs of a guinea-pig dead of plague for three days at 46° C., rubbing the material to a powder, and further drying at 37° C. for three days. Of this dry powder 15–16 mgrm. protected a rat, and 25 mgrm. a monkey.

With reference to experimental immunity and protection in plague, Klein [2] found that a guinea-pig which had been three times injected with an amount of living culture insufficient to kill was still capable of being infected; that the blood of a guinea-pig which had twice passed through an attack of plague did not contain an appreciable amount of germicidal substances; and that the immunisation of guinea-pigs by sterilised cultures is an extremely slow and difficult process. Calmette also found that the guinea-pig was extremely difficult to immunise.

Calmette, from laboratory experiments, surmised that protection with a vaccine is not attained for some days, and that in the interval susceptibility to infection is increased. These observations are not borne out in practice, for Bannerman [3] found that

[1] *Rep. Med. Off. Loc. Gov. Board* for 1905–06.
[2] *Ibid.*, 1896–97, App. B., p. 2.
[3] *Centralblatt f. Bakt.* (2te Abt.), Bd. xxix. p. 873 (Bbiliog.).

so far from there being an increase in mortality among those who have been inoculated and who develop plague within ten days of inoculation the reverse is the case, and that in a small community where the population had been partly vaccinated and partly not vaccinated, the incidence of plague during the week following vaccination was less among the vaccinated than among the unvaccinated, pointing to the rapid production of protection.

Anti-plague serum.—This is prepared by growing the *B. pestis* on the surface of agar in plate bottles, washing off and emulsifying the growth, and for the earlier injections the emulsion is heated to 65° C. for one hour, and the commencing dose is $\frac{1}{24}$ part of a flask. The injections are given intravenously at intervals of a week. At the end of three months the bactericidal power of the blood will have become very marked, and living cultures are then injected for a further period of about three months until a whole flask-culture is given at a dose. An interval of a fortnight is allowed to elapse between the last dose and the bleeding of the animal. The serum is tested upon mice.

The anti-plague serum, which is mainly anti-microbic, is not very potent, and to be of service large amounts and early treatment are essential.[1]

Epidemiology.—The mode of infection in man has been a matter of controversy. The pneumonic form arises generally from aërial infection by the respiratory tract. It is extremely fatal and infectious, while the bubonic and septicæmic varieties are hardly even contagious. Although a gastric and intestinal form of the disease has been described, and there is evidence to show that food or drink may be the vehicle of infection, this must be a rare mode of infection. Yersin claimed to have isolated the bacillus from the dust and earth of a native dwelling, and Hankin from the brackish water in a field. The observations of Hankin and others indicate, however, that contagion is

[1] See Hewlett's *Serum Therapy*, 1910.

likely to occur only from immediate contact with man or animals, or their excretions, infected with plague, and not from a saprophytic form of the organism.

Certain animals, especially the rat (*Mus rattus* and *Mus decumanus*), are important agents in spreading the disease. The association of sickness and of death among rats with an epidemic of plague has been established by a number of observations, and in some instances the epizootic among rats has been definitely shown to precede the epidemic in man. The epidemics at Sydney are perhaps the most striking instances of rat-borne plague; discussing the first one Tidswell says: "The one clear fact in our epidemic was that human beings were not becoming infected from one another." In the first epidemic the mode of introduction of the disease was never traced to any human source. During an epidemic rats may be found in all stages of illness and plague bacilli can be found in large numbers in their carcases. In the various epidemics at Sydney, cases of plague first occurred among the rats and mice, followed after an interval of days or weeks by human cases. Other animals may occasionally be the means of disseminating the disease, *e.g.* the ground squirrel in California and the marmot in Manchuria. The experiments of the Advisory Committee on Plague Investigation in India have conclusively shown the important part played by rats in the dissemination of the disease, though the origin of the primary infection in rats is obscure. They may possibly become infected from the dust of earthen floors of the native houses soiled with excreta or discharges of plague patients, or from their clothing, poultices or dressings, but the readiest method is probably by feeding on the dead. Once the epizootic has started, further infection is simple; rats fight, and so may directly inoculate one another; sick rats may soil grain or other food-stuffs, and dead rats are eaten by their fellows.

Moreover, parasitic insects, especially fleas, undoubtedly may transmit the disease from one animal to another. Thus it is found that if guinea-pigs be placed in a plague-infected compound, many of the animals contract plague; but if the animals be placed in cages of wire-gauze, the mesh of which is small enough to prevent access of fleas, the animals do not contract plague. The transmission of the disease from rats to man is similarly due to transmission by fleas (except in the pneumonic forms in which infection is direct from the sick to the healthy). The great majority of rat fleas are *Xenopsylla cheopis*, *Ceratophyllus fasciatus*, *Cer. anisus*, *Ctenopsylla musculi*, and *Ctenophthalmus agyrtes*, of which the first is most prevalent in the tropics and subtropical regions, the second in cooler regions and in this country.[1] Walker [2] has found that bed-bugs may occasionally transmit plague. The bacilli multiply in some of the fleas to such an extent as to occlude the entrance to the stomach. Such fleas will still bite, but on ceasing to suck, some of the blood with numerous bacilli in it regurgitates into the wound and thus infects.[3] The seasonal prevalence of plague coincides with the prevalence of rat-fleas. Humidity favours the longevity of the rat-flea, and the decline of plague epidemics in Northern India with the onset of the hot weather is probably correlated with this factor. The manner in which the periods in the year when human plague does not occur are bridged over is unknown. In such periods rats suffering from plague have been found, but these are regarded as having a retrogressive form of the disease rather than a chronic infection. The destruction of rats, either by trapping, poisoning, or

[1] See Chick and Martin, *Journ. of Hygiene*, vol. xi, 1911, p. 122.
[2] *Ind. Med. Gaz.*, May 1910.
[3] Bacot and Martin, *Journ. of Hygiene*, XIII, Plague Supp. III, 1914, p. 423.

asphyxiating, or by the use of the Danysz rat virus (see p. 417), is, therefore, one of the means to be adopted in fighting the disease. The extermination of rats seems quite impossible, but by rat destruction there is a likelihood of destroying infected animals and the subsequent development of a healthy race. On the other hand, objection has been taken to rat-destruction, it being surmised that if the epizootic be allowed to proceed, the susceptible rats will be exterminated and a race of rats relatively insusceptible to plague will ultimately be established.

On Plague, see Simpson, *Treatise on Plague* (Cambridge University Press); Klein, *Bacteriology of Oriental Plague*; "Reports on Plague Investigations in India," *Journ. of Hygiene* (extra numbers), vols. vi, *et seq.*

Clinical Examination

If it cannot be examined immediately, plague material may be placed in a solution containing glycerin 20 c.c., distilled water 80 c.c., calcium carbonate 2 grm. The bacilli retain their vitality and virulence in this for thirteen days (Albrecht-Ghon method).

(1) Withdraw a little of the fluid from the bubo by means of an antitoxin syringe. Make smears and stain with methylene or thionine blue. Search for short plump bacilli, often in pairs, with polar staining and unstained centres. They are not stained by Gram's method.

N.B.—There may be a mixture of organisms in the buboes.

(2) Make agar plates and broth cultures. Incubate the cultures at 25°–27° C., *not* at 37° C. From colonies on the agar plates the organism may be isolated and its cultural and pathogenic characters ascertained. The appearance of the broth cultures, if characteristic, would be very suggestive of plague, but if uniform turbidity develops this may be due to contaminating organisms, *e.g.* micrococci.

(3) Inoculate mice, rats, or guinea-pigs subcutaneously with the fluid or with the culture. Some of the animals should be inoculated by the cutaneous method—rubbing a little of the material on the shaved abdomen, and also as in (4). Inoculation of rats

serves to distinguish the *B. pseudo-tuberculosis* from the *B. pestis.* If the animals die, investigate for the *Bacillus pestis* by staining and culture methods.

(4) In the pneumonic form, dilute the sputum with a little boiled water, inoculate several agar tubes, and incubate at 25°–27° C. Examine in two to three days. Also daub the nostrils of a guinea-pig or rat with a brush or pledget of wool dipped in the diluted sputum, avoiding wounding the mucous membrane. Smears of the sputum may also be made, stained, and examined. Gram's method will distinguish the *B. pestis* from the *Streptococcus pneumoniæ ;* the latter stains well by Gram.

(5) *Agglutination reaction.*—Agglutination and other serum reactions do not seem to be of much value in the diagnosis of plague. A modified method of agglutination is considered by Dunbar [1] to be of use and is carried out as follows :

A small quantity of peptone water is inoculated with the juice from the bubo (or other organ), post mortem, and is mixed with an equal quantity of plague-serum of such a strength that the dilution reduces it to 1 : 200 (approximately). A second dilution of 1 : 400 and a third of 1 : 800 are also prepared.

As a *control,* an equal quantity of the inoculated peptone water is mixed with normal serum (rabbit or horse serum), the dilution being 1 : 100.

In a few minutes a distinct difference is observable. The " control " shows with the oil-immersion lens a few isolated non-motile bacteria, while the plague-serum dilution 1 : 200 shows larger and smaller masses of agglutinated bacteria.

After two hours' incubation the same result is obtained with the plague-serum dilution of 1 : 400. No agglutination, however, is observed after incubation for twenty-four hours of the dilution of 1 : 800. This agglutination reaction, in conjunction with other suspicious phenomena, justifies notification of suspected plague.

In the examination of rats suspected to be suffering from plague infection, it is essential not only to take the naked-eye characters into account, but to make microscopical preparations and cultures, and to test the cultures by animal inoculations. *Care must be taken not to mistake hæmorrhagic septicæmic bacilli* (see pp. 445, 458) *and other organisms for the plague bacillus.* The

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[1] *Centralbl. f. Bakt.,* xli (Originale), 1906, p. 860.

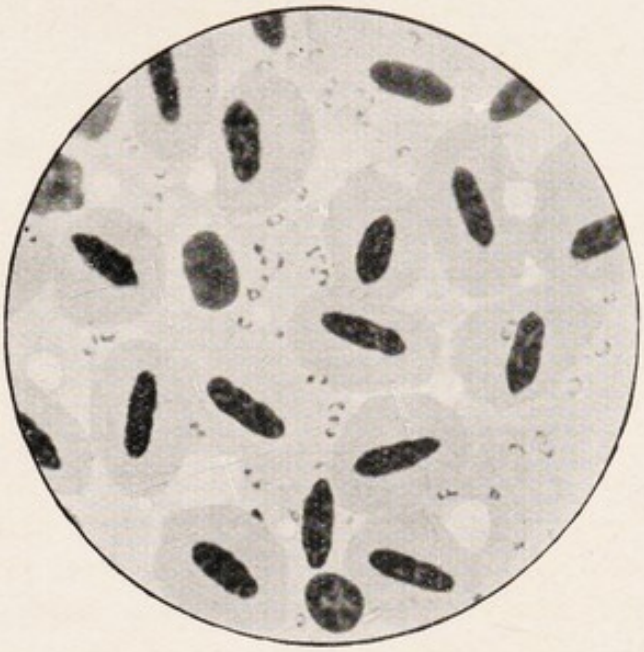
B. coli, *B. proteus*, and other organisms are recorded by Klein (*loc. cit.*) as simulating the *B. pestis*.

Chicken Cholera

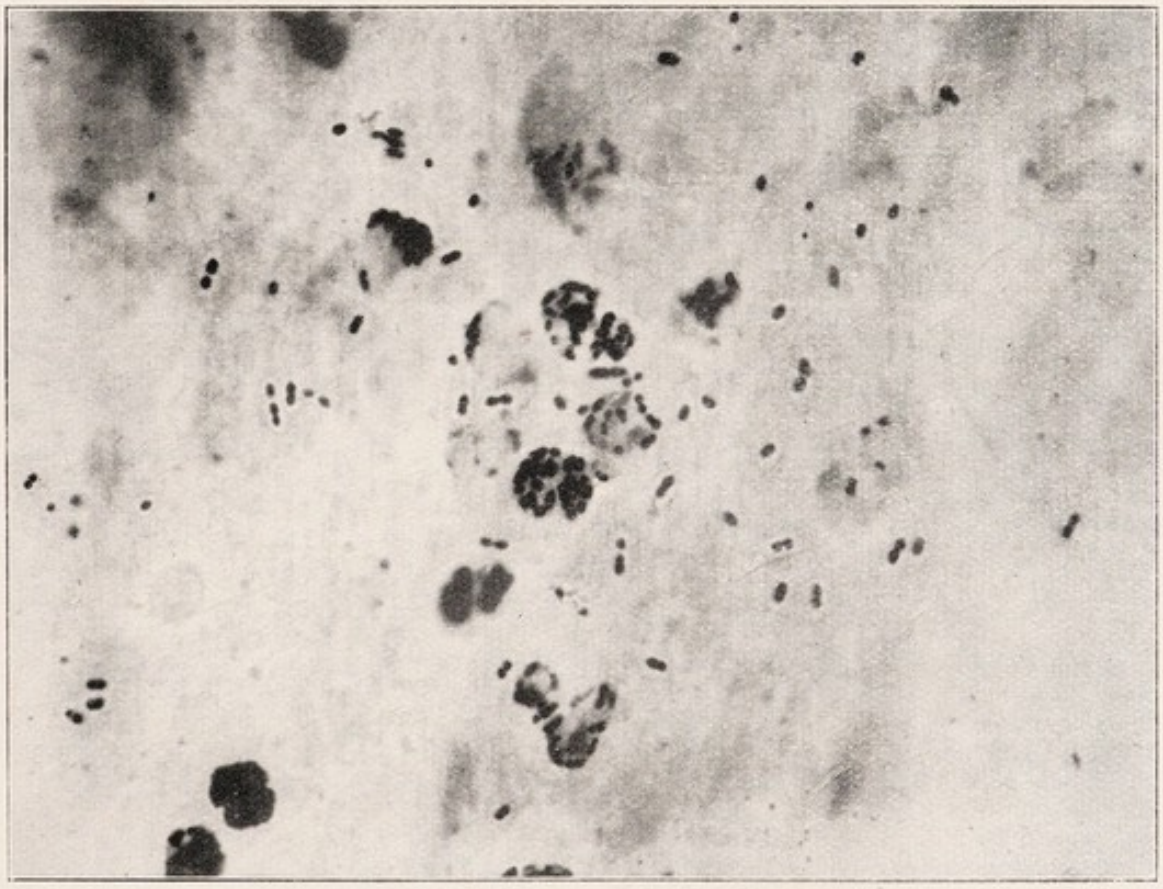
Chicken cholera is a disease of poultry characterised by profuse diarrhœa; its course may be very rapid, and the bird found dead without having shown signs of illness. The organism (*B. cholerœ gallinarum*) is a very short rod, non-motile, so short that it is almost ovoid, 0·6 to 0·8 μ in length, and 0·4 to 0·5 μ in diameter. It stains by the ordinary anilin dyes, but not by Gram's method, and the staining tends to be polar, so that Pasteur, who first investigated the disease, described it as a diplococcus (Plate XVI, *a*). It is aërobic and facultatively anaërobic, non-sporing, and is easily killed by heat and antiseptics. The organism grows freely on the various culture media from 20° to 38° C., on agar forming a thick, moist, cream-coloured layer, on gelatin a shining, white, expansive growth without liquefaction. In broth a general turbidity forms, but growth on potato is indifferent. Some strains appear to produce acid only, other strains acid and gas, from most of the sugars. If dried it dies in a few days, but retains its vitality for a considerable time in damp earth or in water, and so infection is readily conveyed. Fowls die after subcutaneous, intramuscular or intravenous inoculation and by feeding, the organisms being found abundantly in the blood. Post-mortem, the serous membranes may be inflamed and hæmorrhagic, the liver large and soft, and the intestine shows hæmorrhagic spots, and is sometimes ulcerated and contains a mucoid fluid stained with blood. Other birds, pigeons, pheasants, sparrows, wild and domestic ducks are also susceptible to the disease, and rabbits and guinea-pigs can be successfully inoculated; in the latter animal a local abscess sometimes forms instead of a general infection. By continuous cultivation with free access of oxygen the virus becomes attenuated, and Pasteur was able thus to prepare a vaccine which protected fowls.

The bacillus of chicken cholera belongs to the group of *hæmorrhagic septicæmic bacilli* known to the French under the generic name of *Pasteurella*, and may be identical with Koch's bacillus of rabbit septicæmia. The bacillus of swine *plague B. suisepticus* (see p. 417), also belongs to this group. These organisms tend to form a stalactite growth in butter broth.

PLATE XVI.



a. Chicken cholera. Film preparation of blood of fowl. × 1000.



b. *Diplococcus pneumoniæ*. Smear of sputum. Gram and eosin. × 1000.

Mouse Septicæmia

This disease may be conveniently described here. Koch first obtained a minute bacillus (*B. murisepticus*) by injecting putrefying material subcutaneously into mice, the organism being met with in large numbers in the blood and tissues. It seems to be identical with the bacillus found in swine erysipelas, measures only 1 μ in length, and occurs in considerable numbers in the leucocytes. The bacillus stains well by Gram's method, and is stated by some writers to be motile. It grows readily, forming on agar extremely delicate, almost invisible colonies ; in stab gelatin cultures after some time a delicate cloudiness radiates from the central puncture. From an agar culture the bacilli are somewhat larger than those found in the animal body, and form filaments. It is pathogenic for swine, rabbits, and mice.

CHAPTER XII

PNEUMONIA, INFLUENZA, AND WHOOPING-COUGH

Pneumonia

PNEUMONIA is of two types, lobular, catarrhal, or broncho-pneumonia, and lobar or croupous pneumonia. The former may be primary, or may be secondary and arise in connection with many of the specific fevers, as in measles, whooping-cough, diphtheria, enteric fever, influenza, plague, etc. The broncho-pneumonia occurring in the course of other diseases may be due to the causative organism of the disease, or may be due to other organisms. Eyre [1] examined 62 cases of broncho-pneumonia occurring in the course of other diseases and 102 cases in which the broncho-pneumonia was the primary lesion. Of these 164 cases, 52·4 per cent. yielded pure cultivations of some one or other of six bacteria—pneumococcus, *Strep. longus*, *M. pyogenes* var. *aureus*, *M. catarrhalis*, *B. pneumoniæ*, and *B. influenzæ*; whilst 47·5 per cent. gave a mixed growth of one or more of these six in association with one or more of five other bacteria—*M. tetragenus*, *B. pertussis*, *B. pyocyaneus*, *B. typhosus*, *B. diphtheriæ*. The *B. coli* also occurs in broncho-pneumonia. Acute croupous or lobar pneumonia in many of its characters resembles an acute specific infection, and while frequently a primary disease, may also occur secondarily in almost any condition, and occasionally in epidemic form.

Friedländer in 1882–83 first described organisms in cases of pneumonia. In 1883–85 Talamon, Klein and Sternberg each described in pneumonic sputum an oval encapsuled organism, which induced pneumonia in animals; it was termed by the former the *Micrococcus lanceolatus*, and by Sternberg the *Micrococcus Pasteuri*. This and Friedländer's organisms were at first believed to be identical, but Fränkel and Weichselbaum subsequently showed

[1] *Journ. Path. and Bact.*, vol. xiv, 1910, p. 160.

that they are quite distinct, and that the former is the etiological agent of acute croupous pneumonia.

The majority (95 per cent.) of cases of acute croupous pneumonia are caused by the *Streptococcus pneumoniæ*, and Friedländer's organism, now termed Friedländer's pneumo-bacillus, or *B. pneumoniæ*, is of etiological significance in only a small minority, if at all. The latter organism is, however, associated with certain pathological processes which will be referred to below.

From pleuro-pneumonia of cattle, Nocard and Roux succeeded in cultivating in broth in collodion sacs in the peritoneal cavity of rabbits an organism just visible as minute granules with a magnification of 2000 diameters (*B. bronchisepticus*). Bordet [1] states that it may be grown on the medium employed by him for the cultivation of the *B. pertussis* (p. 473), and then appears as fine, straight, curved, undulating, or even spirillar filaments not unlike spirochaetes.

The Streptococcus (Diplococcus) pneumoniæ

Synonyms, Fränkel's pneumococcus, *Micrococcus Pasteuri* (Sternberg), *Micrococcus lanceolatus* (Talamon), *Micrococcus pyogenes tenuis* (Rosenbach).

Morphology.—The *Streptococcus pneumoniæ* in the sputum and tissues occurs as an oval or lance-shaped coccus united in pairs, occasionally in chains of three or four elements, and then often almost spherical, and is generally surrounded by a well-marked capsule (Plate XVI, *b*). In order to isolate the organism several tubes of glycerin agar, serum or serum-agar may be inoculated in succession with the same loopful of rusty sputum and incubated for forty-eight hours; in some a pure culture may be obtained. A more certain method is to inject a drop or two of the rusty sputum into the peritoneal cavity of a mouse or young rabbit. The animal will die in from twenty-four to thirty-six hours, and the organism will be found in considerable numbers in the lung and blood (Plate XVII, *a*), from

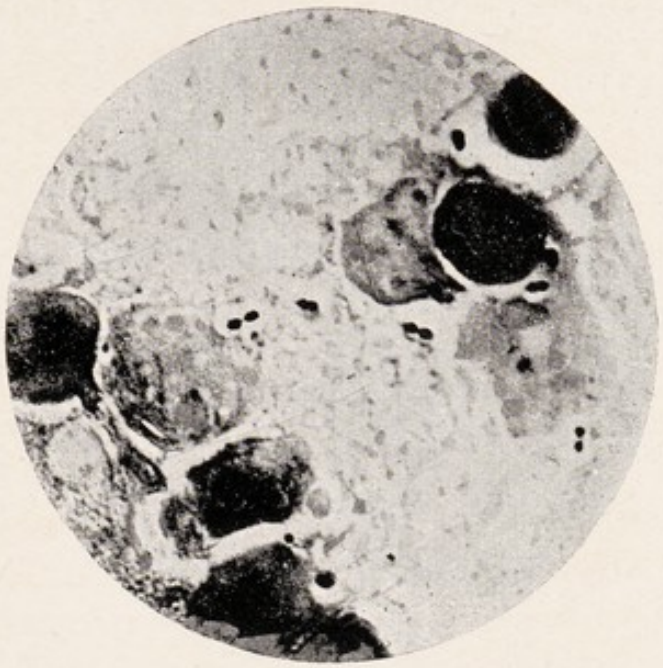
[1] *Ann. de l'Inst. Pasteur*, xxiv, 1910, March.

which cultures may be obtained. It is non-motile, stains with the ordinary anilin dyes and by Gram's method.

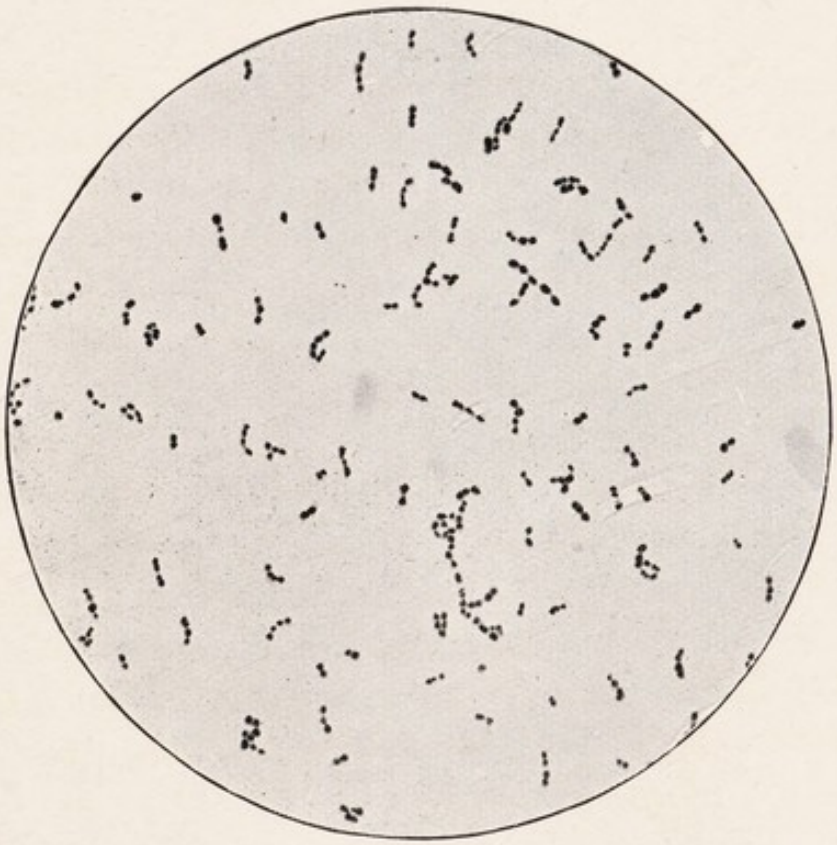
Cultural characters.—The *S. pneumoniæ* is aërobic and facultatively anaërobic. On glycerin agar at 37° C. it forms minute, transparent, almost invisible colonies like droplets of fluid ; on serum the growth has much the same characters, but is somewhat more abundant. It hardly grows on gelatin at the ordinary temperature, but in a 20 per cent. gelatin at 25° C. minute white colonies develop without liquefaction. In broth it produces a slight cloudiness ; it does not grow on potato but develops in milk, which is usually coagulated ; neutral litmus glucose-agar becomes red during growth, indicating the production of acid. The fermentation reactions are given in the Table on p. 257. Hiss's medium (p. 325) with inulin is fermented and coagulated ; most other streptococci fail to ferment inulin. On the ordinary culture media it retains its vitality for a short time only, not more than about a week ; but if a little blood be smeared over the surface of the agar the vitality may be prolonged for a month or even longer. Washbourn recommended an agar rendered alkaline to the extent of 4 c.c. of normal caustic soda per litre, after neutralisation, rosolic acid being the indicator. This medium is smeared with blood, placed in the incubator for twenty-four hours to ascertain whether it be sterile, then inoculated, capped, and kept at 37° C. Foa's method for keeping Fränkel's pneumococcus alive and virulent is to take up the infected blood of an inoculated animal into a small glass pipette, so that the blood completely fills the tube, which is then sealed and kept away from the light at the ordinary temperature. If inoculated on to ordinary gelatin, which is then kept in the *blood heat* (37° C.) incubator, the organism retains its vitality for a month or six weeks.

Under cultivation the *S. pneumoniæ* usually assumes

PLATE XVII.



a. Diplococcus pneumoniæ. Film preparation of blood of inoculated animal. × 1000.



b. Diplococcus pneumoniæ. Film preparation of a pure culture. × 1500.

the form of a short streptococcus (Plate XVII, *b*) (included by Gordon in his *S. brevis* class) and the capsule is lost, but is regained on passage through a susceptible animal, or by growing in fluid serum. A good deal of variation occurs in the morphology of the organism obtained from different sources and under cultivation. The thermal death-point of the *S. pneumoniæ* according to Sternberg is 52° C., the time of exposure being ten minutes, and it is readily destroyed by the ordinary germicides, by light, and by desiccation; but in dried sputum it may retain its vitality and virulence unimpaired for weeks.

Dochez and Gillespie [1] have shown that the pneumococci may be divided into groups by agglutination and saturation tests (*cf.* the meningococcus). They divide them into two general groups; the larger of these contains about 80 per cent. of the strains encountered and may be further subdivided into three smaller groups—I., II., and III. Group III. consists of the type known as the *Pn.* (*Streptococcus*) *mucosus*. The smaller of the two general groups—Group IV.—consists of a series of independent varieties which possesses no cross immunological reactions with one another, nor with the members of the other three groups. The following table shows the relative frequency of these groups among cases treated at the Hospital of the Rockefeller Institute: [2]

	1912-1913		1913-14.	
	No.	Per cent.	No.	Per cent.
Group I	35	47	21	30
Group II	13	18	28	39
Group III (*mucosus*) . .	10	13	6	8
Group IV (heterogeneous) .	16	22	16	23

Lister [3] has also shown that pneumonia on the Rand is associated with at least four groups of pneumococci

[1] *Journ. Amer. Med. Assoc.*, 1913, LXI, p. 727.
[2] *Journ. Exper. Med.*, vol. xxi, 1915, p. 114.
[3] South African Institute for Medical Research, *Rep.* No. viii, 1916.

which are additional to those recognized by Dochez and Gillespie.

The *Strep. mucosus* may here be referred to. The organism was first obtained by Howard and Perkins from a case of peritonitis, and has since been met with in pneumonia and otitis media. It occurs as a capsulated organism but tends to form longer chains and to grow more freely than the *S. pneumoniæ*. The chief points of difference between the two organisers are (1) though apparently capable of causing pneumonia, it is infrequent in this disease, (2) on subcutaneous inoculation into an animal it tends to cause a mucoid œdema at the site of inoculation, (3) it forms a capsule when grown in lactose serum broth, which the *S. pneumoniæ* does not do.[1]

Pathogenic action.—The *S. pneumoniæ* is pathogenic for a number of animals, the most susceptible being mice, then in decreasing order, rabbits, rats, guinea-pigs, and dogs. Pigeons and fowls are immune. Death follows after subcutaneous, intravenous, intraperitoneal, or intrathoracic injection of a virulent culture, or of rusty pneumonic sputum, into mice and rabbits in twenty-four to forty-eight hours. The virulence of the organism varies considerably; under cultivation it may be completely lost, while by a series of passages through a susceptible animal it may be much increased. The less virulent it is the longer it tends to retain its vitality under cultivation. Except when injected into the lung or into the trachea, pneumonia does not result, but the disease runs the course of a septicæmia with high temperature and dyspnœa, death being generally preceded by a subnormal temperature and often convulsions. The post-mortem appearances are much œdema and inflammatory infiltration at the seat of inoculation, hæmorrhages in the serous membranes, enlargement and congestion of the spleen, and congestion

[1] See Holman, *Journ. Path. and Bacter.*, vol. xix, 1915, p. 478.

of the lungs. The organisms occur in large numbers in the blood, lungs, and spleen, usually in the form of oval diplococci with well-marked capsules (Plate XVII, *a*), but sometimes as short chains of streptococci. When injected into the lung or trachea a typical fibrinous or croupous pneumonia results.

The *S. pneumoniæ* is the cause of acute croupous pneumonia in man, and occurs in large numbers in the rusty sputum and hepatised lung, and in 20 per cent. of the cases can be isolated from the blood if 5–10 c.c. be cultured. The production of a typical pneumonic process experimentally and the presence of the diplococcus in a large proportion of cases of acute croupous pneumonia point to its specific relationship to the disease. With regard to the latter observation, Weichselbaum obtained it in 94 cases out of 129 examined, Wolf in 66 out of 70 cases, and Netter in 75 per cent. of the cases examined. In America the disease has of late been much on the increase, in Chicago the mortality having reached as high as 20 per 10,000 inhabitants. Acute croupous pneumonia sometimes occurs in epidemic form and has decimated the native labourers in the Rand mines.

The organism is frequently present in the saliva of healthy individuals, as shown by Netter, Sternberg, and others, and the generally accepted idea of the relationship of " catching cold " to an attack of the disease is explicable on the theory that the action of cold lowers vitality, and renders the tissues vulnerable to the attacks of the organism already in close proximity to them.

Besides acute croupous pneumonia, more than half the cases of broncho-pneumonia, both primary, and secondary in the course of other diseases, are due to the *S. pneumoniæ*, which is also associated with a number of other important pathological conditions in man. It is a pyogenic organism, producing abscesses when inoculated

into a relatively insusceptible animal such as a dog, and has been met with in abscesses, empyema, suppuration in the antrum, and purulent arthritis. It is also found in about half the cases of purulent meningitis, sometimes causing a cerebro-spinal meningitis, in about a third of the cases of otitis media and infective endocarditis, sometimes in purulent pericarditis, and occasionally in peritonitis. The pneumococcus is also frequent in chronic bronchial catarrh. An agglutination reaction with patient's serum on the pneumococcus is only very irregularly obtained and normal serum rarely exerts any bactericidal effect upon the organism.

As regards opsonic determinations, freshly isolated strains frequently fail to give any phagocytosis, and every strain of pneumococcus gives a different amount of phagocytosis. For the control, the pooled serum of several individuals should be used, and the culture should be emulsified in distilled water. The serum of the Rand native seems to have a very low opsonic content for the pneumococcus compared with that of the European.[1]

Toxins.—Auld separated a proteose and an organic acid from the blood and organs of infected animals, and from cultivations of the *S. pneumoniæ* in alkali-albumin the same products were apparently obtained, the alkaline medium soon becoming permanently acid. The proteose on subcutaneous or intravenous injection produced some fever ; on intra-thoracic injection fever and dyspnœa, and post-mortem pleurisy and consolidation of the lung were found. The organic acid produced slight rise of temperature, but no other symptom. Macfadyen [2] obtained an endotoxin by triturating cultures with liquid air.

Anti-serum.—Immunity can be conferred on susceptible animals by treating them with attenuated cultures, or

[1] Wright, *Lancet*, 1914, i, p. 1 *et seq.*

[2] *Brit. Med. Journ.*, 1906, vol. ii, p. 776 (Refs.).

by inoculation with increasing doses of filtered broth cultures of the virulent organism followed by doses of the living organism. The blood-serum of such immunised animals will protect other animals when injected, and an anti-pneumococcic serum has been prepared by the foregoing method. This anti-serum has been used in the treatment of pneumonia and other pneumococcic infections, but the results have not been very encouraging. The protective serum seems to produce aggregation of the cocci when added to a culture of the diplococcus. Klemperer and Washbourn found that the serum of convalescent patients possesses some degree of protective power. The serum, however, withdrawn during the pyrexial stage of the disease rather increases the susceptibility of animals to pneumococcic infection.

Vaccine.—A vaccine prepared from cultures killed by heat and standardised has been found of service in chronic pneumococcic infections, and has also been employed in acute croupous pneumonia [1]; the doses range from 20 to 50 millions. Wright (*loc. cit.*) advised a vaccine for *prophylactic* inoculation against pneumonia on the Rand, and Lister (*loc. cit.*) recommends for this purpose three inoculations at weekly intervals, each dose consisting of 6000 million cocci of each group against which immunity is desired.

Friedländer's Pneumo-bacillus

This organism, already referred to above in the general discussion of pneumonia, and originally believed by Friedländer to be the cause of the disease, has been obtained by recent observers in only a small proportion of cases of pneumonia. It is one of the capsulated bacilli (p. 291).

Morphology.—The *B. pneumoniæ* is a very pleomorphic organism, occurring in sputum or in the blood of an inocu-

[1] Willcox and Morgan, *Brit. Med. Journ.*, 1909, vol. ii, p. 1050.

lated animal generally as a short rod with rounded ends surrounded by a marked capsule. It is non-motile, does not form spores, and is readily stained with the ordinary anilin dyes, but not by Gram's method—an important distinction from the *S. pneumoniæ*. In cultivations it forms short rods, long rods, chains, and even filaments, the capsule being absent, but this is regained on passage through a susceptible animal.

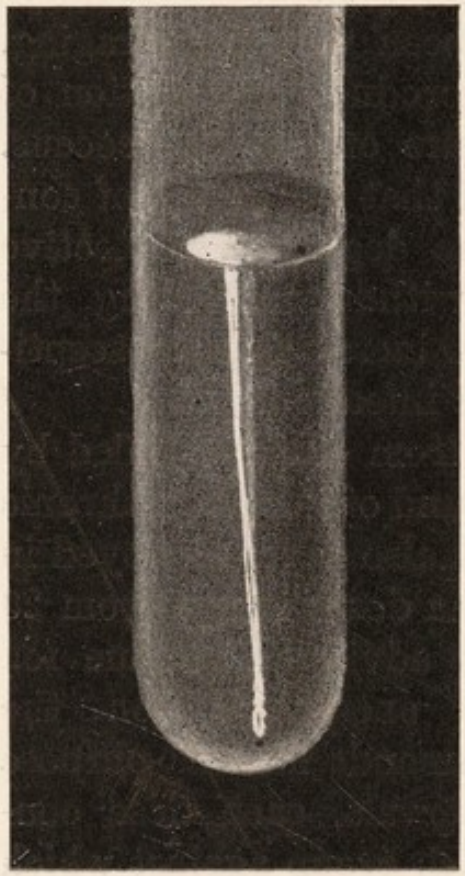


FIG. 48.—Friedländer's pneumo-bacillus. Gelatin stab-culture, seven days old.

Cultural characters.—The *B. pneumoniæ* is aërobic and facultatively anaërobic, and may produce indole. It grows readily on the various culture media from 20° to 37° C., on agar and blood-serum forming a copious, viscid, greyish growth; on gelatin, a thick, white, shining, porcelain-like growth without liquefaction; and in stab-cultures in gelatin a so-called nail-shaped growth is developed (Fig. 48), consisting of a white growth along the needle-track, tapering from above downwards, and at the surface heaped up and expanded, forming the "head" of the nail, gas-bubbles frequently forming. On potato a copious whitish growth develops. Milk is curdled and the organism is an active fermenter of carbohydrates; the fermentation reactions are given in the Table, p. 432.

Pathogenic action.—The pneumo-bacillus of Friedländer is pathogenic to mice and guinea-pigs, but rabbits are immune. Post-mortem, the spleen is enlarged, the lungs

are congested and consolidated in patches, and the organism is found in large numbers in the blood. In a small percentage of cases of croupous pneumonia Friedländer's bacillus may be associated with the *S. pneumoniæ*. Friedländer's bacillus may sometimes set up a broncho-pneumonic or bronchitic process, and is occasionally associated with anginal conditions, which are characterised by the formation of a false membrane, with an absence of any general symptoms. A microscopical examination of the membrane will show the organisms surrounded with a capsule and unstainable by Gram's method. If a culture be made on serum, the large, round, greyish colonies of the bacillus will be recognisable in fifteen to twenty hours, and should be examined microscopically. To obtain a pure culture a white mouse should be inoculated from a colony ; it will die in twenty-eight to sixty hours. Friedländer's pneumo-bacillus has also been met with in water by Grimbert. According to him, it is identical with the *B. capsulatus* of Mori.

Clinical Examination (Pneumonia)

1. Make smear specimens from the rusty sputum, and stain some with Löffler's blue, and others by Gram's method with eosin. By a microscopical examination the oval diplococci will be readily recognised, the *B. pneumoniæ* and *B. pestis* being distinguished from the *S. pneumoniæ* by being decolorised by Gram's method. The latter organism is the only one, moreover, which is likely to be ordinarily met with.

2. If the diplococci are found to be fairly abundant in the sputum, and other organisms nearly absent, an attempt may be made to cultivate by inoculating several glycerin-agar and serum tubes and incubating at 37° C. for forty-eight hours.

3. If the diplococci are scanty, or so mixed with other organisms that it is difficult to identify them, and probably impossible to obtain a pure culture, a drop or two of the sputum should be injected into the peritoneal cavity of a mouse or rabbit. The

animal will die in from twenty-four to thirty-six hours, and the *S. pneumoniæ* will be found plentifully in smears prepared from the blood or lung-juice, and pure cultures can be readily obtained by inoculating glycerin-agar tubes with the blood or lung-juice.

4. The culture or inoculation method, preferably both, will probably have to be adopted for the recognition and isolation of the *S. pneumoniæ* in pus from empyemata, abscesses, etc.

5. Friedländer's pneumo-bacillus can be readily isolated by making gelatin-plate cultivations, in which its colonies form white, shining, heaped-up points.

Epidemic Influenza

A minute bacillus was first described in this disease by Pfeiffer in 1892, who found it in large numbers in the bronchial secretion. In order to isolate the organism a patient with bronchial expectoration should be chosen; he rinses his mouth and gargles his throat with hot water several times, and then, after coughing, the expectoration is obtained. A little of this expectoration is washed by shaking in a test-tube with sterile salt solution, then repeating the washing with sterile salt solution in a second and finally in a third test-tube. By means of a platinum needle a number of glycerin-agar and blood-agar tubes are inoculated with the sputum after the last washing, and incubated at 37° C.

Morphology.—The influenza bacillus measures 0·5–1·5 μ in length, and is non-motile and non-sporing. It does not stain by Gram's method, and not very readily with the ordinary dyes, dilute carbol-fuchsin or prolonged staining with Löffler's blue yielding the best results, the poles tending to stain more deeply than the centre. In the sputum it occurs singly, in short chains, in small groups, or in larger masses, being most numerous early in the acute stage of the disease. In culture, it forms short and long rods and short filaments.

Cultural characters.—The bacillus is strictly aërobic, and no growth occurs on media at 22° C. On glycerin-agar and blood-serum at 37° C. it forms very small, transparent, drop-like colonies in from twenty-four to forty-eight hours, which, according to Kitasato, never become confluent. There is no growth on potato. The organism grows best on media containing blood, such as agar smeared with sterile human, rabbit's, or pigeon's blood. In broth it grows at the surface in fine white flakes which subsequently sink.

It soon dies out in cultivation, but according to Klein can be kept alive for some weeks in gelatin incubated at 37° C. The melted gelatin remains clear, the growth forming a delicate flocculent precipitate at the bottom. Preparations from cultures show long twisted chains and threads of bacilli, aggregated so as to form dense networks and convolutions. These chains or threads are composed of bacilli placed end to end, and united by a continuation of the cell-membrane. Involution forms occur. It is stated to grow better in association with the *M. pyogenes* var. *aureus* than alone. The organism does not seem to be able to live outside the body for any length of time, and is readily destroyed by desiccation, weak antiseptics, and by a temperature of 60° C. acting for five minutes.

Pathogenic action.—Cannon stated that he obtained this bacillus from the blood in a number of cases, but many other investigators have failed to find it. Klein also obtained it in six cases out of forty-three examined. According to Pfeiffer the bacillus is pathogenic only to monkeys and rabbits. Klein, however, was unable to obtain any definite effects in these animals by the injection either of sputum rich in bacilli or of pure cultures.

The influenza bacillus is met with in all uncomplicated cases of influenza in the nasal and bronchial secretions, often almost in pure culture, and in the bronchial tubes

and lung in the pneumonic complications accompanying the disease. The organisms disappear with convalescence, and are not met with in other diseases. The pneumonia often complicating the disease is sometimes directly due to the bacillus. The typical influenza pneumonia is of the lobular type with a cellular rather than a fibrinous exudate. True lobar pneumonia, due to the *S. pneumoniæ*, may, however, often complicate the influenzal attack. The organism also occurs in bronchial catarrh and bronchitis, broncho-pneumonia, and whooping cough, and occasionally in meningitis.

A vaccine prepared with killed culture is frequently useful in the treatment and prevention of catarrh of the respiratory tract, either alone or in combination with *M. catarrhalis*, *B. coryzæ*, etc., according to the organisms found to be present in the expectoration.

Although epidemic influenza may be due to the *B. influenzæ*, many febrile conditions attended with pulmonary catarrh and frequently termed "influenza" are not due to this organism. In an epidemic simulating influenza occurring in Essex in 1905, the examination was negative as regards streptococci, *B. diphtheriæ* and *B. influenzæ*, but the *M. catarrhalis* was present in number in most cases (twenty-two out of twenty-four). This organism was originally isolated by Seifert in a small epidemic of infectious bronchitis, afterwards by Pfeiffer in cases of broncho-pneumonia in young children (see p 280). Two other Gram-negative cocci were also isolated from three other cases (see Table, p. 279).

So-called "influenzal meningitis" may be due to organisms resembling, but not identical with, the *B. influenzæ* (see "Meningitis," Chap. xx.).

Clinical Examination

In cases of influenza, accompanied with bronchitis or pneumonia, the influenza bacillus may be met with in large numbers in the sputum, and its presence may aid in confirming the diagnosis. Film preparations may be stained with carbol-methylene blue.

Whooping-cough (Pertussis) [1]

An influenza-like bacillus has been isolated by Koplik, Czaplewski and Hensel, Davis and others in this disease, but the researches of Bordet and Gengou have shown that it is distinct from the influenza bacillus.

The *B. pertussis* is a minute bacillus, very like the *B. influenzæ*, non-motile, non-sporing, and Gram-negative. It is scanty in the bulk of the expectoration, but is abundant in the viscid exudate, rich in leucocytes, coming from the depth of the bronchi, and voided at the end of a paroxysm of coughing.

It is best isolated on a medium consisting of defibrinated blood (human or rabbit), thoroughly mixed with an equal volume of 3 per cent. agar containing a little extract of potato made with 4 per cent. aqueous glycerin. It forms on this a fairly thick whitish streak, the subjacent blood being hæmolysed. It may also be grown in serum or blood broth in shallow layers. After acclimatisation to artificial media it will develop on the ordinary laboratory media.

The *B. pertussis* is agglutinated feebly by the blood of patients, but complement-fixation is marked.

Monkeys are stated to develop a typical whooping-cough on inoculation, but the ordinary laboratory animals are susceptible only to massive intraperitoneal or intravenous inoculation, death ensuing from a septicæmic process.

Freeman has treated the disease with a vaccine. Doses of 5–20 millions were used, and about 68 per cent. of the cases improved.

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[1] See Bordet, *Brit. Med. Journ.*, 1909, vol. ii, p. 1062.

CHAPTER XIII

ANAËROBIC ORGANISMS

TETANUS—THE ANAËROBES OF WOUNDS—B. BOTULINUS—BLACK QUARTER—B. PUTRIFICUS—ANAËROBIC BUTYRIC ACID ORGANISMS

ANAËROBIC organisms vary in their oxygen requirements from facultative anaërobes to strict anaërobes. Even the strict anaërobes in some circumstances (*e.g.* in sulphindigotate broth), seem to be able to develop more or less aërobically, and may also be "educated" to grow aërobically. An anaërobe, *e.g. B. perfringens*, which will not grow in glucose broth under aërobic conditions, may do so if a piece of potato be added to the medium (Wright). It is probable that the organism locates itself in nooks and crannies in the potato where it finds approximately anaërobic conditions and so is able to develop.

The anaërobic organisms have assumed considerable importance of late owing to the occurrence of several species in septic wounds, particularly lacerated wounds and compound fractures caused by shrapnel, etc., where they induce serious conditions—tetanus, gas gangrene, septic infection and the like.

Anaërobic organisms are common in soil, in decomposing organic matter and in the intestine of man and animals, and it is chiefly from the soil, particularly if highly cultivated and manured, that wounds derive their infection.

The anaërobes seem to play a considerable part in Nature in the breaking down of organic matter and are regarded as the principal agents in liquefying the solid material of the sewage in the septic tank of a bacterial system of sewage purification.

The study of anaërobic organisms is beset with pitfalls and difficulties—their cultural characters are not well defined, and are less distinctive than those of many other organisms, their staining reactions are liable to vary according as they are under natural or artificial conditions of growth, the pathogenicity of different strains of the same organism varies enormously, the same organism has sometimes been described under a number of synonyms, and lastly extreme difficulty may be experienced in obtaining pure cultivations. Thus cultures which for months appear to be pure ones and continuously give the same appearances and reactions, may ultimately be found to consist of two distinct species.

Ordinary media may be employed for the culture of many anaërobes, glucose agar is particularly useful, and blood-broth, inspissated serum, Dorset's egg medium, and meat broth are valuable for the cultivation of anaërobes from the animal body. Meat broth is prepared as follows :

Eight ounces of bullock's heart are minced and then pounded in a mortar ; add eight ounces of hot tap-water, bring slowly to the boil and boil for one hour to cook the meat. Add normal sodium hydrate solution sufficient to render alkaline to litmus, fill into tubes *without filtration* and autoclave to sterilise. The meat broth so prepared may be covered with a layer of sterile liquid paraffin (it is preferable *not* to add the paraffin to the broth before autoclaving). Immediately before use, the tubes should be boiled in a water-bath for half an hour.

It will be convenient first to describe some of the more important pathogenic anaërobes, and then to discuss their classification and identity and to tabulate their principal characters.

Literature on the Anaërobes of Wounds.—Muriel Robert-

son, *Journ. of Pathol. and Bacteriol.*, vol. xx, 1916, p. 327 (Refs.); Weinberg, *Proc. Roy. Soc. Med.*, vol. ix, 1916, No. 9, p. 119 (Refs.); Goadby, *Lancet*, 1916, vol. ii, pp. 89, 586 and 851 (Refs.); Dean and Monat, *Journ. Roy. Army Med. Corps.*, 1916, February and March; Fleming, *Lancet*, 1915, vol. ii, p. 638; Distaso, *Lancet*, 1916, vol. i, p. 74; Emery, *Lancet*, 1916, vol. i, p. 948.

Tetanus

The causation of tetanus was for a long time involved in mystery. No obvious or characteristic changes being met with after death, the disease was regarded by many as "functional." Others believed that a primary lesion of the central nervous system might be the cause of the affection, while a few classed it with the specific diseases.

It had long been noticed that wounds soiled with earth were specially prone to be followed by tetanus, and Sternberg in 1880, and Nicolaier in 1884, produced tetanus in rabbits by introducing a little garden earth beneath the skin. The latter observer found at the seat of inoculation and in his impure cultures—for he was unable to obtain pure ones—a distinctive bacillus, and he was able with these cultures, and with the pus from the seat of inoculation, to induce tetanus in other animals. Carle and Rattone subsequently showed that the bacillus of Nicolaier was present in the tissues of, and secretions from, the wound, in cases of traumatic tetanus in man, and that inoculation with the pus from such a wound produced tetanus in the lower animals—observations which were confirmed by Rosenbach in 1885. The bacillus was isolated in pure culture by Kitasato in 1889 by taking the impure cultures obtained from the wound in a case of traumatic tetanus, heating to 80° C., and plating the heated cultures, the plates being incubated anaërobically in hydrogen.

The Bacillus tetani

Morphology.—The *Bacillus tetani* is a straight, slender rod with rounded ends, but under cultivation the rods may grow into longish filaments. It is somewhat motile and possesses a large number of flagella, three or four of which are generally thicker than the rest. Spores are freely formed; they are spherical and completely ter-

minal, and their diameter being much greater than that of the rod, the spore-bearing organism has been likened to a " pin " or " drum-stick " (Plate XVIII, *a*). It stains with the ordinary anilin dyes, and also by Gram's method. " Drum-stick " bacilli are not necessarily tetanus ; other anaërobic bacilli, *e.g. B. putrificus* (*coli*) and Hibler IX, may also have large terminal spores (Plate XIX, *b*).

Cultural characters.—The *B. tetani* is a strictly anaërobic organism, and will not grow in the presence of a trace of free oxygen, nor in an atmosphere of carbon dioxide. It grows well in deep stabs in glucose agar and gelatin. In a gelatin stab-culture at 22° C. the growth radiates from the central puncture, and the gelatin is slowly liquefied. In a glucose agar stab-culture it forms feathery, radiating outgrowths from the central puncture, a small amount of gas being formed (Fig. 49). Broth becomes turbid with the formation of some gas and the development of a foul odour ; there is no film formation. Meat-broth is not blackened. The colonies on agar have a central opaque portion surrounded by diverging rays. It grows on serum without liquefaction (Macé and many authors), or with slight pitting (Dean and others) and in milk without curdling.[1] The tetanus bacillus remains alive for some time, possibly indefinitely, in cultures, and the spores retain their vitality for years in the dried state, withstand a temperature of 80° C. for an hour, but are killed by boiling for five minutes. Carbolic acid (1 : 20) does not destroy the spores under about fifteen hours.

Occurrence and pathogenic action.—Man and the horse are most subject to tetanus ; cattle and sheep are rarely affected, while the fowl, frog, triton, snake, and tortoise

[1] There is some variation in the descriptions given of the changes produced in serum and milk by *B. tetani*, probably owing to the use of impure cultures, for even old laboratory strains may prove to be mixed cultures.

are immune. Mice, guinea-pigs and rabbits are all very susceptible. The bacillus is present in the superficial layers of the soils in many localities, but not in all, and this accounts for the fact that tetanus is rare in some places and frequent in others. The natives of the Solomon Islands have made use of this fact for the preparation of poisoned arrows. The arrows are tipped with a viscid fluid, then rubbed in the soil from a mangrove swamp containing tetanus spores, and afterwards dried. Individuals wounded with these arrows generally develop tetanus.

FIG. 49.—Tetanus bacillus. Stab culture in glucose agar, seven days old.

The tetanus bacillus is particularly frequent in highly cultivated soil and cases of dirty lacerated and contused wounds soiled with earth are most prone to develop tetanus; this is the form known as "traumatic tetanus." There is also the so-called idiopathic or rheumatic form.

Tetanus spores are frequently present in the dejecta of cattle, horses, and other animals, and occasionally of man (p. 479).

The bacillus is confined to the seat of inoculation, or at most is met with in the nearest lymphatic glands, so that the general symptoms are due to the absorption of toxin. The researches of Ransom and Meyer have shown that the tetanus toxin is mainly absorbed by the motor nerve-trunks (see also p. 173). The organisms associated with the tetanus bacillus in earth are probably of considerable importance in the production of the disease, for it has been shown that if the tetanus bacilli and their spores be

carefully washed so as to remove all adherent toxins, they fail to set up tetanus on inoculation, while if the same washed bacilli be injected, together with a little lactic acid, tetanus follows, the explanation being that the bacilli are unable to multiply unless the surrounding tissues are damaged and phagocytosis is prevented. The associated organisms in the wound probably effect this, and do not act by producing a condition of anaërobiosis as has been suggested.

There is also the so-called "idiopathic" or rheumatic" tetanus in which there is no obvious wound. This may be due to infection of some slight wound which has practically healed. Hamilton suggested that tetanoid organisms in the intestinal tract might be the cause of this form of tetanus. An alternative suggestion is that of Semple [1] who found that tetanus spores are occasionally present in the human intestinal tract. He injected guinea-pigs with washed spores, and tetanus did not ensue, but the tissue at the site of inoculation, examined five to seven months later, still contained the living spores. Semple suggested that such latent spores may in some instances be disturbed and become active by trauma or by the hypodermic or intra-muscular injection of quinine, owing to the tissue necrosis and inhibition of phagocytosis produced by the drug (tetanus has followed on many occasions injections of quinine).

Toxins.—Cultivated anaërobically in broth, the tetanus bacillus forms a most potent extra-cellular toxin, so that if the culture be filtered through a porcelain filter, 0·001 c.c., 0·0001 c.c., or even 0·00001 c.c. of the filtrate is a fatal dose for a guinea-pig.

Tetanus toxin broth contains a tetanising substance, termed tetano-spasmin, and also a hæmolysin, tetanolysin. The toxin has a special affinity for nerve-tissue

(see p. 170). Injected into animals such as the mouse, guinea-pig and rabbit, the toxin broth produces tonic, not clonic, spasm and with small doses the muscles at or near the seat of inoculation tend first to be affected, so that the spine may be curved, the leg paralysed, etc. (Fig. 50). The same effect is occasionally seen in man, a local tetanus developing in a limb or group of muscles in the neighbourhood of the wound ; it is sometimes met

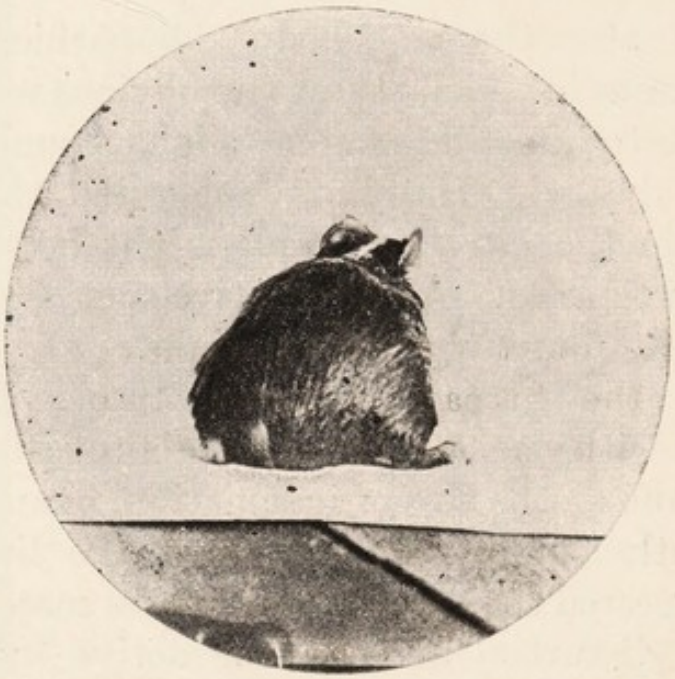


FIG. 50.—Guinea-pig inoculated with a small dose of tetanus toxin, showing paralytic condition of right hind leg due to spasm.

with after the prophylactic use of tetanus antitoxin where only a single dose has been given.

By treatment with carbon disulphide, tetanus toxin broth becomes practically non-toxic, though it still retains its power of immunising on inoculation and of combining with antitoxin—that is to say, bodies are formed analogous to the toxoids of diphtheria toxin.

Brieger, from impure cultures of the tetanus bacillus, obtained two basic bodies which he termed "tetanine" and "tetano-toxin, the former producing tetanic symp-

toms in mice, and the latter tremor, paralysis, and finally convulsions. Brieger also isolated tetanine from the amputated limb of a tetanic patient. Brieger and Fränkel obtained a tox-albumin from bouillon cultures which induced tetanus in guinea-pigs. Brieger and Cohn subsequently investigated the tetanus poison obtained by precipitating veal-broth cultures with ammonium sulphate added to saturation, and purifying by re-dissolving, precipitating the protein with basic lead acetate, and removing other soluble impurities by dialysis. The purified product forms yellow flakes, soluble in water, but not giving the Millon and xanthroproteic reactions. It is not precipitated by most metallic salts, and is not carried down by Roux and Yersin's method of precipitation with calcium phosphate. It contains no phosphorus and only traces of sulphur. Of the most active preparation 0·00000005 grm. killed a mouse.

In a case of tetanus examined by Sidney Martin, an albumose, chiefly deutero-albumose, was extracted from the blood. Injected into an animal, it produced depression of temperature, followed by progressive wasting, but no spasm or paralysis.

Antitoxin.—If an animal is cautiously injected with tetanus toxin, commencing the treatment with a weakened toxin, and increasing the dose very gradually, a high degree of immunity is ultimately obtained, and the blood-serum acquires marked antitoxic properties. The toxin is obtained by growing the tetanus bacillus in glucose broth in an atmosphere of hydrogen, or in sulphindigotate broth, for about three weeks, and filtering through porous porcelain. To obtain an active serum treatment has to be prolonged, a horse immunised by the writer requiring six months.

The antitoxin may be standardised by the Roux or by the Behring method (see p. 313). Methods analogous

to those used for standardising diphtheria antitoxin have also been introduced.[1]

The American immunity unit is now commonly employed; this is defined as being ten times the least quantity of anti-tetanic serum necessary to save the life of a 350 gram. guinea-pig for 96 hours against the official test dose of a standard toxin furnished by the Hygienic Laboratory of the Public Health and Marine Hospital Service. The test dose of the American standard toxin (which is a dry powder) is 0·0006 gram, and it contains 100 mimimal lethal doses for guinea-pigs of 350 grams weight. This is then the L + dose of toxin, analogous to the L + dose of diphtheria toxin used for testing diphtheria antitoxin (but note that whereas the unit of diphtheria antitoxin corresponds approximately to 100 lethal doses of diphtheria toxin, the unit of tetanus antitoxin corresponds to 1,000 lethal doses of tetanus toxin).

Tetanus antitoxin for treatment should contain not less than 150 U.S.A. units per c.c.

The antitoxic treatment of declared tetanus has not proved so successful as that of diphtheria. Two reasons may explain this difference: firstly, tetanus toxin is much more potent than diphtheria toxin, and secondly, tetanus is recognised only when grave injury to the central nervous system has already occurred. In fact, tetanus at an early stage corresponds with diphtheria at a late stage. There can be no question, however, that antitoxin should always be administered in a case of tetanus. In mild cases, 10,000 U.S.A. units may be given intramuscularly, followed at three-day intervals by two doses of 5,000 units. In severe cases intrathecal administration should be practised—15,000–20,000 units, which may be repeated

[1] On the standardisation and therapeutic use of tetanus antitoxin, see Hewlett's *Serum Therapy*, 1910; McConkey, *Brit. Med. Journ.* Oct. 10, 1914; various authors, *Lancet*, 1917; vol. i, p. 673 *et seq.*

if no improvement is manifest after the first dose ; intravenous and intramuscular doses may also be given, and the latter should be continued so long as any symptoms persist. There is little doubt of the superiority of intrathecal administration over all other methods. Intracerebral administration was practised in the past, but the intrathecal route is safer, easier, and as efficient.

The chief value of tetanus antitoxin is, however, as a prophylactic. This has been proved in America where wounds received during Independence Day celebrations were frequently followed by tetanus, but not a single case of tetanus has occurred in a person who has received a timely prophylactic dose of antitoxin. In the earlier stages of the present war, too,cases of tetanus were numerous, but since prophylactic doses of antitoxin have been given to the wounded the disease has almost disappeared.

For prophylaxis, 1500 U.S.A. units should be given as soon as possible after receipt of the wound, followed by a second dose fourteen to twenty days later. As the incubation period of tetanus may be a month or more, and as a dose of anti-toxin does not immunise for more than three weeks, a second dose should always be given if possible. Lack of this precaution may lead to the development of tetanus, local or general. Even if tetanus does develop after a single prophylactic dose, it generally tends to be mild.

Clinical Examination

The symptoms of declared tetanus are so obvious that a bacteriological examination is not needed to establish the diagnosis.

(1) Prepare several smears of the pus or discharge, and stain by Gram's method. Examine microscopically, looking for the spore-bearing rods or " drum-sticks." A " drum-stick " bacillus is, however, not necessarily the tetanus bacillus (see p. 491).

(2) If " drum-sticks " be found, an attempt may be made to

isolate the bacillus by making anaërobic plate cultivations from the discharge after heating it in capillary pipettes to 80° C. for half an hour.

(3) Inoculate mice and guinea-pigs with the heated discharge. If they die with tetanic symptoms, treat the pus at the seat of inoculation as in (2).

Septic Wounds and Gas Gangrene

The micrococcal, streptococcal and aërobic bacillary infections of wounds and sepsis have already been dealt with (Chap. VI., p. 245). We now have to consider the anaërobic bacilli which play so large a part in dirty septic war and other wounds and in gas gangrene.

These organisms may be present as an infection in the wound giving rise to suppuration and sepsis without gas gangrene, or they may induce in addition the serious complication known as gas or emphysematous gangrene. In this condition the tissues surrounding the wound become infected, necrosis and gangrene result, with the presence of more or less gas in the tissues, and the gangrene may spread rapidly and widely causing a state of profound sepsis. Frequently two or three species of anaërobes may be present at the same time, *B. tetani* may also be present, together with, commonly, micrococci and streptococci, and sometimes *B. proteus*, *B. coli*, *B. pyocyaneus*, etc. The complete bacteriological study of such a wound may therefore prove a very complicated matter.

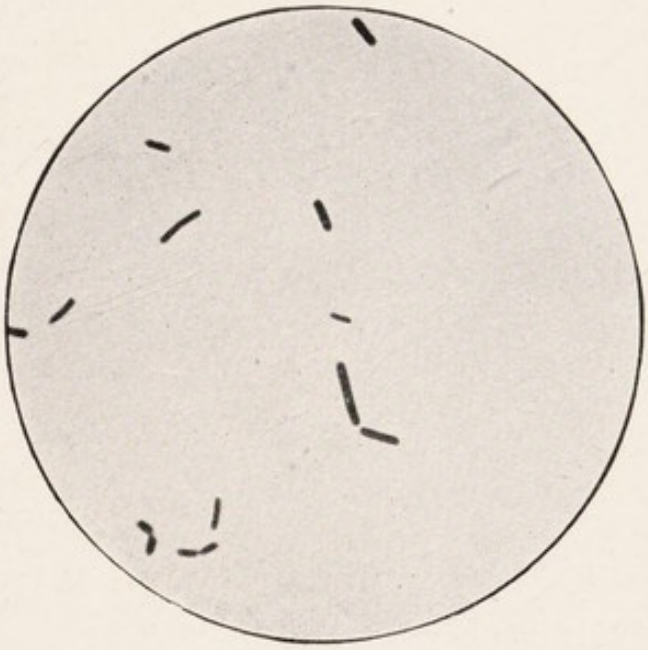
The principal anaërobes occurring in wounds will first be described, after which a brief survey of this class of wound infection will be given. The subject is a very complicated one and the descriptions of the organisms given must be regarded as somewhat tentative.

B. Perfringens (Veillon and Zuber)

Synonyms.—B. aërogenes capsulatus (Welch and Nuttall), *Granulo-bacillus saccharo-butyricus immobilis liquefaciens* (Grass-

PLATE XVIII.

a. Bacillus tetani. Film preparation of a pure culture. × 1500.



b. Bacillus perfringens. Film preparation of a milk culture. × 1000.

c. Milk culture of *B. perfringens.*

berger and Schattenfroh), *B. Welchii*, gasphlegmon bacillus (Fränkel), bacillus of acute rheumatism (Achalme : see "Rheumatism").

This organism was described by Welch and Nuttall under the name *B. aërogenes capsulatus* [1] in conditions accompanied by much development of gas in the tissues, as in cases which might be described either as phlegmonous erysipelas or as emphysematous gangrene, especially after injuries. It is also met with occasionally in perforative peritonitis and in various septicæmic and pyæmic conditions, in the puerperal state,[2] complicated stricture, etc.

The *B. perfringens* is widely distributed, and has been cultivated from the soil, dust, and contents of the intestine. It has either been described under a variety of names, or more probably a group of closely related bacilli exists ; this group may be designated *B. Welchii*. Gas-bubbles found in the blood and internal organs ("foamy organs") at an autopsy seem generally to be due to this organism, but perhaps may occasionally be caused by other putrefactive bacteria.

Morphology.—The *B. perfringens* is a non-motile, sporing, anthrax-like bacillus, variable in size, being 3 to 6 μ in length (Plate XVIII, *b*). It occurs singly, in short chains, or in clumps, and occasionally in long threads. It stains well with the ordinary anilin dyes and also by Gram's method in the exudate and in young cultures, but in older cultures many individuals are Gram-negative. In

[1] See Welch and Nuttall, *Bull. Johns Hopkins Hosp.*, vol. iii, 1892, p. 81 ; Welch, "Shattuck Lecture," *ibid.*, vol. xi, 1900, p. 185 ; Dunham, *ibid.*, vol. viii, 1897, p. 68 ; Welch and Flexner, *Journ. Exper. Med.* vol. i, 1896, p. 5 ; Herter, *Bacterial Infections of the Digestive Tract*, 1907 ; Kamen, *Centr. f. Bakt.*, Orig. xxxv, 1904, pp. 554, 686 ; *Archiv. f. Hyg.*, vol. liii, 1905, p. 128 ; and Blake and Lahey, *Journ. Amer. Med. Assoc.*, vol. liv, 1910, p. 1671.

[2] See Little, *Bull. Johns Hopkins Hosp.*, vol. xvi, 1905, p. 136.

the exudate it is capsulated, but the capsule is lost under cultivation except in serum. In the exudate the organism spores freely, the spores being large and ovoid and central or subterminal. Under cultivation, spores are formed only in media rich in protein and poor in fermentable sugar, *e.g.* serum, fluid or coagulated.

Cultural characters.—The *B. perfringens* grows well on all the ordinary culture media, slowly at 20° C., rapidly at blood-heat, but is strictly anaërobic, and heavy implantations are usually necessary for success. It forms greyish-white colonies on agar, and gelatin is liquefied. In glucose-broth it produces at first a diffuse cloudiness, but later the fluid becomes clear and a whitish viscid sediment settles. Milk is coagulated with a strong odour of butyric acid, the casein forming a thick, stringy, honeycombed mass on the surface of a clear watery whey; growth ceases after a few days (Plate XVIII, *c*). On potato the growth is almost invisible. There is abundant formation of gas in culture media, the gas both in dextrose media and in milk, according to Theobald Smith, consisting of hydrogen and carbon dioxide in the ratio 2 : 1 or 3 : 2.

Inspissated serum is never liquefied. In meat broth a bright pink colour is produced with considerable gas formation, the medium becomes acid and there is a sour smell, but no putrefactive odour. Glucose and lactose are actively fermented with gas and acid production, there is a tendency to ferment both inulin and glycerin, but starch is not fermented.

The surface colonies on glucose agar are round and raised, but do not grow downwards, and do not develop filamentous outgrowths; the deep colonies are lenticular.

Toxins.—A toxin is formed in broth culture to which serum or fresh muscle has been added. The toxin is a complex of a hæmolysin and another poisonous body.

The filtered toxin injected intravenously into a rabbit produces great blood destruction. Injected subcutaneously into a guinea-pig, 2 c.c. causes œdema and sloughing and death in three days. Rabbits can be immunised with the toxin and yield an antitoxic serum (Bull and Pritchett). Butyric acid is freely formed by the organism.

Pathogenicity.—The virulence of various strains of *B. perfringens* varies. It is pathogenic for guinea-pigs and mice, but slightly so for rabbits. The whey of a milk culture in quantities of 0·5–2 c.c. per 100 grm. of body-weight produces death in a guinea-pig within forty-eight hours. Post-mortem, if injected subcutaneously, the hair strips readily from the skin, which may be green and gangrenous ; the subcutaneous tissue may also be green and gangrenous, or more or less digested, so that the skin hangs loose, and the sac formed contains gas and exudation, sometimes scanty, sometimes abundant, thin and sanguinolent, and containing numbers of bacilli. Around the gangrenous area the tissues are markedly œdematous. If the post-mortem be delayed, or if the heart-blood be taken up into tubes, and these are sealed and incubated for some hours, many of the bacilli will spore. Pigeons, by intra-muscular inoculation, are also susceptible. Injected intravenously into a rabbit, the animal killed immediately and the carcase incubated at 37°C.for twenty-four hours and examined, there is an abundant formation of gas, particularly in the liver, which is riddled with gas bubbles. Monkeys fed with considerable numbers of *B.perfringens* are unaffected, and the organism is a normal inhabitant of the intestinal tract of man.

In man the *B. perfringens* is the principal cause of gas gangrene. The condition arises subsequent to a wound, within a period from a day or two up to two or three weeks after infliction. The tissues become hard, tense and cre-

pitant, gangrene ensues which may be limited to a muscle or group of muscles or even to an area of skin or may spread widely, a repulsive sickening odour emanates from the patient, who in the bad cases rapidly passes into a state of profound toxæmia and death may ensue within twenty-four to forty-eight hours.

Many different conceptions of the mode of action of *B. perfringens* have been advanced. Some have held that the general symptoms are due to an intoxication with the products of tissue disintegration, others that they are caused by intoxication with bacterial toxin. Kenneth Taylor ascribed the gangrene to the mechanical action of the gas formed by the organism in the tissues, the necrotic tissue being then attacked by putrefactive bacteria. Wright holds that *B. perfringens* induces an acidæmia whereby the antitrypsin is diminished and the organisms are then able to multiply explosively. Bull and Pritchett[1] hold the view that infection by *B. perfringens*, like infection by *B. tetani*, essentially resolves itself into an intoxication, in which an exotoxin yielded by the multiplying organisms constitutes the chief danger.

Although the organism is so deadly in many cases, it may be present in enormous numbers without gas gangrene ensuing. Emery[2] attributes this difference largely to the influence of the toxin upon leucocytic emigration. Provided the amount of toxin is not too great, leucocytes emigrate in numbers and by their phagocytic action are protective; if however the toxin becomes concentrated, leucocytes no longer emigrate. Concentration of toxin depends on several factors—virulence of the organism, facilities for rapid multiplication of the organism (*e.g.* dead and lacerated tissues or blood-clot heavily infected), retardation of escape of toxin (*e.g.* in a badly drained

[1] *Journ. Exper. Med.*, vol. xxvi, 1917, p. 119.
[2] *Lancet*, 1916, vol. i, p. 948.

wound and by interference with the blood supply). Bacterial associations may also play a part. Thus Douglas, Fleming and Colebrook [1] find that proliferation of *B. perfringens* is much more rapid when it grows in symbiosis with streptococcus, staphylococcus, diphtheroid organisms, *B. proteus*, *B. pyocyaneus* and a coliform organism, and Emery regards the prognosis as being decidedly better if *B. perfringens* be present alone, than if streptococci or staphylococci be also present.

Bacillus Œdematis Maligni (Koch)

Synonyms.—Vibrion septique (Pasteur), *B. septicus* (Macé)

An anaërobic bacillus varying in morphology in the tissues and in culture. In a wound definite bacillar forms are present, many sporing, the spores being spherical or ovoid and central or subterminal and clostridial forms are frequent (Plate XIX, *a*). In an inoculated animal, the organism may be found in the liver, spleen and blood as long filamentous forms and spores may be observed as early as twenty-four hours after inoculation. In a glucose agar culture the organism may vary from 3 μ to 15 μ in length and chains of slender bacilli may be present in which the subdivisions are distinguished with difficulty. The malignant œdema bacillus is actively motile and multi-flagellate and is Gram-positive in the tissues and for the most part in quite young cultures, but in older cultures many individuals are Gram-negative.

It grows and spores freely on the ordinary culture media. Surface colonies on glucose agar consist of a tangle of filaments which grow out from the centre giving a woolly appearance ; in deep plates and shakes the colonies are similarly woolly. Gelatin and serum are rapidly liquefied, milk is digested with little curdling, meat broth

[1] *Lancet*, 1917, vol. i, p. 604.

is digested and blackened with a marked putrefactive odour. In a glucose agar stab gas-bubbles are formed and the odour is offensive.

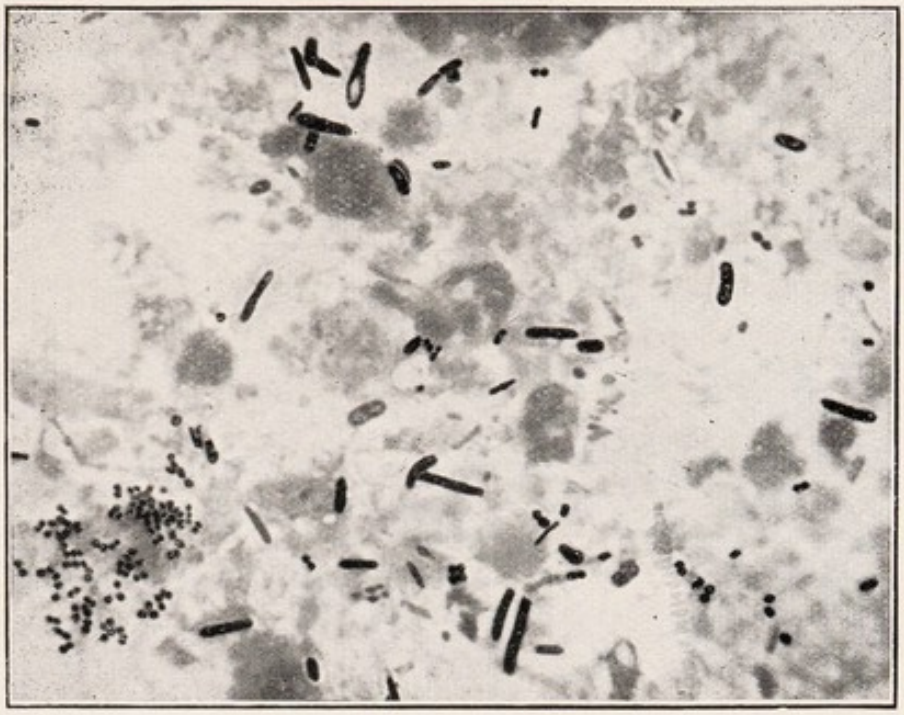
Miss Robertson states that a "vibrion septique" culture from the Pasteur Institute did not liquefy serum. Macé, however, states that the "vibrion septique" of French authors liquefies serum. Other forms have been described. The malignant œdema bacillus occurs in the soil and is pathogenic for many animals—guinea-pig, rabbit, rat, mouse, sheep, goat and horse, but the ox is refractory. The ass, fowl and pigeon are also somewhat susceptible.

While Pasteur and the earlier French investigators regarded the malignant œdema bacillus as one of the most virulent of organisms (possibly their cultures were not pure), Miss Robertson states that none of the cultures tested killed a guinea-pig, and 5–8 c.c. of a broth culture was necessary to cause local gangrene. Mixtures of *B. perfringens* and *B. œdematis maligni* were, however, very toxic, causing extensive œdema with some gas and very fetid odour.

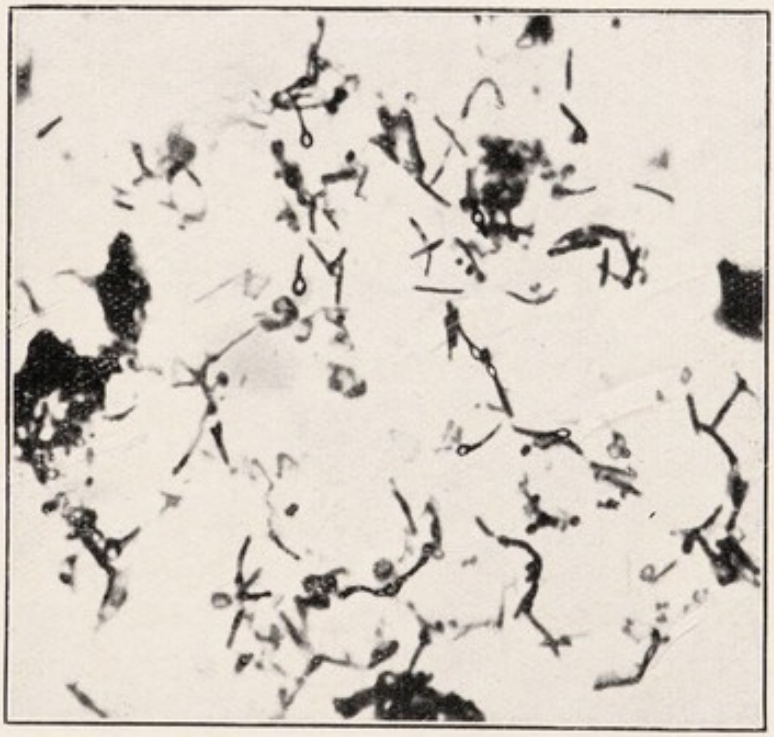
B. Hibler IX

A long slender anaërobic bacillus forming terminal slightly ovoid spores: sometimes spores appear at both ends (Plate XIX, *b*). It is slightly motile and Gram-positive in the tissues, but becoming largely Gram-negative in culture. It grows well in the ordinary culture media. The surface colonies on glucose agar are flat, round or slightly irregular in outline with crenated margins, but no outgrowths; the organism tends to spread over the plate if the agar be at all moist. The colonies in deep shakes are generally more or less lenticular. Neither gelatin nor serum is liquefied. In meat broth there is considerable gas formation, but no darkening. Milk is changed slowly, a soft

PLATE XIX.



a. Smear from a septic gunshot wound. Note clostridial form at top. Gram and eosin. × 1000.



b. Film preparation of a meat-broth culture of same wound. Note slender sporing bacilli, probably *B. Hibler*. × 1000.

clot being formed in eight to ten days. Acid and gas are formed from glucose, but inulin and glycerin are not fermented. *B. Hibler IX.* is non-pathogenic to guinea-pigs. It is frequent in wounds and may contribute to the gas formation in gas gangrene. Its presence is important in wounds owing to its similarity to *B. tetani;* the rods of the latter are stouter and the spores rounder. *B. Hibler IX*, is probably identical with *B. Rodella III.*

Bacillus œdematicus (Weinberg).—A large anaërobic Gram-positive bacillus 0·8 μ broad by 4–10 μ long. While the organisms in the wound and in *young* cultures are Gram-positive, in older cultures a number are Gram-negative. It is motile (in the wound exudate), possessing several long flagella, and spores freely in all media within twenty-four to forty-eight hours. The spores are large, ovoid and sub-terminal. It grows well in most media, in sugar-media producing gas. The cultures have a peculiarly strong fetid odour. White of egg is not attacked. Milk is curdled after a few days, forming a soft clot, with gas formation. Gelatine is not liquefied and the colonies on this medium are delicate and woolly. It is pathogenic to guinea-pigs, rabbits, rats and mice, a twenty-four hour glucose broth culture injected subcutaneously or into the muscles, killing a guinea-pig in from six to thirty hours. The muscle at the site of inoculation is hyperæmic but not gangrenous, contains gas bubbles, and has a putrid odour. A considerable gelatinous œdema surrounds the area of inoculation.

Bacillus fallax (Weinberg).—An anaërobic bacillus, 0·6 μ broad by 2–5 μ long. It is motile in the wound exudate and possesses several very long spiriliform flagella. Spores have not been observed. It is Gram-positive in the wound exudate and in *young* cultures, but in older cultures tends to become Gram-negative. It grows freely in glucose broth, giving a general turbidity and much gas. The odour of the culture is acid and not putrid. Milk is curdled in a few days, the curd being soft and mostly at the bottom. It does not attack egg-white. It does not liquefy gelatin and the colonies in this medium are yellowish and heart-shaped. It ferments glucose, maltose, galactose and lævulose, but not saccharose, with the production of much gas. Much gas is formed in a glucose agar stab. It is pathogenic for guinea-pigs and mice, but not for rats. If inoculated into a muscle in a guinea-pig, 1 c.c. of a broth culture kills in twelve

to sixteen hours and at the site of inoculation a large local lesion forms—the muscle is red and hyperæmic and contains numbers of gas bubbles and a considerable gelatinous œdema occurs at the margin of the lesion.

Bacillus histolyticus (Weinberg).—A motile, multi-flagellate, anaërobic diplobacillus, forming large subterminal spores. It develops well in ordinary culture media; no gas production in sugar media. The deep colonies in gelatin show branching. Gelatin is liquefied. Milk is coagulated and the clot becomes digested in eight to fifteen days. The organism produces a toxin; 1–2 c.c. of a filtered broth culture injected intravenously kills a 2–3 kilogram rabbit often in a few minutes.

Cultures injected subcutaneously in a guinea-pig cause extensive destruction and liquefaction of the tissues. While incapable itself of producing gas gangrene, it plays a part when present with *B. perfringens* and *B. œdematicus* in bringing about softening of the tissues.

Bacillus Enteritidis Sporogenes

Under the name *B. enteritidis sporogenes*, Klein [1] isolated an anaërobic bacillus similar to the *B. perfringens* from the evacuations of, and from milk consumed by, patients suffering from an epidemic diarrhœa which occurred in St. Bartholomew's Hospital; as did Andrewes,[2] from cases of diarrhœa admitted into the same hospital. Klein believed this organism to be the cause of the diarrhœa, and stated that it could not be found in the intestinal evacuations of healthy individuals. Klein also found it in water, sewage, manure, and milk. The writer, however, showed that it could generally be found in the normal dejecta, also in road and laboratory dust and frequently in milk, and the opinion he formed was that it was probably a ubiquitous organism and had little to do with the diarrhœa.[3] Glynn also found the organism to be very widely distributed, and fed guinea-pigs with, and himself ingested, cultures without result.[4]

The *B. enteritidis sporogenes* in its morphology, staining re-

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[1] *Rep. Med. Off. Loc. Gov. Board*, 1895–96, p. 197; *ibid.*, 1897–98, p. 225.

[2] *Ibid.* for 1896–97, p. 225.

[3] *Trans. Jenner Inst. Prev. Med.*, vol. ii, 1899, p. 70.

[4] *Thomson Yates Lab. Rep.*, vol. iii, Pt. ii, 1901, p. 131.

action, and cultural characters is almost, if not quite, identical with the *B. perfringens*. The only point of difference between them is that the former is motile and flagellated, while the latter is non-motile and non-flagellated. Miss Robertson remarks that it seems to be an ill-defined species. Spores are only formed in serum or gelatin, not on agar. It is abundantly present in sewage and sewage-contaminated water (see Chap. XXI).

B. enteritidis sporogenes is often found in wounds and sets up putrefactive changes in them (Weinberg).

Other Anaërobes present in Wounds

Goadby describes the *B. necrosis* as being frequently present. It is a long, non-motile, Gram-negative, slender and often pointed-end anaërobic bacillus. No spores are formed. Meat undergoes peptonisation. Neither gas, acid nor clot are formed in milk. The colonies are diffuse and woolly with long tangled filament formation. Grows with difficulty.

Fleming describes *Bacillus X*, *Bacillus Y*, "*Wisp*" *Bacillus* and an anaërobic streptococcus as frequent. *Bacillus X* is the *B. anaërobicus alkaligenes* (De Bono) and is long, non-motile, Gram-positive and spores freely, the spores being large, ovoid and subterminal. There is much gas production in glucose media. Milk is not clotted but is peptonised in three to four days. Causes much local œdema at site of inoculation in a guinea-pig. *Bacillus Y* is a long, slender, Gram-negative bacillus, feebly motile with a subterminal ovoid spore. Produces less gas than *Bacillus X*, liquefies albuminous media, gives rise to a putrid odour and is non-pathogenic to the guinea-pig. The "*Wisp*" *Bacillus* is probably *B. ramosus*. It is a small Gram-positive, non-motile diphtheroid bacillus growing well, producing no gas, clotting milk, with acid production in three to four days. It is markedly pyogenic.

The anaërobic streptococcus formed long chains in culture, did not redden neutral-red egg medium, and did not seem to ferment any of the sugars. Fleming also notes the occasional presence of an anaërobic influenza-like bacillus [? *B. fragilis* (Veillon)]. It is a very small Gram-negative bacillus growing well and producing acid but no gas in glucose media. It clots milk with an intense acid reaction.

The Course of Wound Infection and the Occurrence of the foregoing Organisms

Wound infection probably passes through three principal phases: (1) A phase of numberless microbes amongst which coliform organisms are predominant and active; this phase is one which passes quickly into (2): A phase in which the coliform organisms disappear and the field is occupied by the anaërobes, (3) A phase of reduction in the number of microbes, more or less disappearance of the anaërobes, with the presence of staphylococci and streptococci only (Distaso).

The *B. perfringens* is relatively frequent and is generally accompanied with other organisms; it is the cause of the condition in nearly two-thirds of the cases of gas gangrene. *B. œdematis maligni* is infrequent. Weinberg met with it only four times in 100 cases of gas gangrene.

B. (*enteritidis*) *sporogenes* is fairly common, and may often have been mistaken for the *B. œdematis maligni* (Weinberg). *Hibler IX* is common in association with *B. perfringens*, etc. *B. fallax* is rare in gas gangrene. *B. œdematicus* occurs particularly in the toxic forms of gas gangrene, associated with other organisms and occasionally with *B. perfringens* and *B. œdematis maligni*.

The three most pathogenic organisms would seem to be *B. perfringens*, *B. œdematis maligni* and *B. œdematicus*, and bacterial associations probably play a considerable rôle in determining the severity of the infection.

Bacillus botulinus

In certain forms of meat poisoning (see Chap. XXI) van Ermengem isolated an anaërobic bacillus, the *B. botulinus*. It is chiefly met with in ham and sausage, and the symptoms are caused by the absorption of toxin, which has a special effect on the nerve centres.

The organism is a large Gram-positive sporing anaërobic slightly motile bacillus, often occurring in pairs or in short chains. The spores are oval, larger in diameter than the rods and generally terminal. In glucose gelatin it forms a whitish streak in the line of the stab, with lateral outgrowths, liquefaction of the medium, and gas-formation. The cultures have a rancid odour, due to butyric acid production. The colonies in gelatin are semi-transparent spheres, later becoming fluffy; on agar the colonies are woolly. Meat broth is rendered alkaline and blackened. Milk is unchanged. The optimum growth is from 20°–30° C. The source of the organism is unknown, but it has once been isolated from the excreta of a healthy pig.

The *B. botulinus* in broth cultures forms a potent extracellular toxin, which is toxic both by injection and by ingestion. The toxin is also produced in the infected ham, sausage, etc. With the toxin an antitoxin can be prepared. The organism is somewhat pathogenic for guinea-pigs.

Black Quarter

Syn.: Black Leg, Quarter Evil, Symptomatic Anthrax, Rauschbrand.

Black quarter is a disease affecting sheep and oxen, and is unknown in man. The names black quarter, black leg, and quarter evil are derived from the dark discoloration of the muscles of the leg and flanks or quarters of the affected animals. When the muscles are cut into, a thin sanguineous fluid exudes, and in this fluid slender bacilli are present, some of which are swollen or club-shaped from the presence of spores. The muscles are dark, slightly crepitant owing to the presence of gas, and have a rancid odour.

The organism, the *B.* (*Clostridium*) *Chauvœi*, is a slender rod never forming long threads, is strictly anaërobic and motile but loses its motility in the presence of oxygen. Some of the rods are cylindrical throughout, others form slender spindles, others are oval or lemon-shaped. It stains in the tissues by Gram's method, but tends to be Gram-negative in culture. The organism forms endogenous spores, the spore-bearing rods being enlarged or club-shaped, and therefore should be termed a "clostridium."

It can be grown in deep stabs in gelatin and agar. Gelatin is rapidly liquefied. In glucose-agar it forms a thick, irregular,

greyish growth, with much development of foul-smelling gas. It forms acid and clot in milk and acidifies meat broth without blackening. The colonies are round or lenticular with regular margins. The writer has found extreme difficulty in isolating and in maintaining cultures of the organism. The guinea-pig is susceptible if inoculated subcutaneously or into the muscles, the bacilli being found at the seat of inoculation, but not in the blood or internal organs. Artificial immunity can be induced in various ways: by bacilli attenuated by heat or by successive cultivations, or by heating the dried muscle to 85° to 90° C. for six hours (Kitt), also by inoculating the susceptible animal at the tip of the tail. Hanna,[1] by growing the organism in a mixture of blood-serum and broth, obtained toxins which, by careful injection, conferred immunity on rabbits, the animals after injection yielding an antitoxic serum.

Hamilton described anaërobic bacilli in braxy, louping-ill, and other diseases of sheep and deer, but they are probably putrefactive and non-specific.

Bacillus Putrificus

The *B. putrificus* (*coli*) of Bienstock is found in the intestine and in sewage and manured soil. It is a slender rod 5–6 μ in length, actively motile, Gram-positive and sporing. The spores are round and terminal and their diameter is greater than that of the rods producing them. The organism is strictly anaërobic and grows readily. It forms gas from glucose and liquefies gelatin. The colonies on agar are woolly. Milk is digested without clotting. Meat broth is rendered alkaline and blackened with putrid odour. It rapidly digests fibrin with a putrid odour. It is hardly pathogenic. The *B. cadaveris sporogenes* of Klein[2] is probably identical with this organism.

Anaërobic Butyric Acid Bacteria

Anaërobic organisms occur in milk, in which they produce a marked butyric acid fermentation with changes like those of the *B. perfringens*. Such are the *B. butyricus* (*Vibrion butyrique*, Pasteur), *Clostridium butyricum* (Prazmowski), and *Bacillus*

[1] *Journ. Path. and Bact.*, vol. iv, 1897, p. 383.
[2] *Centr. f. Bakt.* (1te Abt.), xxv, p. 278.

amylobacter (Van Tieghem), which are not clearly distinguished from one another. They form short and long rods and filamentous forms. They do not liquefy gelatin nor blacken meat broth which is acidified. The surface colonies are smooth, the deep ones filamentous. Spore-formation takes place freely in enlarged segments or *clostridia*. They are non-pathogenic.

CHAPTER XIV

ASIATIC CHOLERA—SPIRILLUM METCHNIKOVI—SPIRILLUM OF FINKLER AND PRIOR—SPIRILLUM TYROGENUM—SPIRILLUM RUBRUM

Asiatic Cholera

THE bacteriological study of Asiatic cholera may be said to date from the researches of Koch, who in 1884 was sent by the German Government to investigate the disease in Egypt and India. He described an organism present in the intestine and in the dejecta which he believed to be the specific contagium, and named it the "comma bacillus" from its curved shape like a German comma. "Koch's comma bacillus," as it is commonly termed, is a curved rod or vibrio, by some placed in the genus *Spirillum*.

Spirillum (Vibrio) choleræ asiaticæ

Morphology.—Curved rods with rounded ends 1 to 2 μ in length, sometimes forming half a circle, sometimes united in pairs forming an S-shaped curve (Plate XX, *a*). It is present in the intestine and in the alvine discharges, especially in the rice-like flakes. In the rice-like flakes it is frequently so numerous that in a film the "commas" are arranged in "ranks and files" parallel to one another; this is also known as the "fish-in-stream" arrangement. Greig [1] found in six recently passed stools from 145 to 2000 million vibrios per c.c.

Greig has isolated the vibrio post-mortem from the lungs,

[1] Greig's papers on cholera will be found in the *Indian Journ. Med. Research*, vol. i *et seq.*

liver, spleen, kidneys and gall-bladder, bile and urine, but never from the blood during life. He suggests that dissemination is by the lymphatics. Cholera-like vibrios have also been found in the tissues after death.

The vibrio stains well with ordinary anilin dyes, especially with dilute carbol-fuchsin, but is decolorised by Gram's method. It is actively motile, and typically possesses a single terminal flagellum at one end only, but there is some variation in this respect. Spores are not formed, though in old cultures Hueppe described bodies which he believed to be arthrospores. In such cultures the bacilli lose their regular shape, and swollen and distorted involution forms are seen.

The majority of the organisms in a young agar culture assume the vibrio form, but in broth or peptone water cultures two or three days old they are longer and there is a tendency for them to become somewhat spirillar.

Cultural characters and biology.—The Koch vibrio is aërobic and facultatively anaërobic, and grows well on the ordinary culture media from 20° to 37° C. It grows readily in an atmosphere of hydrogen, but does not develop in one of carbonic acid gas.

In gelatin plates at 22° C. small cream-coloured colonies appear in about twenty-four hours, soon accompanied by liquefaction, so that in two or three days the plate becomes pitted. Microscopically, the young colonies are rounded with irregular margins, cream-coloured, and coarsely granular. In stab-cultures development occurs all along the stab as a whitish, opaque, punctate growth, thicker above than below. Liquefaction commences about the second day and progresses slowly; in the early stage it is confined to the surface, and looks like a little bead or air-bubble (Plate XX, *b*), but in a fortnight or so the greater part of the gelatin may be liquefied. Liquefaction varies greatly both in rate and in extent in different

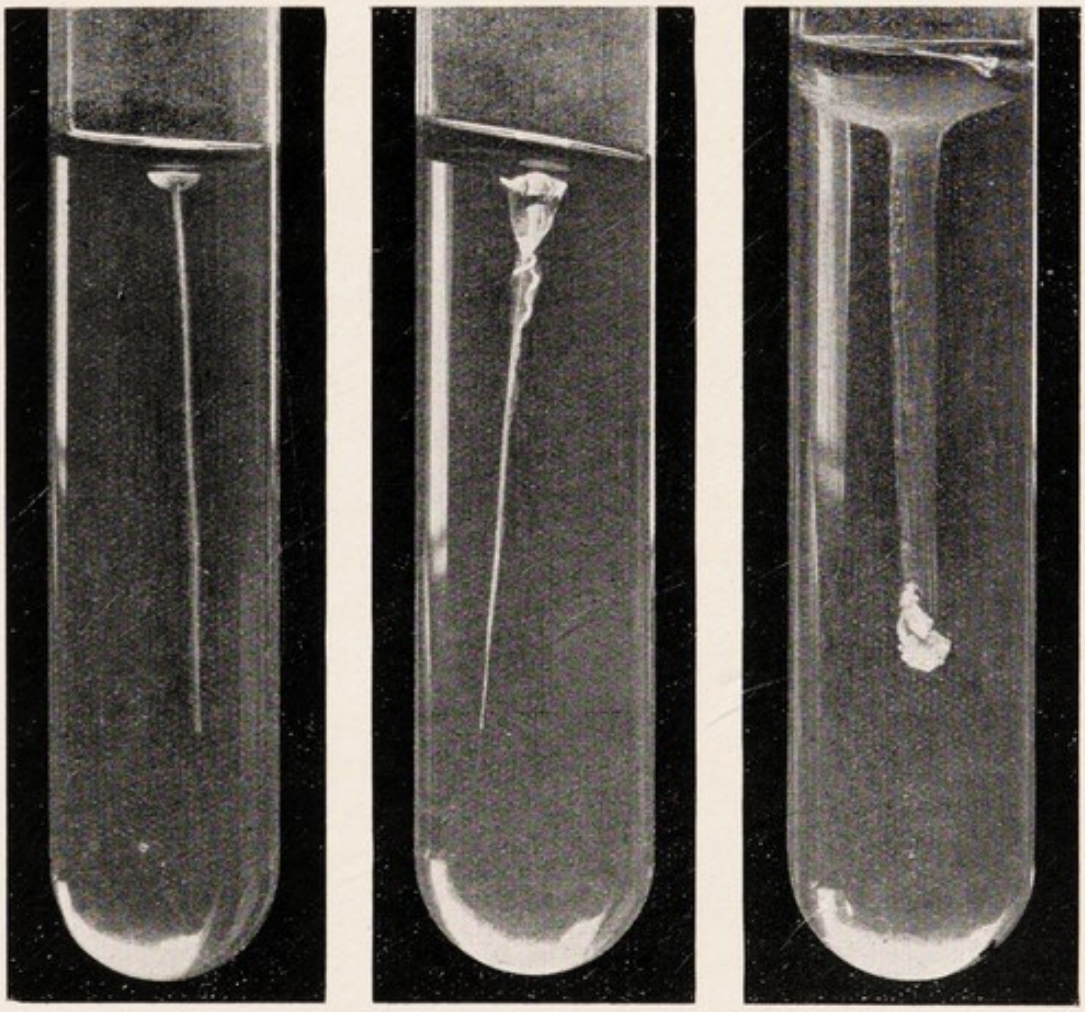
cultures and stocks ; in some old laboratory cultures it may be almost absent. On surface agar a thick, moist, shining, greyish growth quickly develops with more or less crenated margins, often becoming brownish when old. On blood serum much the same growth occurs with slow liquefaction. A thin brownish layer is formed on potato at 37° C., and broth becomes turbid, a delicate film forming on the surface. Peptone water, or Dunham's modification of it (1 per cent. NaCl), is a good cultivating medium, and a delicate film forms on the surface. In milk it multiplies rapidly with more or less acid-production and sometimes curdling ; neutral litmus glucose-agar is reddened from the development of acid, but no gas is produced under cultivation. Acid, but not gas, is produced from glucose, maltose, saccharose, lactose, mannitol and starch.

An important characteristic of the cholera vibrio is the rapid formation of indole in considerable quantity, and the reduction of nitrates to nitrites, especially in peptone water. This forms the basis of the important cholera-red reaction ; a few drops of pure sulphuric or hydrochloric acid added to a peptone-water culture, eight to twelve hours old, give a pink colour, and the colour is intense when the culture is two to three days old, and of a purplish-red colour, like that of potassium permanganate. Some specimens of " peptone " are unsuitable for preparing the peptone water used for obtaining the reaction, on account of the absence either of a tryptophane nucleus, or of nitrates and nitrites. The medium should be sugar-free, and the addition of 0·01 per cent. potassium nitrate to it is an advantage. Some believe that two pigments are formed in the reaction, a cholera-red and the nitroso-indole pigment.[1] The reducing action

[1] Wherry, Bureau of Government Laboratories, Manila, *Bulls.* 19 and 31, 1904 and 1905.

PLATE XX.

a. Spirillum choleræ. Film preparation of a pure culture. × 1500.



b *c* *d*

Gelatin stab-cultures, two days old, of (*b*) *Sp. choleræ*, (*c*) *Sp. Metchnikovi*, (*d*) *Sp. Finkleri.*

of the cholera vibrio can also be shown by growing in litmus broth, which becomes decolorised (Cahen's test).

Kraus and Prantschoff [1] noticed that certain vibrios dissolved red blood-corpuscles, but came to the conclusion that no recently isolated true cholera vibrio is hæmolytic.

The question of the formation of hæmolysins by the cholera and allied vibrios is important.

Strong,[2] in the Philippines, found that all vibrios which agglutinated well with a cholera serum were genuine cholera vibrios and that none of them was hæmolytic. On the other hand, Baerthlein [3] found that seven freshly isolated strains of the cholera vibrio were definitely hæmolytic in suspensions of sheep's corpuscles in from twenty-four to forty-eight hours. Van Loghem [4] employs goat's blood-agar plates in hæmolytic tests for the cholera vibrio. He asserts that goat's blood is quickly hæmolysed by hæmolysing cholera-like (*e.g.* El Tor, p. 506) vibrios, but that recently isolated cholera strains, if they hæmolyse at all, do not do so for some time—twenty-four to forty-eight hours.

With regard to this important question of hæmolysis and the cholera vibrios, Van Loghem [5] distinguishes two types of blood solution, viz. hæmolysis proper and hæmo-digestion. He asserts that the apparent hæmolysis on a blood-agar plate occasionally occurring with the true cholera vibrio is really hæmo-digestion. He distinguishes the two conditions by the tint of the hæmolytic zone—red in true hæmolysis and greenish in hæmo-digestion—and spectroscopically the affected zone shows oxy-hæmoglobin in hæmolysis but not in hæmo-digestion. The

[1] *Wien. klin. Woch.*, 1906, p. 299.
[2] *Philippine Journ. of Science*, vol. v, 1910, p. 403.
[3] *Arb. aus dem kaiserl. Gesundheitsamte*, xxxvi, 1911.
[4] *Centr. f. Bakt.*, Abt. I (Originale), lvii, 1911 p. 289.
[5] *Ibid.* lxx, 1913, p. 70.

blood agar used for the plates is composed of ordinary nutrient agar with an addition of 11–12 per cent. of defibrinated goat's blood.

Greig, using 1 c.c. of a 5 per cent. suspension of goat's corpuscles added to quantities of a 3-day-old broth culture varying from 0·01 c.c. to 1·0 c.c. (the smaller quantities made up to 1 c.c. with saline), the mixtures incubated for two hours at 37° C., and then kept in the ice chest over night, found that *not one* of 333 different strains of cholera hæmolysed, while of 100 cholera-like vibrios 19 gave no hæmolysis, 16 gave a trace of hæmolysis, and the remaining 65 hæmolysed more or less strongly. Grown for twenty-four hours on 12 per cent. goat's-blood agar plates, of 161 cholera vibrios, one gave distinct, nine gave a trace of, and the remainder no, hæmolysis, while of 43 cholera-like vibrios, one gave no, one gave a trace of, and the remainder gave decided, hæmolysis.

The cholera vibrio retains its vitality in cultures for a month. It can multiply in water and on the surface of moist linen, but rapidly dies on drying. Its thermal death-point, according to Sternberg, is 52° C. with an exposure of four minutes ; according to Kitasato, 55° C. in about ten minutes. It is easily destroyed by the ordinary germicides.

Dempster found that the comma bacillus lived from three to five days in dry soil, but only one day in an artificially dried soil, while in moist soil it lived from twenty-eight to sixty-eight days. In peat, however, it was invariably dead within twenty-four hours. In sterilised salt solution (0·75 per cent.) the comma bacilli were alive on the 159th day, and in fresh urine (sterilised) they lived fourteen days at 37° C. and twenty-nine days at 22° C.

Greig found that in Calcutta in the stools kept at room-temperature, the vibrio lived for just over one day in

June (hot season), and for just under eight days in February (cold season).

In sterilised distilled water the cholera vibrio usually rapidly dies, as a rule within twenty-four hours. The addition of sodium chloride greatly increases the length of time it may remain alive, a survival of five or six weeks having been recorded. In ordinary sterilised potable waters it may survive many months. In unsterilised potable waters its survival is greatly influenced by the presence of salts; in some cases it dies out rapidly; in others, especially in those containing a large proportion of salts, it may remain alive for some time. Houston [1] found that cholera vibrios die very rapidly in *raw* Thames, Lee, and New River waters as the result of storage in the laboratory. At least 99·9 per cent. perish within one week, and it was not possible to isolate any, even from 100 c.c. of the water, three weeks after infection. Klein [2] found that the cholera vibrio could retain its vitality for at least fourteen days in unsterilised sea-water, while from the interior of oysters, kept in water infected with the vibrios, it was obtained up to nine days after infection. In sterilised sewage the cholera vibrio multiplies and survives for months; in unsterilised sewage it may survive for two to four weeks (Houston).

Pathogenicity.—The disease is spread mainly by infected water; milk, salads, vegetables and flies are other sources of infection. The organism has been found in the dejecta of contacts not suffering from the disease, and it may sometimes persist for long periods after convalescence. In these cases the vibrio may sometimes be located in the biliary tract. Crendiropoulo examined the stools of 34,461 persons on ships coming from cholera-infected ports. Cultures of vibrios were obtained from 63 of

[1] Metropolitan Water Board, *Fifth Rep. on Research Work*, 1910.
[2] *Rep. Med. Off. Loc. Gov. Board for* 1896, p. 135.

these, of which 23 were agglutinated, and 40 were not agglutinated, by a high-titre cholera serum.

The relation of the cholera vibrio to the disease has been a very vexed question in the past, but the outcome of the voluminous researches which have been made is to confirm Koch's work. The organism is found in all cases of cholera, and several instances of laboratory infection from cultures have been recorded.

None of the lower animals suffers from or contracts a disease in any way comparable to Asiatic cholera, so that the test of animal experiment cannot be applied except in the case of young suckling rabbits (see below, " Anti-serum "). By first neutralising the acidity of the gastric juice by an injection of sodium carbonate solution into the stomach, then diminishing peristalsis by an injection of tincture of opium into the peritoneal cavity, and finally injecting a broth culture of the cholera vibrio into the stomach, Koch succeeded in inducing in guinea-pigs a condition somewhat similar to cholera in man—namely, indisposition with falling temperature, weakness of the extremities, and death in forty-eight hours. Post mortem, the small intestine was congested and filled with a watery fluid containing large numbers of the vibrios. Injected into the peritoneal cavity of mice, guinea-pigs and rabbits, the vibrio produces death from a general septicæmia, and intra-muscular inoculation into pigeons is sometimes fatal. The virulence varies much and is lost under cultivation.

Metchnikoff ascribed the immunity of animals to intestinal cholera as largely due to the inhibitory action of the other organisms present in the digestive tract. In man digestive disturbances are often an important predisposing cause of an attack. The acidity of the gastric juice is also probably a means of defence (see " Water ").

The blood-serum of an animal immunised by injections of the cholera vibrio gives a typical agglutination reaction

with recent cultures of the organism. The reaction can also be obtained with the serum-blood of cholera patients.

According to Greig, in non-fatal cases the cholera agglutinins rarely develop so early as the second day of the disease, from that date they increase and about the sixth day are marked. The titre remains high until the seventeenth day and then drops, and by the twentieth day or soon after the agglutinins disappear. The majority of cases agglutinate up to a dilution of 1 in 60, some in dilutions of 1 in 200, 400, 800, or 1,000. The majority of fatal cases, even if they live for several days, do not develop agglutinins. In no case did the serum agglutinate a cholera-like vibrio isolated from the stool. In carriers agglutinins are generally present. Normal serum may agglutinate the vibrio up to a dilution of 1 in 20.

Occurrence of the vibrio and of allied vibrios.—That the cholera vibrio is etiologically associated with the disease seems to be beyond any doubt. The bacteriological diagnosis, however, has become complicated owing to the presence in stools and in various natural waters of pathogenic vibrio which, although not identical with the cholera vibrio of Koch, resemble it closely. The principal cholera-like vibrios isolated from natural waters are those of Sanarelli from the Seine (*Sanarelli*), Dunbar from the Elbe (*Elwers*), Neisser from the Spree (*Berolinensis*), Heider from the Danube (*Danubicus*), and one isolated by Ivanoff (*Ivanoff*). Cholera-like vibrios are also not infrequent in choleraic diarrhœa and in true cholera, and Greig has isolated a number from these conditions. Lastly, there is the *Vibrio Massowah*, isolated from an epidemic of cholera at Massowah, which differs from the Koch vibrio in having two terminal flagella at each end.

Applying the Pfeiffer and agglutination tests to some of the vibrios in question, the following results were obtained. In the first place, each of the organisms gives a complete

positive reaction to both tests with its own serum ; this, of course, is only to be expected. Pfeiffer found that, using his reaction, the variety *Ivanoff* gave a positive reaction with cholera serum, and Durham found that *Ivanoff* and *Berolinensis* reacted completely with cholera serum. Conversely, positive reactions with cholera vibrios were obtained with *Massowah*, *Danubicus*, and *Elwers* sera, while *Massowah* and *Elwers* react completely to each other. From these considerations it would therefore seem probable that some of these vibrios—*Sanarelli*, *Berolinensis*, and *Ivanoff*—may be varieties of the Koch vibrio.

Ruffer [1] in 1905 at El Tor isolated vibrios, which may be distinguished as " El Tor vibrios," from the intestine of pilgrims returning from Mecca and suffering from various diseases (dysentery, diarrhœa, pneumonia, rheumatism), but among whom there had been no cholera, and who had not been in contact with cholera. These vibrios were subjected to detailed examination by the agglutination, saturation and fixation tests, and Pfeiffer's reaction with Berlin cholera-immune serum, and also by the hæmolysis test. Vibrios isolated from a previous epidemic of cholera (referred to as Group 1), and other vibrios isolated from cholera and other stools (Groups 3 and 4), were also compared with the El Tor vibrios. Ruffer's results were as follows :

Group 1 (undoubted cholera vibrios).—Those which react positively to the four principal tests with cholera serum—namely, the agglutination, saturation, and fixation tests and Pfeiffer's reaction. They do not hæmolyse, even when remaining in contact with red corpuscles for three days at the temperature of the laboratory.

Group 2.—The second group contains the vibrios agglu-

[1] *Researches on the Bacteriological Diagnosis of Cholera.* Sanitary, Maritime, and Quarantine Council of Egypt, Alexandria, 1907. (Also *Brit. Med. Journ.*, 1907, vol. i, p. 735.)

tinated by, and giving the saturation and Pfeiffer's reactions with, cholera serum, but not fixing the cholera-immune body. These vibrios are strongly hæmolytic. This group consists of the El Tor vibrios only.

Group 3.—The third group is formed by vibrios which are not agglutinated by immune serum, nor give the saturation or Pfeiffer's reaction, but fix the cholera-immune body. These vibrios also hæmolyse, but feebly and late, often only after thirty-six to forty-eight hours.

Group 4.—The last group is formed by strongly hæmolytic vibrios not reacting at all to cholera-immune serum.

Ruffer concluded that the El Tor vibrios are not genuine cholera vibrios. He says : " The only possible classification is to group together all the vibrios reacting in the same way to all tests, separating them from those which, under the same conditions, behave in a different way. If this method be applied to the vibrios found at El Tor, there is no difficulty in distinguishing them from the true cholera vibrios, in spite of several of the reactions of both being similar. And it follows also that the agglutination, saturation and Pfeiffer's tests are not in themselves of absolute diagnostic value for cholera vibrios."

Neufield and Haendel,[1] however, after a re-examination of some of these vibrios, consider that they are true cholera vibrios. The matter therefore remains undecided.

Klein found that the cholera vibrio kept in sea-water showed marked variation from the original strain. In the East many cases of cholera are mixed " vibrionic " infections ; the stools may contain several varieties of vibrios, some agglutinating with cholera serum, others not ; some monociliate, others multiciliate.

It may be that the cholera vibrio, like the *B. dysenteriæ*, is not a single definite organism, and that cholera may be caused by any one of a group of closely allied vibrios.

[1] *Arbeit. a. d. kais. Gesundheitsamte*, xxvi, 1907, p. 536.

This view is supported by Castellani and by some of Greig's observations. Castellani suggested the term "paracholera" to denote choleraic cases in which the true *V. choleræ* is absent but which seem to be caused by some of these cholera-like vibrios.

Toxins.—Brieger in 1887 obtained cadaverin and putrescin and two other basic bodies from cholera cultures. Brieger and Fränkel isolated a tox-albumin, and Gamaleia a ferment-like body. Hueppe believes that the cholera poison is a tox-albumin formed in the culture medium, but that immunising substances are derived from the bacterial cells.

Rontaler compared the chemical products of the ordinary cholera and of the Massowah spirilla, and could find little difference between them.

Wesbrook [1] obtained albumoses and other bodies from alkali-albumin, egg, and Uschinsky medium, cultures. This observer also found aërobic cultures of the cholera vibrio to be much more toxic than anaërobic ones.

Pfeiffer found that cholera cultures killed with chloroform vapour contained a toxic substance fatal to guinea-pigs in small doses, with extreme collapse. He believed the substance to be an integral part of the bacterial cells.

Metchnikoff,[2] Roux and Salimbeni demonstrated the existence of a soluble cholera-poison in a very ingenious manner. Collodion sacs of 2 c.c. to 3 c.c. capacity were sterilised, filled with peptone solution, inoculated with the cholera spirillum, and closed. The closed sac was then introduced into the peritoneal cavity of a guinea-pig, which died in three or four days from the effects of the soluble toxins dialysing through the walls of the sac (see also next page).

Brau and Dernier [3] obtained a toxic filtrate by culti-

[1] *Journ. of Path. and Bact.*, vol. iv, 1896, p. 1.

[2] *Ann. de l'Inst. Pasteur*, x, 1896, p. 257. [3] *Ibid.*, xx, 1906

vating the cholera vibrio in a medium consisting of horse-serum with an addition of 10 per cent. of defibrinated horse-blood.

Macfadyen obtained a highly toxic endotoxin by triturating cholera cultures with liquid air.[1]

Emmerich [2] advanced the view that the cholera intoxication is not a toxin intoxication, but is due to nitrite poisoning, the nitrites being produced by the reducing action of the vibrios on nitrates present.

Anti-serum.—By growing the cholera vibrio in a shallow layer with free access of oxygen in a peptone gelatin-salt medium, Metchnikoff and his co-workers obtained a toxic fluid after three or four days' growth. After filtration, 0·25 c.c. killed a 300-grm. guinea-pig in eighteen hours. Goats, inoculated with increasing doses of this toxin, commencing with 10 c.c. and reaching 200 c.c. in six months, become immunised and yield an antitoxic serum, 1 c.c. of which will neutralise four times the lethal dose of toxin. Metchnikoff had previously found that young suckling rabbits suffer from an intestinal cholera when fed with cultures, so that the effect of the cholera antitoxin in preventing intestinal cholera could be tested on these animals. Experiment showed that of the treated rabbits, 51 per cent. survived, of the untreated only 19 per cent. Salimbeni employed a serum prepared in this manner in the treatment of cases of cholera in the Russian epidemic, 1910.

Animals may be inoculated with dead and living cultures and an immune serum so prepared, but no practical value has yet attended the use of anti-sera in the treatment of cholera. Macfadyen immunised a goat with cholera-cell juice, and obtained a serum of which $\frac{1}{500}$ c.c. protected a guinea-pig against three lethal doses of cholera culture.

[1] *Lancet*, 1906, vol. ii, p. 494.

[2] *Munch. med. Wochenschr.*, 1911, No. 18, p. 942.

The writer prepared an anti-endotoxic serum in this manner, with which a few cases of cholera were treated in Russia.[1]

Vaccine.—Ferran in 1885 first prepared a vaccine by making cultures (mixed) in broth from cholera stools and injecting 0·3–0·5 c.c. subcutaneously, but the reports of the commissions sent to investigate the method were unfavourable.

Haffkine subsequently prepared a vaccine against cholera which has been extensively used. In the Haffkine method two vaccines are made use of. The first or weak vaccine is prepared from cultures of the cholera vibrio attenuated by growing on the surface of agar, with free aëration, for several generations. The second or strong vaccine is prepared by enhancing the virulence of a cholera culture by a succession of passages through the peritoneal cavity of guinea-pigs. The virulence of this culture must be maintained in the same manner.

For making both vaccines, "standard" agar cultures are employed. These are tubes in which the sloping surface of agar measures 15 cm. in length, and the cultures are incubated for twenty-four hours. The whole growth on such a tube is emulsified in 8 c.m. of broth or salt solution; the dose of this is 1 c.c., and the living vaccines are injected into the flank, the second or strong being given seven to ten days after the first or weak. Haffkine[2] in a more recent study on cholera inoculation suggests the use of the strong vaccine "devitalised." The devitalised vaccine may be prepared by two methods, (*a*) prolonged cultivation in broth and treatment of the culture with heat and carbolic acid, (*b*) cultivation on agar and treatment with carbolic acid.

[1] *Lancet*, 1910, vol. ii, October 22.

[2] *Preventive Inoculation against Cholera* (W. Thacker & Co., 1913).

Besredka [1] claims that an immediate and lasting (six months) immunity may be produced by making a mixture of cholera culture and cholera-immune serum, allowing this to stand for twelve hours, heating to 56° C. for one hour and then injecting subcutaneously.

Strong [2] prepares a vaccine from autolysed cultures. The cholera vibrio is grown on surface agar for twenty-four hours at 37° C.; the growth is then washed off with sterile water, the suspension is kept at 60° C. for twenty-four hours, and then at 37° C. for two to five days, and is finally filtered through a porcelain filter.

Fox [3] concludes from an experimental study that a heated vaccine without phenol is the most efficient and that next in order come (*a*) living culture, (*b*) a modification of Strong's vaccine, (*c*) a sensitised vaccine. Castellani has introduced the use of a polyvalent vaccine containing typhoid, paratyphoid A and B, and cholera.

The most extensive statistics of the value of anti-cholera inoculation are those of the Balkan war 1913–14. Of 114,803 men, 8,968 were not inoculated and the incidence of cholera among them was 93 cases per 1000; 14,613 were inoculated once, and the incidence was 42 per 1000; 91,224 were inoculated twice, and the incidence was 7 per 1000. The case-mortality was among the uninoculated 27·5 per cent.; among the once inoculated 12·2 per cent., among the twice-inoculated 10·2 per cent.

Clinical Diagnosis

1. Prepare films from the stool and stain with dilute carbol fuchsin. If on examination large numbers of curved rods lying in groups parallel to one another are observed, the dia-

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[1] *Ann. de l'Inst. Pasteur*, 1902, p. 918.

[2] *Bureau of Gov. Laboratories, Manila*, Bull. No. 16, 1914 (Bibliog.).

[3] *Indian Journ. Med. Research, vol.* iv, No. 2, 1916, p. 335.

gnosis of Asiatic cholera may be made with some degree of certainty. (Single, or a few, vibrios are of no diagnostic significance; they may occur in normal and diarrhœa stools. The presence of numbers of vibrios having the "fish-in-stream" arrangement is also not absolutely characteristic.

2. (*a*) Inoculate about 40 c.c. of Dunham's peptone water—contained in a small Erlenmeyer flask—with one to two c.c. of the stool. Incubate at 37° C.

(*b*) After six hours' incubation make films and hanging drop preparations and, if necessary, inoculate a second flask of peptone water from the surface fluid of flask No. 1. Incubate for eight to twelve hours.

(*c*) At the end of incubation period, from the surface of the fluid make films and hanging-drop preparations, and plate out a loopful on Endo- or Conradi agar medium. Incubate the plates until growth can be distinctly seen; usually eight to twelve hours. Search for and examine suspicious colonies. If vibrios be found, test for agglutination by taking up a trace of the colony on a straight platinum needle and mix with a drop of a 1/200 dilution cholera serum of high titre (1 in 5–10,000). If agglutination be positive the remainder of the colony is picked off the plate and transferred to an agar or peptone water tube for further growth and examination if necessary.

(In many instances it is not necessary to proceed beyond the peptone water culture referred to in (*b*), and it is occasionally possible to isolate with ease the vibrio from plates prepared directly from the stool. The stained films and hanging drops prepared at the specified times give an indication as to the number (if any) of vibrios present at that particular time, and plates are made immediately they are found to be fairly numerous, the routine being designed to complete the examination in the shortest possible time.) [1]

A better medium to employ for plating is Dieudonné's blood alkali agar. Equal parts of defibrinated ox-blood and normal caustic potash solution are mixed and sterilised in the steamer. Of this 30 c.c. are mixed with 70 c.c. of 3 per cent. peptone-agar (neutral to litmus), previously melted. Plates are poured and kept at 60° C. for half an hour, and are then allowed to stand for forty-eight hours for ammonia to evaporate. On this medium few organisms

[1] The writer is indebted to Mr. Edwin Burgess, of the Bacteriological Institute, Colombo, for the foregoing technique.

except the cholera vibrio develop (but cholera-like vibrios develop equally well).

The Dieudonné medium requires fresh blood, but Lentz has devised a dry blood-alkali powder for its preparation. Freshly obtained and defibrinated ox-blood is mixed with an equal quantity of normal caustic potash solution. The mixture is steamed for half-an-hour and the fluid is then evaporated to dryness *in vacuo* over sulphuric acid at 37° C. The dry mass is finely powdered and preserved in a stoppered bottle. For use 3 grm. of the powder are dissolved in 30 c.c. of distilled water and the solution is mixed with 70 c.c. of melted neutral agar as above, and plates are poured and are ready for immediate use.

3. If the case has lasted some days, the agglutination reaction may be applied, testing the patient's serum on a known strain of cholera vibrio.

Spirillum Metchnikovi

Isolated by Gamaleia from the intestinal contents of chickens dead of an infectious gastro-enteritis which occurred in certain parts of Russia. The disease, although resembling chicken-cholera in some respects, is quite distinct from the latter. This spirillum forms curved rods and spiral filaments, generally slightly shorter, thicker and more curved than the Koch vibrio. It is decolorised by Gram's method, and is best stained with weak carbol-fuchsin. It is readily cultivated, and is aërobic and facultatively anaërobic. In gelatin plates it forms small whitish colonies, visible within twenty hours, which grow more rapidly than the cholera vibrio, and in two or three days produce marked areas of liquefaction. In a stab-culture in gelatin a whitish granular growth occurs along the line of puncture with liquefaction, much like that of the Koch vibrio, but the rate of growth and the liquefaction are more rapid (Plate XX, *c*). Grown in eggs by Hueppe's method typical appearances are produced. After ten days the white becomes transformed into a yellowish limpid liquid, while the yolk, though retaining its form and consistence, is quite black. On surface agar a thick cream-coloured layer develops; on potato the growth is brownish, and milk is coagulated. It grows freely in broth and peptone-water, the fluid becoming uniformly turbid, and a slight film forms on the surface, and these cultures give a marked indole reaction on the addition of sulphuric acid alone, in this respect resembling the

Koch vibrio. The *S. Metchnikovi* is pathogenic to chickens, pigeons and guinea-pigs, but not to rabbits or mice except in large doses. It is, however, more pathogenic to guinea-pigs than the cholera vibrio. Pigeons are killed by intra-muscular inoculation, and fowls are susceptible to feeding, whereas the cholera vibrio is not pathogenic to fowls by feeding. It is not agglutinated with cholera-immune serum. Abbott isolated a pathogenic spirillum from the Schuylkill River, Philadelphia, which resembles the *S. Metchnikovi* closely, and is probably identical with it.

Spirillum Finkleri (of Finkler and Prior)

Isolated from the stools in certain cases of cholera nostras, but its etiological significance is doubtful. It occurs as short, thickish, curved or straight rods, and sometimes as spiral filaments. It is aërobic and facultatively anaërobic, does not form spores, and does not stain by Gram's method. In a gelatin stab-culture a yellowish growth forms with rapid liquefaction (Plate XX, *d.*) On agar a thick, slightly brownish, moist layer develops. Serum is rapidly liquefied. On potato a slimy brownish growth occurs even at room temperature. It grows in broth and peptone-water, producing a general turbidity. It does not as a rule give the indole reaction with sulphuric acid alone, but the ordinary laboratory cultures after three to four days' growth occasionally give a slight reaction. It is stated to be pathogenic to guinea-pigs by intraperitoneal inoculation.

Spirillum tyrogenum

Obtained by Deneke from old cheese, and frequently spoken of as Deneke's spirillum. It forms curved rods and spiral filaments somewhat closely resembling the Koch vibrio. It grows well on the ordinary culture media at room temperature, but development is usually slight or absent at 37° C. In a gelatin stab-culture a yellowish growth occurs with liquefaction, which is much more rapid than that of the Koch vibrio, but less so than that of the Finkler-Prior spirillum. On agar a thinnish, brownish, somewhat membranous and coherent layer slowly develops at room temperature. On potato a yellowish growth occurs. It is stated to be slightly pathogenic to guinea-pigs by intra-peritoneal inoculation

Spirillum rubrum

A chromogenic spirillum obtained by Koch from the putrefying tissues of a mouse. In a gelatin stab-culture a dark red growth slowly develops along the line of puncture without liquefaction; at the surface, however, the growth is colourless. In broth at 37° C. it grows freely, producing a general turbidity with a red deposit at the bottom of the tube; there is no film formation. In such a broth culture large numbers of typical spirillar filaments can be seen, which are thin and delicate, of varying length, and actively motile. It is non-pathogenic.

Vibrios are common in the mouth, and may be met with in the discharge of septic ulcers.

CHAPTER XV

STREPTOTHRIX INFECTIONS—ACTINOMYCOSIS—MYCETOMA —LEPTOTHRIX BUCCALIS—CLADOTHRIX DICHOTOMA —MYCOSIS TONSILLARIS

Streptothrix Infections (Streptothricosis)

The terms *Streptothrix*, *Leptothrix* and *Cladothrix* have been loosely used to denote an organism forming long unsegmented filaments, in the two former the filaments are unbranched, in the latter the filaments show pseudo-branching. Some of them may be filament-forming Schizomycetes, but the majority are probably true Fungi.

A number of pathological conditions is caused by this class of organism. These conditions are infective granulomata and they may be included in a group entitled *Mycetoma*, of which the following definition may be given [1]: "The term *Mycetoma* includes all growths and granulations producing enlargement, deformity and destruction in any part of the body of man or animals, brought about by the invasion of the affected area by certain species of Fungi, belonging to different genera, which give rise to variously shaped and coloured bodies known as *grains*, which are either embedded in the granuloma or are found in the discharge from the affected area. The grain is composed of hyphæ (p. 539), and sometimes of chlamydospores (p. 541) embedded in a matrix, which on germination give rise to

[1] See Chalmers and Archibald and Chalmers and Christopherson, *Ann. Trop. Med. and Parasitology*, vol. x, No. 2, 1916, pp. 169 and 223 (*Bibliog.*), whose description is here largely adopted; Musgrave, Clegg and Polk, *Philippine Journ. of Science*, vol. iii, 1908, p. 447; Foulerton, *Lancet*, 1910, vol. i, p. 551, *et seq.*; Pinoy, *Bull. de l'Inst. Pasteur*, vol. xi, 1913, pp. 929, 977.

mycelial filaments." (The "grains" must be distinguished from sclerotia. A "sclerotium" is a hard body formed by a dense lignified mass of hyphæ, as in ergot.) The Mycetomas may be divided into two classes :

A. The *Actinomycoses* in which the grains are composed of fine non-segmented mycelial filaments, the walls of which are usually not clearly defined from the contents, and without chlamydospores.

B. The *Maduramycoses* in which the grains are composed of large segmented mycelial filaments, possessing well defined walls and, usually, chlamydospores.

As types of the *Actinomycoses* we have Actinomycosis of man and animals and the classical white variety of Madura disease, while the classical black form of Madura disease is a type of the *Maduramycoses.*

The Actinomycoses are caused by organisms belonging to the *Fungi Imperfecti* (p. 540), Sub-class *Hyphales* (Vuillemin, 1910), Order *Microsiphonales* (Vuillemin, 1912), in which the mycelium is composed of fine bacilliform hyphæ, usually one micron or less in diameter, with a thickened hyphal wall and septa. This contains at present only two genera which are distinguished as follows :

A. *Nocardia*, grows aërobically and produces arthrospores (De Toni and Trevisan, 1889).

B. *Cohnistreptothrix*, grows best anaërobically, usually difficult to cultivate and does not produce arthrospores (Pinoy, 1911).

The Maduramycoses may be classified according to the colour of their granules into (1) the Black Maduramycoses, and (2) the White Maduramycoses. They are caused by a variety of fungi.

The Student is advised to read through the introduction to chapter xvii on the Hyphomycetes (p. 539) before reading this chapter.

Actinomycoses

Actinomycosis in man clinically and pathologically often closely resembles tuberculosis, with which in the past it was frequently confounded.

Actinomycosis in cattle (*Actinomycosis bovis*) has long been known, but its exact pathology was involved in

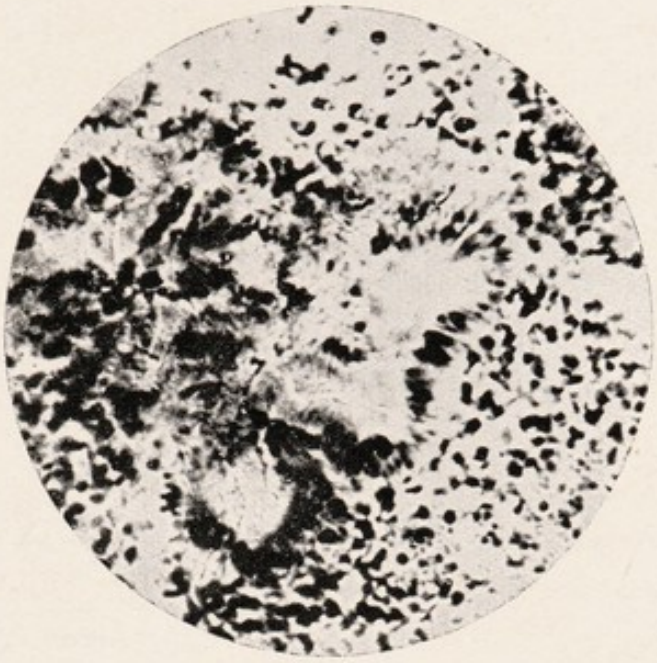
considerable doubt until the researches of Bollinger in 1876. It forms tumours chiefly affecting the tongue, jaw, face, and throat, and was described under such varied names as wen, scrofula, scirrhus, osteo-sarcoma, cancer, wooden tongue, etc.

The tumours after a time break down and discharge, the tongue often protrudes from the mouth, the saliva drips, and the animal becomes much emaciated.

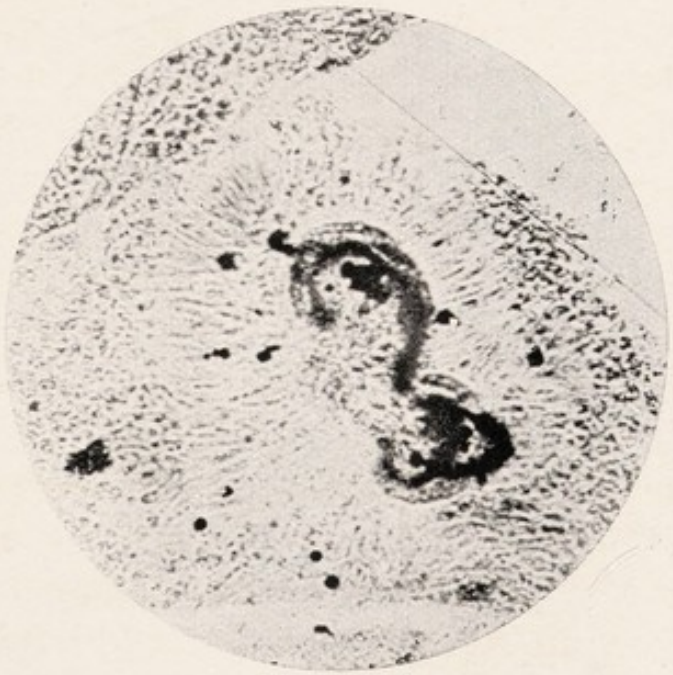
On cutting into a "wooden tongue," or wen, a grating sensation is felt, such as that experienced in cutting a turnip or unripe pear; on examining the section little rounded, yellowish, frequently almost caseating areas will be noticed, resembling old tubercles. On making sections and examining with a low power, these rounded areas are found to be composed of masses of small round-cells, with occasionally giant-cells, surrounded by a capsule of fibrous tissue. The growth may be so soft as to be practically purulent, and abscesses varying in size from a pin's head to that of an orange may be present in the affected areas. Like tubercles, the growths may become caseous, calcified, or fibrous. In the growth or in the pus from abscesses, when examined fresh with a low power, yellowish or yellowish-white granules will be found here and there, which may be very minute, or as large as a small pin's head, are somewhat soft in consistence, and on slight pressure flatten out. Examined with a high power, these granules are found to contain round, ovoid, or reniform bodies which have a rosette-like appearance, a more or less structureless centre with club-shaped bodies radially arranged around the periphery (Plate XXI, *a*). These peculiar structures are the cause of the disease, and are the form assumed in the animal body by an organism *Nocardia bovis* (*Actinomyces*, *Streptothrix* or *Oospora*, *bovis*), or, from its appearance, the ray fungus.

Sections of the diseased tissues show the structure of

PLATE XXI.



a. Actinomycosis bovis. Section of tongue. Gram. × 350.



b Mycetoma. Section of tissue, white variety. Gram. × 350.

the organism still better. Gram's method usually gives good results, and it will generally be found that the following appearances can be observed : Surrounded by the round-cells are the reniform or ovoid bodies, situated at the periphery of which are radially arranged, club-shaped structures deeply stained with the gentian violet, while the central portion is unstained and structureless, or contains granular matter or calcareous particles. Various appearances may be met with in different parts of the section, according as the actinomycotic nodule is cut through its centre or periphery ; when the latter is the case, the clubs are shown in transverse section and appear as closely packed, deeply stained dots. Sometimes, however, in addition to the clubs, the centre of the rosette is occupied by numerous interlacing filaments, also stained by the gentian violet.

Nocardia bovis grows well aërobically at 22° C., but better at 37° C. Anaërobic growths are, as a rule, but poorly developed.

It may form a dry pellicle on the surface of broth, but more usually it gives rise to cohering colonies at the bottom of the tube, and in either case the medium remains clear.

It grows slowly on gelatine, producing a yellowish-white growth and slow liquefaction, beginning about the seventh day. The resulting fluid may or may not be dark coloured. On blood serum it produces poor growths, and no liquefaction or pigmentation of the medium.

On agar and glycerine agar it forms hard spherical white colonies, which give rise to an undulating crateriform growth, having a yellowish or greyish tint, which in its turn becomes a lichenoid ashen grey or yellowish mass with a powdery efflorescence. Microscopically the growth consists of masses of filaments (Fig. 51), the ends of which may be somewhat clubbed and segmented, but

which never show rosette arrangement of clubs such as occurs in the tissues. On maltose agar it forms discrete fawn-coloured colonies, later becoming yellow, dark brown or even black, while the medium may be slightly darkened.

On potato it forms confluent, hard, raised, variously-coloured masses, at first white but becoming greenish-

Fig. 51.—Actinomyces. Film preparation of an agar culture. Gram. × 750

yellow, brown, greyish-black or even black, with more or less erosion and pigmentation of the medium to which the growth is very adherent (Fig. 52). No diastatic action has been observed. Litmus milk is first reddened, but later it becomes a clear brown alkaline liquid.

Nocardia bovis infects cattle, swine, horses and occasionally man, while experimentally, rabbits and guinea-pigs have been infected by intraperitoneal inocula-

tion. Much calcification sometimes occurs in the nodules, particularly in swine. The mode of infection is uncertain, but it is generally believed that the organism occurs on grasses or grain and gains access to the tissues through some abrasion.

"Farcin des bœufs," a disease of cattle occurring in Guadeloupe, and characterised by infection first of the skin and afterwards of the lymphatic glands and viscera, is caused by *Nocardia farcinica*. The organism is a strict aërobe, forms yellowish-white grains and is without sheath.

Fig. 52.—Actinomyces. Potato culture, three months old.

In Argentina, Lignières and Spitz described a form of actinomycosis of cattle caused by a Gram-negative bacillus-like organism, the *Actinobacillus*. Griffith [1] has shown that this condition is frequent in this country in cattle. The organism grows well aërobically and anaërobically on various media, does not liquefy gelatin or serum, and is pathogenic for many animals. One case of human infection with this organism has been described in a man from the Argentine. The organism was obtained from the cerebro-spinal fluid and on glucose peptone formed clubs, and this being so its name becomes *Nocardia lignieresi*.

Actinomycosis in man (*Actinomycosis hominis*) is usually associated with suppuration, and may occur in almost any part of the body—jaw, lungs, liver, intestine, skin and pelvic organs. If a little of the pus be examined it will probably contain tiny yellowish or sulphur-yellow granules, which, microscopically, are

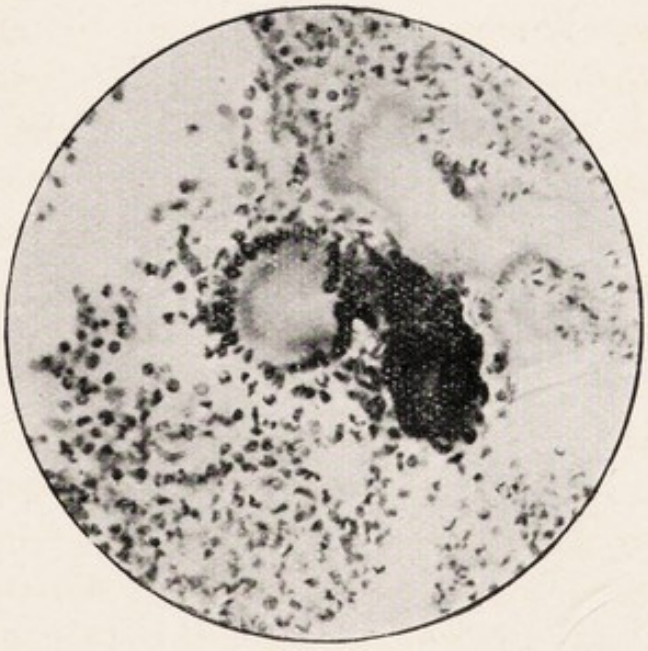
[1] *Journ. of Hygiene*, vol. xv, 1916, p. 195.

found to consist of tufts of fine tangled filaments, the ends of which may be continued into little swellings or clubs. In teased-up specimens, or in sections stained by Gram's method, an appearance is observed very different from that of the bovine variety, viz. tufts of interlacing filaments stained by the gentian violet, but a complete absence of purple clubs (Plate XXII, *a*). Clubs, however, are frequently present around the periphery of the filamentous tufts in a stunted condition, although they do not usually stain by Gram's method. These clubs are often seen better in fresh specimens of the pus or in unstained sections, or by staining with orange-rubin, or the Ehrlich-Biondi reagent (Plate XXII, *b*). The conditions in cattle and man, at first sight so very different, are thus seen to be similar, a similarity which is further established by the occasional occurrence in cattle of filamentous tufts, staining by Gram's method, within the rosettes, and by the clubs in man now and then taking on the gentian-violet stain.

Actinomycosis in man in Europe is commonly caused by the *Cohnistreptothrix israeli*, occasionally by *Nocardia bovis*. *C. israeli* is composed of short and long rods, some of which show club-like swellings, while in old cultures spores which resemble cocci in appearance can be seen. It grows but poorly in the presence of air, but much better anaërobically at 37° C. on agar, on which it forms dew-like drops, which later become yellowish and generally remain discrete. In broth it forms a deposit of small scaly particles. It does not grow on gelatine at room temperature, but egg cultures show typical branched filaments with club-like ends, which later break up into bacillary and coccal forms, but true arthrospores (*i.e.* resistant spores) are not produced. It forms granulation tumours when inoculated intraperitoneally into rabbits and guinea-pigs, after an interval of four to seven

PLATE XXII.

a. *Actinomycosis hominis*. Section of liver showing a mycelial mass. Gram. × 500.



b. *Actinomycosis hominis*. Section showing a ring of stunted clubs. Gram. × 350. Same material as Fig. *a* above.

weeks. In these tumours typical actinomycotic grains can be found, containing branched filaments with clavate ends. The source of infection of man is unknown, though cases have been reported where the disease has occurred after eating grains of barley, etc.

It is important to note that tuberculin may cause a reaction in actinomycosis, similar to that which occurs in tuberculosis, and as the actinomycotic lesions are very like those which are found in the latter disease, mistakes may easily be made, and can only be avoided by a microscopical examination. It is of considerable practical importance to distinguish actinomycosis from tuberculosis, for in many cases of the former, both in man and in animals, iodide of potassium exerts a specific curative action. Vaccine treatment has also been employed with a certain amount of success.

Other forms of Actinomycosis occasionally occur in man. Eppinger obtained an organism, *N. asteroides* (*Streptothrix Eppingeri*), from a case of pseudo-tuberculosis of the lungs and glands with cerebral abscess. The fungus was Gram-positive and acid, but not alcohol, fast, grew well aërobically on laboratory media and was pathogenic for laboratory animals. The growths are yellowish-orange to brick-red. It has been met with elsewhere, and in a case of white Madura disease in the Philippines (p. 526). The classical white variety of Madura disease in India is an Actinomycosis (p. 526). Birt and Leishman isolated an acid-fast organism, *N. leishmani*, pathogenic to man and animals. Various saprophytic forms of *Nocardia*, some of them chromogenic, occur in air, soil, and water, and may gain access to sputum, etc.

Clinical Examination

1. Pour out the pus or discharge into a large capsule or Petri dish so that it forms a thin layer, look for any yellowish or other

granules, pick them out with a needle, and place on a clean slide in a drop of 50 per cent. glycerin. If no granules can be found, a little of the discharge may be spread on a slide with a drop of 50 per cent. glycerin. Cover with a cover-glass, and apply a little pressure. Examine with a $\frac{2}{3}$-in. objective. If any actinomycotic tufts are present they will be seen as yellowish or pale brownish, spheroidal, ovoid, or reniform masses, and with a higher power will be found to consist of tufts of filaments with, perhaps, stunted clubs (in the bovine form rosettes of clubs will be seen).

2. Stain films of the discharge, by Gram's method, with eosin. The actinomycotic tufts will generally be found to consist of little masses of tangled filaments stained violet, and surrounded by a pink zone which has an indistinct radiating structure.

N.B.—In most instances the clubs in Actinomycosis hominis do not stain by Gram's method. The reverse is the case in Actinomycosis bovis.

3. Sections of actinomycotic tissue are best prepared by the paraffin method. If frozen, the actinomycotic nodules are very apt to fall out. Sections may be stained by any of the following ways :

(*a*) By Gram's method, with eosin or orange-rubin.

(*b*) With the Ehrlich-Biondi triple stain. Stain for from half an hour to two hours. Place in methylated spirit until the sections appear greenish, then pass through absolute alcohol and xylol. The clubs are stained yellowish-brown, and are sometimes shown in human cases when unstained by Gram's method.

(*c*) By Plaut's method. Stain in warm carbol-fuchsin for ten minutes, rinse well in water, stain in a saturated solution of picric acid in methylated spirit for five to ten minutes, rinse well in water, place in 50 per cent. alcohol for ten minutes, pass through absolute alcohol and xylol.

(*d*) Good preparations may be obtained by staining in Ehrlich's hæmatoxylin and counter-staining with orange rubin. This may also show the clubs when they are unstained by Gram's method.

Madura Disease or Mycetoma

Madura disease, otherwise known as madura foot, mycetoma, or the "fungus disease of India," is a chronic local affection generally attacking the foot, occasionally the hand, sometimes extending up the leg, but rarely to the trunk. The disease was originally described in India, where it is fairly common in certain districts,

but similar affections (due to a variety of organisms) are met with in other parts of Asia, in Europe, Africa and America. A "madura" foot appears enlarged, and numerous sinuses with raised mammilated apertures open on the surface (Fig. 53). On making a section into the diseased tissues the bones are found to be more or less carious, while the soft structures are tough and hypertrophied from the occurrence of chronic inflammatory changes. Numerous small cavities are present, sometimes filled by yellowish granules resembling fish-roe, and hence termed "roe-like particles," at others containing black particles of irregular shape, coal-like consistence, and variable size, exceptionally as large as a marble or walnut. The presence of the white or black granules, which may be discharged from the sinuses before mentioned, divides the disease into two classes—the so-called white and black varieties. Lewis and Cunningham also described a third variety, in which the granules are red like cayenne pepper.

Vandyke Carter [1] first called attention to the similarity between the white variety and actinomycosis in their microscopical characters. In sections stained by Gram's method more or less crescentic or reniform bodies are noticeable, divided into wedge-shaped areas, which contain masses of fine filaments stained purple. Surrounding the crescentic bodies is a zone of radially arranged elements, many of which are fan-shaped owing to branching; they are indistinct, as they do not stain with the gentian violet, but they are very suggestive of the club-shaped structures present in actinomycosis, and they resemble the *Actinomycosis hominis* inasmuch as they do not stain by Gram's method (Plate XXI, *b*). By staining with hæmatoxylin and orange rubin, or with the Ehrlich-Biondi triple stain, here and there in the radial zone well-defined clubs can be demonstrated. It seems, therefore, that the radial zone is composed of degenerate club-shaped structures, and the disease evidently closely resembles actinomycosis.

From a case of the white variety Boyce [2] cultivated a streptothrix which differed somewhat from the human and bovine *Actinomyces*, as it grew slower, produced no dark pigment, and on agar formed white raised colonies with radial grooves, not

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[1] *Bombay Med. and Phys. Soc.*, vol. ix, 1886 (new series), p. 86, Also Hewlett, *Trans. Path. Soc. Lond.*, vol. xlii, 1893.

[2] *Hygienische Rundschau*, 1894, No. 12.

unlike the tiny barnacles found on wooden piles in the sea. Vincent [1] also isolated a streptothrix, perhaps identical with that of Boyce, which differed from the Actinomyces in growing feebly in broth, in not liquefying gelatin, and in not being inoculable in the rabbit. He describes it as forming on glycerin agar umbilicated colonies, first white and afterwards red. Shattock [2] suggests that the red, cayenne-pepper-like grains occasionally met with in mycetoma may be due to colonies of the streptothrix which have produced their pigment. Microscopically, this organism (*Nocardia indica*, *Streptothrix* or *N. maduræ*, *Oospora*

FIG. 53.—A foot affected with madura disease. (White variety.)

indica), is identical with the Actinomyces. Musgrave and Clegg in a case of the white variety isolated a streptothrix (*S. freeri*) differing from the *N. indica*, but identical with the *S. Eppingeri* (*Nocardia asteroides*).

The relation of the black to the white variety of madura disease has been debated. Kanthack [3] described the black variety as being probably a late stage of the white. It seems, however, that the co-existence of the two conditions in the same specimen is very rare, and Boyce and Surveyor,[4] after a critical examination of a large number of specimens, came to the con-

[1] *Ann. de l'Inst. Pasteur*, 1893.
[2] *Trans. Path. Soc. Lond.*, vol. xlix, 1898, p. 294.
[3] *Journ. Path. and Bact.*, 1892.
[4] *Proc. Roy. Soc. Lond.*, 1893, and *Phil. Trans. Roy. Soc. Lond.*

clusion that the black variety is a distinct disease, and due to an organism belonging to the group of the higher fungi, the black particles or masses being the lignified mycelium or "sclerotium" such as is met with in ergot.

By planting out the granules from an early case of the black variety Wright succeeded in cultivating a hyphomycete.[1] It formed long branching hyphæ, but no spore-bearing organs were produced, and inoculation experiments on animals were negative. It grew on potato as a dense, widely spreading, coherent, velvety membrane, in colour pale brown with white periphery. Small drops of brown, coffee-coloured fluid appeared on the surface, and the potato became brown throughout. On agar the growth formed a meshwork of widely spreading greyish filaments; in old cultures (also in potato infusion) black hard granules, or "sclerotia," were observed. In broth little balls of radiating filaments developed.

According to the classification here adopted, the classical white variety of mycetoma (Madura disease) is, therefore an Actinomycosis; while the classical black variety is a Maduromycosis. The common European, Asian and African forms of the latter are caused by *Madurella*[2] *mycetomi* which grows well aërobically at 37° C., forming when young a greyish-white mycelium which darkens when older and stains the medium. The hyphæ are 2–8 μ, the spores 2–5 μ, in diameter. Black sclerotia 0·5–1·0 mm. in diameter may be formed in the depths of the culture medium. It is non-pathogenic to animals. But *Madurella mycetomi* is by no means the only organism causing Maduramycosis. Bouffard found an Aspergillus (*A. bouffardi*) in an African black Maduramycosis, Pepere in a similar condition in Sardinia *Monosporium sclerotiale*, Nicolle and Pinoy in Tunis, *Madurella tozeuri* and Chalmers and Archibald in the Soudan, *Glenospora khartoumensis*. There are also *white* Maduramycoses (i.e. the grains are white), caused by fungi, placed by

[1] *Journ. Exp. Med.*, vol. iii, 1898, p. 421.

[2] The genus *Madurella* (Brumpt) contains fungi belonging to the *Fungi Imperfecti*. They are mucedine with white thallus, living parasitically in animal tissues, possessing during vegetative life filaments with a diameter greater than 1 μ. The filaments are septate and branch from time to time and secrete a brown substance. When old, sclerotia are formed, which may become brown, and in which rounded corpuscles 8–30 μ in diameter are present. These are chlamydospores.

Brumpt in the genus *Indiella*, e.g., *Indiella mansoni* in an Indian form and *Indiella somaliensis* in India and Somaliland.

It is difficult experimentally to reproduce mycetoma in animals, but Pinoy has succeeded in doing so with an *Aspergillus*, and Nicolle with *Madurella tozeuri*, both in pigeons.

Mycosis tonsillaris (Mycosis pharyngis leptothricia)

A chronic disease attacking young adults, resistant to treatment, and characterised by the presence of small, white, tough, adherent excrescences on the mucous membrane of the pharynx. Microscopically, the patches consist of collections of epithelial cells and *débris*, infiltrated with leptothrix filaments and bacteria. The disease, however, seems to be a keratosis, infection with the organisms being secondary.

But occasionally a true "mycosis" apparently occurs, readily amenable to treatment, and due to a leptothrix.[1]

Leptothrix buccalis

Four somewhat similar thread forms occur in the mouth, viz. *Leptothrix racemosa*, *L. buccalis maxima*, *L. innominata*, and *Bacillus maximus buccalis*. The first is very common, forms large threads, shows a peculiar beaded appearance on staining which has been regarded as sporulation, and may be a fungus form. *L. buccalis maxima and L. innominata* differ from each other in that the former gives a blue granulose reaction when treated with iodine and dilute sulphuric acid, while the latter does not. All these three organisms are very similar, and the filaments are either unsegmented, or the segments are of considerable length. The *B. maximus buccalis* is very like the *L. buccalis maxima*, but does not give the granulose reaction, and its segments are shorter. It is motile, flagellated, and sporing, and stains by Gram's method.

Some confusion exists respecting the thread forms of the mouth.[2]

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[1] See *Glasgow Medical Journal*, No. 2, 1896, p. 81 *et seq.* (Brown Kelly).

[2] See Goadby, *Mycology of the Mouth*,

Cladothrix dichotoma

An organism not unfrequently met with in natural waters. It forms long threads, straight, or sometimes slightly undulating, or even spiral and apparently branched, though the branching is not dichotomous. It can be cultivated on the ordinary laboratory media at room temperature, forming on agar a brownish, wrinkled, tough, membranous layer, very adherent, and staining the medium beneath it a pale brown, not unlike the Actinomyces in these respects. It is non-pathogenic.

CHAPTER XVI

THE SACCHAROMYCETACEÆ

The Pathogenic Blastomycetes—Yeasts and Fermentation

The Yeasts

The Saccharomycetaceæ or Yeasts are characterised by a vegetative reproduction by budding or gemmation. If a cell of ordinary brewer's yeast be watched under conditions favourable to growth and reproduction, it will be found that a slight protuberance makes its appearance at one pole of the organism; this increases in size, and ultimately a daughter-cell resembling the parent is reproduced and separates off.

The true yeasts also reproduce by spore-formation by ascospores (p. 535); in some there is a fusion of cells before sporulation, in others the first cell formed by germination of the spore undergoes *fission*, forming what is known as a pro-mycelium, after which the cells multiply by gemmation. The Saccharomycetaceæ may therefore be divided into:

1. Zygosaccharomyces, in which pairs of cells fuse before sporulation.

2. Saccharomyces, in which there is no fusion of cells before sporulation, and in which the spores germinate by ordinary budding.

3. Saccharomycoides, in which the spores germinate by means of a promycelium.

Besides the true yeasts, there are a number of budding forms known which do not spore. These have been termed "Torulæ" (any yeast-like cell is frequently called a "torula"). Some form films on saccharine liquids and are known as Mycoderma. Organisms are also known having a yeast-like form and multiple spores but multiplying by fission; these have been termed *Schizosaccha-*

romyces. The position of these forms is uncertain and they are classed by the botanist among the Fungi Imperfecti (p. 540).

In addition to reproduction by gemmation, the Saccharomycetaceæ are also distinguished from the Bacteria by their larger size, by having a cellulose cell-wall, and in those forms in which endospores occur by the spores being multiple and not single in each cell. From the Hyphomycetes, or moulds, the Saccharomycetaceæ are distinguished by being unicellular, and by the reproduction being generally asexual. The Saccharomycetaceæ, however, are probably much more nearly allied to the Hyphomycetes than are the Bacteria, for many of the moulds have a stage in which the mycelium (see next chapter) resembles an aggregation of yeast-cells, and the yeasts in old cultures form films in which the cells become much elongated, like those in the mycelium of a mould. Jörgensen and others have attempted to show that some of the yeasts are stages in the development of a fungus, but it cannot be said that this has yet been satisfactorily demonstrated.

Pathogenic Yeasts and Blastomycosis [1]

Organisms apparently belonging to the Saccharomycetaceæ and termed *Blastomycetes* have been isolated from certain tumours, and have been regarded as having an etiological significance in connection with malignant disease. Sanfelice cultivated yeast forms from fermenting fruits, which, on inoculation into guinea-pigs, produced death in about a month with the formation of a tumour at the seat of inoculation and embolic growths in the spleen and liver. He also obtained a similar yeast from an ox affected with carcinoma, which on subcutaneous inoculation killed guinea-pigs in about two months, and inoculated into the peritoneum in a month, with multiple embolic growths in the lungs, spleen and mesenteric glands. A good deal of calcification was present in the growths, from which fact Sanfelice named this yeast *Saccharomyces litogenes*. Rabinowitch and also Foulerton [2]

[1] See Le Count and Myers, *Journ. of Infectious Diseases*, vol. iv, 1907, p. 187.

[2] *Journ. Path. and Bact.*, vol. vi, 1899, p. 37.

have found that some of the ordinary yeasts give rise to tumour formation on inoculation, especially in the rabbit. These tumours produced by yeasts are probably granulomata and not true malignant tumours.

Curtis [1] obtained a yeast from an apparently myxomatous tumour in a young man. The organism was met with in two forms—free and encapsuled. The free form appeared in young agar cultures as round or ovoid cells measuring 3 to 6 μ in diameter, often showing budding. The encapsuled form was met with in the original tumour and in the tissues of inoculated animals, and occurred as a large sphere 16 to 20 μ in diameter, enclosing the yeast cell, the capsule being hyaline and 4 to 6 μ in thickness. On agar at 37° C. the organism formed whitish, opaque, creamy colonies in two to three days, becoming a thick creamy growth at the end of a week, on gelatin white colonies or growth in four to five days without liquefaction, and in broth a flocculent deposit, the broth remaining clear. It was aërobic, did not grow on serum, and formed a small quantity of acetic acid and alcohol when grown in beerwort and sugar solutions. It was not pathogenic for guinea-pigs, but inoculated into rabbits, rats, mice, and dogs it produced tumours and caused death. The tumours to the naked eye appeared to be myxo-sarcomata, and in them the yeasts were found.

Bousse also obtained a pathogenic yeast from a young woman who suffered from a tumour of the tibia, and ultimately died with diffused growths in the bones and organs. The yeast-like cells were observed in the affected parts, and were isolated by cultivation, and the cultures, inoculated into mice and rabbits, produced death with growths in the organs. As in Curtis's case, the cells in the tissues appeared to be encapsuled.

Gilchrist described a case of blastomycetic dermatitis.

[1] *Ann. de l'Inst. Pasteur*, x, 1896, p. 449 (Refs.).

Small miliary abscesses were present in the rete and corium, in the pus of which the parasitic cells were observed. These were usually in pairs of unequal size, the largest measuring about 16 μ, surrounded by a well-defined capsule, and containing a granular protoplasm in which a vacuole was present. Clinically, the case had been regarded as one of scrofuloderma, but no tubercle bacilli could be found.

Numerous cases of blastomycetic dermatitis have now been recognised, and several instances of general systemic blastomycetic infection have been recorded.

Granulomatous tumours occurring in epidemics among horses in Japan, France, and Italy are also caused by Blastomycetes.

Stoddard and Cutler [1] have critically examined the condition known as Blastomycosis. They consider that this is caused by three classes of organisms, viz. *Oidia*, *Torulæ*, and *Coccidioides*. The last named (not to be confounded with *Coccidium*) is an ascomycetous fungus, *C. immitis*. They give the following, among others, as points of distinction between the three :—

	Torula Infection.	Oidiomycosis (Blastomycosis).	Coccidioidal Granuloma.
Clinical Course	A chronic disease of the nervous system.	A chronic skin disease or general infection.	A chronic skin disease or general infection.
Cells of Parasite	1–13μ. Reproduction by budding. Buds may form daughter-buds.	3–4μ–20μ. Reproduction by budding. The bud grows to size of parent before again budding.	5–85μ. Never buds. Produces ascospores.
Pathogenicity	Marked for mice and rats.	Slight or absent for all animals.	Marked for all animals.
Culture . .	No mycelium.	Mycelium sooner or later.	Mycelium and aërial hyhae.

[1] *Studies from the Rockefeller Institute* (Reprints), vol. xxv, 1916, p. 1.

Clinical Examination (Pathogenic Yeasts, etc.)

The cells can be well seen in the fresh state in the teased-up tissues mounted in water or glycerin.

Curtis recommends staining in carbol-thionine blue, and for sections, picro-carmine.

Busse's method for sections is as follows :

1. Hæmatoxylin solution for fifteen minutes.
2. Wash in distilled water.
3. Counter-stain in weak carbol-fuchsin (1 : 20) for thirty minutes to twenty-four hours.
4. Decolorise in 95 per cent. alcohol for fifteen seconds to one minute.
5. Absolute alcohol, xylol, mount in Canada balsam.

Gilchrist recommends treating the sections with 10 per cent. caustic potash solution and examining in 50 per cent. glycerin without staining.

Brayton recommends that small pièces of the tissues should be excised from the growing margin, treated with ether for two to five minutes, macerated in 20 to 30 per cent. caustic potash solution for five to ten minutes, and then examined without staining. Cultures may be readily obtained, with a little care, preferably on beer-wort gelatin or maltose agar.

Fermentation

The yeasts are of great importance in inducing many chemical changes, especially alcoholic fermentation, beer and wine being almost exclusively due to their activity.

Taking brewer's yeast, *Saccharomyces cerevisiæ*, as a type, the yeast cell is observed to be slightly ovoid in shape, measuring 8 to 9 μ in diameter. The protoplasm is granular, contains one or more clear spaces or vacuoles, frequently bright, refractile globules of fatty matter, and is surrounded by a cell wall of cellulose. It has been repeatedly stated that a nucleus is present, but this is doubtful. When the yeast-cell is freely supplied with nutriment, reproduction by gemmation proceeds rapidly, and a whole string of cells may form owing to the daughter-cells budding again before they have separated from the parent. When the cell is starved, gemmation ceases, fat-globules and vacuoles increase in number, and the cell may finally become little more

than a large vacuole, the protoplasm forming a thin coating over the inside of the cell wall. Within the vacuoles are often seen minute spherical bodies of a doubtful nature in rapid movement. In ordinary circumstances endospore formation does not occur, but by deprivation of nutriment, as by growing on a block of plaster-of-Paris, the cells develop spores. First the cell becomes divided by the development of membranes, the so-called "partition-wall formation," into several chambers in which the spores form. In the different yeasts the number and arrangement of the spores vary ; in the *S. cerevisiæ* the typical number is four, arranged close together, three on one plane and one resting on these, like a pyramid of billiard balls.

Although the reproduction of yeasts by gemmation or ascospore formation is usually asexual, ascospore formation is sometimes preceded by conjugation of sister-cells, or conjugation may occur between neighbouring cells at the moment of germination (Guilliermond, Nadson, and Marchand).

The spores are of considerable importance in the identification of species of Saccharomyces, as the form of the cells alone and the growths on culture media are not sufficiently distinctive. In fact so little can these two characters be relied upon that in order to isolate in pure cultivation it is necessary to grow from a single cell. This can be done by making a miniature plate cultivation with wort-gelatin on a large sterilised cover-glass, and, after the layer of gelatin has set, mounting, gelatin downwards, on a large cell on a glass slide. The cover-glass should be divided into small squares by cross-lines etched on the glass and numbered. The preparation is carefully examined with a $\frac{1}{6}$ or $\frac{1}{8}$ inch objective, and the positions of single isolated cells are noted. This is not a difficult matter on account of the comparatively large size of the yeast-cells, and their position is determined by the numbered squares on the cover-glass. The preparations are kept in a moist chamber in a warm place, and when visible colonies have developed, those which are derived from a single cell can be inoculated into tubes or flasks of a suitable culture medium.

It is found that the various yeasts form spores in different periods of time when grown under similar conditions, and on this fact is based what is known as the analysis of yeast—a most valuable method, which we owe to Hansen. The chief "diseases" of beers and yeast—*i.e.* abnormal fermentations giving rise to inferior products—are due to admixture of certain "wild yeasts," as they are termed, with the brewer's yeast, chiefly the *S. ellip-*

soideus and *S. pastorianus ;* and, in order to detect these "disease" species, the analysis consists in determining at what time ascospores appear. The mode of procedure is as follows :

The yeast is sown in a flask of sterile wort, and incubated at 25° C. for twenty-four hours. The yeast revives, and from the deposit of young cells two cultures are made on plaster-of-Paris blocks. These cultures are kept, one at 25° C., the other at 15° C., and are examined twice daily. In an uncontaminated brewing yeast ascospores should not be detected in less than thirty hours in the culture kept at 25° C., and seventy-two hours in that kept at 15° C. The plaster-of-Paris blocks are sterilised by careful flaming in the Bunsen, and are then placed in sterile glass capsules with lids, containing sufficient sterilised water thoroughly to moisten the whole of the blocks ; unless this is done no growth occurs. By this method of analysis as little "wild yeast" as one two-hundredth of the whole can be detected.

Besides the distinct species of yeasts, there are also a number of varieties employed in brewing, etc., differing but slightly in morphological and cultural characters, yet giving rise to varied products. These varieties may be divided into two groups—the surface, high or top, and the sedimentary, low or bottom, fermentation forms. In this country beer is brewed by fermenting an infusion of malt ("wort") with yeast, which, during fermentation, *rises to the surface*, and belongs to the first group ; while the German beers are obtained by yeast, which *sinks to the bottom*, and belongs to the second group. The floating of the yeast in the high fermentation process seems to be due to the attachment of minute bubbles of carbonic acid gas to the cells, and it has not yet been possible to convert the one form into the other.

Characters of some of the more important yeasts.—Hansen divides the important yeasts into groups having the same general characters, and distinguishes the varieties in each by Roman numerals (I, II, etc.).

Cerevisiæ Group.—These are the yeasts producing the normal fermentations resulting in beer, etc. They are round or slightly ovoid cells, and four ascospores are produced. In old cultures long sausage-shaped or even filamentous cells may be met with.

S. cerevisiæ I and II.—These are bottom fermentation forms in use at the Old Carlsberg Brewery ; the cells of No. II are rounder and slightly larger than those of No. I, and ascospore formation is more abundant.

There is also a top fermentation form described by Hansen (*S. cerevisiæ I* top), which is the yeast employed in the breweries of London and Edinburgh.

The yeasts of the *cerevisiæ* group can invert cane sugar, select dextrose from lævulose, and ferment maltose, but they cannot ferment lactose, nor decompose malto-dextrin.

Pastorianus Group.—These are wild yeasts. The cells are elongated or sausage-shaped, and six or eight ascospores are produced in a cell.

S. pastorianus I.—A bottom fermentation yeast producing a bitter taste in beer.

S. pastorianus II.—A feeble top fermentation form. Surface cultures on yeast-water gelatin have smooth edges, which distinguishes it from the next species.

S. pastorianus III.—A top fermentation form producing turbidity in beer. Surface cultures on yeast-water gelatin have woolly margins.

Ellipsoideus Group.—These are wild yeasts. The cells are usually ovoid, or pear-shaped, sometimes round, rarely elongated.

Five or six ascospores are produced in a cell.

S. ellipsoideus I.—A bottom fermentation yeast occurring on ripe grapes.

S. ellipsoideus II.—A bottom fermentation yeast causing turbidity in beer.

Both the *pastorianus* and *ellipsoideus* groups resemble the *cerevisiæ* group in their chemical actions, but they are able in addition to decompose malto-dextrin.

S. anomalus is a yeast forming small ovoid cells. It is curious in that the spores are hemispheres with a projecting rim at the base like a bowler hat.

Another point in the identification of species of yeasts is the period of formation of films. If the yeast is grown in wort with free access of air and is undisturbed, *e.g.* in a beaker capped with filter-paper, after a varying period a film composed of a zoogloeal mass of cells appears on the surface.

If yeast, or disintegrated yeast-cells, be injected into animals, the blood acquires specific agglutinative properties, agglutinating the yeast-cells of the species with which the inoculation has been carried out.[1]

On the yeasts of fermentation, see Jörgensen, *Micro-organisms*

[1] See Macfadyen, *Centr. f. Bakt.* (1te Abt.), xxx, 1901, p. 368.

and Fermentation 4th ed., 1911 (C. Griffin and Co.), (full bibliog.); Klöcker, *Fermentation Organisms.*

Examination of Yeasts

The yeasts can be readily examined in the fresh state in hanging-drop preparations. The cells should be young or they will not be of the typical form; a two or three days' old culture in wort or grape-sugar solution may be used. The yeasts grow well at 20°–30° C. on the ordinary gelatin, agar, and potato, but wort gelatin or wort agar is to be preferred. The elongated cells, common to all old cultures of yeasts, may be obtained from the films which form on wort cultures in wide flasks or beakers after two or three weeks.

In order to stain yeasts, a dilution of the culture should be made in a watch-glass of water, so that the cells may be isolated, as they become distorted if groups form in the preparations.

If the yeast has been grown in wort, it is best, before staining, to pour off the fluid from the deposit of cells at the bottom of the flask or test-tube, add some physiological salt solution and shake, then allow the vessel to stand for an hour for the cells to sediment, or centrifuge, and the process of washing may be repeated once. Films may be prepared in the ordinary way and stained for five minutes in Löffler's methylene blue, washed in water, dried, and mounted. Or the films, after air-drying, may be fixed by immersion in equal parts of alcohol and ether for ten minutes, dried in the air, and stained as before. The preparations can also be stained in gentian violet or fuchsin, or by Gram's method.

Ascospores may be double stained by preparing films of a sporing culture in the ordinary way, staining with carbol-fuchsin for two minutes, rinsing in water, decolorising with 5 per cent. sulphuric acid and with alcohol, rinsing in water, counter-staining with Löffler's blue for five minutes, washing, drying, and mounting. The spores are red, the remainder of the cells blue.

CHAPTER XVII

THE HYPHOMYCETES—ASPERGILLOSIS—RINGWORM

The Hyphomycetes

THE moulds are, for convenience, collectively termed the *Hyphomycetes*, but this is not a strict botanical group. They are Fungi having as a common character a plant body made up of hyphæ. They are multicellular individuals, composed of filaments, simple or branched, jointed or unjointed, which are termed *hyphæ*, and are formed by the end-to-end union of elongated cells. When the hyphæ project upwards into the air they are known as aërial hyphæ, and when downwards into the fluid or medium on which the organism is growing as submerged hyphæ, and the compact tufts or masses resulting from interlacing hyphæ are termed mycelia. A mycelium may form a hard lignified mass or pseudo-parenchyma, which is known as a sclerotium, such as is met with in ergot and in the black variety of mycetoma.

Any piece of the mycelium will grow, but in addition moulds reproduce by multiple spores, which may be asexual or sexual. Practically all moulds produce asexually formed spores; some produce sexually formed spores by the fusion of two cells or gametes. The two principal sexually formed spores are *zygospores* and *ascospores*. Zygospores occur in *Mucor* (see p. 541). In ascospore formation, after conjugation of the gametes, instead of immediately developing into a spore, the fertilised cell grows into a mass of branching hyphæ, some of the cells of which produce spore sacs or *asci*, each of which contains two or more *ascospores* (see *Penicillium*, p. 542).

Asexual spores are either free, borne at the ends or sides of hyphæ—*conidia*—as in *Penicillium*, or are formed in specialised spore cases—*sporangia*—as in *Mucor*.

Usually the spore-bearing hyphæ are specially differentiated, and one bearing conidia is known as a *conidiophore*, one bearing a

sporangium as a *sporangiophore*. Some moulds produce spores by segmentation of hyphæ, these conidia being known as *oïdia*.

The Fungi are divided into the Phycomycetes, Ascomycetes, Basidiomycetes, and Fungi Imperfecti. The Phycomycetes are distinguished by non-septate or slightly septate hyphæ and zygospore-formation, as in the Mucors. The Ascomycetes are characterised by the development of the cell resulting from fertilisation into cells, some of which become spore sacs or *asci* containing several spores. Asexual spores are usually produced as well. The Basidiomycetes have spore-bearing structures known as *basidia ;* the rusts, smuts, toadstools, puff-balls, and mushrooms belong to this group. All fungi which do not fall into one of these three groups are placed among the *Fungi Imperfecti*, most of them probably belong to the Ascomycetes. *Mucor mucedo*, *Penicillium glaucum*, and *Aspergillus niger* may be taken as types and more fully described.

Mucor mucedo

The *Mucoraciæ* belong to the Phycomycetes, and are divided into some eighteen genera.

Mucor mucedo, the common white mould which appears like tufts of cotton-wool on various substances, may be obtained by exposing some moistened bread or horse-dung to the air for a short time, and then keeping it moist under a bell-jar. It consists of a mycelium composed of hyphæ, and its fluffy appearance is caused by aërial hyphæ. The aerial hyphæ are at first of even diameter throughout, but later on their free ends become swollen and ultimately form spherical bodies, which become filled with spores, the sporangia. In the early stage the whole organism forms but a single cell, the protoplasm of which is granular and contains vacuoles and numerous small nuclei. As it grows, and the sporangia form, these become separated by a septum from the hyphæ, and when it becomes older still the mycelial hyphæ may be divided into elongated cells. The development of a sporangium takes place as follows : The distal end of an aërial hypha swells, and immediately below the swollen part a division occurs in the protoplasm and a cellulose septum is formed, so that the swollen part is separated off from the rest of the hypha, forming the rudimentary sporangium. The sporangium continues to grow, and its protoplasm undergoes multiple fission into numerous ovoid masses, the spores, each of which becomes surrounded with a

cellulose capsule. The septum separating the sporangium from the hypha projects upwards into the interior of the sporangium as a club-shaped knob known as the columella. When the sporangium is ripe the slightest touch causes its wall to rupture, so liberating the spores. When placed under favourable conditions the spore germinates, and the buds increase in length and ultimately form hyphæ.

Occasionally a process of conjugation occurs. Two adjacent hyphæ send out lateral branches which come in contact with one another, and a septum forms in each, separating a small portion of protoplasm from the rest of the hypha. The apposed walls of the two cells become absorbed and the contents mingle. The mass of protoplasm so formed becomes surrounded with a thick cell-wall, giving rise to an inactive spore-like body, the zygospore, which under favourable conditions develops like an ordinary spore. Some Mucors form thick-walled resting cells, known as *chlamydospores*, in the vegetative mycelium. These are storehouses of reserve material which is ultimately expended in the production of shoots.

Certain *Mucors* form appreciable amounts of alcohol from carbohydrates, and *M. rouxii* has been used for the commercial production of alcohol.

Penicillium glaucum

Penicillium belongs to the Ascomycetes, and bears conidiophores. *Penicillium glaucum* forms the bluish-green mouldy patches familiar to every one. It is by far the commonest of all species, and may be obtained from moist bread or jam or by exposing a gelatin plate to the air for a short time. If the mouldy patch be rubbed a fine greenish dust comes away. This dust consists of myriads of spores ; if a little of it be transferred with a moistened needle to a gelatin plate, or, better still, to a hanging-drop preparation, the growth of the organism can be studied. After two or three days little *white* specks will be observed, which microscopically are found to consist of tufts of delicate interlacing hyphæ ; these, becoming interwoven, ultimately form a tough mycelium. The patches of growth are circular, and the hyphæ will be found to radiate from the centre. As the patch increases in size it changes in colour, becoming bluish-green, though the margin for some time still remains white. From the upper surface of the mycelium delicate aerial hyphæ

grow upwards, and from the under surface short submerged ones project downwards.

The hyphæ are composed of elongated cells arranged end to end, the cell-walls of which consist of cellulose enclosing a more or less vacuolated protoplasm containing several nuclei.

The aërial hyphæ are unbranched filaments, but as development proceeds the distal ends branch dichotomously, the branches remaining short and nearly parallel to one another, so that a kind of brush is produced. The ultimate branches are known as sterigmata. The ends of the sterigmata become constricted so that little globular masses, the spores, are formed ; this process is repeated until a chain of spores results, *the proximal one* being *the youngest.* A spore when placed under favourable conditions germinates, a little bud appearing, elongating, and forming a hypha, just as in *Mucor.*

Brefeld, by sowing spores on moist bread, inverting the bread, and examining at intervals, observed a sexual method of reproduction in *Penicillium.* Two sets of spiral cells develop on a thick hypha, they intertwine, their contents probably mingle, and from the union or carpogonium a tube-like hypha develops, which becomes surrounded and enclosed by branching hyphæ from the mother cell. By further development and thickening of the cell-walls a sclerotium forms ; it is a hard solid body, yellowish in colour, and resembles a grain of sand, the carpogonium being at the centre. If placed in favourable conditions the sclerotia germinate after some time. Two forms of hyphæ are produced, one thick, the other thin ; the latter become much twisted. The thick hyphæ become branched, and ultimately a number of pear-shaped bodies are produced. The contents of these bodies then become broken up and form spores ; the bodies are known as asci and the spores as ascospores. From the ascospores the ordinary mycelial form again develops.[1]

Aspergillus niger

Aspergillus also belongs to the Ascomycetes, and representatives of this genus are common on damp and decaying vegetable matter. The asci occur as golden-yellow bodies in the mycelium. It forms conidiophores which are unbranched and are swollen at the tip. Short unbranched stalks (sterigmata) grow on this

[1] See Brefeld, *Quart. Journ. Microscop. Soc.*, vol. xv, p. 342.

swelling and on the tips of these the spores develop. A process of sexual reproduction occurs very like the one observed in *Penicillium*. *Aspergillus niger* grows well on the ordinary laboratory media, producing on potato a powdery, sooty growth after a time. *Aspergillus glaucus* is a common green-spored species.

With the exception of the ringworm and allied fungi, which produce parasitic skin affections, the Hyphomycetes are not of very great pathological importance. In the ear and nose mucors and aspergilli may be met with, but in these situations they are epiphytes rather than parasites, and the same species occur in bronchiectases and pulmonary vomicæ. Occasionally, however, a pneumono-mycosis has been met with, the mycelium of the fungus ramifying in the lung tissue and setting up irritative and other changes. "Pneumono-mycosis" or "pulmonary aspergillosis" is especially a trade disease among bird-rearers. Grain is taken into the mouth and the bird is fed with it, and in the course of this operation the mould spores are inhaled. The course of the disease is much like chronic bronchitis or pulmonary tuberculosis. The species met with in this condition seems generally to have been the *Aspergillus fumigatus*.

The Maduramycoses, as already stated (p. 517), are due to various fungi, one of them to an *Aspergillus* (p. 527)

Sporotrichosis [1]

A rare disease clinically resembling syphilis or tuberculosis, characterised by indurated granulomata like gummata, which subsequently break down, suppurate and ulcerate. Potassium iodide has a curative action on the condition.

In the pus of the lesions large ovoid refractile bodies

[1] See Walker and Ritchie, *Brit. Med. Journ.*, 1911, vol. ii, p. 1; Gougerot, *Journ. of State Med.*, xxi, 1913, p. 614 *et seq.*

suggestive of yeasts or of large spores may be detected, but no mycelium.

Cultures are best obtained on maltose agar, from non-ulcerated lesions ; agar and potato may also yield growths. The organism (*Sporotrichon Beaumanni*) grows as small raised woolly colonies, at first white, afterwards becoming brown. The growths consist of a felted mycelium of filaments with spores and yeast-like cells. It produces granulomata in inoculated mice. The botanical position of the organism is uncertain ; by some it is regarded as a true fungus. It is stated to occur on decaying vegetable matter, and to be the cause of epizootic lymphangitis in the horse—a disease having a superficial resemblance to farcy—in the pus of which oat-shaped bodies are found, the " cryptococcus " of Rivolta.

Accladiosis is an ulcerating dermatomycosis occurring in Ceylon and elsewhere in the East, and somewhat resembling sporotrichosis. The fungus was isolated by Castellani [1] and is named *Accladium castellanii*.

Thrush

Thrush is due to an organism (*Oïdium* or *Monilia albicans*) which is usually classed among the Hyphomycetes. There are probably several species distinguished by their fermentation reactions (Castellani). It forms the whitish patches so frequently seen on the mucous membrane of the mouth and pharynx in children and in those suffering from wasting diseases, it also causes bronchitis and a general infection has occasionally been produced by it. If one of these patches is removed and teased up, it will be found to consist of masses of tangled mycelial threads with yeast-like budding. The organism can be readily cultivated on all the ordinary laboratory media, and will also

[1] See *Proc. Roy. Soc. Med.* (Dermatolog. Sect.) 1917.

grow on slightly acid media such as wort gelatin. It produces whitish, membranous, adherent growths, in which it appears morphologically under two forms—as masses of tangled filaments or hyphæ and as yeast-like cells. On acid media the latter exclusively occur, on alkaline the former predominate. It liquefies gelatin and serum, stains by Gram's method, produces an alkaline reaction by the formation of ammonium carbonate, and does not ferment lactose. It forms acid and curd in milk, and acid and gas in glucose. Inoculated on to a damaged mucous membrane the " thrush " patches appear, subcutaneously it produces an abscess, and injected into the peritoneum a general infection, followed by death and accompanied by a seropurulent peritonitis.

Cultivation and Examination

The Hyphomycetes can be cultivated on the ordinary laboratory media, but wort-agar, or wort-gelatin, potato, bread, or maltose agar is to be preferred.

They can be examined by removing a portion of the growth, teasing up gently with needles in a little 50 per cent. alcohol containing a trace of ammonia, removing the surplus fluid with blotting-paper, and mounting in Farrant's solution or in glycerine jelly. If desired, they may be stained by the irrigation method with fuchsin. Thrush may be examined in this way.

In the tissues they may be stained with hæmatoxylin or methylene blue, or by Gram's or by Weigert's method.

Ringworm

The ringworm fungi must probably be included in the group of the Hyphomycetes. Human ringworm, formerly regarded as a single disease, has been proved to comprise at least two affections through the researches of Sabouraud. These two forms are distinguished from each other clinically and by differences in the parasitic organisms.

The first variety is an affection of early childhood,

forming 80 to 90 per cent. of the ringworms met with in London ; it never attacks the scalp of adults, never affects the beard or nails, is very intractable, and frequently epidemic. The parasite is characterised by small round or ovoid spores measuring 3 μ to 4 μ in diameter. Affected hairs are generally broken off, forming relatively long stumps, greyish in colour, and possessing a whitish sheath. When suitably prepared in potash this sheath is seen to be composed of the spores agglomerated together without apparent order, and the hairs themselves are filled with delicate parallel mycelial threads (Fig. 54). The fungus is named the *Microsporon Audouini*.

The second variety comprises the ringworms with large spores, and is divided into two groups by Sabouraud. The first of these groups is exclusively of human origin, and has a marked tendency to affect the interior of the hairs only, and hence the organism has been termed the *Trichophyton megalosporon endothrix*. The other group is of animal origin, and the spores are met with chiefly on the outside of the hairs, and the fungus is hence termed the *Trichophyton megalosporon ectothrix*.

The *endothrix* form occurs later in childhood, is not so persistent as the *Microsporon*, and does not attack the nails or beard. Microscopically, the fungus is seen to consist of beaded threads, which are rounded or ovoid spores arranged end to end. The *ectothrix* form rarely attacks the scalp, but is responsible for all the tinea sycosis and ringworm of the nails and half the cases of tinea circinata. Suppuration is common in this form. Microscopically appearances differ ; generally the spores are arranged in chains, but the sporulation is less regular than in the *endothrix*. The spores in the *endothrix* and *ectothrix* varieties measure 4 μ to 12 μ in diameter.

The ringworm fungi can be readily cultivated on all the ordinary media—beer-wort agar and beer-wort gelatin

being especially favourable. They form whitish fluffy growths with rapid liquefaction of gelatin. In order to obtain cultivations the diseased hairs or stumps are removed by forceps and placed on a sterile glass slide. The aërial portion of the hair is then cut away by means of a sterile scalpel, and the diseased portion is divided into small fragments. These can be picked up with a moistened platinum needle and transferred to the culture media, preferably beer-wort agar. In some cases a pure culture

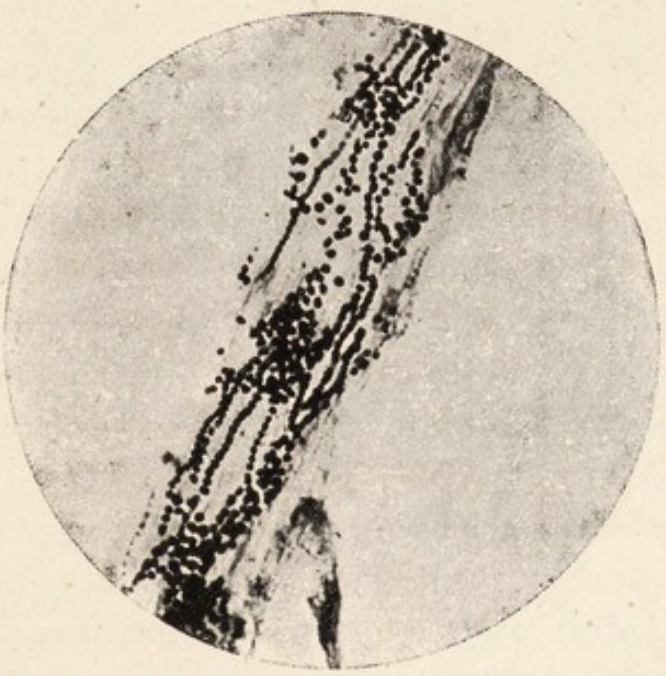


FIG. 54.—Ringworm in a hair (*Microsporon*) × 350.

is thus obtained, but in others further treatment is necessary. When the *Trichophyton* or *Microsporon* has thrown up its aërial hyphæ the plug of wool is removed from the tube and the mouth well flamed; the tube is then held inverted over a Petri dish containing solidified maltose agar. A sharp tap or two is given to the tube, sufficient to cause the spores to drop, and the dish is re-covered. A growth of the organism from single isolated spores thus ensues, and pure cultures can be obtained (Blaxall).

The various forms of the ringworm fungi can be differen-

tiated by cultures, but it is necessary when comparing them to employ media of identical composition, because slight differences in the latter are liable to induce marked changes in the characters of the cultures. A favourite medium, used by Sabouraud and by Blaxall, is maltose agar :

Peptone	0·5 grm.
Maltose	3·8 grm.
Agar-agar	1·3 grm.
Water	100 c.c.

Blaxall found that different samples of maltose materially influenced the characters of the cultures.

Characters of the cultures.—Cultures are incubated at 30° C. The colonies of the *Microsporon* do not show any growth until about the seventh day ; little white downy tufts then appear. The fully developed growth on maltose agar forms a large white downy patch with a small central boss ; on potato white downy patches appear with brown discoloration.

The *endothrix* variety commences to grow in six or seven days, and on maltose agar in about a month forms a rounded patch with a central crateriform depression, the whole being dusted with fine white powder (Fig. 55) ; on potato, powdery stars develop tinged with yellow and usually without discoloration of the medium.

The cultures of the *ectothrix* form are variable. They commence on the third or fourth day ; some develop whitish smooth or wrinkled growths ; others, from the dog, form dry, brown, wrinkled, powdery growths ; others, of bird origin, form purplish growths.

Microscopically, all the fungi show masses of mycelial threads with spores. They stain with the ordinary anilin dyes and also by Gram's method, and can be mounted in glycerin jelly in the manner described at p. 545.

Macfadyen found that the ringworm organism produces an active peptonising enzyme, and seems to increase the

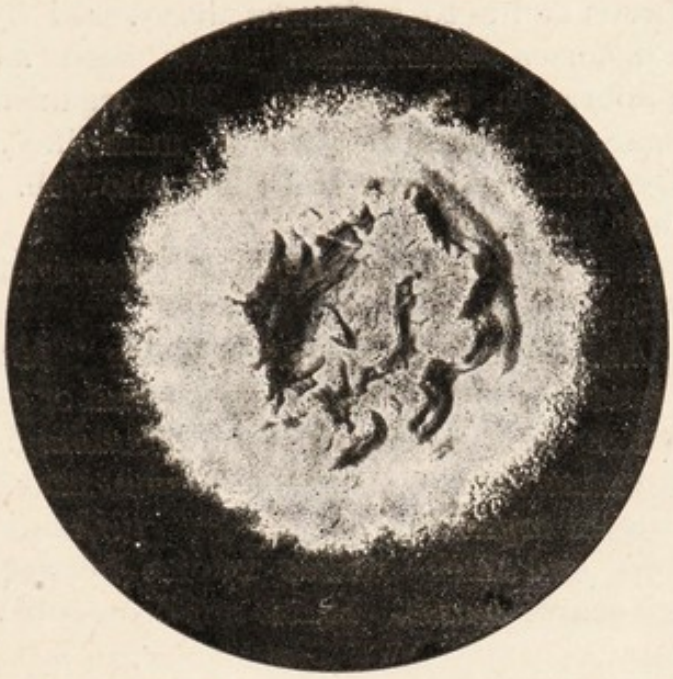


FIG. 55.—Culture of the ringworm organism. Endothrix form.

solubility of keratin when grown on it; no inverting enzyme could be isolated.

Clinical Examination

The hairs should be treated first with ether for not less than 15 minutes and then with caustic potash solution of about 7 per cent. strength. In this reagent they may remain for from half-an-hour to a few hours; they are then floated on to a slide and carefully covered with a cover-glass. Permanent preparations may be mounted in Farrant's solution or in glycerine jelly.

Hairs, after treatment with ether for half-an-hour, may be stained by the following method:

(1) Stain in anilin-gentian violet for one to two minutes, and blot.

(2) Treat with Gram's iodine solution for one to two minutes, and blot.

(3) Decolorise carefully (watching microscopically) with anilin oil containing 1 per cent. of hydrochloric acid.

(4) Treat with anilin oil and then with anilin oil and xylol.

(5) Clear in xylol, and mount in Canada balsam.

Erythrasma.—Due to infection with a fungus (*Microsporon minutissimum*), very difficult to cultivate, which occurs as extremely long, fine filaments.

Favus.—Favus is due to a fungus discovered by Schoenlein in 1839—the *Achorion Schoenleinii.* It is seen as a mycelial growth with spores in the patches. The organism grows well on maltose agar, forming fluffy, woolly, moss-like colonies with radiating outgrowths, first grey and then yellowish. It occurs on mice and other animals.

Dhobie itch.—Castellani has isolated three trichophyton-like organisms in this disease.

Pityriasis Alba.—In this disease Unna's "bottle bacillus" is invariably present. It occurs as large round or oval bodies like yeast-cells, which may occasionally show budding.

Pityriasis versicolor.—In the epidermal scales of this skin affection a fungoid organism (*Microsporon furfur*) is present. It occurs as short and thick curved hyphæ between which are masses of large coarse spores. It has not been cultivated (or very rarely).

Pinta.—A skin disease met with in South America. In the scales short mycelial filaments with large (8–12 μ) spores are seen. Various organisms have been cultivated belonging to the genera *Penicillium* and *Aspergillus.*

Piedra.—A disease of the hairs met with in South America. The nodosities on the hairs are composed of masses of very large refractile spores. The fungus is supposed to be a *Trichosporium*

CHAPTER XVIII

THE PROTOZOA [1]

The General Structure of the Protozoa—Pathogenic Amœbæ—Trypanosomata—Leishman-Donovan Body—Spirochaetæ—Syphilis—Coccidia—Malaria

THE Protozoa are an important group of unicellular organisms, regarded as animal in nature, and sharply and definitely distinguished from the rest of the animal kingdom, to which the names of Metazoa and Enterozoa are applied. The latter consists of many cells, differentiated to perform different functions, and arranged in two layers—endoderm and ectoderm—around a central cavity, the enteron.

"It is true that some protozoa consist of aggregates of cells, and should therefore be entitled to be called multicellular; yet an examination of the details of structure of these cell-aggregates and of their life-history establishes the fact that the cohesion of the cells in these instances is not an essential feature of the life of such multicellular protozoa, but a secondary and non-essential arrangement. Like the budded 'persons' forming, when coherent to each other, undifferentiated 'colonies' among the polyps and corals, the coherent cells of a compound protozoon can be separated from one another and live independently; their cohesion has no economic significance. Each cell is precisely the counterpart of its neighbour; there is no common life, no distribution of function among special groups of the associated cells, and no corresponding differentiation of structure. As a contrast to this, we find in the simplest enterozoa that the cells are functionally and structurally distinguishable into two

[1] See Lankester's *Treatise on Zoology*, Part I, first and second Fascicles, 1907 and 1909; Minchin in Clifford Allbutt's *System of Medicine*, ed. 2, vol. ii, pt. ii; Hartog in *Cambridge Natural History*, vol. i.

groups—those which line the enteron or digestive cavity, and those which form the outer body wall. The cells of these two layers are not interchangeable, but are fundamentally different in properties and structure" (Ray Lankester). In some instances there may be a difficulty in deciding whether an organism is vegetable or animal, and Haeckel proposed to include all indeterminate unicellular organisms in a distinct kingdom, the Protista.

The cytoplasm of a protozoon is commonly differentiated into an outer, clearer, denser layer or ectosarc, and an inner, granular, more fluid portion, the endosarc. The cytoplasm is sometimes naked, or may be covered with a cuticle, usually protein in nature. The cytoplasm contains a well-marked nucleus, sometimes a secondary nucleus and occasionally subsidiary chromatin particles or *chromidia*. A contractile vacuole, which is an excretory organ, is frequently present.

In most protozoa reproduction takes place by simple division or fission, and by a process of spore-formation; in others reproduction is exclusively by spores, which are often formed by a complicated process of development. In many of the protozoa a simple form of sexual reproduction by conjugation occurs. Two dissimilar cells (*gametes*) are produced, the larger comparable to female cells or ova and termed *macrogametes*, the smaller comparable to male elements or spermatozoa and termed *microgametes*. The cells from which the gametes are derived are known as *gametocytes*. The gametes conjugate and form a zygote, which usually divides into a number of spores from which the adult is reproduced.

In certain cases sexually differentiated individuals reproduce by fission without conjugation; this phenomenon is termed *parthenogenesis*.

Various classifications of the Protozoa have been suggested. Bütschli divides them into four classes: I. The Sarkodina (p. 552); II. The Mastigophora (p. 558); III. The Infusoria (p. 598); and IV. The Sporozoa (p. 599).

Class I.—Sarkodina

There are Protozoa in which the cell protoplasm is naked, and locomotion and ingestion of food are performed by means of *temporary* protoplasmic processes or pseudopodia.

The Sarkodina includes a number of forms of very varied mor-

phology and habits, such as the Amœbæ, Heliozoa, Radiolaria, and Foraminifera, the three latter groups being characterised by the presence of a siliceous or a calcareous skeleton or shell.

Pathogenic Amœbæ[1]

Three species of *Amœbæ* seem to be parasitic in man, and the generic name of *Entamœba* has been given to them. One, the *E. gingivalis*, occurs in the mouth in dental caries and pyorrhœa (see p. 657), the two others inhabit the intestine, the *Entamœba coli* (*Amœba coli*, Lösch), which appears to be harmless ; the other, the *Entamœba histolytica*, is the cause of amœbic or tropical dysentery.

The *Entamœba coli* inhabits the large intestine and cæcum. It varies in size from 10 to 30 μ, and is sluggishly amœboid even on a warm stage. The cytoplasm tends to be granular and vacuolated, and frequently encloses yeasts and bacteria, but never red blood corpuscles. A single large spherical nucleus is present which is generally clearly discernible. The cytoplasm is not differentiated into ectoplasm and endoplasm (Plate XXIII, *a* & *b*). The organism multiplies by binary fission and also by division into eight small amœbæ after encystment. The cyst is distinctly encapsuled, measures 15–20 μ in diameter, and contains when fully developed eight nuclei (Plate XXIII, *c*).

The *Entamœba histolytica* occurs in two forms in the bowel, a larger known as the *tetragena* form measuring 15 to 30 μ in diameter, and met with during the acute stage when the stool may consist of little else than blood and mucus, and a smaller or *minuta* form, measuring 10 to 20 μ in diameter. The *tetragena* form is also the tissue-invading form and is present in the ulcers and in the wall

[1] Councilman and Lafleur, *Johns Hopkins Hosp. Reps.*, vol. ii, 1891 ; Schaudinn, *A.K. Gesundheitsamte*, xix, p. 547 ; Strong, Musgrave, Clegg, Thomas and Woolley, Bureau of Gov. Laboratories, Manila, *Bulls.* 18 and 32 ; Wenyon, *Lancet*, 1915, vol. ii, p. 1173.

of the liver abscess, which is a frequent sequel of this form of dysentery (Plate XXIII, *d* & *e*). The *minuta* form replaces the *tetragena* form in the bowel as the acute symptoms abate and the stools return to normal.

The cytoplasm of *E. histolytica* is more refractile than that of *E. coli*, frequently encloses red blood corpuscles, and there is distinct differentiation into a clearer ectoplasm and more granular endoplasm. The pseudopodia consist entirely of the clearer ectoplasm and the parasite is fairly actively amœboid on a warm stage. A single spherical nucleus is present in the cytoplasm, but is indistinctly seen. The parasite multiplies by binary fission. It may be difficult to distinguish the amœbæ of *E. coli* and *E. histolytica* from each other, but large active amœbæ, particularly if they contain red blood corpuscles, present in the stool of a case with dysenteric symptoms and blood and mucus, may be assumed to be *E. histolytica*.

The greatest difficulty in diagnosis occurs when the acute symptoms have subsided and the stools are returning to the normal. The *tetragena* forms are now replaced by the *minuta* ones which more nearly resemble *E. coli*. Now, however, cyst formation takes place and the cysts are distinctly different from those of *E. coli*. They are spherical, but not so accurately spherical as those of *E. coli*, are more refractile, and are smaller (10 to 14 μ), and nuclear multiplication leads to the formation of two and then four, very rarely more, nuclei (Plate XXIII, *f*).

Care must be taken not to mistake the cysts of other intestinal Protozoa for amœba cysts, nor a vegetable organism, *Blastocystis hominis*, which sometimes occurs in considerable numbers in the fæces. This is more or less spherical in shape and 5 to 15 μ in diameter. It is a more delicate structure with thinner capsule than cysts of Entamœbæ. The greater part of the cyst is occupied with a vacuole, the cytoplasm being reduced to a narrow

rim at one side, or at opposite sides, of the cysts. In the cytoplasmic rim a varying number of greenish nuclei are present (Plate XXIII, *v*).

The differences between *E. coli* and *E. histolytica* may be tabulated as follows :—

	E. coli.	*E. histolytica.*
Size . .	15–30 μ.	*Tetragena* form, 15–30 μ. *Minuta* form, 10–20 μ.
Cytoplasm .	No differentiation into ectoplasm and endoplasm. Never encloses red blood corpuscles.	Clear differentiation into ectoplasm and endoplasm. Frequently encloses red blood corpuscles.
Nucleus .	Large, spherical, with coarsely granular membrane. Clearly visible.	Smaller, spherical, with finely granular membrane. Indistinctly seen.
Refractility	Less refractile.	More refractile.
Amœboid movement	Sluggishly amœboid.	Fairly actively amœboid
Cysts . .	Accurately spherical, 15–20 μ, less refractile. Contains more than four, and up to eight, nuclei.	Not so accurately spherical, 10–14 μ, more refractile. Two to four nuclei present.

With both parasites, cysts are responsible for the spread of infection. The *E. histolytica* cysts may persist for long periods in the individual, constituting a " carrier " state. Cats may be infected by feeding with the cysts of *E. histolytica*, and a typical dysentery produced. Amœbic dysentery has also been produced in man by feeding with the cysts. Ipecacuanha and emetine have a specific amœbicide action upon the amœbæ and cysts, though the latter are far more resistant than the former.

As mentioned above, abscess of the liver is a not infrequent sequel of amœbic dysentery.

The presence of the amœba in the pus, and especially in the walls, of tropical abscesses is diagnostic. The amœbæ are not usually observed in the abscess pus at the time of operation, but make their appearance in the discharge about the third day, *i.e.* when the wall of the abscess-cavity is contracting. In the true tropical abscess the ordinary pyogenic organisms are absent, unless a secondary infection has occurred, which is the exception. The abscess is usually single, and Rogers suggests that the amœbæ reach the liver through adhesions between it and the bowel. Saprophytic amœbæ, *e.g. A. limax* (Plate XXIII, *g* & *h*), may occasionally occur in the bowel, and it was probably one of these that Musgrave and Clegg cultivated from dysenteric stools and not the *E. histolytica*. The method was to take material rich in amœbæ, and to smear it over plates of agar (nutrient or made with water only), on which some bacterium had been grown, *e.g. B. coli*, cholera vibrio, etc. The plates are grown at 25°–30° C. for twenty-four to forty-eight hours, and are then examined with a low power. At any spot where isolated amœbæ are observed, with a little dexterity the organism may be lifted up with a fine needle and transferred to a fresh plate, and by a repetition of the process pure cultures may be obtained. The cultivated amœbæ were pathogenic for monkeys, and induced abscess on inoculation into the liver. Musgrave and Clegg are of opinion that all amœbæ are, or may become, pathogenic.

Clinical Diagnosis

1. Intestinal Protozoa are best examined in the fresh and living condition and as soon after leaving the body as possible: the encysted forms, however, do not alter for days after they have been passed.

A thin suspension of the fæces should be made with saline solution and several thin preparations examined. A warm stage should be used if it be desired to observe amœboid movement

PLATE XXIII.

Intestinal Protozoa (after Wenyon)

Entamœba coli.

a. Small entamœba, nearly spherical, with vacuolated cytoplasm.
b Large entamœba of irregular form.
c. Encysted form with eight nuclei as it appears in the fæces.

Entamœba histolytica.

d. Large tissue-invading form (*tetragena* form) containing the remains of five red-blood corpuscles.
e. Large tissue-invading form, with pseudopodium and containing the remains of two red-blood corpuscles.
f. Encysted form with four nuclei as it appears in the fæces.

Amœba limax.

g. Form without pseudopodium showing characteristic nucleus.
h. Form with pseudopodium.

Trichomonas intestinalis.

i. Flagellate of normal structure and free flagella.
k. Degenerate form which has lost the flagella and axostyle and has the appearance of an amœba with an undulating border.

Lamblia intestinalis.

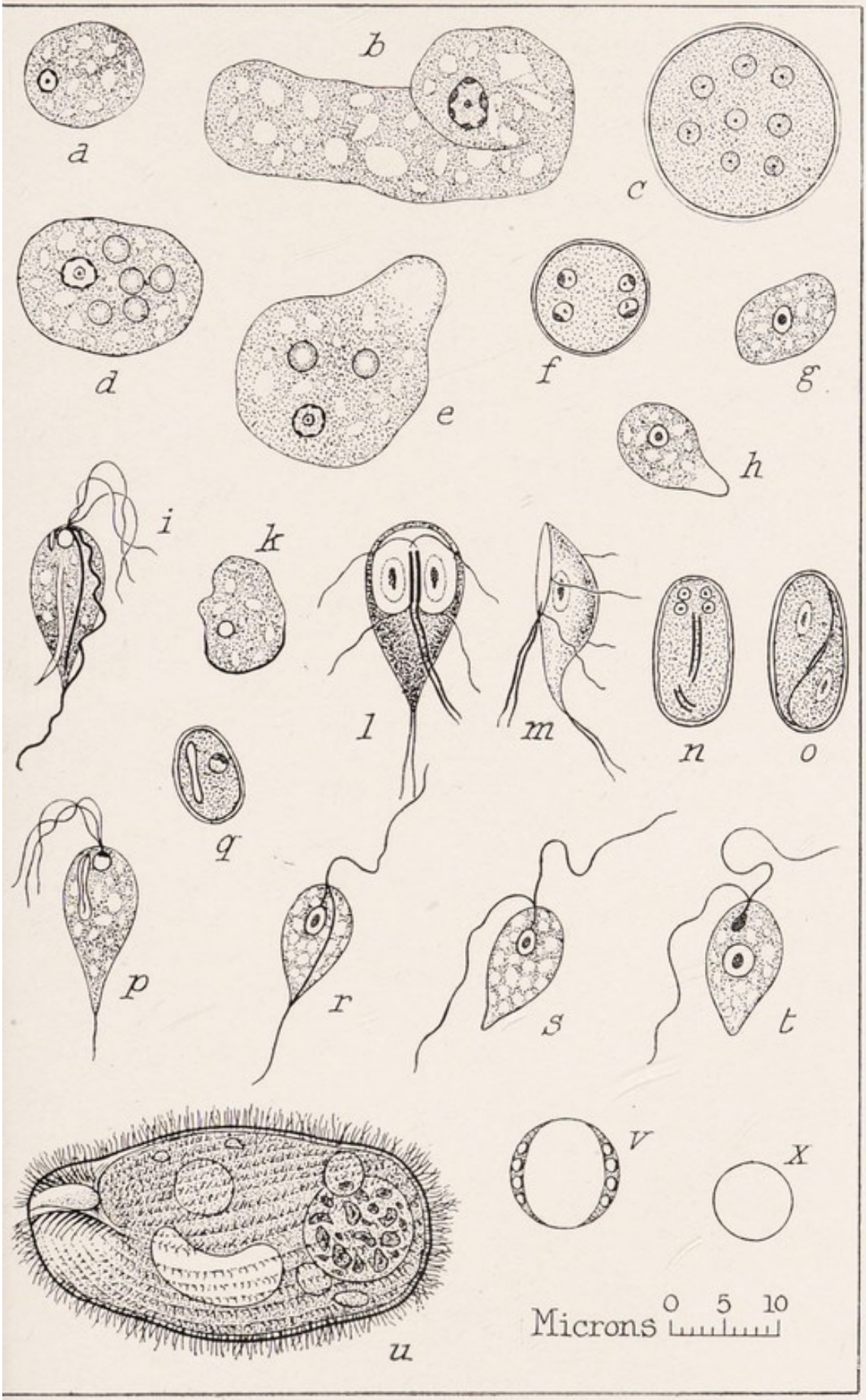
l. Surface view showing sucking disc, two nuclei and eight flagella.
m. Side view of thick form.
n. Encysted form with four nuclei.
o. Encysted form containing two flagellates.

Tetramitus mesnili.

p. Form showing flagellum in cytostome.
q. Encysted form with single nucleus and visible cytostome.
r. *Cercomonas.* *s*. *Bodo.* *t*. *Prowazekia*
u. *Balantidium coli* as met with in the bowel and tissues.
v. *Blastocystis.* *x*. Red-blood corpuscle.

All the figures are drawn to the scale shown, except *u*, which is *half* the size it should be.

PLATE XXIII.



of amœbæ or motility of flagellates. It may be difficult to distinguish amœbæ unless amœboid movement is observed, and for this purpose the stool should be quite fresh, free from urine, and collected in a warmed bed-pan. In order to render the nucleation of the cysts more evident the fæces may be rubbed up into a thin suspension with a drop of Weigert's iodine solution on a slide. A preliminary examination of the preparation may be made with a $\frac{1}{6}$ in. or $\frac{1}{8}$ in. objective with a high eye-piece, and subsequently a more critical examination with the $\frac{1}{12}$ in. oil immersion objective. A micrometer eye-piece by which exact measurements may be made is a useful adjunct. Dark ground illumination is of considerable assistance in the diagnosis of flagellates.

2. Donaldson [1] recommends the following method for examination of fæces for entamœba cysts. The following solutions are used :

A. Five per cent. aqueous solution of potassium iodide saturated with iodine. At time of using mix with an equal volume of either of the following solutions, B:

B. (1) A saturated aqueous solution of rubin s. ; or
(2) A saturated aqueous solution of eosin ; or
(3) Stephens's scarlet writing fluid.

A few loopfuls of one of the above stain mixtures are placed on a clean slide, a loopful of fæces is taken and is rubbed up with the stain to form a uniform suspension, and a clean cover-glass is gently lowered over the drop. No gross particles must be left in the emulsion. Amœbic cysts (both varieties) stand out as brilliant yellow or greenish-yellow spheres in a more or less uniform red background. The nuclear structures in the cyst stand out prominently. Other protozoal cysts, plant hairs and mould spores also stain yellow, *Blastocystis* stains reddish.

3. Cropper and Row [2] describe a method for concentrating Entamœba cysts. One gramme of fæces is shaken in a mechanical shaker with 30 c.c. of saline in a bottle of 120–150 c.c. capacity for at least half an hour until thoroughly disintegrated. The suspension is then poured into a separating funnel, and shaken up by hand for half a minute with 10 to 20 per cent. of its volume of methylated ether, after which the mixture is allowed to stand for a minute or two in the funnel until the two liquids have

[1] *Lancet*, 1917, vol. i, p. 571.
[2] *Lancet*, 1917, vol. i, p. 179.

separated. The fæcal débris float in the upper ethereal layer, the cysts remain in the lower saline layer. The latter is then drawn off from the funnel and is centrifuged at low speed for two or three minutes ; experience will give the precise time and speed. The cysts are now concentrated in the deposit, and a concentration of 15 times may thus be obtained.

4. Probably Heidenhain's iron-hæmatoxylin method is the best for staining this and other protozoa :

(*a*) Make smears of the material and drop while *wet* into the fixative—two parts of saturated aqueous mercuric chloride solution, one part of alcohol, with a few drops of glacial acetic. They remain in this for ten minutes.

(*b*) Wash in weak spirit and then in weak spirit coloured with iodine, and finally wash in distilled water.

(*c*) Treat with 4 per cent. iron-alum solution for six to ten hours.

(*d*) Stain in Heidenhain's hæmatoxylin for at least six hours.

(*e*) Differentiate in 1 per cent. iron-alum, watching microscopically.

(*f*) Wash well in tap-water, pass through alcohol and xylol, and mount.

Allusion may here be made to the Mycetozoa (Myxomycetes). These are masses of protoplasm resembling huge amœbæ, which are found on decaying vegetable matter. By some they are regarded as vegetable, by others as animal, in nature, and belonging to the *Amœbæ* of the Sarkodina.[1] Some important plant diseases, such as the "finger-and-toe" of cabbage roots, are due to their activity. The finger-and-toe disease is due to an amœboid parasite (*Plasmodiophora brassicæ*, by some included among the *Amœbæ*), the cycle of which begins with spores from which small flagellulæ are set free. Similar organisms have been supposed to be present in cancer.

Class II.—Mastigophora

These are protozoa in which one or more permanent organs serving for locomotion or food capture are present in the form of flagella. As a rule the body is limited by either a cuticle or a differentiation of the protoplasm into a firmer external portion or *periplast*. One, two, or more flagella may be present, and when multiple are arranged in various ways. Food-vacuoles may occur

[1] See Lankester's *Treatise on Zoology*, Pt. 1, First Fascicle, p. 37.

in the protoplasm, also contractile vacuoles, but not in the parasitic forms. Various other granules, including *chromatophores*, which generally contain chlorophyl, may be present. The nuclear apparatus is usually double, consisting of a large principal or macronucleus, and a small or micronucleus or blepharoplast; the latter is not, as in the Infusoria, composed of generative chromatin, and is in relation with the locomotor apparatus. An undulating membrane, a thin protoplasmic membrane attached to one aspect of the body like a dorsal fin, may be present. *Euglena* is a common form in ditches, and *Noctiluca* is the chief cause of phosphorescence in the sea; both are uniflagellate. *Volvox* and *Protococcus* are also placed by some in this group. The chief parasitic genera are:

Trypanosoma and *Trypanoplasma*, both of which have an undulating membrane, but the former has one flagellum, the latter two flagella, one at each end of the body, but both starting from the blepharoplast, which is situated at the non-flagellated end of the body.

Herpetomonas is like *Trypanosoma*, but has no undulating membrane. The flagellum runs the length of the body.

Leptomonas has a single flagellum starting from the end of the body (Fig. 56).

Crithidia has a single flagellum and a short undulating membrane running half the length of the body.

The trypanosomes and other forms living in the blood-plasma are known as hæmoflagellates.

Trichomonas intestinalis

This is the commonest of all the intestinal parasites of man. It inhabits the large intestine and cæcum and may occur in enormous numbers, though the infection does not seem to be of long duration, and may quickly disappear. Ordinarily it appears to be quite non-pathogenic, though Escomel in South America states that a dysenteric condition may be induced by it.

The organism has a pear-shaped body 5–15 μ in length. At the blunt anterior end a spherical nucleus is present, just anterior to which is a chromatin granule

from which three long free flagella arise, which are directed forwards, and a fourth thicker flagellum which passes backwards in a slightly spiral manner attached to the border of an undulating membrane, beyond which it is continued as a free posterior flagellum. Other structures present are the cytostome, a slight conical depression near the nucleus, a stiff rod running along the base of the undulating membrane, and the axostyle, a clear refractile bar arising near the cytostome, and continued through the body towards the posterior end where it protrudes through the surface as a sharp point (Plate XXIII, *i*). The cytoplasm is often vacuolated, the vacuoles containing bacteria. The flagellate is actively motile. The flagella are best seen with dark ground illumination. For diagnosis, it is important to count the three anterior flagella, as only by this character can it be distinguished from two closely allied, though much rarer forms—*Tetratrichomonas* which possesses four anterior flagella, and *Pentatrichomonas* which has five.

Reproduction takes place by longitudinal fission. Encystment does not appear to occur, but by a casting off of the flagella an amœboid cytoplasmic mass is formed, still possessing the undulating membrane at one edge (Plate XXIII, *k*). It was this form which Castellani probably described as *Entamœba undulans* of the human intestine.

Trichomonas is common in rats, mice, fowls and other animals. Hadley [1] holds that it is the cause of dysenteric affections in turkeys (" blackhead disease ") and poultry, in which it causes necrosis of the cæcal epithelium and necrotic foci in the liver.

A very similar, though larger (15–30 μ), form, *Trichomonas vaginalis*, is common in the vagina.

[1] *Bulls.* 166 and 168, Agricultural Experiment Station of the Rhode Island State College, U.S.A., 1916.

Lamblia intestinalis

This flagellate is fairly common and inhabits the upper part of the small intestine. Infection is often abundant, and is very persistent, and though for the most part unassociated with symptoms, from time to time abdominal uneasiness and attacks of diarrhœa may ocur. In the rabbit, the parasite may invade the glands of the small intestine.

Lamblia in shape somewhat resembles the half of a pear split longitudinally, and measures 12–18 μ in length. There is a rounded flat surface on which is a sucking disc with raised edge and convex surface and which terminates in two flagella. Three other pairs of flagella are also present, the arrangement of which is depicted in the figure (Plate XXIII, *l* & *m*). The flagella originate from a paired rod-like structure occupying a central position in the body of the parasite. Two nuclei are present, one on either side of the rods, and these give the organism a peculiar owl-like appearance when viewed on the flat surface. The flagellate is actively motile, is able to curl the tapering tail over its back, and attaches itself by the sucker-like disc to the surface of the intestinal epithelium. Reproduction appears to take place only in the encysted condition. The cyst is clear, oval in shape and measures 14 μ in length. The two nuclei of the parasite migrate to one end of the cyst and divide so that four nuclei are formed (Plate XXIII, *n*). The contents of the cyst then divide so as to form two *Lamblia* and the division between the two appears as a line running obliquely and longitudinally across the cyst (Plate XXIII, *o*).

Other Intestinal Flagellates

Tetramitus mesnili has a wide distribution in tropical and sub-tropical countries. Small epidemics of diar-

rhœa have been attributed to it. In general appearance, shape and size it resembles *Trichomonas intestinalis*, and like the latter has three long anterior flagella, but it has no axostyle and no undulating membrane. The cytostome is large and forms an elongated slit (Plate XXIII, *p*). Encysted forms are produced (Plate XXIII, *q*).

Cercomonas, Bodo and Prowazekia are of rare occurrence, and it is doubtful if they be true parasites : they may be free-living forms accidentally introduced into the host. The general characters of these three organisms are sufficiently indicated by the figures (Plate XXIII, *r*, *s* & *t*).

Trypanosomata [1]

The trypanosomes are all parasitic in the blood of vertebrates, and a blood-sucking invertebrate is almost invariably concerned in their transmission. In the case of each pathogenic trypanosome, some indigenous wild animal, tolerant to that form, serves as a reservoir from which infection is derived.

A trypanosome has a slender, flexible, flattened body, one extremity of which is pointed, the other passes into a single flagellum. A delicate undulating membrane runs along one edge of the body. The organism lives in the plasma, in which it is actively motile, the flagellated end being usually anterior, and measures 15–30 μ, or even 40–50 μ, in length. The protoplasm of the organism is finely granular, and near the centre of the body is a large macronucleus, and generally between it and the non-flagellated end is a smaller micronucleus or blepharoplast. From the latter a chromatin filament starts, runs along the free edge of the undulating membrane and passes into the flagellum. Reproduction takes place by longitudinal division, occasionally probably by transverse division, and amœboid and plasmodial masses may be found in the internal organs and bone-marrow. The trypanosomes have great morphological similarity, which renders them practically indistinguishable by structural characters. They can usually be differentiated into three forms —indifferent, male, and female—which in some cases may all

[1] For current literature on Trypanosomes and trypanosome diseases see *The Tropical Diseases Bulletin*.

occur together, but only become fully differentiated in an invertebrate host. The males are slender, active, only slightly granular, and with an elongated nucleus ; the females are bulky, sluggish, granular, and have a rounded nucleus ; the indifferent forms are intermediate. The males usually soon die off unless they conjugate ; the indifferents are more hardy, the females most so. The sexual forms conjugate in an invertebrate host, but if the males have died off, both male and female forms may be reproduced from the females by a process of parthenogenesis.

Trypanosoma Gambiense

In human trypanosomiasis and sleeping-sickness of West and Central Africa, a trypanosome *Tr. Gambiense* is the causative agent (Plate XXIV, *a*). It is usually present, though scanty, in the blood, but can often be found in numbers in the fluid aspirated from the enlarged cervical glands. In the later stages, when cerebral symptoms ensue, it is found in the cerebro-spinal fluid, but scantily, centrifuging being necessary in order to demonstrate the parasites. The blood may give the Wassermann reaction. The *Tr. Gambiense* is pathogenic to monkeys, and to a less extent to white rats and guinea-pigs. Cattle and certain antelopes and other wild game may act as reservoirs of the parasite, and it has been seriously suggested to kill off all the big game in the affected areas. It is conveyed by a tsetse-fly (*Glossina palpalis*), possibly by other tsetses.

The tsetse (and possibly other biting flies) may rarely convey the disease by direct inoculation. Generally a cycle of development is passed in the tsetse. The stages of this are not known with certainty, but Roubaud has observed multiplication of the parasites in the fly and the development of *Herpetomonas* forms. According to the observations of Kleine and Bruce, the flies become infective about thirty-four days after feeding and remain infective for at least 70–80 days, and probably for the rest of their lives.

In Rhodesia, a human trypanosome (*Tr. Rhodesiense*) has been found which is probably distinct from *Tr. gambiense*, and the

G. palpalis does not occur in the district; it may be conveyed by the *G morsitans*. The macronucleus of the parasite is frequently situated between the blepharoplast and the posterior end.

In Brazil another human trypanosome-like parasite has been discovered by Chagas (*Tr.* or *Schizotrypanum cruzi*), which is conveyed by a bug (*Conorhinus megistus*).

Tr. Brucei is the causative parasite of nagana or tsetse-fly disease of horses in Africa.

Nagana is met with in large tracts of country in Zululand and West Africa. It especially attacks the equines—horse, mule, and ass—in which it is very fatal. The animals become anæmic and emaciated, there is a discharge from the eyes and nose, staring coat, swelling of the legs and neck, and fever. The animal dies two to six weeks after infection. Oxen are also attacked, but a small proportion recover. The dog, cat, rabbit, guinea-pig, mouse, and rat may be infected by inoculation with the fresh blood of a diseased animal. In infected animals the trypanosome is generally abundant in the blood and spleen. The *Tr. Brucei* can be cultivated, though with difficulty, on rabbit-blood agar—equal parts of melted agar cooled to 45° C. and of defibrinated rabbit's blood warmed to 45° C., allowed to solidify in the sloping position (Novy and McNeal). The disease is conveyed through the bites of a tsetse-fly (*Glossina morsitans*). The trypanosome is believed to live in the big game, from whence it is transmitted to horses entering the infected localities. The blood loses its infective properties usually within twenty-four hours after being withdrawn.

Surra attacks horses in Burma, Mauritius, and the Philippines, and is pathogenic to the same animals as nagana, and in the blood a parasite (*Tr. Evansi*) similar to that in nagana, but more active, was observed by Evans. Surra is probably spread by certain biting flies belonging to the *Tabanidæ*.

The tsetse flies (*Glossina*) belong to the house-fly order (Muscidæ)

and have a general resemblance to a house-fly, but when at rest the wings fold completely over each other. The proboscis is long and straight and the wing venation is characteristic, especially the fourth longitudinal vein, which makes two bends. Instead of laying eggs, the female extrudes a single full-grown larva. They are confined to Africa and Arabia; some sixteen species have been differentiated, and they occur in forest land in the vicinity of water ("fly-belts").

Tr. equinum attacks horses in South America, causing weakness and paresis of the hindquarters ("*mal de caderas*"). Cattle are immune, most other animals susceptible.

Tr. Theileri, the largest trypanosome known (50–60 μ in length), is found in cattle in South Africa, and is not pathogenic to any other animal.

Tr. dimorphum occurs in two forms, large and small, in horses in Africa. Is pathogenic to most animals.

Dourine, a venereal disease of the horse met with in North Africa, Spain, and Hungary, is due to the *Tr. equiperdum*, which is conveyed by direct contact, and is mainly confined to the lesions, being scanty in the blood. It is pathogenic to the ordinary laboratory animals.

In rats a non-pathogenic trypanosome was found by Lewis (*Tr. Lewisi*). It is especially met with in sewer-rats, but also occurs in field-rats (Crookshank). It is somewhat shorter and thinner than the *Tr. Brucei*, and there are other small differences between the two forms. With the exception of rats and mice, and to a less extent guinea-pigs, other animals cannot be infected with the *Tr. Lewisi*. It may be kept alive for long periods in the blood placed in a refrigerator, whereas the *Tr. Brucei* soon dies under the same conditions. The two forms do not protect against each other. The *Tr. Lewisi* is readily cultivated on rabbit-blood agar and is transmitted by the rat-flea, in which it seems to penetrate into the epithelial cells of the gut and there undergoes a process of multiplication.[1] It is passed in the fæces of the flea and a rat ingesting the infected fæces becomes infected.

A number of other trypanosomes have been found in the lower animals, birds, fish, reptiles, and amphibians. A large and characteristic one is generally present in the blood of the eel.

The trypanosomes are usually agglutinated when mixed with the serum from an infected animal.

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[1] Minchin and Thompson, *Brit. Med. Journ.*, 1911, vol. ii, p. 361.

Hewlett was unable to obtain any toxic or immunising substance from ground-up trypanosomes (*Tr. Brucei*).[1]

Levaditi and Twort [2] found that the filtrate of broth cultures of *B. subtilis* is markedly trypanocidal *in vitro* but not *in vivo*.

Examination of Trypanosomes, etc.

The trypanosomes, if numerous, are readily observed in the fresh blood. A very shallow cell may be formed on a slide by ringing with melted paraffin. For stained preparations the Leishman stain (see "Malaria") or the Heidenhain method (p. 558) may be employed.

Leishmaniasis

This term is applied to a group of diseases, caused by a similar parasite, and widely distributed in tropical and sub-tropical countries of the old and new world.

In kala-azar or tropical splenomegaly, a disease met with in India, Assam and the East, a small parasite, the Leishman-Donovan body, occurs in large numbers in the spleen and liver, also in the lymphatic glands, lungs, and intestinal submucosa, and in large mononuclear leucocytes and endothelial cells. The bodies are small (2–3 μ), round, ovoid, or oat-shaped masses of protoplasm, apparently encapsuled, and contain two chromatin masses, one large and oval, staining pale red with Leishman's stain, the other small and rod-shaped, and staining deep red with Leishman (Fig. 56, *a*). They sometimes occur in masses (Fig. 56, *c*). Leishman considered the bodies to be degenerate trypanosomes, but the organism is now considered to belong to a distinct genus, and is termed *Leishmania Donovani*. Rogers succeeded in cultivating it in citrated blood at 20°–25° C., in which it develops into a flagellated form like *Leptomonas* (Fig. 56, *b*). The parasite is inocul-

[1] *Proc. Roy. Soc. Lond.*, B., vol. lxxxiv, 1911, p. 56.

[2] *Comp. Rend. Soc. Biol.*, vols. lxx and lxxi, 1911.

able into certain animals, provided large doses of virus be injected into the loin or peritoneal cavity. It has been stated to be transmitted to man by a bug (? a *Conorhinus*), but this is very doubtful.

The bodies are well shown in smears stained with the Leishman stain.

In Oriental sore, or Delhi boil, a parasite practically identical with the Leishman-Donovan body is present,

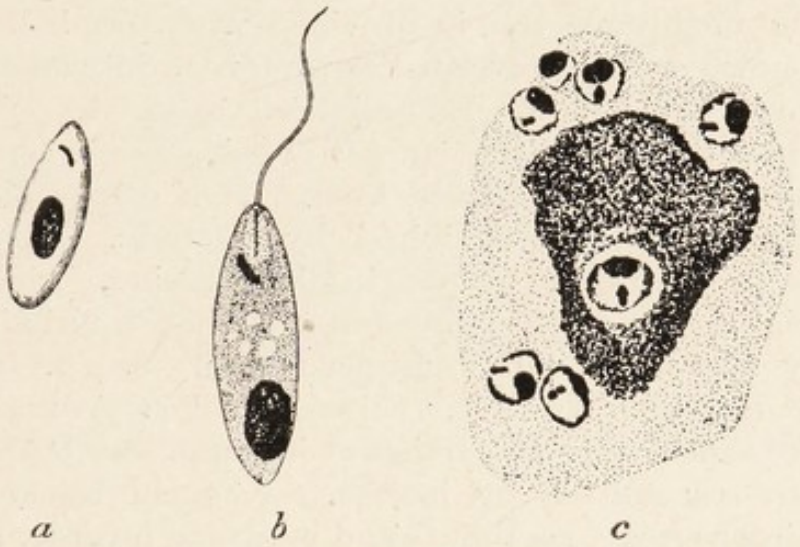


FIG. 56.—*a*. The Leishman-Donovan body. *b*. The flagellated form (*Leptomonas*) developing in citrated blood. *c*. Seven parasites in a large mononuclear leucocyte. (After James, Patton, and Rogers.)

but as the two diseases run a totally different course, it is probably a distinct species (*L. tropica*). On cultivation it develops a flagellated form. The disease has a seasonal prevalence, and Wenyon suggested that it is conveyed by a mosquito, a species of *Stegomyia*.

In N. Africa Nicolle has observed a Leishmaniosis of children due to another species (*L. infantum*). It is transmissible to the dog and monkey, and can be cultivated. The disease has recently been found all along the Mediterranean littoral. It may be transmitted by dog fleas.

About 30 per cent. of cases of kala-azar give a slightly positive Wassermann reaction.

Spirochaetosis [1]

Diseases caused by infection with spirochaetes.—The spirochaetæ are delicate, undulating, or somewhat spirillar, filiform parasites occurring in the blood of man, mammals, birds, shell-fish, etc. The filaments taper to a point at the ends, are flexible and motile, coiling and uncoiling, are described as having two nuclear masses, and some possess an undulating membrane, like typranosomes, but in the smaller forms no definite structure can be made out. (Strictly a *Spirochæta* possesses an undulating membrane which may be quite rudimentary, but no flagella ; a *Treponema* has no undulating membrane but is provided with terminal flagella.) They are now generally regarded as protozoa, but some still consider them to be bacteria. Bacterial cells are never pointed, nor do they show the coiling movements of spirochaetes; motility is produced by flagella, which are absent from many spirochaetes and periodicity is not exhibited by bacteria. Spirochaetes are said to multiply by longitudinal fission, while fission in bacteria is transverse (Dobell states that multiplication is always by transverse, but multiple, fission. See p. 19) ; they react in some cases to drugs (*e.g.* salvarsan) like trypanosomes, are much more sensitive to the action of immune sera than bacteria are, and are transmitted by insects. Noguchi has cultivated certain spirochaetes of the mouth and relapsing fever by a method similar to that which he employed for syphilis (p. 569). For the saprophytic spirochaetes a small quantity of oxygen is required, for the blood spirochaetes absolute anaërobiosis is necessary as in the case of syphilis.

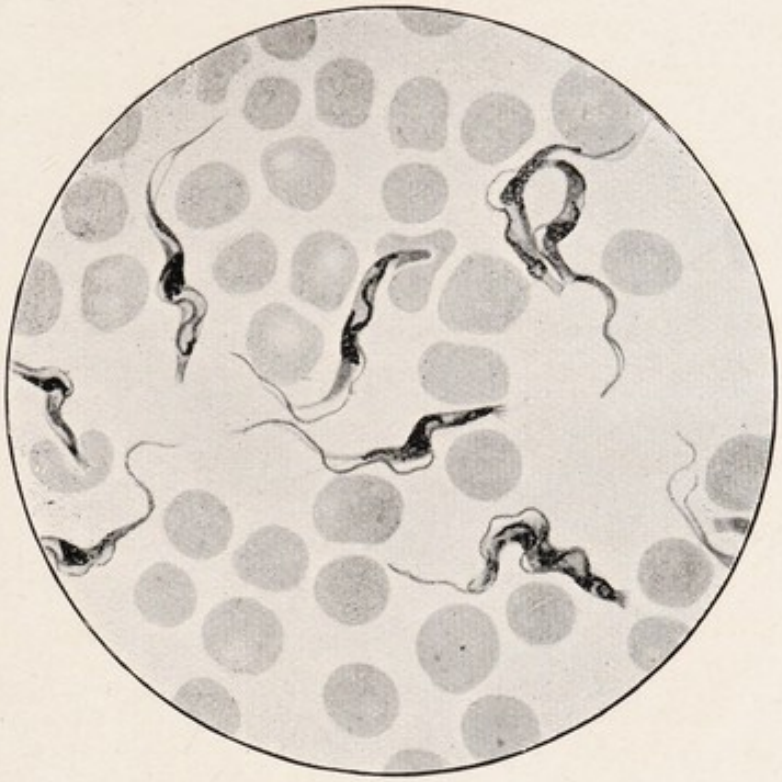
Schaudinn believed that many so-called spirochaetes may be connected with the trypanosomes.

In the case of a *Halteridium* parasite of the little owl (*Athene noctua*), Schaudinn claimed to have shown that it is a stage of a trypanosome (*T. noctuæ*) which is disseminated by the common gnat. His observations have not been confirmed, and Novy and McNeal believe that Schaudinn was dealing with a double infection of both a trypanosome and a *Halteridium*, not that one was transformed into the other.

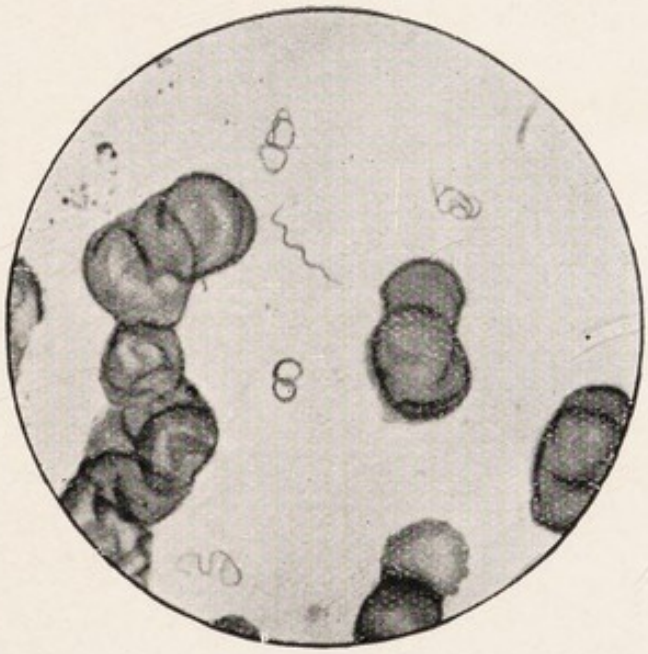
Spirochaeta recurrentis (*Obermeieri*).—Found in the blood-plasma, not in the corpuscles, in relapsing fever during the febrile paroxysms. It is very slender and

[1] See Nuttall, *Journ. Roy. Inst. Pub. Health*, xvi, 1908, p. 449.

PLATE XXIV.



a. Trypanosoma Gambiense. Smear of blood of inoculated rat. × 1500.



b. Spirochaeta recurrentis (Obermeieri). Smear of blood. × 1500.

delicate, measuring 12 to 16 μ in length, and actively motile (Plate XXIV, *b*). Bugs were formerly supposed to transmit this parasite, but Nicolle, Blaizot and Conseil have established the body louse as the agent of transmission. Infection is however not due to the bite of the louse, but to lice being crushed by the victim's scratching and the contents of the lice rubbed into the abrasions. The lice not only retain the infection for the rest of their lives, but the spirochaetes pass into their eggs, and these eggs and the larvæ hatched from them may similarly be infective to man. It is inoculable into monkeys, and less readily into rats.

Noguchi and Hata [1] have cultivated this form: the latter in a medium consisting of one part of horse-serum and two parts of saline. This mixture is placed in tubes to a depth of 7 cm., which are then heated slowly in a water-bath from 58° C. to 70° C., at which they are kept for thirty minutes. Small pieces of rabbit kidney are then pushed to the bottom of the tubes and the incubation must be carried out anaërobically.

It is probable that the spirochaetes of relapsing fever in different countries are distinct species.

Spirochaeta Duttoni.—Found in the blood-plasma in African relapsing, or tick, fever. It closely resembles the *S. recurrentis*, but is more readily inoculable into rats, mice, and guinea-pigs, and the one does not protect against the other. It is conveyed by a tick, *Ornithodoros moubata*, the malpighian secretion of which is the principal infective agent. The eggs of infected ticks are also infected, and the infection may be transmitted to the third generation of ticks.

Duval and Todd [2] state that multiplication of *S. Duttoni* takes place *in vitro* in a culture medium made with hens'

[1] *Centr. f. Bakt.*, Abt. I (Originale), vol. lxxii, 1913, p. 107.
[2] *Lancet*, 1909, vol. i, p. 834.

eggs and mouse blood. Leishman believes that certain chromatin bodies present in the eggs and nymphs of the ticks are the developmental forms of the spirochaete.

Spirochaeta icterohæmorrhagiæ.—This is the cause of Weil's Disease, Febrile or Infective Jaundice (spirochaetosis icterohæmorrhagica).

This disease occurs in epidemic and endemic form in Japan and other localities, and is met with occasionally in Europe—in the present war in Germany and on the Western front. In 1914 Inada [1] and co-workers discovered a spirochaete in the blood of a patient, and this has been confirmed by other observers. The organism is present in the blood only in the early stage of the disease and then only in small numbers. It is most abundant in the liver (Plate XXV, *a*), sections of which may be stained by the Levaditi method (p. 581), and may be met with in the spleen, adrenals, kidneys and urine. It averages 6–9 μ in length, but shorter (4–5 μ), and longer (12–20 μ) forms occur. Guinea-pigs may be infected by intraperitoneal inoculation with blood taken in the first three to six days of illness, and succumb in from five to eight days. The spirochaete may be cultivated by inoculating melted blood-agar or gelatin with blood, covering with a layer of paraffin and incubating at 20°–25° C. for 10 to 14 days. The mode of infection is uncertain, probably it is by water or by contact, possibly by insects.

Trench Fever clinically resembles a spirochaetosis and spirochaetes are stated to be found in the blood and in the urine (but they seem to occur in normal urine).

Spirochaeta bronchialis.—First described by Castellani and met with in a form of bronchitis occurring in the Tropics.

Spirochaeta pertenuis.—Castellani [2] found in the yaws

[1] *Journ. Exper. Med. vol.* xxiii, 1916, pp. 377 and 557.

[2] *Brit. Med. Journ.*, 1907, vol. ii, p. 1511.

(frambœsia) granulomata a delicate spirochaete resembling the *S. pallida* of syphilis closely, but even more delicate and difficult to stain than the latter organism, and named the *S. pertenuis*. It is present also in the spleen and lymphatic glands in the disease and in inoculated monkeys. Rabbits can be inoculated in the testicle and Noguchi has obtained cultures.

Some observers have supposed yaws to be a manifestation of syphilis, but (1) syphilitic patients can be inoculated with yaws; (2) syphilis may supervene on yaws; (3) Neisser and Castellani showed that monkeys inoculated with syphilis are not immune to yaws, and *vice versâ;* and (4) Castellani showed that the yaws antigen and anti-bodies are distinct from the syphilis antigen and anti-bodies, though the ordinary Wassermann test may react with yaws.

Spirochaetes are also present in rat-bite and typhus fevers (which see), in the ulcerating granuloma of the pudenda of Guiana (Wise) and Australia, in malignant growths, in ulcers, in the mouth (p. 657), and in Vincent's angina (p. 331).

Blood spirochaetes have been found in many animals, *e.g.* cattle (*S. Theileri*), mice (*S. muris*), fowls (*S. gallinarum*), and geese (*S. anserina*).

Staining methods.—Blood-smears may be stained with the Leishman or Giemsa stain (p. 615 *et seq.*).

Syphilis

Various bacterial organisms were described in this disease, *e.g.* by Lustgarten, Eve and Lingard, Van Niessen, de Lisle and Jullien, etc., and bodies regarded as protozoa by Siegel, de Korté, and others. In March 1905, Schaudinn [1] noted the constant presence of a spiriform

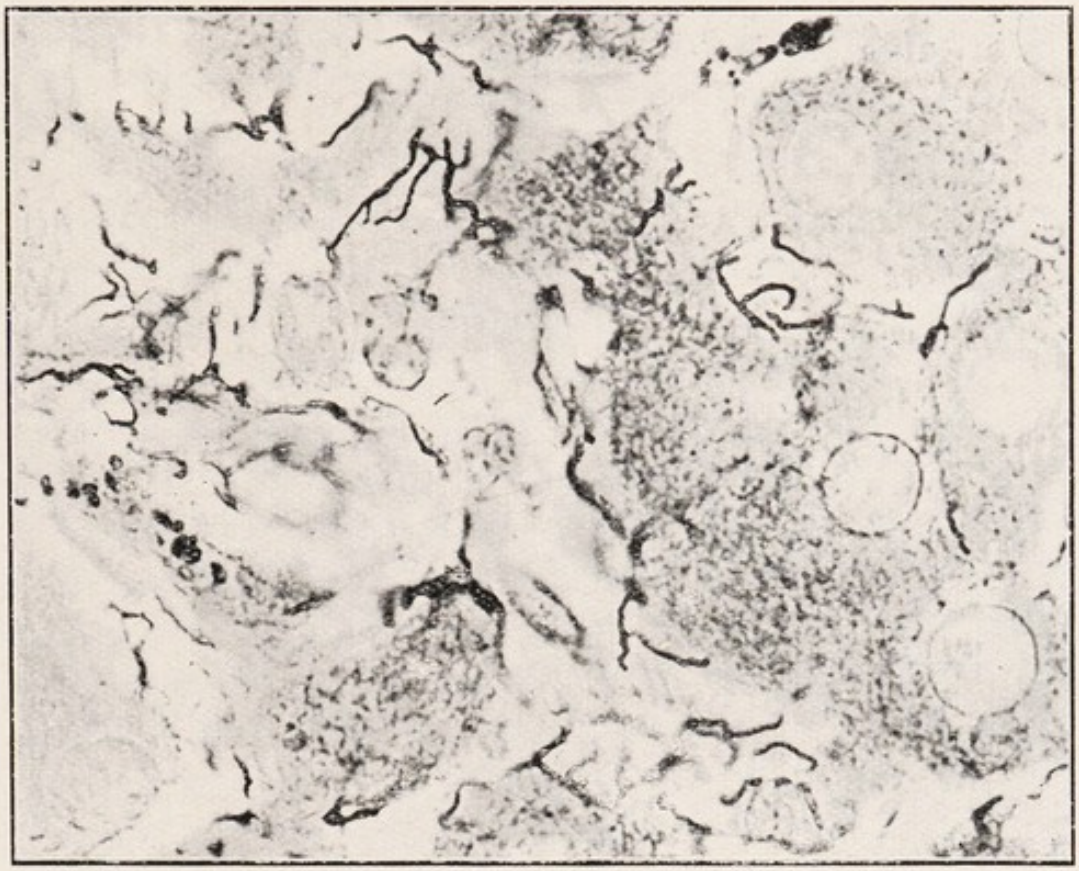
[1] *Arbeit. a. d. kaiser. Gesundheitsamte*, xx, 1905.

organism or spirochaeta (*S. pallida*, or *Treponema* or *Spironema pallidum*) in various lesions in acquired and congenital syphilis. The *T. pallidum* varies from 6 to 15 μ in length, averaging 8–9 μ (Plates XXV, *b* & XXVI, *a*). It is much more attenuated than the majority of spirochaetes, having a maximum thickness of 0·3 μ, has from three to twelve, usually from six to eight, twists, forming a close, regular, and narrow spiral, is actively motile, and possesses a single delicate flagellum at each end. It stains feebly and with difficulty. Another spirochaete, the *S. refringens*, frequently accompanies, and must not be mistaken for, the *T. pallidum* in ulcerating lesions ; the former is more refractile and coarser, has fewer twists and forms a wider spiral, and stains deeper and more readily than the latter. The *T. pallidum* is found generally in all primary and secondary lesions of syphilis, *e.g.* the primary sore and adjacent lymphatic glands, in the papular and roseolar eruptions, in condylomata and mucous patches. It has also occasionally been found in the spleen and blood. In congenital syphilis the *T. pallidum* is met with in the bullous eruptions, blood, and organs, and is particularly abundant in the spleen and liver (Plate XXVI, *b*).

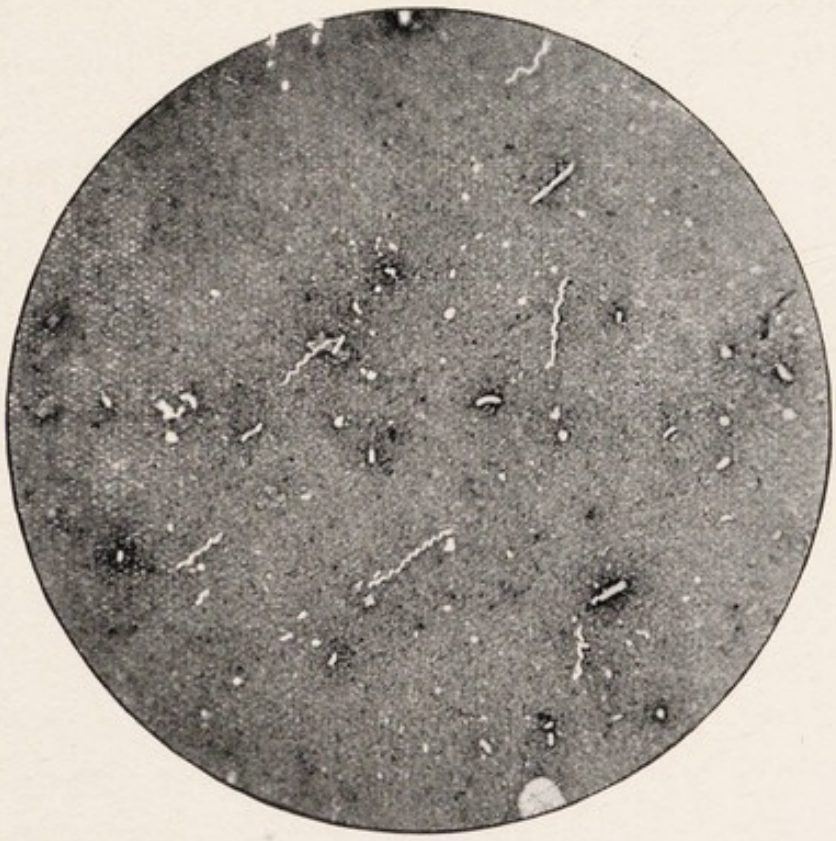
Tertiary lesions are generally considered to be non-infective, unless ulcerated, and the *T. pallidum* is usually difficult to find in them. It has, however, been detected in the peripheral portions of gummata and in syphilitic aortitis, and may persist in the body for years after the primary lesion. Noguchi, after a careful search, has detected the spirochaete in the brain in cases of general paralysis (in 48 cases out of 200 examined) and also in the posterior columns in a case of tabes.

The *T. pallidum* is now universally regarded as the specific organism of syphilis, being present not only in the human lesions but in experimental lesions of inoculated apes (see below). It must be recognised that spirochaetes

PLATE XXV.



a. Spirochaeta icterohæmorrhagiæ in liver. Levaditi's method. × 1200.



b. Treponema pallidum from condyloma (*T. pallidum* with *Spirochaeta refringens*). Indian-ink method. × 1000.

are of frequent occurrence in various non-syphilitic ulcerating and other lesions, *e.g.* in the mouth and in pyorrhœa, in yaws and ulcerating granuloma (in yaws they are specific forms, see p. 571), in ordinary ulcers and in carcinomatous tumours. Generally the *T. pallidum* can be distinguished microscopically from the other species, but care is necessary.

When material from a *rhesus* monkey inoculated with syphilis is placed in collodion sacs which are introduced into the peritoneal cavity of another monkey, a great multiplication of the organism takes place in the contents of the sacs a month after the operation.[1] Noguchi has obtained cultures of the *Treponema pallidum* by making use of serum water (serum 1 part, water 3 parts), sterilised for fifteen minutes at 100° C. on three days, to which fragments of fresh sterile tissue of a rabbit (kidney, heart-muscle) were added. Rabbits are inoculated with syphilis in the testicle and the spirochaete-containing testicular material is employed to inoculate the tubes, which are then incubated at 35°–37° C. under strictly anaërobic conditions. Multiplication of the spirochaetes commences forty-eight hours after inoculation. The primary cultures are somewhat difficult to obtain, but once obtained sub-cultivation is easy. Both thick and thin forms of the Treponema were obtained, which Noguchi considers may be distinct varieties.

Metchnikoff and Roux (also Grünbaum) found that the chimpanzee is very susceptible to syphilis, and can readily be inoculated from man, the *T. pallidum* being found in the lesions.

Macacus rhesus is also somewhat susceptible, likewise the *M. cynomolgus* and the Chinese bonnet monkey, but not the mandril. By several passages through a *rhesus* monkey the syphilitic virus becomes attenuated, so that

[1] Levaditi and McIntosh, *Ann. de l'Inst. Pasteur*, xxi, 1907.

in man it produces merely a local lesion.[1] Syphilis may also be inoculated on the eye or testicle of the rabbit.

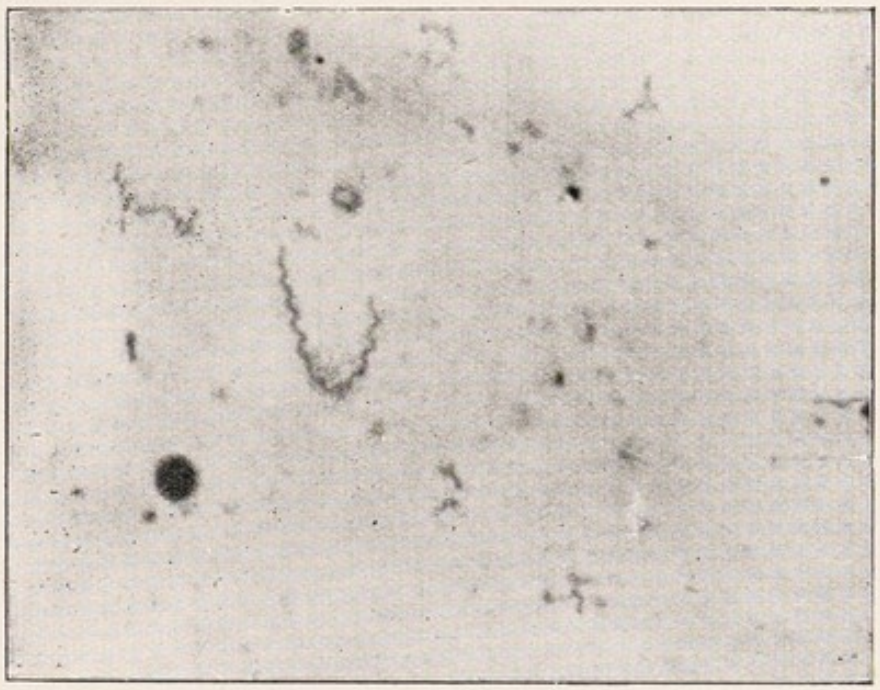
Although the central nervous systems of rabbits and monkeys are refractory to direct inoculation with *T. pallidum*, Noguchi has succeeded in inducing some of the symptoms (convulsions) and lesions of general paralysis in these animals by the following method. Intravenous inoculations of dead spirochaete cultures were given every five days over a period of five months, an interval of five months was then allowed to elapse, and finally the living spirochaetes were introduced into the brain, subdurally or intra-cerebrally.

Attempts by Metchnikoff and Roux to prepare an anti-syphilitic serum by inoculating apes and goats with syphilitic virus proved unsuccessful (as did earlier experiments with other animals by Héricourt and Richet). The syphilitic virus as ordinarily introduced into man by sexual intercourse probably takes some hours to become generalised, for Metchnikoff found experimentally in apes that if the seat of inoculation were treated with a calomel ointment up to eighteen hours after inoculation infection was prevented.

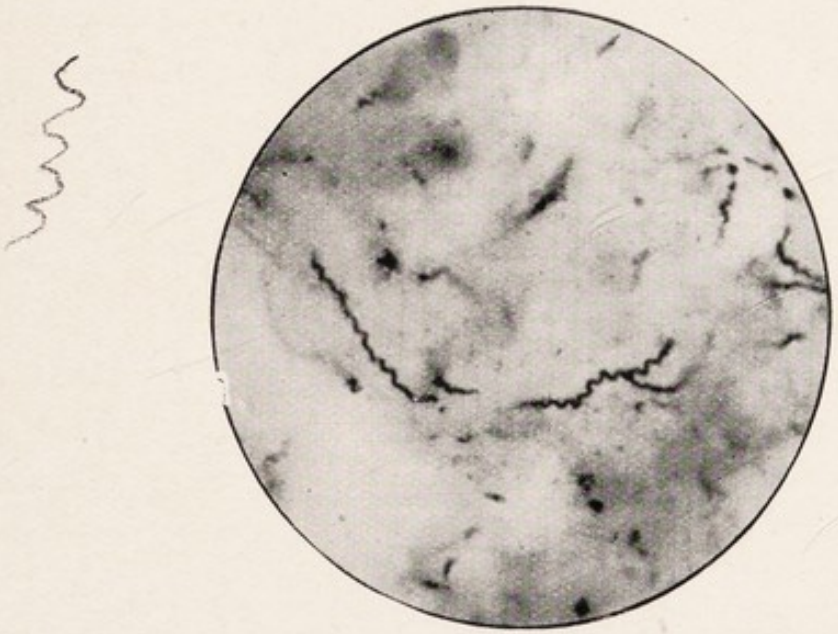
By triturating cultures of the *Treponema* in salt-solution, heating to 60° C. for sixty minutes, and adding 0·5 per cent. of carbolic acid, Nogouchi has prepared an agent, termed *Luetin*, which can be used for a cutaneous reaction for the diagnosis of syphilis. In syphilitic infection redness, sometimes becoming pustular, develops at the site of inoculation.

The syphilitic virus does *not* pass through a Berkefeld filter, and hence is not ultra-microscopic. It is readily destroyed by heat (52° C.) and antiseptics. Treatment with mercury and with salvarsan (" 606 "), neo-salvarsan,

[1] Metchnikoff, *Journ. of Prev. Med.*, 1906, August.



a. Treponema pallidum. Smear from condyloma. Giemsa. × 1500.



b. Treponema pallidum. Section of liver of fetus (congenital syphilis.) Levaditi's method. × 1500.

karsivan, galyl, etc., causes diminution or disappearance of the spirochaetes.

In central nerve lesions salvarsan, etc., is more effective when injected into the central nervous system, but this procedure is not free from danger. To obviate this, the salvarsan may be injected intravenously and then some of the patient's serum is injected into the spinal canal by lumbar puncture.

The *Wassermann reaction*, originally described by Wassermann, Neisser and Brück, is now extensively employed for the diagnosis of syphilitic infection in all its stages. It is a test based on complement-fixation (p. 201), for amboceptor-like bodies are present in the syphilitic blood-serum which, in the presence of specific antigen fix complement, this being determined by the use of a hæmolytic system. When first devised, the spirochaete had not been cultivated, so for the antigen Wassermann, Neisser and Brück made use of a saline extract of an organ rich in spirochaetes, viz., the liver of a syphilitic fetus, assuming that in this way a specific antigen was obtained. As a matter of fact, however, the Wassermann reaction is apparently not a true antigen reaction, for various alcohol-soluble and non-specific substances may be used as antigen. Moreover, the nature of the substances which act as amboceptor and together with antigen fix the complement is uncertain ; some regard them as globulins, others as lipoids, and while Wassermann considered them to be specific anti-bodies, others believe them to be derived from a peculiar degeneration or breaking down of the tissues in syphilis. Again, the reaction is not confined to syphilis : it may also be obtained (with the antigen employed for syphilis), in malaria (p. 614), yaws (p. 571), trypanosomiasis, leishmaniasis, leprosy (p. 384), and scarlatina in the early stage. Of the value of the Wassermann reaction as a means of diagnosis there is now no question,

it is widely employed and forms a part of the recent scheme for the control of venereal disease in this country.

The Wassermann reaction is a quantitative one, and as the amount of the amboceptor-like bodies which fix complement will vary from *nil* at the time of infection up to a considerable amount when the disease is fully developed, and *vice versâ* during the period of treatment and cure, border-line reactions will at times occur. That is, while some departure from the normal is present this is not sufficiently marked to form a basis for a definite diagnosis except in combination with the history and with the clinical aspect of the case. It may, however, be confidently stated that a well marked positive Wassermann reaction, obtained with the full technique, justifies a definite diagnosis of syphilitic infection, provided that leprosy, yaws, malaria, leishmaniasis and trypanosomiasis be excluded.

The Wassermann reaction is positive in all cases in the primary stage of syphilis, though it does not become manifest until at least a fortnight, usually from three or four to six weeks, after infection. It is similarly positive in all cases during the secondary stage ; in the tertiary stage, some 80–90 per cent. of the cases are positive. In parasyphilitic affections, *e.g.* tabes and general paralysis, some 50 per cent, of the cases are positive. In latent intervals, *i.e.* when there are no active symptoms, some 50 per cent. of the cases are positive. In cases with lesions of the nervous system, the cerebro-spinal fluid frequently yields a positive Wassermann reaction when the serum gives a negative one.

In congenital syphilis a large proportion of the cases yield a positive reaction. Adequate treatment of the syphilis leads to a condition in which the Wassermann reaction, previously positive, becomes negative. In such a case the reaction usually becomes weaker and weaker, and finally two or three months after the commencement

of treatment it becomes entirely negative. The reaction is valuable for estimating the efficiency of treatment ; if the treatment is successful the reaction remains permanently negative, but an interval of six months should be allowed to elapse after the cessation of the course of treatment before a negative reaction should be accepted as proof that the syphilitic virus has been destroyed and a further test after a lapse of twelve months is desirable. Some American authors assert that chronic alcoholism induces a condition in which the syphilitic individual does not give a positive Wassermann reaction.

The details for carrying out the Wassermann reaction are given at p. 582.

The essentials for carrying out the Wassermann reaction are (1) Antigen, (2) complement, (3) hæmolytic system, and (4) the fluid to be tested.

Many modifications of the reagents used and of the method of carrying out the test have been introduced and a brief survey of some of these may now be given.

(*a*) *Antigen.* The various substances which have been used as antigen include :

1. A watery or alcoholic extract of syphilitic fetal liver.
2. Alcoholic extract of normal liver or heart-muscle [1] (human, ox, sheep or guinea-pig), with or without previous extraction with acetone.
3. Alcoholic extract of normal heart-muscle with the addition of cholesterin.
4. Various artificial mixtures, *e.g.* lecithin and cholesterin, sodium glycocholate or taurocholate.
5. Extracts of pure cultures of the *Treponema pallidum* obtained by Noguchi's method.

Probably the most widely employed antigen at the present day is number (3), the so-called " Sachs antigen."

(*b*) *The Complement.*—Fresh guinea-pig serum is generally employed as complement, the patient's serum being inactivated to destroy its complement. In some methods the patient's serum

[1] Heart muscle is peculiar in that it contains a high content of lipoid substances.

is not inactivated and the complement present in it is that used. Fresh rabbit or human serum has also been used as complement.

The general opinion is that it is better to add a foreign complement than to make use of the complement present in the patient's serum, and this is the recommendation of the Royal Commission on Venereal Disease.

The reason for this is that the content of complement present in different sera is liable to considerable variation; if, however, guinea-pig serum be used, its complement content can be estimated and the same amount of complement may therefore always be employed in the test.

(*c*) *The Hæmolytic System.*—This may be serum hæmolytic for ox, sheep, human or other red blood corpuscles, with the homologous corpuscles (see p. 202). The hæmolytic serum should have a fairly high titre and the corpuscles be fresh and well washed with saline.

Serum hæmolytic for sheep's corpuscles with sheep's corpuscles is the hæmolytic system usually employed. The hæmolytic serum is inactivated.

As human serum in low dilution is generally (75 per cent.) hæmolytic for sheep's corpuscles, the patient's serum itself together with sheep's corpuscles is used as the hæmolytic system in some methods, *e.g.* Fleming's. In the ordinary methods, the serum is too diluted for this hæmolytic effect to be exerted.

(*d*) *The Fluid to be Tested.*—This will be either the blood-serum or the cerebro-spinal fluid of the patient. Either should be free from corpuscles. If foreign complement is to be used, the serum is inactivated by heating to 56° C. for half an hour. The cerebro-spinal fluid is *not* inactivated.

The Method.—The Wassermann reaction to be accurate must be carried out quantitatively and the particular volumes of the various constituents adopted must always be used. Thus Fildes and McIntosh employ the following volumes: 0·1 c.c. of patient's serum to be tested, 0·5 c.c. of antigen dilution, 0·5 c.c. of complement dilution, and 0·5 c.c. of hæmolytic system. In different methods the total number of volumes of all the constituents varies from ten to fifteen, the volume of the patient's serum being taken as one. This variation has no influence on the *accuracy* of the results obtained, but it may entail some slight adjustments of the *strengths* and some variation in the *volumes*, of the reagents employed. This, however, is determined by the

standardisation of the reagents, which is always carried out in any method by the same technique as employed in the test itself. Although particular *volumes* of the constituents must be adhered to, these need not be definite *quantities* (*i.e.* c.c.'s or fractions of a c.c.), so long as the same *ratio of volumes* is maintained. Thus, in the Fildes and McIntosh technique, the volume V may be any quantity (but not necessarily a *known* quantity) so long as the ratio of constituents 1 V serum + 5 V antigen + 5 V complement + 5 V system is adhered to.

The technique for carrying out the test may therefore conveniently be divided into the "large volume" method, in which no volume will be less than 0·1 c.c., and the "small volume" method in which the unit volume used in the test will be under 0·05 c.c. The Writer is unable to discern that the "large volume" method can claim any advantage over the "small volume" one, while the "small volume" method possesses advantages, (1) a sufficiency of the patient's blood may be obtained from a prick and there is no need to puncture a vein, (2) it is more economical of the reagents—antigen, hæmolytic serum, and complement—and it is rarely necessary to kill a guinea-pig, (3) the time of incubation of the mixtures is lessened.

CLINICAL EXAMINATION FOR SYPHILIS

A. EXAMINATION FOR THE T. PALLIDUM

1. *Examination in fresh preparations.*—Scrapings from the deeper layers of the chancre, etc., may be emulsified in physiological salt solution and examined microscopically, particularly with dark-ground illumination.

Another useful method is the *Indian-ink method.* A scraping is obtained from the lesion as above, and the fluid thus obtained is placed on a slide and an equal quantity of ink added. The ordinary commercial Indian inks may be used, Günther Wagner's being particularly good. The ink must be previously examined microscopically to prove the absence of spirillar forms, which sometimes occur in it. The serum and the ink are then rapidly and thoroughly mixed and smeared over the slide so that a pale brown colour results. The material dries in a minute or slightly less, and may be examined directly with the oil-immersion lens, or the wet preparation may be covered with a cover-glass and examined. Benian's method (p. 118) may be similarly used.

The preparations, which keep for a considerable time, show the red blood-cells as large clear circular areas in a brownish-black field, the bacteria and *débris* as white rods, dots, etc., and spirochaetes as clear white spirals (Plate XXV, *b*).

It is particularly important in using this method that in so far as possible serum be used alone, and that a minimal amount of mucous material or fibrin be mixed with the ink. The presence of mucus results in the taking up of a large amount of the colouring matter of the ink, with the result that a smear of the requisite colour and thickness cannot be made. If too much serum is used the albuminous material appears to precipitate the colour from the fluid and a finely granular appearance is seen microscopically, which is practically worthless for diagnostic purposes. Again, if too much ink is used, the surface of the smear is increased in size to such an extent that the task of examining it thoroughly is greatly lengthened.

Coles [1] notes a useful point in the recognition of the treponema, namely, that if the number of turns of the spiral of the syphilitic spirochaete be counted, six or seven turns will be found in a length equal to the diameter of a red blood-cell. The distance from the top of one spiral to the next is from 1 to 1·2 μ. As red blood-cells measure about 7·5 μ in diameter, on an average six or seven turns will be equal to the diameter of a red blood-cell. The treponema varies in length from 6 to 15 μ, or even more, and consequently contains from six to fourteen and sometimes twenty or more turns. This measurement of the length of the spiral is usually possible, and is of the greatest value in identifying the treponema.

2. *Stained preparations.*—*Smears* from chancres, etc., may be stained by the *Giemsa method.*

The smears are fixed for ten minutes in absolute alcohol. The preparations are then stained in a dilute solution of the Giemsa solution for two to twenty-four hours, washed in distilled water, dried, and mounted. (The dilute Giemsa is prepared by adding one drop of the Giemsa stain to a cubic centimetre of "best" distilled water, and rendering alkaline with one drop of 0·01 per cent. potassium carbonate solution.) The preparations may also be stained in the undiluted Giemsa stain for half to six hours. Leishman's solution may also be used for the Giemsa method described under "Malaria."

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[1] *Brit. Med. Journ.*, May 8, 1909.

Fontana's method is probably the best. Three solutions are required :

1. Fixing Fluid :
   Acetic Acid, 1 c.c.
   Formalin, 20 c.c.
   Distilled water, 100 c.c.
2. Mordant :
   Tannic Acid, 5 grm.
   Aqueous Carbolic Acid (1 per cent.) 100 c.c.
3. Silver Solution :
   Prepare a 0·25 per cent. solution of silver nitrate in distilled water and add just sufficient ammonia to it to cause a slight permanent turbidity.

Prepare films and allow them to dry spontaneously. Fix by flooding with the fixing solution for a sew seconds and then pouring it off and repeating the process several times for not less than a minute. Wash well with distilled water. Flood the film with the mordant, heat until it steams and treat for half a minute. Wash thoroughly with distilled water. Flood with the silver solution and warm gently for half a minute. Wash with distilled water, blot and dry. The spirochaetes are stained jet-black.

Sections may be stained by Levaditi's method :

(1) Fix pieces of tissue about 1 mm. thick in 10 per cent. formalin for twenty-four hours.

(2) Wash in water, and harden in 96 per cent. alcohol for twenty-four hours.

(3) Wash in distilled water for some minutes (until pieces sink).

(4) Place in 3 per cent. silver nitrate solution at 37° C. for three to five days in the dark.

(5) Wash in distilled water for some minutes, and then place in the following solution at room temperature for twenty-four to forty-eight hours.

Pyrogallic acid	2–4 grm.
Formalin	5 c.c.
Distilled water	100 c.c.

(6) Wash in distilled water, dehydrate in absolute alcohol, clear in xylol, embed in paraffin, cut, and mount.

The spirochaetes are stained black or brown (Plate XXVI, *b*), the tissues yellow.

Some have asserted that the spirochaetes seen in the tissues

after staining by this method are artifacts or are composed of filaments of elastic tissue,[1] but this is not the general opinion.

B. THE WASSERMANN REACTION.

As already mentioned (p. 577) many modifications of the method of carrying out the Wassermann test and of the reagents employed have from time to time been introduced. The method here described is a "small volume" one as adopted by the Writer, the essential details of which were originally planned by Dr. F. E. Taylor.

In this method eleven unit volumes are employed and the total volume of the reagents, etc., in standardisation tests and in the test itself is always made up to eleven volumes.

The unit volume adopted by the writer is 20 cb. mm. for reasons given later, and calibrated Wright's pipettes are used to measure the volumes.

For the method of carrying out a "large volume" test consult Fildes and McIntosh, *Lancet*, vol. ii, 1916, p. 751.

APPARATUS REQUIRED

(*See* Fig. 57)

(*a*) Ordinary graduated pipettes, viz. one $\frac{1}{10}$ c.c. in tenths, three 1 c.c., one 5 c.c. and one 10 c.c. in tenths of a c.c. These may stand in a suitable cylinder.

(*b*) An ordinary test-tube rack with front and back rows of six holes each.

Fill the back row with 4 in. $\times$ $\frac{5}{8}$–$\frac{3}{4}$ in. test tubes, the front row with 3 in. $\times$ $\frac{5}{8}$ in. test tubes (which will contain 10–12 c.c.) for antigen dilution and hæmolytic system. Thick-walled tubes are to be preferred.

(*c*) Three graduated throttled pipettes (one of each form described below) provided with rubber teats. The unit volume is 20 cb. mm. These may stand in three of the larger tubes in the rack (*b*).

(*d*) A number of quill tubes for the test. These should be 2 in. long made from glass-tubing with thinnish walls of about 4 mm. internal diameter.

(*e*) Two or three small tubes 1–2 in. $\times$ $\frac{3}{8}$–$\frac{1}{2}$ in. These are for complement and complement dilution.

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[1] See Saling and Mühlens, *Centr. f. Bakt.* (Orig.), xlii and xliii.

(*f*) Two or three tubes 3 in. × $\frac{5}{16}$ in. These fit the large holes in the special water-bath and are used to contain the hæmolytic system.

(*g*) Metal racks with two rows of numbered holes suitable for containing the quill-tubes; each rack should hold 6–12 pairs of quill-tubes. They are used to hold the quill-tubes during filling,

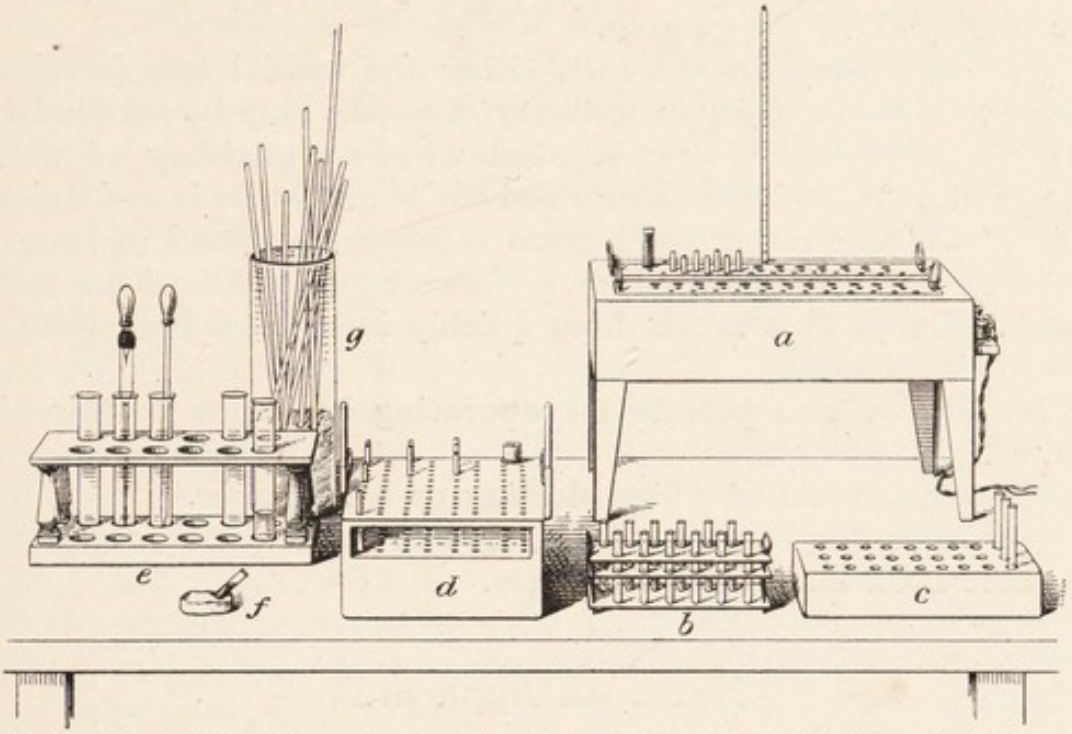

FIG. 57.—Apparatus arranged for Wassermann test (small-volume method):—

a. Electrically-heated water-bath.
b. Metal rack for small tubes.
c. Wooden block drilled with holes for small tubes.
d. Another form of metal rack for small tubes; it may be immersed in a water-bath.
e. Test-tube rack with test-tubes and throttled pipettes.
f. Small tube containing complement set in a block of plasticine.
g. Cylinder with pipettes.

and, if the special water-bath is *not* available, they may be immersed in an ordinary water-bath for inactivation of the specimens of blood-serum to be tested and for incubation of the mixtures in the test. In the latter case, one of the racks should have a couple of larger holes suitable for containing the tubes (*f*) used for the hæmolytic system. If the special water-bath *is* available, blocks of wood drilled with suitable holes may be used instead of metal racks as stands to contain the quill-tubes while these are being filled.

(*h*) One metal rack with holes to contain quill-tubes. The holes should be numbered in order. This is used to hold the specimens of blood-serum, etc., to be tested and the stand should have a sufficient number of holes to contain the largest number of specimens that are likely to be examined at one time.

(*i*) Inactivating bath. This may be either a special inactivating bath with regulator, or the stew-pan mentioned in (*j*) may be employed for the purpose.

(*j*) Water-bath for the test, either the special one described below, or, failing this, an ordinary stew-pan may be adapted for the purpose. The latter is filled with a sufficiency of warm water at 38° C. or thereabouts and the temperature is maintained at 37°–38° C. with a small flame regulated by hand (a Bunsen with by-pass may be used) or preferably controlled with a regulator. Tubes heat much more quickly in a water-bath than in an air-incubator.

(*k*) One or two porcelain evaporating basins to contain the saline.

(*l*) Some sheets of white blotting or filter paper.

(*m*) A Bunsen burner, forceps, file and grease-pencil, and paper or note-book and pencil for notes.

Notes on Apparatus

Duplicates of all glass apparatus should be kept at hand, so that there may be no delay in replacing breakages. If many specimens have to be examined as a routine, the special water-bath for the test and a special inactivating bath should be provided.

Section (*a*).—The $\frac{1}{10}$ c.c. pipette divided into tenths (*i.e.* each division = 0·01 c.c.) is used for measuring out the hæmolytic serum when this has a high titre. Failing this a Wright's pipette having a bulb on the stem is prepared by drawing out a piece of glass tubing in *two* places. The pipette will therefore consist of stem, undrawn out portion or bulb, stem and barrel. The lengths are so adjusted that the first portion of stem may contain 10 volumes and the 100 volume mark may be on the second portion of stem. By graduating the first portion of stem into 10 volumes the 100 volume mark may readily be ascertained by sucking up ten ten-volumes. It may be provided with a throttle.

Section (*c*).—Throttled pipettes (Fig. 8, p. 56) are much more convenient than simple Wright's pipettes (Fig. 37, p. 237).

They must be calibrated and the volumes be marked upon the stem with grease-pencil, or, better, with blue-black ink burnt in. The volumes are best determined with a globule of mercury. The pipettes are provided with stout rubber teats. It is very convenient to adopt some particular volume which can be easily reproduced at any time so that fresh pipettes can be prepared to replace breakages, etc., and be graduated into volumes the same as in the old ones. The Writer has adopted 20 cb. mm. (0·02 c.c.), as the unit volume ; *this is the volume of the pipette of the ordinary hæmoglobinometer*, and also happens to be a very convenient volume to use. To calibrate the pipettes, therefore, 20 cb. mm. of mercury are measured out with the hæmoglobinometer pipette and the pipettes are calibrated with this as described at p. 35. If a hæmoglobinometer pipette is not available, a *weighed* quantity of mercury may be used (20 cb. mm. of mercury weighs 0·270 grm.). It is convenient to have three different sizes of throttled pipettes, viz. one of somewhat fine bore and with a fine throttle, graduated at one and two unit volumes only ; a second of somewhat coarser bore and with looser throttle and graduated at the fourth and fifth unit volumes only ; and a third with long stem divided into ten unit volumes. They are made from glass tubing $\frac{3}{8}$ in. external diameter with not too thin a wall and the stem measures in the first two about $2\frac{1}{2}$–3 in., in the third about 5 in., in length. The first form is used for measuring out the patient's serum and for adding the complement to each tube of the test, the second form is used for measuring out the volumes of saline, antigen and hæmolytic system into the tubes of the test, the third form is used for preparing the dilution of complement. Care must be taken that the barrel of the pipette is sufficiently long so that the point of the throttle does not become wetted by the fluid sucked up ; if this happens the throttle becomes blocked and it must be rinsed with alcohol and then with ether to remove the moisture. With careful usage (and luck) these pipettes will last for hundreds of tests, comprising thousands of measurements. Each time after use these pipettes should be rinsed with saline, distilled water and absolute alcohol in succession, the rubber teats removed, and the pipettes kept in a suitable box.

Section (*j*).—If many tests have to be done a special water-bath is almost essential. It should be provided with a regulator (an electrically-heated one is the most convenient) and trays with suitable holes. In the one used by the Writer there are

two trays, and each tray has three rows of twelve holes each, a back row with large holes of about 10 m.m. diameter in which tubes containing the hæmolytic system may be kept warm (or a "large-volume" test may be carried out), and a middle and a front row of small holes, numbered, of 6 mm. diameter to contain the quill-tubes used for the test. The bath is in copper and measures 13 in. long, 6 in. wide and 4 in. deep. (Fig. 57).

Preparation of the Reagents, etc.

(*a*) *Saline Solution.*—This should be freshly prepared with good distilled water. For sheep's corpuscles it is usually made with about 0·85 per cent. of sodium chloride. If too weak it will hæmolyse, if too strong (*e.g.* 1·2 per cent.) it will fix complement.

(*b*) *The Antigen.*—The Writer has found the Fildes and McIntosh [1] antigen to be very satisfactory. The following is the method of preparation : *Solution* A. Take the muscular tissue, free from fat, from the ventricles and elsewhere of a fresh human heart, mince and weigh. Grind the mince up in a mortar with a little sand and absolute alcohol, using 9 c.c. of absolute alcohol to 1 grm. of heart. Transfer the whole to a well-stoppered bottle and shake occasionally for an hour and a half. Filter through paper into another bottle with well-fitting stopper. A deposit forms in time and may be filtered off. *Solution* B. Take 1 grm. of pure cholesterin and place in a bottle with well-fitting stopper. Add 100 c.c. of absolute alcohol and stopper tightly. Heat in a water-bath with occasional shaking until the cholesterin is dissolved. The two solutions should be preserved in separate bottles in the ice-chest.

For use, with a 1 c.c. pipette rinsed with absolute alcohol, take 0·3 c.c. of A and 0·2 c.c. of B, mix and add 7 c.c. of Saline and well mix. The Antigen may weaken in time and must be standardised occasionally (see p. 597).

If human heart is unobtainable, guinea-pig heart may be used instead.

(*c*) *The Complement.*—Guinea-pig serum is used. It is obtained shortly before the test is performed by bleeding a large buck

[1] "A method of applying the Wassermann Reaction in large Numbers," *Lancet*, vol. ii, 1916, p. 751. This method is very satisfactory and is widely used and the Writer has taken many of the details therein contained and embodied them in this account.

guinea-pig, by preference, by pricking an ear-vein and collecting the blood in a small tube. In this way 2 c.c. or 3 c.c. of blood may easily be obtained. The blood is allowed to coagulate and is then centrifuged and the serum is pipetted off into another similar tube. The serum so obtained is kept in the ice-safe until required for use. It must always be standardised immediately before the test is performed.

(*d*) *The Hæmolytic System* (see p. 578).

α. The Hæmolytic Serum.—This will usually be purchased. The Writer uses Messrs. Burroughs and Wellcome's serum hæmolytic for sheep's corpuscles. It has proved quite satisfactory and when fresh has a titre of 1 in 2,000 or thereabouts.

As it slowly (very slowly in the ice-chest) loses strength, it must be standardised from time to time (see p. 595).

β. The Corpuscles.—Sheep's blood is obtained from the slaughter-house. The blood as it flows from the animal is caught in a wide-mouthed stoppered bottle containing a coil of iron-wire. Fill only half full and shake sufficiently to defibrinate: the defibrinated blood will keep in the ice-safe for 3–4 days. When required for use, fill the centrifuge tubes with blood and centrifuge until the corpuscles are deposited. Remove the supernatant fluid and fill up with saline and mix. Centrifuge until the corpuscles are deposited, remove the fluid and fill up again with saline and repeat the process once more so that the corpuscles receive in all three washings with the saline.

The corpuscles are then suspended in saline by mixing 1 volume of the well-deposited corpuscles with 3 volumes of saline, thus making a 25 per cent. suspension of the corpuscles. The corpuscles are best used not more than two days old. With a *trace* of formalin the saline suspension will keep for five or six days in the ice-safe.

In a mixture of citrate solution (3·8 per cent. sodium citrate in distilled water) two parts, dextrose solution (5·4 per cent. dextrose in distilled water) five parts, and deposited corpuscles, three parts, the corpuscles will remain intact for three to four weeks in the ice-safe.[1]

(*e*) *The Fluid to be Tested.*—If the cerebro-spinal fluid is to be tested, this is obtained by lumbar puncture. If it contain blood or leucocytes it should be centrifuged to remove these; no further preparation is required.

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[1] Rous and Turner, *Journ. Exper. Med.*, vol. xxiii, 1916, p. 219.

Blood-serum is obtained by bleeding the patient ; a quarter to half a c.c. of blood is ample. This may be obtained by first swinging the arm quickly so as to drive the blood towards the finger tips, rapidly applying a ligature of small rubber tubing round the thumb and then stabbing the congested tissue on the side of the thumb near the base of the nail with a surgical needle in two or three places. The blood as it flows is collected in one or two Wright's capsules, or by means of a short pipette with rubber teat by which it is transferred into a short wide tube (*e.g.* a short Durham's tube 8 mm. in diameter). The tubes should be carefully labelled as they are filled so that no mistake can be made.

If the blood has been badly collected or has been sent by post it is better to centrifuge at once and to pipette off the serum from the clot and corpuscles. The tubes of blood or serum should be preserved in an efficient ice-safe, as the occurrence of bacterial or other growth in the serum tends to render it fixative of complement, *i.e.* to react positively when actually it may be negative. An excess of blood-pigment present acts similarly.

The Test

It is customary in laboratories attached to clinics where many specimens have to be dealt with weekly to collect the specimens so that they may all be examined on the same day. This saves much labour.

All solutions and dilutions are made with freshly prepared saline.

Some additional details amplifying the description given will be found on p. 592.

The Routine

1. *On the day previous to the test.*—(*a*) Collect the specimens of blood and cerebro-spinal fluid from the patients.

(*b*) Procure the sheep's blood from the slaughter-house.

2. *On the day of the test.*—(*a*) Wash or complete the washing of the sheep's corpuscles and make a 25 per cent. suspension.

(*b*) Centrifuge the specimens of blood. Pipette off the serum of each into quill-tubes and label or number. Inactivate the specimens at 56°–57° C. for half an hour.

(*c*) Bleed a guinea-pig and prepare the complement.

(*d*) Make up the dilution of Antigen (*b*, p. 586).

(*e*) Make up the hæmolytic system. We will assume that the hæmolytic serum has been tested and possesses a titre of 1 in 2,000 or thereabouts. With the $\frac{1}{10}$ c.c. pipette measure out carefully 0·01 c.c. into a test tube. Add 4·8 c.c. of saline and mix. Add 1·2 c.c. of the 25 per cent. corpuscle suspension and mix. Place in the water-bath for a few minutes for the amboceptor to become fixed. Then fill one or two of the tubes which fit the large holes in the special water-bath (see *f*. p. 583) and keep in the water-bath. With the volumes taken the hæmolytic system will contain 5 per cent. corpuscles and $3\frac{1}{2}$ times the minimal hæmolytic titre.

(If the $\frac{1}{10}$ c.c. pipette be not available, the special throttled pipette with bulb described at p. 584 may be used for making up the solution of hæmolytic serum.)

(*f*) Proceed to standardise the complement: Take 7 quill-tubes. To each add 5 volumes of saline. Prepare dilutions of complement, and to each tube in order add 1 volume of complement dilution, viz.

Tube 1.	Complement	diluted	1 in 2.
,, 2.	,,	,,	1 in 4.
,, 3.	,,	,,	1 in 6.
,, 4.	,,	,,	1 in 8.
,, 5.	,,	,,	1 in 10.
,, 6.	,,	,,	1 in 12.
,, 7.	,,	,,	1 in 16.

Add to each tube 5 volumes of hæmolytic system, mix and incubate for 15–20 minutes. Note the highest dilution of complement which gives complete hæmolysis.

For the test, use a dilution of complement about $2\frac{1}{2}$ times as strong as this. For example, if the highest dilution of complement giving complete hæmolysis be 1 in 8, for the test use complement 1 in $3\frac{1}{4}$ (*i.e.* complement, 1 volume; saline, 2·25 volumes).

As a check, put up another series with antigen solution. Take 7 quill-tubes. To each add 1 volume of saline and 4 volumes of antigen solution.

To each tube in order add 1 volume of complement and complement dilution, viz.:

Tube 1. Undiluted complement.
„ 2. Complement diluted 1 in 2.
„ 3 „ „ 1 in 3.
„ 4. „ „ 1 in 4.
„ 5. „ „ 1 in 5.
„ 6. „ „ 1 in 6.
„ 7. „ „ 1 in 7.

Mix and incubate for five minutes to allow of fixation of complement. Then add 5 volumes of hæmolytic system to each tube, mix and incubate for fifteen to twenty minutes. Note the highest dilution of complement which gives complete hæmolysis.

If the reagents are working properly, the lowest strength of complement dilution producing complete hæmolysis in the *antigen* series will be about double that acting similarly in the *saline* series. Thus, if the saline series shows complete hæmolysis in the complement dilution 1 in 8, but not beyond, the antigen series should show complete hæmolysis in the complement dilution 1 in 4 or 1 in 5, but not beyond. Any greater difference than this between the two series indicates that the antigen solution is too weak (or if in the other direction, too strong) and the antigen must be re-standardised (p. 597).

(In actual practice, it is preferable first to put up the antigen series, and while this is incubating for fixation of complement the saline series may be prepared.)

(*g*) Put up a control with a known negative non-syphilitic serum and a known positive syphilitic serum.

Make up a few volumes of the proper complement dilution as determined by the standardisation in (*f*) (*e.g.* 1 in $3\frac{1}{4}$ from the example given).

Take 6 quill-tubes in two rows of 3 each, arranged as follows :

	Negative Serum Undiluted.	Positive Serum Undiluted.	Positive Serum Diluted 1 : 2.
Back Row. Saline .	(1)	◯	(3)
Front Row. Antigen .	(1)	(2)	(3)

The *saline* series contains :

Four vols. Saline + 1 vol. Serum + 1 vol. Complement Dilution.

The *antigen* series contains :

Four vols. Antigen Solution + 1 vol. Serum + 1 vol. Complement Dilution.

After mixing, incubate all the tubes for five minutes. Then add 5 volumes of hæmolytic system to every tube and incubate for a further period of fifteen to twenty minutes.

If everything is working properly, all the saline tubes (1, 2 and 3) of the back row should show complete hæmolysis. Of the antigen tubes in the front row, Tube 1 with the non-syphilitic serum should show complete hæmolysis, while Tubes 2 and 3 with the syphilitic serum should show *no* hæmolysis.

If this be so, the specimens may then be tested. Supposing there are twelve specimens to be tested, proceed :

(*h*) Prepare a sufficiency of the complement dilution to make 24 volumes and some over (*e.g.* if the proper complement dilution is 1 in $3\frac{1}{4}$, take 12 volumes of complement and 27 volumes of saline).

Every serum to be tested is put up both in saline and in antigen so that for the twelve specimens 24 quill-tubes, arranged in two rows of 12 each, will be required. Proceed as follows :

Make the following mixtures with every specimen :

Back Row.	Front Row.
Saline, 4 vols.	Antigen Solution 4 vols.
Serum, 1 vol.	Serum 1 vol.
Complement Dilution, 1 vol.	Complement Dilution, 1 vol.

Incubate for five minutes for fixation to take place. Then add to every tube 5 volumes of hæmolytic system, mix, and incubate for a further period of fifteen to twenty minutes.

At the end of this time all the saline tubes in the back row should show complete hæmolysis.

Of the antigen tubes in the front row, the negative sera will show complete hæmolysis, the positive sera will show *no* hæmolysis.

A certain number of sera (perhaps 5 per cent. of all sera) will show fixation with saline as well as with antigen. If this be the case make two or three dilutions of the serum, *e.g.* 1 in 2, 1 in 4 and 1 in 6, put up each dilution in a saline tube and treat in the same manner as in the test. In this way the lowest dilution of serum which does not fix complement in saline is ascertained and this same dilution is then tested in antigen for fixation.

(*i*) It is well now to test the *degree* of fixation of the positive sera. Proceed :

With each positive serum make dilutions of 1 in 2, 1 in 4 and

1 in 8. Put up 1 volume of each of the respective dilutions in a separate antigen tube only and treat as for the test.

A serum which gives complete fixation undiluted only may be marked +, one which gives fixation undiluted and in dilution of 1 in 2 as + +, one which gives fixation undiluted and in dilutions of 1 in 2 and 1 in 4 as + + + and so on.

A serum which gives fixation undiluted only and none in a dilution of 1 in 2 (provided of course that the saline tube gives no fixation) must be regarded as a somewhat doubtful positive. It is very exceptional for a definite positive not to give some fixation in a dilution of 1 in 2.

Frequently an intermediate dilution may give partial fixation, *i.e.* it is partially but not completely hæmolysed. We may report the results, therefore, in some such fashion as the following, the figures referring to the dilutions :

PATIENT X"

Wassermann Reaction. Positive + +.
Complete Fixation, 1–1, 1–2.
Partial Fixation, 1–4.
Complete Hæmolysis, 1–8.

When cerebro-spinal fluid is to be tested, the procedure is the same as described in Section *h*, with the exception that *two* volumes of the fluid are taken instead of one volume.

Notes on the Test

General Remarks.—Either complete fixation without any hæmolysis or complete hæmolysis is obvious enough. Partial hæmolysis is also soon readily determined with the naked eye ; in cases of doubt the tubes may be centrifuged for two to three minutes ; hæmolysis of even slight degree leads to some staining of the fluid above the deposited corpuscles.

A porcelain basin containing saline is kept at hand. From it the saline for making up antigen solution, hæmolytic system and complement dilutions and for the quill-tubes is pipetted. The throttled pipettes are also rinsed with it between each different reagent pipetted and each specimen of serum, etc. ; a little experience will soon teach when rinsing is required.

Two or three thicknesses of white filter-paper should be placed in front of the worker. On this background the measurement

of the volumes with the throttled pipettes is much facilitated and the traces of fluid left in the pipettes are easily got rid of by placing their points upon it.

If many specimens have to be tested, two individuals working together saves much time and labour. Thus at the commencement, while one is making up the antigen solution and hæmolytic system, the other may be filling quill-tubes with saline and be preparing dilutions of complement for the standardisation.

The times of incubation in the water-bath recommended are ample. Fixation of complement is actually completed with incubation for one minute, and after the addition of the hæmolytic serum hæmolysis is completed within 20 minutes. Care must be taken to mix the constituents thoroughly in the quill-tubes.

Section (*f*).—It is not absolutely necessary to put up an antigen series as well as a saline series. The antigen series does help, however, to control the working of the antigen and saves the trouble of *frequent* standardisation of the antigen. The series of dilutions of complement suggested generally covers the range of activity of the complement. Now and then a complement may be met with of exceptional activity and in that case higher dilutions would have to be prepared and tested, but personally the Writer has not met with such an one.

In order to prepare the dilutions of complement for the saline series, we may proceed in some such fashion as the following:

I.	Dilution 1 in 2.	Take 3 vols. complement + 3 vols. saline.
II.	,, 1 in 4.	Take 2 vols. of I + 2 vols. saline.
III.	,, 1 in 6.	Take 1 vol. of I + 2 vols. saline.
IV.	,, 1 in 8.	Take 2 vols. of II + 2 vols. saline.
V.	,, 1 in 10.	Take 1 vol. of I + 4 vols. saline.
VI.	,, 1 in 12.	Take 1 vol. of III + 1 vol. saline.
VII.	,, 1 in 16.	Take 2 vols. of IV + 2 vols. saline.

In this way 3 volumes of complement serve to prepare all the dilutions required. The dilutions may be made in watch-glasses. A series may be worked out in a similar way for the antigen series.

As regards the dilution of complement to employ in the test, this will be in the neighbourhood of $2\frac{1}{2}$ times the highest dilution which gives complete hæmolysis in the saline series. For an active complement giving complete hæmolysis in dilutions of from 1 in 8 to 1 in 12, the proper dilution for the test will generally be $2\frac{1}{2}$ times that giving complete hæmolysis. For a less active complement giving complete hæmolysis in 1 in 3 to 1 in 6 the

proper dilution for the test will frequently be only twice or $2\frac{1}{4}$ times that giving complete hæmolysis. The reason for this is that the difference between the dilutions is of a less order with the higher dilutions than with the lower ones (between 1 in 2 and 1 in 4 there is a 50 per cent. difference, between 1–8 and 1 in 10 the difference is only one of 12·5 per cent.). Thus if 1 in 4 gave complete hæmolysis, and 1 in 6 gave only partial hæmolysis, it is quite likely that a 1 in 5 might give complete hæmolysis and a dilution for the test of 1 in 2 though only twice that found (viz. 1 in 4) would really be $2\frac{1}{2}$ times the actual working strength, 1 in 5.

Supposing no hæmolysis occurred in any of the complement dilutions in the saline series, this might be due to a variety of causes, such as :

1. Very poor complement. This may be tested by putting up a saline tube with undiluted complement, or, better still, by using the serum from another guinea-pig. So weak a complement should be discarded and fresh complement be obtained.

2. Too weak hæmolytic serum. This may be tested by making a stronger solution of hæmolytic serum or by using another specimen.

3. Too strong saline.

4. The wrong corpuscles are being used, *e.g.* ox corpuscles instead of sheep's corpuscles may have been obtained from the slaughter-house (this has actually happened) :

In the antigen series, the same causes might act or the antigen might be too strong.

Section (*g*).—Again, though it is not absolutely necessary to put up a control with negative and positive sera, this procedure serves as an additional safeguard that everything is working properly, and the proper complement dilution to be used may be further adjusted. Thus, if hæmolysis proceeds slowly in the salines, the strength of the complement dilution for the test may be increased by one-quarter part. In ordinary practice, a positive serum from the last batch of specimens is kept in the ice-chest for the test the following week. It is preferable to choose as a control a positive serum possessing moderate fixative powers, *e.g.* fixing in dilution of 1 in 2 or 1 in 4 but not beyond. The negative serum may be similarly reserved from the last batch of specimens, or a serum may be obtained from one of the laboratory staff on the morning of the test and be inactivated along with the other sera.

Section (*h*).—It is not necessary to put up a saline tube as well as an antigen tube with each specimen, but any specimen which reacts positively in antigen must also be tested in saline to ascertain that it does not fix without antigen. This may be done when the three dilutions are being tested in antigen for the degree of "positiveness." If a third or more of the specimens are expected to be positive, it takes little more time to put up a saline control for every specimen.

In hot weather, the tubes containing the complement and the complement dilution prepared should be kept in iced water. If many specimens have to be tested mixtures of saline and of antigen with complement dilution may be prepared by mixing 4 parts of saline or of antigen with 1 part of the proper complement dilution, a sufficiency of each being prepared so that 5 volumes of each mixture may be allowed for every specimen. In this case, 1 volume of the specimen is put into each of two quill-tubes and then 5 volumes of the saline-complement mixture are added to one and 5 volumes of the antigen-complement mixture are added to the other. If this procedure be adopted, the antigen-complement mixture should be used as quickly as possible after preparation.

For preparing the dilutions for ascertaining the degree of "positiveness," 2 volumes of the serum are placed in a watch-glass and 2 volumes of saline are added, giving a dilution of 1 in 2. Two volumes of this dilution are then placed in a second watch-glass and 2 volumes of saline are added, giving a dilution of 1 in 4. Finally 2 volumes of the second dilution are placed in a third watch-glass and 2 volumes of saline are added, giving a dilution of 1 in 8.

Instead of watch-glasses, artists' sunk porcelain palettes may be used for preparing these dilutions.

Standardisation of Hæmolytic Serum and Antigen

Any new specimen of hæmolytic serum and of antigen must be standardised before use, and subsequently at occasional intervals.

For standardisation the same apparatus will be required as for the test itself and the total volume of the mixtures should total eleven unit volumes.

Standardisation of the Hæmolytic Serum.

Method.—Dilutions of the hæmolytic serum are prepared

above and below what is expected to be its minimal hæmolytic titre. To each dilution fresh guinea-pig serum, diluted with saline 1 in 2, is added, together with sheep's corpuscles, and the mixtures are incubated. In this way the highest dilution of the hæmolytic serum which completely hæmolyses under the conditions of the test is ascertained.

If nothing is known as to the titre of the hæmolytic serum, a preliminary test may be done with dilutions of the serum at wide intervals, *e.g.* 1–100, 1–500, 1–1,000, 1–1,500, 1–2,000, and 1–2,500. Having thus found between which limits the titre lies, another test is done with dilutions between these limits.

Assuming that the titre of the hæmolytic serum may be 1 in 2,000 or thereabouts, proceed :

Obtain guinea-pig serum, preferably from two guinea-pigs, and dilute with saline 1 in 2. With the $\frac{1}{10}$ c.c. pipette measure out accurately into a small test-tube 0·05 c.c. of the hæmolytic serum and add 4·95 c.c. of saline ; this gives a dilution of 1 in 100. Measure out 1 c.c. of this dilution into a small test-tube and add 9 c.c. of saline ; this gives a dilution of 1 in 1,000.

Into each of 5 small test-tubes measure out 1 c.c. of this 1 in 1,000 dilution and add to the tubes the following amounts of saline and of 20 per cent. suspension of corpuscles :

	Saline	Corpuscles	Total Volume =
Tube A.	0·2 c.c.	0·4 c.c.	1·6 c.c.
,, B.	0·35 c.c.	0·45 c.c.	1·8 c.c.
,, C.	0·5 c.c.	0·5 c.c.	2·0 c.c.
,, D.	0·65 c.c.	0·55 c.c.	2·2 c.c.
,, E.	0·8 c.c.	0·6 c.c.	2·4 c.c.

There will now be present in all the mixtures 5 per cent. of corpuscles, and hæmolytic serum in strengths respectively of 1 in 1,600, 1 in 1,800, 1 in 2,000, 1 in 2,200 and 1 in 2,400. In this way five hæmolytic systems, A–E, will be prepared, differing only in their content of hæmolytic serum. To each of 5 quill-tubes (numbered 1 to 5) add 5 volumes of saline and 1 volume of the complement (1 in 2). Then to the tubes in order add 5 volumes of the respective hæmolytic systems, 5 volumes of hæmolytic system A to Tube 1, 5 volumes of B to Tube 2, and so on. The constituents are well mixed in each tube and the tubes are then incubated for fifteen to twenty minutes.

If the hæmolytic serum has a titre of 1 in 2,000 or thereabouts, some such result as the following may be expected :

Tube 1.	Dilution	1 : 1,600 =	Complete hæmolysis.
,, 2.	,,	1 : 1,800 =	,, ,,
,, 3.	,,	1 : 2,000 =	,, ,,
,, 4.	,,	1 : 2,200 =	Partial ,,
,, 5.	,,	1 : 2,400 =	Trace of ,,

From this we may assume that the titre of the hæmolytic serum is actually 1 in 2,100. For the test, therefore, it would be used 1 in 600, *i.e.* three and a half times the minimum hæmolytic titre.

If no, or only partial, hæmolysis were given with these dilutions of the hæmolytic serum, then stronger dilutions would have to be tested ; if there were complete hæmolysis in all the tubes, then weaker dilutions would have to be tested.

Standardization of Antigen

The McIntosh and Fildes antigen, which is recommended to be used, remains remarkably constant for months at a time. This or any other antigen may be standardised by the following method.

Method.—Make up with saline several dilutions of the antigen above and below *twice* the strength which would probably be used for the test. Make mixtures of saline, antigen dilution and complement (the proper dilution of the latter as determined by the standardisation in saline described in Section (*f*), p. 589 in quill-tubes, incubate for five minutes for fixation to take place. Then add hæmolytic system (as used for the test) and incubate for fifteen to twenty minutes. Note which is the highest dilution of antigen which gives complete fixation.

Requisites.—Complement dilution and hæmolytic system as used in the test.

The Process.—Assume that the antigen will work properly in a dilution of about 1 in 14. Prepare hæmolytic system and standardise complement in saline and prepare the proper dilution of complement indicated thereby, as described under the test. Make dilutions of antigen with saline 1 in 5, 1 in 6, 1 in 7, 1 in 8 and 1 in 9. Into each of 5 quill-tubes measure out 1 volume of saline, add to the tubes in order 4 volumes of the respective antigen dilutions, then add to each tube 1 volume of complement dilution and mix. Incubate for five minutes and finally add 5 volumes of hæmolytic system, mix, and incubate

for fifteen to twenty minutes. The following illustrates the result that may be obtained :—

Tube 1.	Dilution of	Antigen	1 : 5 =	No hæmolysis.
,, 2.	,,	,,	1 : 6 =	,, ,,
,, 3.	,,	,,	1 : 7 =	,, ,,
,, 4.	,,	,,	1 : 8 =	Partial hæmolysis.
,, 5.	,,	,,	1 : 9 =	Nearly complete hæmolysis.

From this it is seen that the highest dilution of the antigen which completely fixes complement is 1 in 7. *For the actual test the antigen is used about half this strength*, viz. 1 in 14–1 in 16.

Porges' reaction.—If syphilitic serum be added to a solution of lecithin or other lipoid substances, in many cases it gives a white precipitate. Normal or non-syphilitic serum gives no precipitate. This has been tried extensively as a substitute for the Wassermann reaction, but it is not so delicate.

Class III.—Infusoria (Ciliata)

The Infusoria are protozoa the locomotive organs of which consist of cilia, and in which the nuclear apparatus is differentiated into a vegetative macronucleus and a generative micronucleus. The cytoplasm is enclosed within a cuticle, an oral aperture is present in the form of a slit or pore, and waste matter is extruded by a pore, constant in position, but, as a rule, visible only when in use. A contractile vacuole is generally present. Reproduction usually takes place by fission, which is preceded by division of the two nuclei, the micronucleus by mitosis, the macronucleus by direct division.

The Infusoria are not of much pathological importance, but are common in ponds and ditches, *e.g. Paramecium* and *Vorticella*.

Balantidium (Paramecium) coli

This is an intestinal parasite of swine, occasionally met with in man in conditions associated with chronic diarrhœa and dysentery.

It is somewhat ovoid in shape, the ends being bluntly pointed, is covered with cilia, measures 65 to 85 μ in length, and has a superficial resemblance to the ordinary *Paramecium*.

According to Saville Kent, the *Balantidium coli* is to be distinguished from the ordinary forms of water paramecia by the

following characters : The *Bal. coli* is somewhat spindle-shaped or ovoid, and bluntly pointed at each end, one and a half to twice as long as broad, measuring $\frac{1}{3\,6\,0}$ in. to $\frac{1}{1\,6\,8}$ in. in length ; the *paramecium* is more cylindrical, four times as long as broad, measuring $\frac{1}{1\,2\,0}$ in. to $\frac{1}{9\,6}$ in. in length. The oral aperture in *Bal. coli* is near one extremity (Plate XXIII, *u*) ; in *paramecium* it is situated at about the middle of the ventral surface. In *Bal. coli* the cilia round the oral aperture are as long again as those over the body generally ; in *paramecium* the whole of the cilia are of the same length.

The *Bal. coli* seems undoubtedly sometimes to be a cause of dysentery.[1] *Bal. coli* is a common parasite of pigs and man may contract infection from these animals.

Examination of Flagellated and Ciliated Forms

(1) These may be examined fresh in the fluid in which they are present, by mounting on a slide, and covering with a cover-glass one edge of which rests on a bristle to avoid pressure.

(2) Permanent mounts may be made by the Heidenhain method (p. 558).

(3) Films may be made in the ordinary way, and stained with weak carbol-fuchsin or Leishman's stain. (The organisms are apt to be distorted.)

(4) The following method, devised by Rousselet (*Journ. Quekett Microscop. Club*, 2nd series, vol. vi, no. 36, p. 5, March, 1895) for preserving Rotatoria, may be tried. In those forms which are non-contractile, kill by adding a drop of $\frac{1}{4}$ per cent. osmic acid, wash immediately in water, and preserve in $2\frac{1}{2}$ per cent. formalin. Contractile forms may be first narcotised by adding a drop or two of 2 per cent. cocaine solution, then killed with the osmic and preserved as before.

Class IV.—Sporozoa

The sporozoa are exclusively endoparasitic protozoa, the adult lacking organs for locomotion and for the capture of food, and multiply by some method of sporulation, often very complex. Binary fission is almost unknown in this group. A parasite during the nutritive or "trophic" phase, when it is absorbing

[1] Strong and Musgrave, *Johns Hopkins Hosp. Bull.*, vol. xii, 1901, p. 31 ; Bureau of Gov. Laboratories, Manila, *Bull.* 26, 1904.

nutriment and growing at the expense of its host, is termed a *trophozoite* ; when it is mature and ready for sporulation it is termed a *sporozoite* or *schizont*. The spores are of various kinds, and may develop outside the body or in a second host.

Order.—Coccidiidea

The Coccidiidea, with a single exception, are intra-cellular during the trophic stage, and present a dimorphism or alternation of generations ; the one is endogenous and asporular, determining the reproduction of the parasite within the host, the other exogenous and sporular and permitting of infection.

Coccidial Disease of Rabbits

This is a disease caused by a sporozoon, the *Coccidium* (*Eimeria*) *oviforme* or *cuniculi*, and often met with in warrens and hutches ; in some of the former as many as 90 per cent. of the animals may be affected. The young animals suffer most, and become infected when they cease to suckle and commence to eat green food, the adult animal as a rule resisting the disease. The affected animals waste, suffer from enteritis, and a large proportion die in from one to three weeks, the condition being known as " wet-snout " among the keepers. The parasites occur in the intestine, bile-ducts, and liver in large numbers. Each parasite is ovoid in shape, measuring 36 μ in length and 22 μ in breath, is enclosed in a firm translucent cyst, which encircles a very granular protoplasm. Sometimes this protoplasm becomes condensed so as to form a spherical mass lying free within the cyst (Fig. 58, A). In the intestine and bile-ducts the parasites are attached to the epithelial cells, and in the liver, if the animal lives beyond the acute stage, set up some remarkable changes. The affected liver is studded with greyish-white nodules varying in size from a pin's head to a pea. On making sections and examining them microscopically, it is found that these nodules consist of dilated bile-ducts filled with a much hypertrophied and convoluted mucous membrane, which forms branched projections covered with cubical epithelium, among which the parasites occur in great numbers (Plate XXVII, *a*). A curious fact is that subcutaneous or intravenous inoculation, or inoculation into the liver of a healthy rabbit with the coccidia from another rabbit, fails to induce the disease.

The coccidium has a complicated developmental history, and

infection only seems possible in one of the stages. In order to study the life-cycle the parasite must be placed under suitable conditions, and an infusion of rabbits' fæces, kept at the ordinary temperature, is perhaps as good a cultivating medium as any, the changes being watched by means of interlamellar films. Reproduction may be either asexual or sexual, and may be endogenous,

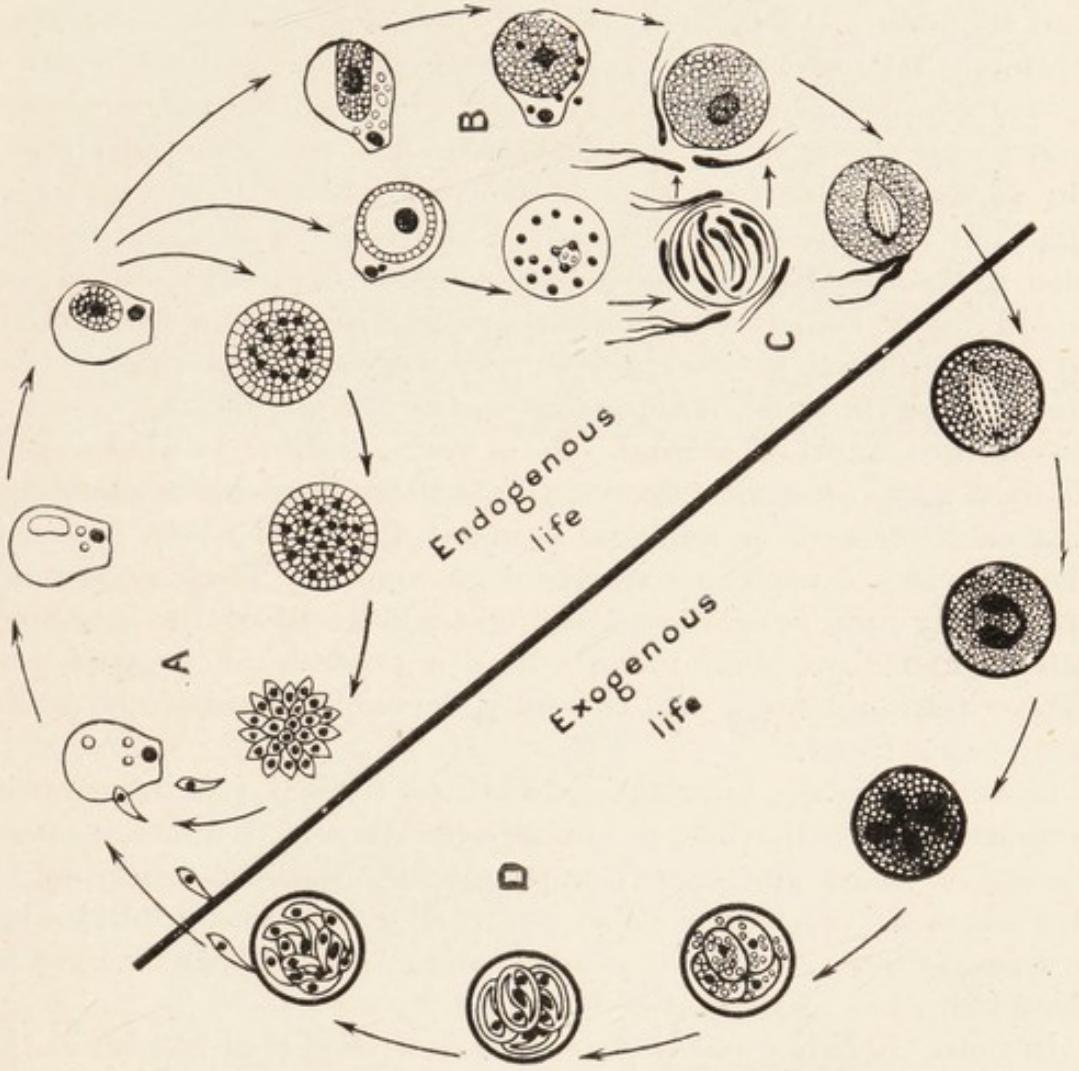

Fig. 58.—Diagram of Development of Coccidia.[1]

within the host, or exogenous, outside the host. In the asexual cycle, division of the protoplasm and nucleus of the coccidium takes place and the cyst comes to contain large numbers of spores (Fig. 58, A). The cyst-wall then ruptures, the spores are liberated, pass into other intestinal or hepatic cells and reproduce the

[1] This diagram is reproduced by permission from Daniel's *Tropical Medicine and Hygiene*, 2nd ed. 1913 (John Bale, Sons, and Danielsson).

coccidium once more (Fig. 58, A). In the sexual cycle, the protoplasm of some coccidia remains undivided with a single nucleus and the cyst has a weak spot, known as the micropyle ; these are the female cells or macrogametes (Fig. 58, B). In other coccidia, the protoplasm having attained maximum growth, divides into a mass of actively motile thread-like bodies, the male elements or microgametes. The cyst-wall then ruptures and the microgametes, penetrating the micropyle of the macrogametes, fertilise them. In the fertilised macrogamete, which is a zyote known as an " oocyst " and is non-motile, the micropyle closes and the cyst is discharged with the fæces of the animal. On damp ground, the nucleus and protoplasm divide into four spherules. Each spherule becomes elongated, and again divides into two somewhat crescent-shaped bodies, around each pair of which a new, somewhat spindle-shaped capsule forms (Fig. 58 D). In this condition the parasite is very resistant, and may remain alive for six months, undergoing no further change unless introduced into another animal. If a young rabbit swallows with its food these crescentic spores, the enclosing capsule is dissolved, and each crescent becomes a rounded amœboid mass, and this again divides up into many crescentic spores. These spores are apparently motile, and enter the epithelial cells of the intestine, gall-bladder, and bile-ducts, where a process of growth and differentiation occurs, and the fully developed parasite is ultimately reproduced.

Coccidial disease, or, as it is sometimes termed, psorospermosis, is occasionally met with in animals, as the sheep, and a wasting disease of young pheasants due to coccidia has been described by McFadyean.[1] Coccidiosis also occurs in grouse and poultry, due to *Eimeria avium ;* in the latter causing " scour," which may be attended with considerable loss.

In man, coccidial disease has been described (but rarely) in the liver, gall-bladder, ureter, etc.[2]

Rixford and Gilchrist[3] described two cases of protozoan infection of the skin and organs, accompanied by great destruction of tissue and ending in death. The organisms were spherical, 7 to 27 μ diameter, surrounded by a thick capsule, enclosing granular bioplasm (*C. immitis*).

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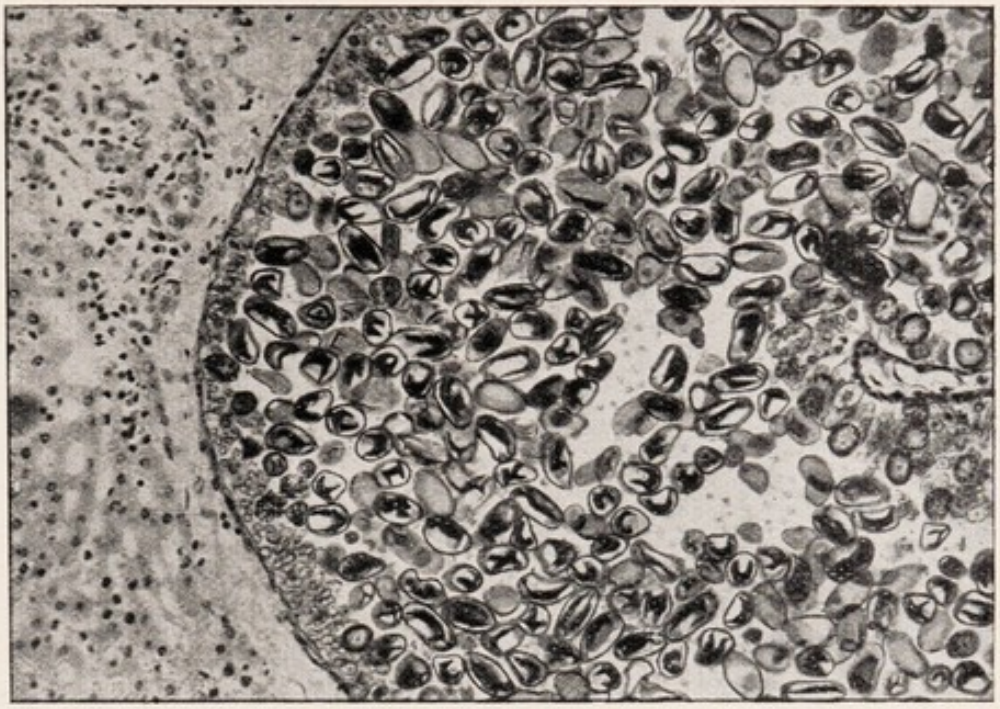
[1] *Journ. Comp. Path and Therapeut.*, 1895.

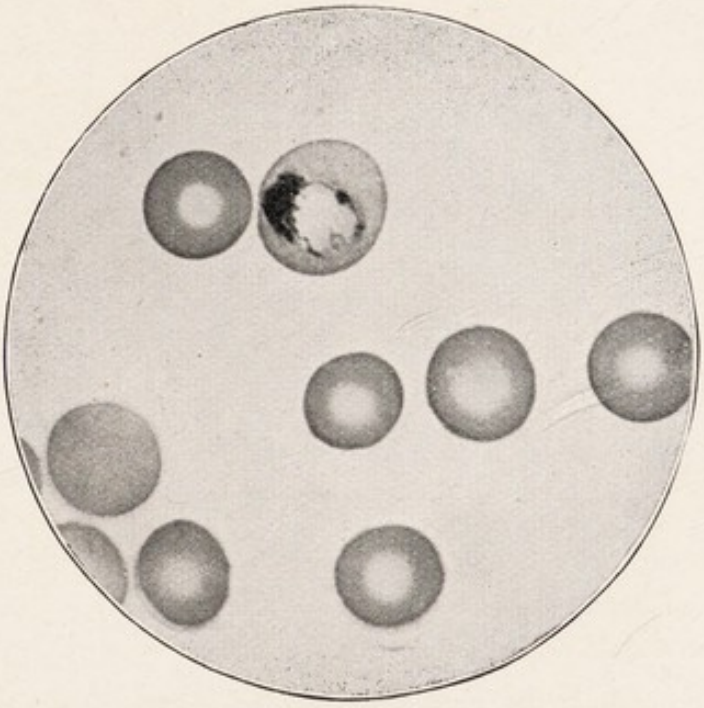
[2] *Journ. Comp. Path. and Bact.*, 1898, June, p. 171.

[3] *Johns Hopkins Hosp. Reps.*, vol. i, 1896, p. 209.

PLATE XXVII.



a. Coccidium oviforme. Section of rabbit's liver. × 350.



b. Malaria. Parasite of benign tertian fever. Note the enlarged corpuscle bearing the parasite. Smear of blood. × 1000.

The Ruffer-Plimmer bodies of cancer were at one time believed to be coccidia (p. 646).

The term "psorospermosis" has been applied to human infection with coccidium, Sarcosporidia (p. 623), etc.

Examination

(1) The coccidial forms are readily examined in the fresh state. The only bodies they are likely to be mistaken for are certain ova.

(2) Paraffin sections of rabbit's liver containing coccidia may be stained much in the same way as tuberculous tissues—viz. warm carbol-fuchsin ten minutes, decolorise *cautiously* in 5 per cent. acid, and counter-stain in methylene-blue. Sections may also be stained in the Ehrlich-Biondi stain for one to two hours.

Order.—Hæmosporidia

The general characters of this group are:

(1) Life at the expense of the red blood-corpuscles, at least during a portion of the life-cycle.

(2) Endogenous multiplication by spores, by which the life-cycle is repeated within the host.

(3) Development of a form which becomes free in the plasma, and which is the commencement of a sexual cycle to be completed in a second host.

(4) Inoculability, but only from one animal to another of the same species.

The group includes the malaria parasite and similar parasites in mammals and birds, the hæmogregarines, *Drepanidium* of the frog, and perhaps the Piroplasmata.

Malaria

Malaria is caused by parasitic protozoa belonging to the genus *Plasmodium* (*Hæmamœba*), the credit of the discovery of which must be given to Laveran.

Infection in malaria is transmitted by certain mosquitoes. Inoculation of malaria-free individuals with the blood of malarial patients reproduces the disease, and the parasites are found in the blood of the inoculated

persons. Inoculation experiments on all animals except man have proved negative, and in the latter the inoculation must be intravenous.

In the various forms of malarial fever the parasites have the same general characters, though there are distinct differences between them, by which they can be recognised and the type of fever differentiated. In each there is an endo-corporeal cycle within the host, through which the recurrent attacks are developed; there is also an extra-corporeal cycle of development outside the body of the host, whereby the infection of fresh individuals becomes possible. Each of these cycles needs separate description.

If the blood of a malarial patient is examined an hour or two before, or at the very commencement of, the febrile paroxysm, the parasite will be recognized as a pale, ill-defined mass of protoplasm within the red corpuscles, of which a variable proportion are infected, the size of the parasite varying in the different types of fever. When some hours old a variable number of blackish pigment-granules of melanin make their appearance. These subsequently coalesce into smaller groups, and the latter again into one or two larger, more or less centrally disposed, masses. The parasites exhibit more or less amœboid movement, and the melanin granules are frequently in a state of tremor. Later on most of the parasites (now schizonts) become divided into a variable number of segments, which separate and become spherical, the blood-corpuscle breaks down, the spherical bodies or spores are set free, and a certain number of them, again becoming attached to red corpuscles, develop into the first stage of the parasite. The melanin granules and some of the spores are ingested by phagocytes, and after some time the melanin is deposited in the spleen and liver.

The parasite, termed a *plasmodium*, or better, an *amœ-*

bula, contains a vesicular nucleus and a nucleolus, and the melanin granules are present in the surrounding protoplasm. When segmentation occurs, each segment contains a portion of both the nucleolus and the protoplasm. The maturation of each " brood " of parasites is coincident with a fresh febrile paroxysm.

In the subtertian (pernicious) form of malarial fever there exist in the blood for some time after the subsidence of the acute paroxysms well-marked non-motile bodies with rounded ends, in shape like a slightly curved sausage ; these are the so-called " crescentic bodies " or " crescents." The long diameter of the crescent is greater ($\frac{4}{3}$) than that of a red corpuscle, the protoplasm is finely granular, and contains at about the centre several well-marked pigment-granules. The extremities of the crescent often appear to be joined by a delicate bowed membrane (Fig. 64, *f* and *j*, and Plate XXIX, *b*) ; this is the remains of the blood-corpuscle in which the parasite has developed.

When a " wet " specimen of malarial blood from a case of pernicious or sub-tertian malaria is kept under observation (p. 618), it not infrequently happens that after a time the so-called flagellated " bodies " make their appearance. These consist of a central protoplasmic mass attached to which are from one to six delicate flagella measuring 20–30 μ in length (Fig. 59, *c*). The flagella are actively motile and disturb the corpuscles, but the body itself does not move much. Frequently one or more of the flagella break away and swim free, remaining active for several hours. The flagellated bodies are never seen in the freshly drawn blood, and Ross found that flagellation does not occur if the finger be pricked through a spot of vaseline, the blood remaining covered with the film of grease. Careful observation has shown that the flagellated bodies develop from " crescents " in subtertian malaria, and from special rounded parasites, difficult to

distinguish from the schizonts, in the benign tertian and quartan fevers.

Various theories were held in the past as to the nature of these flagellated bodies. Through the brilliant researches of Ross, which have been confirmed and extended by observers in all parts of the world, it is now known that these cells are sexual elements. The flagellated body represents the male cell or "male gametocyte," the flagella ("gametes") being analogous to the spermatozoa of higher animals. The female cells or female gametocytes

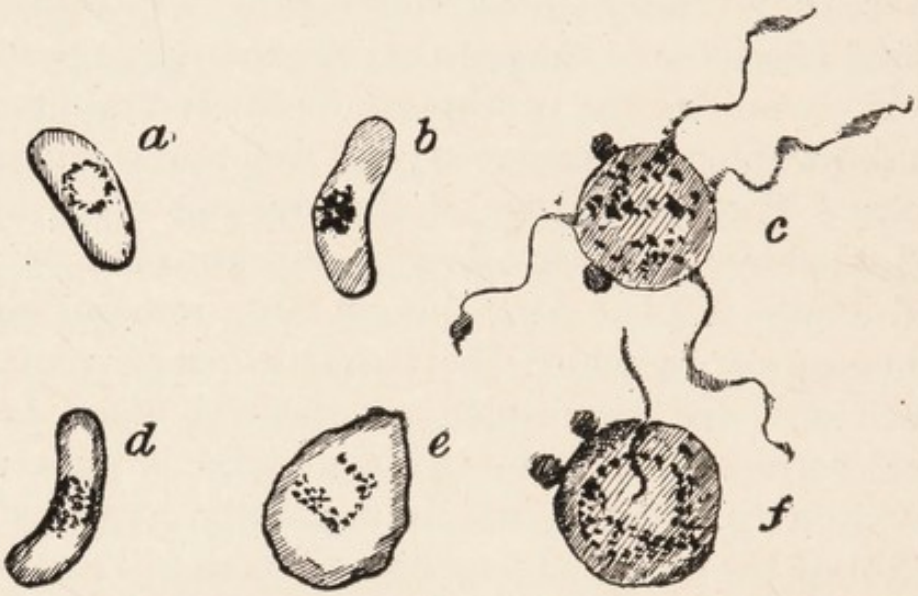


FIG. 59.—Development of the malaria parasite in the mosquito. *a*, *b*, and *c*, the male gametocyte; *d*, *e*, and *f*, the female gametocyte; *f*, fertilisation of the female gametocyte by a microgamete. (After Ross and Fielding-Ould.)

or gametes are non-flagellated, and are fertilised by the entrance of one of the flagella of a male gametocyte. *This fertilisation takes place in the stomach* (middle intestine) *of certain species of mosquito*, and after fertilisation a series of changes ensues resulting in the formation of spore-like bodies, which are injected when the insect bites its victim, and thus the infection of fresh individuals with the malaria parasite takes place. The first demonstration of the nature of "flagellated bodies" was given by Opie and MacCallum

on the *Halteridium*, a parasite of pigeons, and this forms a good example of the value of abstract research to practical medicine (see p. 619. Ross also followed the development of the malaria-like *Proteosoma* of sparrows, etc., in the mosquito, *Culex fatigans*. The development of the malaria parasite of man in the mosquito is as follows, according to Ross and Fielding-Ould.[1] It is not known what determines whether an amœbula will become a sporocyte or a gametocyte. When the sexual cells or "gametocytes" are ingested with the blood by the mosquito, they pass into the middle intestine. Within a few minutes the corpuscles enclosing them break down, the parasites are set free, and quickly become spherical or ovoid (Fig. 59, *c*, *e*, and *f*). One or two spherical granules are often attached to the naked parasites, and may represent polar bodies (Fig. 59, *c* and *f*). Very soon the male cells flagellate (Fig. 59, *c*), and before long the flagella or "microgametes" break away from the parent cell, and by their own motility make their way through the liquor sanguinis. Should one come in contact with a female cell or "macrogamete," it enters the latter and fuses with its nucleus (Fig. 59, *f*), fertilisation is thereby completed, and a "zygote" is formed. As the zygote at this stage is motile it is known as a "travelling vermicule" or "ookinet"; it passes into the outer wall of the mosquito's stomach, where it becomes encysted (Fig. 60, *a*, *b*). At this period the zygote is about 7–8 μ in diameter. If development proceeds, it acquires a distinct capsule and begins to grow rapidly, and when mature at the end of a week or more, according to the temperature, is 60 μ in diameter, and projects into the body-cavity of the insect (Fig. 60, *b*). Its substance next divides into eight to twelve portions, or "zygotomeres," then each zygotomere becomes a spherical body, or "blastophore" (Fig.

[1] *Thompson Yates Laboratories Report*, vol. iii, pt. ii, p. 183.

60, *c*), and each blastophore develops upon its surface a number of spindle-shaped radically disposed bodies, or "zygotoblasts" (Fig. 60, *d*). When the zygote reaches maturity the blastophores disappear, leaving its capsule packed with large numbers ("thousands") of free zygotoblasts. The capsule then ruptures, and the zygotoblasts

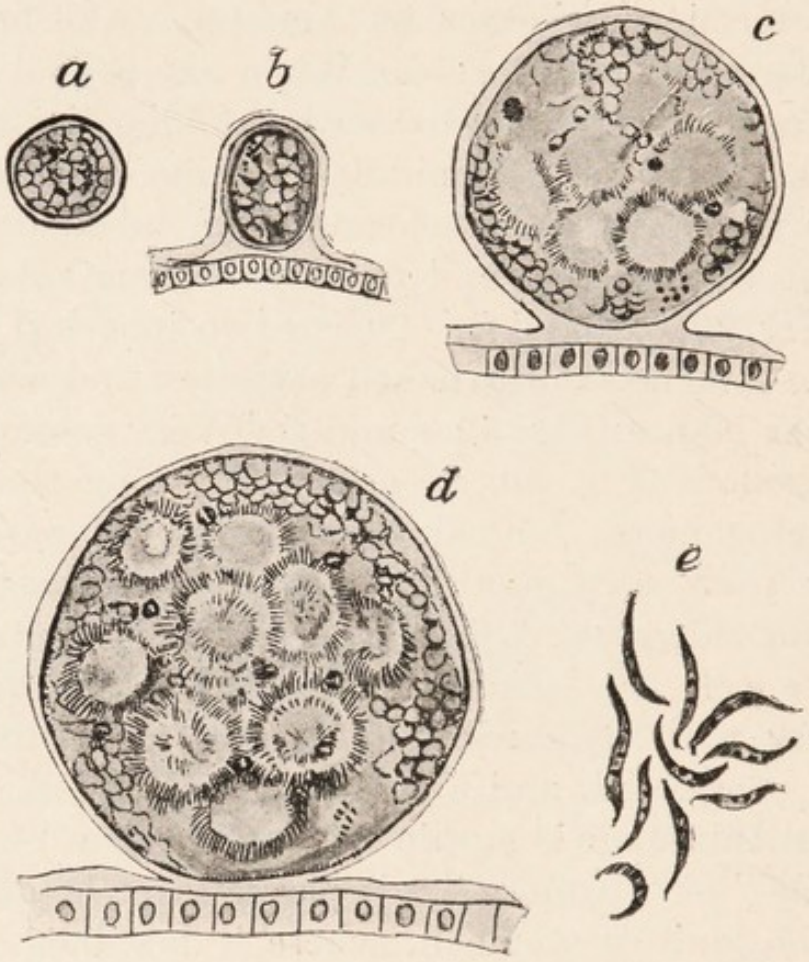


FIG. 60.—Development of the malaria parasite in the mosquito. (After Ross and Fielding-Ould.)

are poured into the body cavity of the mosquito. The "blasts" measure 12–16 μ in length, taper at each extremity, and possess a central nucleus (Fig. 60, *e*), and they make their way to all parts of the body of the host, and accumulate in the salivary or poison glands, whence they are discharged by the middle stylet (hypopharynx)

of the proboscis, when the insect " bites," into the circulation of a fresh vertebrate host. Here, presumably, the blasts become attached to erythrocytes and develop into amœbulæ. The diagram [1] (Fig. 61) represents in graphic

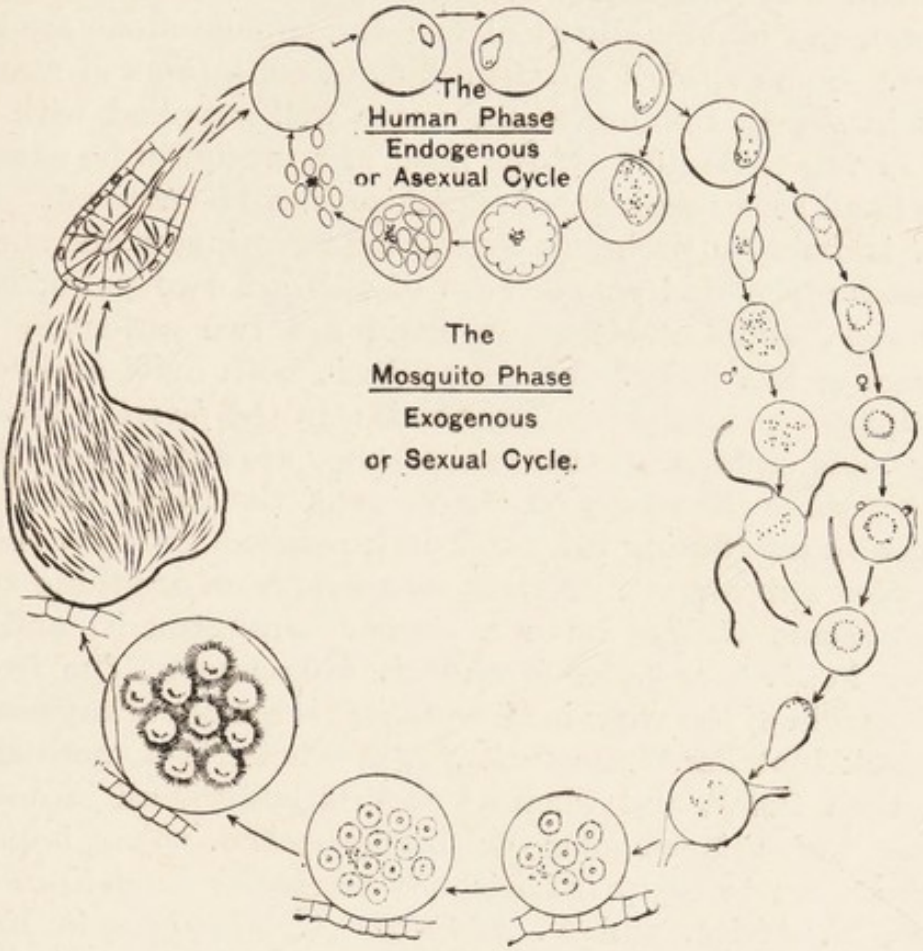


FIG. 61.—Diagram of the asexual and sexual cycle of the malaria parasite.

form the asexual and sexual cycles of reproduction of the malaria parasite.

So far as is known, malarial infection is conveyed only through the bite of infected mosquitoes of the sub-family *Anophelinæ*. It has been repeatedly proved that infected mosquitoes convey infection, and that human beings

[1] This figure is reproduced by permission from Daniels' *Laboratory Studies in Tropical Medicine* (Bale, Sons, & Danielsson, 1908).

protected from mosquito bites may live in the most malarious districts without contracting the disease.

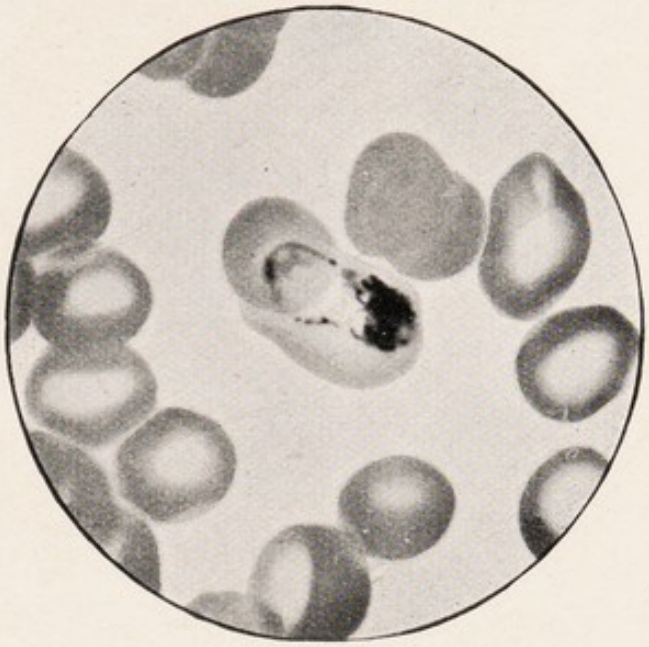
Mosquitoes (*Culicidæ*) are distinguished from other mosquito-like insects by the fringe of scales on the wings. The common mosquitoes belong to the sub-family *Culicinæ*. The *Anophelinæ* are usually less abundant (but there is great variation in different districts), and bite mainly at night ; the females alone are blood-suckers. Some species breed in natural collections of stagnant, others in slowly running fresh, water well supplied with lowly forms of vegetable life. If the head of a mosquito be examined with a hand-lens, three sets of appendages will be noticed. In the middle is the stout proboscis containing the stinging and suctorial apparatus ; situated at the base of this are two palpi, one on either side, and outside these again are two antennæ, which are more or less hairy. In *Anophelinæ*, both male and female, the palpi are as long as the proboscis ; in the *female Culex* (also in *Stegomyia* and many other genera) they are short and stumpy. In *Anophelinæ* the scales on the veins of the wings are usually arranged in alternating light and dark patches, giving a speckled or dappled appearance, different as a rule from anything seen in *Culex*. (Some *Culices* have a similar arrangement, and it is wanting in *A. maculipennis* and *A. bifurcatus*.) The front or costal margin of the wing in *Anophelinæ* is almost always marked with dark blotches. *Anopheles*, as a whole, is a more slender insect than *Culex*, and when at rest its body is all in one line, whereas *Culex* is angular or hump-backed. The important species known to carry malaria are *Anopheles maculipennis* in Europe, N. Africa, and N. America, *A. bifurcatus* in Europe, *Myzomyia funesta* and *Pyretophorus costalis* in Central and W. Africa, and *Cellia argyrotarsis* in tropical America. Other species, *e.g. Myzorhynchus sinensis*, *Cellia Kochii*, and others, are less important carriers.

(On Mosquitoes, see Theobald, *Brit. Museum Monograph*, and Allbutt's *System of Med.*, ed. 2, vol. ii, pt. 2 ; Giles, *Handbook of the Gnats and Mosquitoes ;* Daniels, *Laboratory Studies in Tropical Medicine*, ed. 3, 1908.)

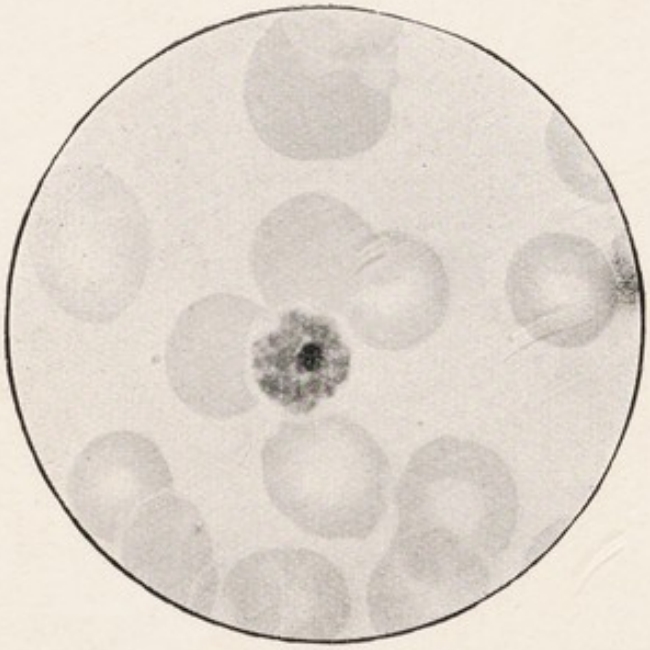
There are probably at least three species of malaria parasite [1] occurring in the various types of malarial fever

[1] Hewlett, *Trans. XIVth Internat. Congress of Hygiene*, vol. ii, 1908, p. 141.

PLATE XXVIII.



a. Malaria. Parasite of benign tertian fever. Smear of blood. × 1500.



b. Malaria. A tertian "rosette." Smear of blood. × 1500.

in man, though some authorities (*e.g.* Laveran) regard the forms as varieties of a single species, and the following are the differential characters between them :

(1) *Benign quartan fever* (Fig. 62).—This is a relatively uncommon form of malaria, being frequent only in certain districts. The quartan parasite (*Plasmodium malariæ*) completes its asexual life-cycle in seventy-two hours ; there are two complete days without an attack, and reckoning the day of the previous attack, an attack occurs every fourth day, hence the name " quartan." It com-

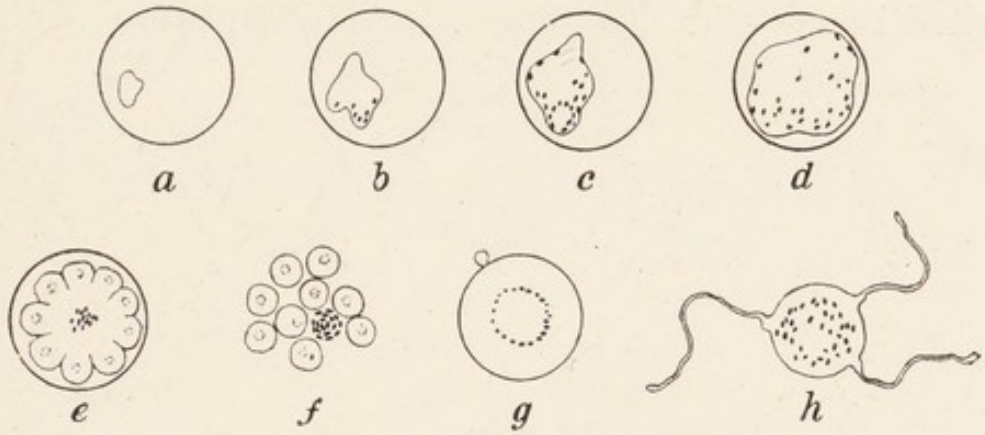


FIG. 62.—The quartan parasite : *a*, *b*, *c*, *d*, amœbulæ ; *e*, sporocyte ; *f*, free spores ; *g*, female gametocyte with so-called polar body ; *h*, male gametocyte. (After Rees.)

mences as a small amœbula, which is feebly motile. It enlarges, becomes pigmented, and motility ceases, the pigment-granules being numerous and coarse. The parasite finally occupies nearly the whole of the corpuscle, which, however, is but little altered (*a–d*).

Towards the end of the apyrexial period the pigment collects in the centre, and segmentation takes place with the formation of a symmetrical rosette (*e*), and afterwards of six to twelve spores (*f*). The quartan parasite does not form crescents, and the flagellated bodies (*h*), which are rarely seen, are developed from large pigmented parasites.

(2) *Benign, or spring, tertian fever* (Fig. 63; Plate XXVII, *b*).—This is the commonest form of malaria. The benign tertian parasite (*Plasmodium vivax*) completes its asexual life-cycle in forty-eight hours, an attack occurring every other day, or, reckoning the day of the previous attack, every third day. In the early stage it resembles the quartan, but shows much more active amœboid movement. The pigment-granules are also finer than in the quartan, and incessantly change their position. The parasite finally invades the whole corpuscle, which becomes enlarged and pale. Enlargement of the corpuscles is a marked feature in the benign tertian infection (*d*).

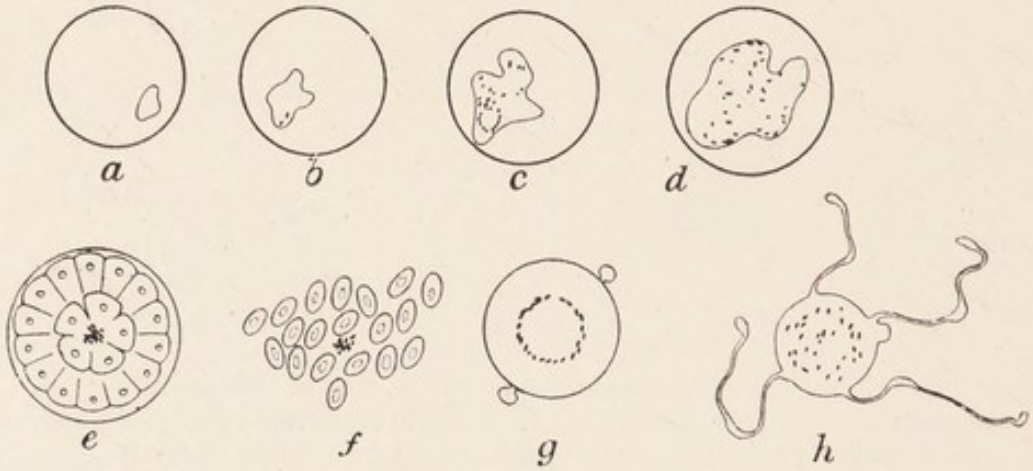
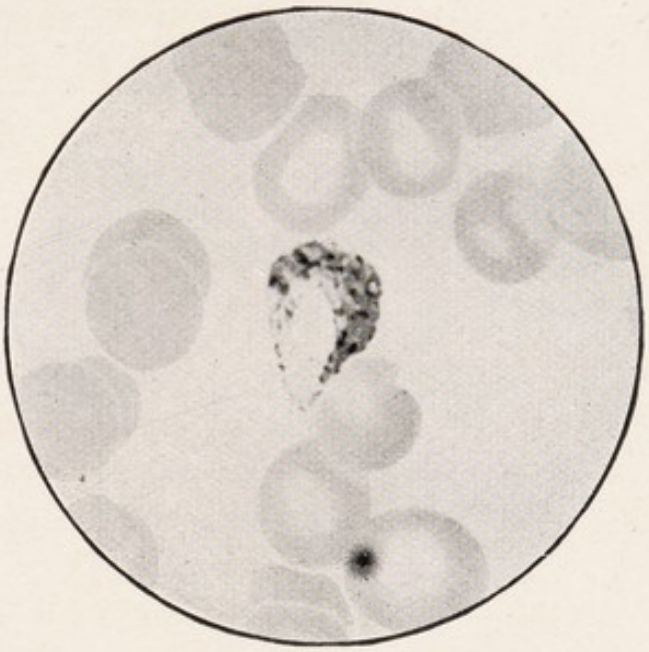


FIG. 63.—The benign tertian parasite: *a*, *b*, *c*, *d*, amœbulæ; *e*, sporocyte; *f*, free spores; *g*, female gametocyte with so-called polar bodies; *h*, male gametocyte. (After Rees.)

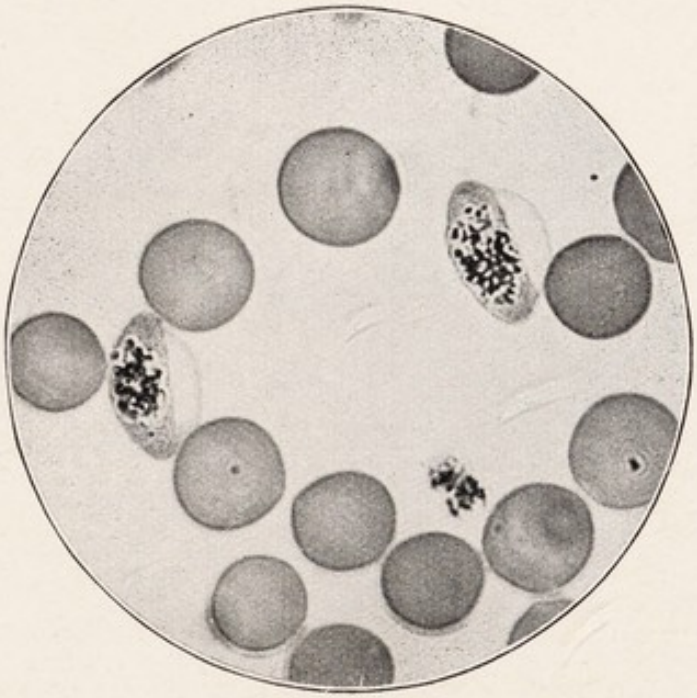
Segmentation takes place, but is unsymmetrical (*e*), resulting in the formation of a grape-like cluster of twelve to twenty spores (*f*). As in the quartan, no crescentic bodies are developed, and the gametocytes (*g*, *h*) are similar to, but larger than, the quartan (Plate XXIX, *a*).

(3) *The æstivo-autumnal, malignant, pernicious, or subtertian, fevers* (Fig. 64).—This is a common form of malaria. This parasite (*Laverania malariæ*) (or parasites, for it has been divided into three species by the Italian observers, viz. the pigmented and the unpigmented quotidian and

PLATE XXIX.



a. Malaria. Gametocyte of benign tertian parasite. Smear of blood. × 1500.



b. Malaria. "Crescents" of sub-tertian parasite. Smear of blood. × 1000.

the malignant tertian, but this is not generally accepted) is much smaller than the quartan or benign tertian, and when it reaches the stage of multiplication it disappears from the peripheral blood and collects in the internal organs, spleen, liver, cerebral capillaries, and bone-marrow. It is actively amœboid, seems to change its position within the corpuscle, and the pigment-granules are very fine in the young parasites, but early aggregate into large

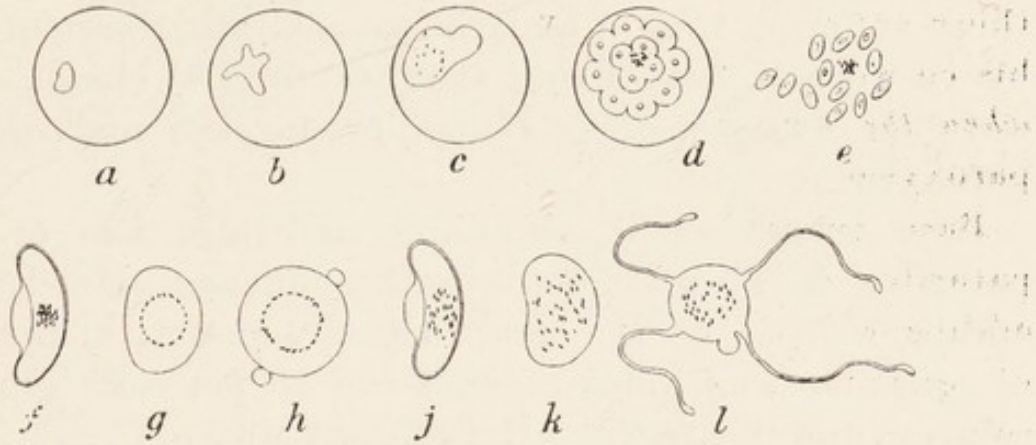


FIG. 64.—The sub-tertian parasite: *a*, *b*, *c*, amœbulæ; *d*, sporocyte; *e*, free spores; *f*, *g*, *h*, female gametocyte; *j*, *k*, *l*, male gametocyte. (After Rees.)

clumps. The fission forms (*d*, *e*) are only met with in the internal organs. Multiple infection of the corpuscles may also occur. The corpuscles often suffer severely from the infection, some being shrivelled and spinous, others dark in colour, "brassy"; they may also be altered or destroyed without being actually invaded by the parasite. It is in this form that the crescentic bodies appear (*f*, *j*, and Plate XXIX, *b*). These, however, are not met with at the very commencement of the attack, but appear in a week or so, and may not disappear until some weeks after the termination of the attack. This parasite is met with in the sub-tertian, or so-called malignant, types of fever, which are characterised by irregularity of the fever, considerable blood destruction, often accompanied by

hæmoglobinuria, and cachexia ; coma is another complication in certain instances, probably caused by massing of the parasites in the cerebral capillaries (Plate XXX, *a*).

The cure of malaria by quinine is regarded as being due to a poisonous action on the parasites analogous to that exerted on numerous protozoa, amœbæ, for example, being injuriously affected by so little as a 1–50,000 solution of quinine hydrochlorate.

No toxin can usually be demonstrated in the blood of those suffering from a malarial attack, but Rosenau and his co-workers have found that the filtered blood, *taken when the temperature is rising*, produces a malaria-like paroxysm.

Bass succeeded in obtaining multiplication of the parasites *in vitro* by defibrinating infected blood, and adding a little of the defibrinated blood to a mixture of equal parts of human serum and 25 per cent. glucose solution and incubating anaërobically at 37° C.

About 12 per cent. of malaria cases *not* suffering from syphilis are stated to give a positive Wassermann reaction, but only during the acute stage.

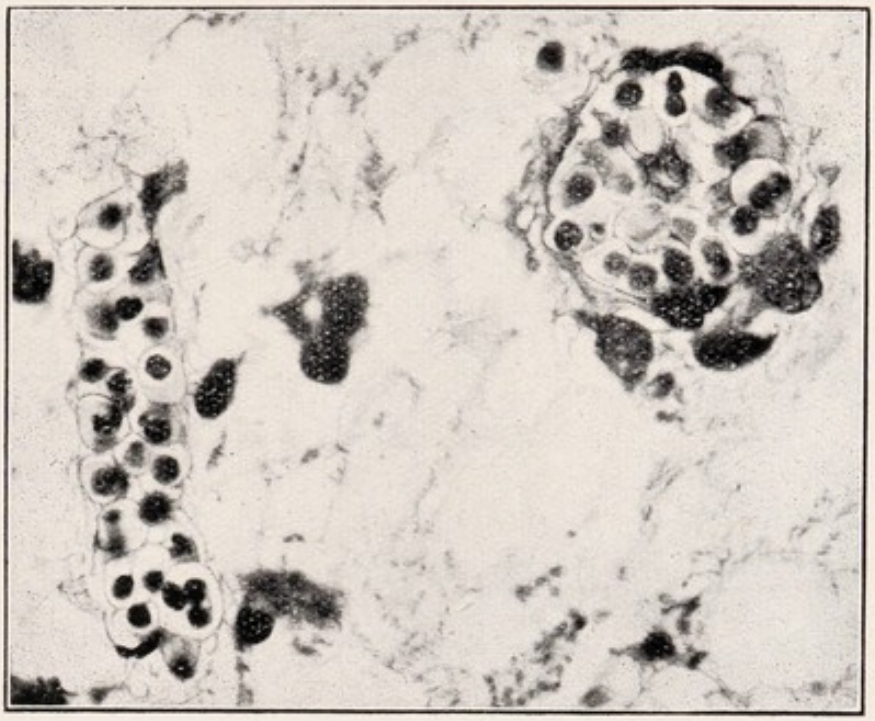
A malaria-like parasite (*Plas. Kochii*) occurs in apes, in which it produces fever.

The nature of Blackwater fever, so called from the presence of hæmaturia and hæmoglobinuria, has given rise to much discussion. By some it is considered to be a disease *sui generis*, of unknown etiology. By others it is regarded as a form of malaria, either of an intense type, or in which the kidneys are especially involved, or as due to malarial infection *plus* quinine. It may be that under particular conditions, of the nature of which we are at present ignorant, hæmolysins may be set free and cause hæmolysis, the blood-pigment being eliminated by the kidneys.

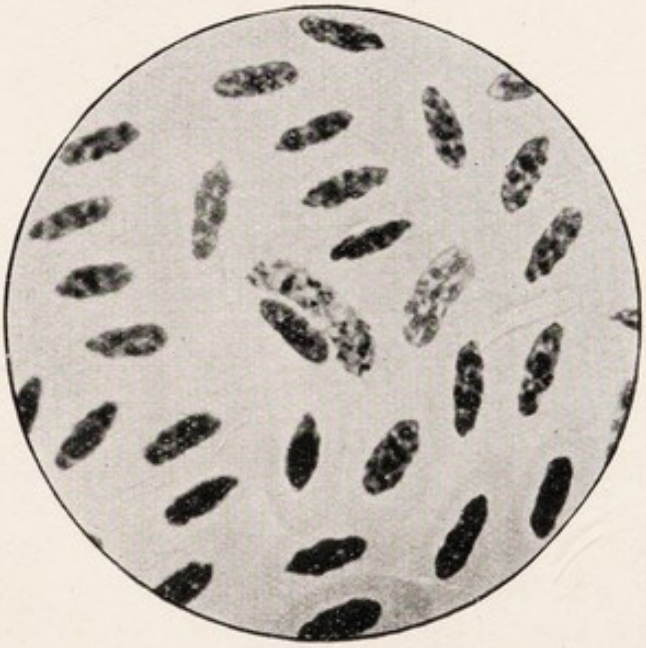
Clinical Examination

The blood of malarial patients may be examined either in the unstained or stained condition.

Examination in the unstained condition.—The finger or lobe of



a. Malaria. Section of brain of a comatose case, showing parasites in the corpuscles in the brain capillaries. × 1000.



b. *Halteridium Danilewskyi*. Smear of pigeon's blood. × 1500.

the ear is pricked, and a droplet of blood taken up on a clean cover-glass, which is then placed upon a slide, so that the droplet of blood spreads out into a thin layer between the two glasses. The cover-glass may then be ringed with oil or vaseline to prevent evaporation. A little practice is required to judge the right quantity of blood. The preparation should be examined with a $\frac{1}{12}$-inch oil-immersion lens.

Examination in the stained condition.—To prepare stained films of the malaria or other blood parasites, *e.g.* trypanosomes, the finger or ear is pricked and a droplet of blood is taken upon the edge of the *end* of a slide (the spreader), which is then applied to the surface of a second slide and, holding the spreader at an angle of 45°, it is *pushed* along the surface of the second slide so that a thin film of the blood is left behind, and the process is repeated for as many films as are required. A little practice is required to gauge the right quantity of blood. Other methods of preparing blood-films are to deposit a droplet of blood on a cover-glass; another cover-glass is applied, and the two are separated so that each is smeared with a thin film of blood, or a droplet of blood on a slide may be spread with a cigarette paper, or with the shaft of a needle. Whatever method is adopted, the film is allowed to dry in the air, and may then be fixed (not if Leishman's stain is used). In order to fix, the smears should be immersed in a mixture of equal parts of absolute alcohol and ether for not less than ten minutes, preferably for half an hour; this gives excellent results. In hot countries a saturated solution of corrosive sublimate may be used. The methods detailed at p. 107 may also be employed.

Staining is usually carried out with Leishman's stain (No. 12, p. 112). The blood films, *unfixed*, are flooded with a few drops (5–10) of the stain, which is spread by tilting, and in hot weather the preparation should be covered with a capsule to prevent evaporation. After a half to one minute distilled water is added and mixed with the stain, in sufficient amount to produce an abundant precipitate, and the mixture should appear pinkish; the water should be about double the amount of stain used, and staining is continued for five, or in some cases for ten, minutes. The staining should be continued until the nuclei of the leucocytes are a rich purple when examined with a low power. The film is then rinsed with distilled water, a little distilled water is left on the film, which is watched under the low power until the red corpuscles appear red; this takes half a minute or more. The

water is now tilted off the film, and the slide on edge allowed to dry, or it may be blotted and dried. Fresh films stain better than old ones ; if the films are old, staining with the diluted stain should be prolonged for ten or fifteen minutes and differentiation with distilled water may take five minutes. Jenner's, Giemsa's or May-Grünwald blood-stain may be similarly used.

The Writer is indebted to Dr. A. C. Coles, of Bournemouth, for the following method of staining blood-parasites.

In order to obtain good stained films of blood containing parasites it is essential to have good slides, well cleaned, a film of blood spread as uniformly as possible, and to avoid any precipitation of the stain on the surface of the film.

Slides are best cleaned with whiting or Creta preparata, made into a paste with water, or with Windowlein, a preparation used for cleaning windows. Rub the whiting thinly over the surfaces of the slide, and when dry rub off with a clean cloth.

The impedimenta required for staining the blood film are :

1. Drop bottle of about ℥iij capacity containing distilled water ;
2. Pipette bottle of about ℥ij to ℥iij capacity for the staining solution ;
3. Bottle of Giemsa's staining solution ;
4. Bottle of Merck's pure methylic alcohol ; both well corked ;
5. A Politzer's bag ; and preferably, though not essential,
6. A curved piece of window glass, 8 inch × 4 inch.

Into the perfectly dry pipette bottle pour some of the Giemsa's solution, and add about twice as much pure methylic alcohol ; shake up and keep well stoppered.

Drop from the pipette bottle just enough of the diluted Giemsa's solution to cover the film. Allow it to act for about ten to twenty seconds [if longer, especially in a hot climate, the alcohol evaporates and precipitates the stain].

Then drop on as much distilled water as the slide will hold—that is, about eight times as much water as stain—allow the stain and distilled water to mix, and stain for the requisite time.

It is better, however, in order to prevent the precipitation of the stain, to pour off the diluted stain and water from the film on to the surface of a piece of slightly curved plate-glass, and immediately place the slide, film side downward, on this. The duration of staining varies according to the temperature of the room and the nature of the film—generally speaking, ten to twenty minutes

give excellent results ; but a good plan is to remove the film, flood off the stain with distilled water, and examine under low power. If the nuclei of the leucocytes are of a ruby-red colour the staining is successful. If they are blue, the film is insufficiently stained, and it should be replaced on the staining fluid ; if they are blackish red, it is too deeply stained for most purposes, and all that is required is to pour distilled water on the surface, watching the effect (easily seen by holding the slide over a piece of white paper), and as soon as the whole film is faintly pink the staining will be good. This method of staining, generally known as Giemsa's new method, closely resembles Leishman's, but very much more distilled water is added.

The exact tint or colour of the objects stained in this way will depend largely on the reaction of the distilled water used to dilute the stain. If the water is acid (as most distilled water is) the red blood-corpuscles are stained reddish, if alkaline they are often bluish in colour.

When the film has been sufficiently stained, do not pour off the stain and then wash, but flood off the stain with distilled water and so avoid any deposition of precipitate.

When the film has been quickly washed, it is essential to dry it as quickly as possible, otherwise decolorisation proceeds. The films should not be dried with filter or blotting-paper ; it tends to leave fluff. They are best dried by blowing on the surface with air from a Politzer's bag.

Films of blood which have been kept for some time, especially in the tropics, will never stain well. Films should therefore be stained at once, and they will keep indefinitely in a dry place. The method of packing stained or unstained films face to face or wrapped in paper is a barbarous one ; the surfaces soon get scratched and dirty. The best plan is to pack them back to back in a racked box, or if this is not at hand, stick a small piece of gummed paper at the end of the slide on the film side, and when this is thoroughly dry, but not before, they can be packed together.

It is essential that the films should be absolutely dry before they are mounted, and if they are mounted in Canada balsam or cedar-oil they will sooner or later fade and be perfectly useless. The best plan is to mount them in parolein or liquid paraffin as described by Coles (*Lancet*, April 1, 1911), which has also been advocated by Giemsa.

If the above-named stains are not available staining may also

be done in a half-saturated aqueous solution of methylene-blue or in Löffler's blue for half an hour, washing in water, and counter-staining with a very weak eosin solution for a few seconds, washing and drying. Manson recommends treating the films with a very weak acetic acid—two or three drops to the ounce of water—to dissolve out the hæmoglobin, and, after washing, staining in the following solution for half a minute :

Borax	5 parts
Methylene-blue	0·5 part
Water	100 parts

washing, drying, and mounting in xylol balsam.

Hæmatoxylin (Ehrlich's, or Mayer's hæmalum) is preferable for permanent preparations, and in hot countries, where methylene-blue rapidly fades. The preparations may be counter-stained with a weak solution of eosin.

Ross recommends for rapid diagnosis the use of *thick* blood films, from which the hæmoglobin is first removed with very dilute acetic acid ; the films are then stained with Leishman's stain, and examined with a $\frac{1}{6}$-inch objective. Practice is required for this method.

In order to demonstrate the flagellated organisms Manson recommends the following procedure : Thirty or forty strips of thick blotting-paper (3 inches by $1\frac{1}{2}$ inch), each having an oblong hole ($\frac{7}{8}$ inch by $\frac{2}{3}$ inch) cut lengthways in the centre, are prepared, moistened with water, and laid on a sheet of window glass. A patient is selected in whose blood the crescentic form is plentiful, and a minute droplet of the blood, about the size of a pin's head, is expressed from a prick. A clean slide is then breathed on, and the droplet of blood picked up on it and spread out with a needle so as to cover an area $\frac{3}{4}$ inch by $\frac{1}{2}$ inch. The slide is immediately inverted over a blotting-paper cell and pressed down sufficiently to secure perfect apposition. The rest of the paper cells are similarly covered with blood-charged slides. In from half to three-quarters of an hour the slides are removed and dried by gentle warming, and then fixed with absolute alcohol for five minutes. The alcohol is allowed to evaporate, and the films are treated with a few drops of 15 per cent. acetic acid to dissolve out the hæmoglobin. The slides are then washed in water and stained with weak carbol fuchsin (20 per cent.) for six to eight hours, washed in water, dried, and mounted.

N.B.—Negative results in the examination for the malaria

parasite must be accepted with caution unless repeated. A single undoubted parasite is sufficient to establish the diagnosis. Quinine causes the disappearance of the parasite. The parasites in the sub-tertian fever disappear during the apyrexial intervals (except the crescents), and are most likely to be found at the commencement of the attack—*i.e.* when the temperature is rising. The parasites of the other forms are larger and more obvious during the apyrexial intervals.

[For further particulars on Malaria and on the demonstration of the malaria parasite, see Daniels' *Laboratory Studies in Tropical Medicine*, 1908.]

Plasmodium præcox

Syn. *Proteosma Grassii*, *Hæmamœba relicta*.

This parasite (commonly called "proteosoma") is met with in sparrows and other birds, in which it invades the red blood-corpuscles, and its structure and development are practically identical with those of the benign malarial parasites of man. It grows from a minute granule into an amœboid plasmodium, which ultimately segments and forms a rosette. In some specimens of blood flagellated male gametocytes make their appearance, similar to those of malaria, the flagella break away from the main mass, fertilise other non-flagellated or female cells, and a series of changes ensues analogous to those occurring in the malaria parasite (p. 605). The fertilisation and development of the fertilised cell take place in the stomach of a mosquito (*Culex fatigans*), by which the infection is transmitted to other birds.

Halteridium Danilewskyi

This is an elongated, curved parasite (also known as *Hæmoproteus* or *Hæmamœba Danilewskyi*), found in the red corpuscles of certain birds (pigeon, crow, etc.), and embracing the nucleus (Plate XXX, *b*). By some it is included among the malaria-like parasites (*Plasmodium*). At an early stage it much resembles the proteosoma, but as it grows it becomes elongated, pigment-granules appear, and are either scattered throughout the protoplasm or collect in two groups, one at each extremity. Finally, the parasite occupies nearly the whole of the corpuscle, dislocating its nucleus. The fully grown parasites may be differentiated into two forms, one of which remains almost completely un-

stained when treated with methylene-blue, the other staining deeply with this dye (Opie). When the blood is withdrawn, the corpuscles disintegrate and liberate the contained parasites, which assume a circular outline, and a certain number become flagellated. *It is only the non-staining form which becomes flagellated.* These two varieties of the parasite are the male and female cells respectively, and the fertilisation of the female cell by a free flagellum has been actually observed by MacCallum.[1] It can hardly be doubted that the development of the fertilised cells takes place in some insect, but the definitive host has not yet been discovered with certainty.

The presence of these parasites induces rise of temperature, deposition of melanin, and changes in, and enlargement of, the spleen and liver, analogous to those occurring in malaria in man.

Somewhat similar parasites are frequent in the blood of the lower vertebrates (see Plate XXXI, *b*).

The Piroplasmata [2]

Syn. *Pyrosoma*, *Babesia*.

The Piroplasmata form a somewhat anomalous group, but are usually included in the Hæmosporidia of the Sporozoa. They differ from the *Plasmodia* in the following respects : absence of pigment, non-fragmenting of the nucleolus, division into two or four only, and frequency of extra-corpuscular forms. They cause many diseases in animals, are conveyed by ticks, but are unknown in man. (A piroplasma was described as the causative organism of Rocky Mountain spotted fever by Wilson and Chowning, but the observations appear to be erroneous, see p. 639). The body of a piroplasma is typically pear-shaped (Plate XXXI, *a*), but rounded and rod forms occur. Two nuclear masses are present, one larger than the other.

The developmental cycle in the ticks has not been worked out, but Koch observed peculiar rayed forms with *P. bigeminum*, and Christophers [3] various developmental forms with *P. canis*. Miyajima states that a piroplasma of Japanese cattle (apparently

[1] *Journ. Exper. Med.*, vol. iii, 1898, pp. 79, 103, 117.

[2] See Hewlett, *Trans. XIVth Internat. Cong. of Hygiene*, Berlin, vol. ii, 1908, p. 146 ; Minchin in Allbutt's *System of Med.*, ed 2, vol. i, pt. 2, p. 86.

[3] *Brit. Med. Journ.*, 1907, vol. i. p. 76.

P. parvum) in blood broth develops into typical trypanosome forms.[1]

Piroplasma bigeminum.—This is the parasite of the well-known Texas fever of cattle, a disease which is characterised by fever, emaciation, anæmia, hæmoglobinuria, and enlargement of the liver and spleen.

The disease causes considerable loss among cattle, and is met with in various parts of the world, America, Australia, South Africa, Malaya, the Philippines, the Roman Campagna, Greece, Roumania, and North Ireland.

In the acute type of the disease a small proportion (1–5 per cent.) of the red corpuscles in the peripheral circulation contain pairs of pyriform bodies 2–4 μ in length and 1·5–2 μ in largest diameter. One end of each body is rounded, and the body gradually tapers to a point at the other end, and the pair lie close together, their tapering ends directed towards each other. A dark spherical body is present at the rounded end of the parasite.

Some of the young parasites exhibit amœboid movements when the blood is examined on a warm stage. In the internal organs the parasites are more numerous; in the kidney and liver 10–25 per cent. of the corpuscles contain them, in the heart-muscle 50 per cent. In the mild type 5–50 per cent. of the corpuscles in the circulating blood may be infected at one time or another, and the parasite appears in some cases as a coccus-like body at the periphery of the corpuscle. This appears to become enlarged and spindle-shaped, then to taper in the middle, divide, and so give rise to the pyriform bodies. Occasionally minute free coccoid bodies are seen in the plasma, and at times two to five minute (0·5 μ) coccoid cells are present in the red cells. After death the pyriform bodies seem to become spherical or angular.

Sexually differentiated gametes are not known with certainty but flagellated forms have been described.

The disease is transmitted through the bites of ticks (*Rhipicephalus annulatus*, *R. australis*). The female tick, after biting an infected ox and sucking its blood, falls off and lays its eggs; the eggs hatch in two to six weeks' time, and the daughter ticks transmit the disease to other animals through their bites. The disease may be to some extent controlled by prophylactic measures designed to destroy the ticks, and to prevent infection thereby.

A partial immunity is enjoyed after an attack of the disease,

[1] *Philippine Journ. of Science*, vol. ii, 1908, p. 37.

but by repeated attacks the immunity may be rendered absolute. By inoculation with the blood of an affected animal in which the fever has subsided, a transient illness in the inoculated animal is produced together with partial immunity, and by a second or third inoculation the immunity may be much increased. The mortality from such a procedure amounts to 3–5 per cent.

P. parvum causes Rhodesian red-water of cattle. It is not directly inoculable, and is conveyed by the tick *R. appendiculatus*.

P. equi causes biliary fever in horses.

P. canis causes epidemic jaundice in dogs (Pate XXXI, *a*). It is conveyed by the ticks *Hæmaphysalis leachi* in South Africa, *R. sanguineus* in India, and *Dermacentor reticulatus* in Europe.[1] (On Ticks, see Nuttall, *Journ. Roy. Inst. of Public Health*, vol. xvi, 1908, p. 385.)

Hæmogregarina

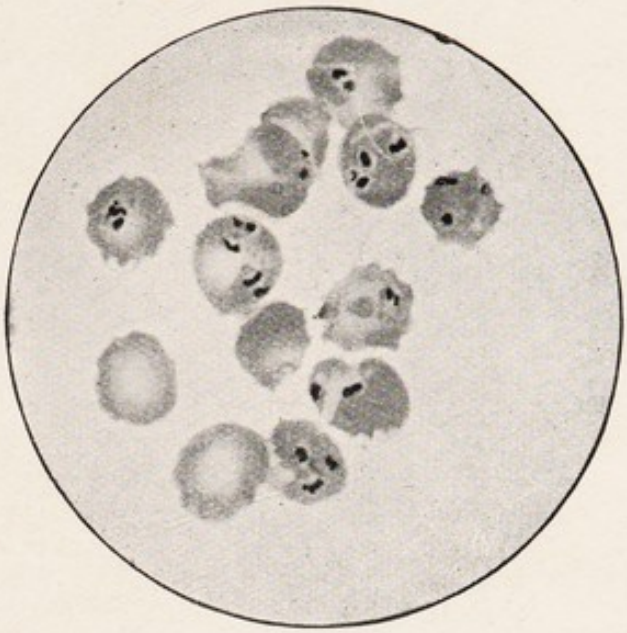
The Hæmogregarines (which must be distinguished from the Gregarines) are unpigmented parasites, not amœboid, typically having an elongated body or vermicule, occurring in the blood, mostly in cold-blooded vertebrates, but several species have of late been found in mammals (dog, jerboa, palm squirrel), though not in man. In the dog, the parasite (*Leucocytozoon canis*) occurs as an elongated, curved or doubled-up body in the polymorphonuclear leucocytes. It is encapsuled and contains a single granular nucleus. Encystment with sporulation occurs in the bone-marrow, and a sexual development is stated to occur in a tick.

Another typical form, *Hæmogregarina* (*Drepanidium*, *Lankesterella*) *ranarum* inhabits frogs (*Rana esculenta*), and possesses both an intra- and an extra-corpuscular phase. In the former the parasite occurs as an elongated gregarine-like body within the red corpuscles. The extra-corpuscular phase, commencing within the corpuscles, ends in an elongated organism possessing a vermicular movement, and free in the plasma. Similar parasites are frequent in the lower vertebrates, *e.g.* snakes.

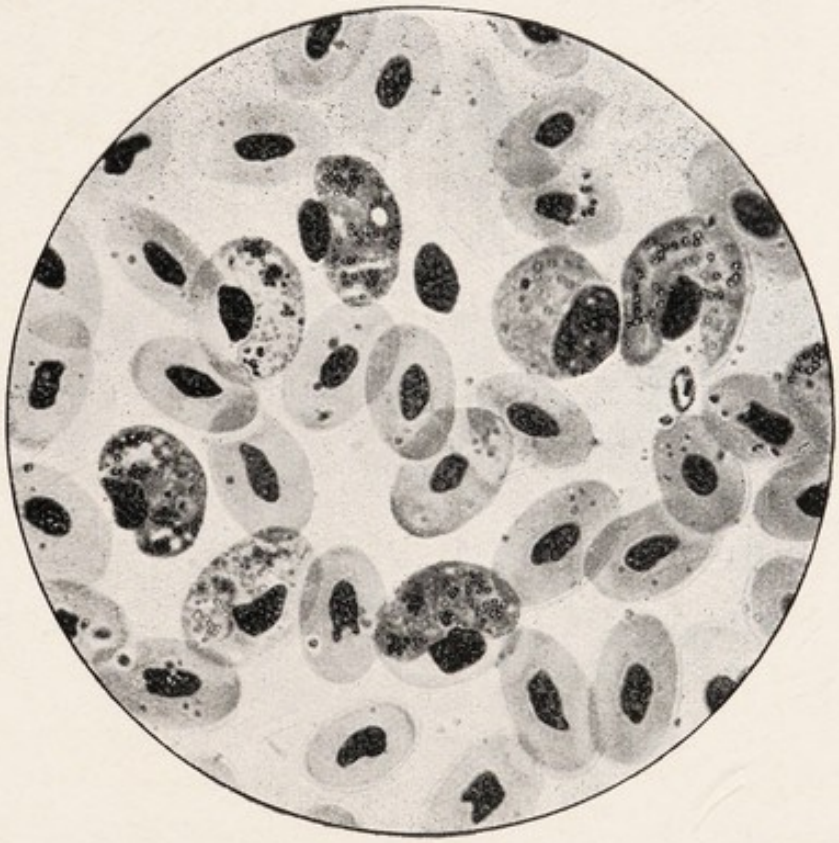
Order.—Myxosporidia

In this group the trophozoite is amœboid, and the species are almost exclusively parasites of fish, in the young stage being intracellular ("fish psorosperms").

[1] See Nuttall and Graham-Smith, *Journ. of Hygiene*, vols. iv to viii, 1904–8.



a. Piroplasma canis. Film of blood. × 1500.



b. Hæmocystidium (Hæmoproteus) najæ. Pigmented parasite of Cobra (*Naja hajæ*).

Order.—Microsporidia

The Microsporidia are cell parasites of invertebrates, especially arthropods, and the trophozoite is more or less amœboid.

Nosema bombycis causes pébrine, a disease of silkworms, which is of considerable importance commercially, for the silk industry in France was once threatened with extinction owing to its ravages. The infected worms do not grow normally, cease to eat, and die, or may form abnormal pupæ. Within the body of the affected worms a large number of roundish, highly refractile corpuscles are found. Pasteur ascertained that the disease was propagated by healthy worms eating with their food the excreta of infected ones. The moths were thus infected, and laid infected eggs. By allowing each moth to lay its eggs separately, and subsequently examining the body of the moth microscopically, he was able to separate the healthy from the diseased, and the eggs of the former were kept, while those of the latter were destroyed.

The Isle of Wight bee disease is caused by *Nosema apis*, which is mainly confined to the alimentary tract. Infection takes place by the ingestion of spores; these give rise to amœbulæ in the gut, which divide and creep about. They later penetrate the cells and become encysted and finally divide into a number of spores and as the cells are cast off they pass out of the bee with the excreta and so the virus is spread.

Another disease of silkworms is known as flacherie, but is due to a bacterium, *Micrococcus bombycis*. It is contagious, and can be transmitted by inoculation.

Order.—Sarcosporidia

The parasites belonging to this order are not thoroughly worked out. They complete their life-history in the substance of striated muscular fibres: such are the well-known Miescher's corpuscles. Few instances of this class of parasite are recorded in man, but it occurs in the monkey [1] and also in the ox. T. Smith [2] describes the characters and development of a species found in mice.

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[1] De Korté, *Journ. of Hygiene*, vol. v, 1905, p. 451.

[2] *Journ. Exper. Med.*, vol. vi, No. 1, 1901, p. 1.

A parasite, *Rhinosporidium kinealyi*, nearly allied to the foregoing, causes a polypoid condition in the nose in the tropics. If a section be made of the mass, cysts (pansporoblasts) will be seen in the deeper layers containing many refractile rounded nucleated bodies, the spores. Neither the life-history nor the mode of transmission of the parasite is known.

CHAPTER XIX

SCARLET FEVER—HYDROPHOBIA—INFANTILE PARALYSIS—TYPHUS FEVER—YELLOW FEVER—DENGUE—PHLEBOTOMUS FEVER—VACCINIA AND VARIOLA—MALIGNANT DISEASE.

Scarlet Fever

THE ætiology of scarlet fever is still unsettled. Many claim a streptococcus to be the causal agent, others a protozoon and others a filter-passer. The disease is inoculable only on apes.

The streptococcus first came into prominence in the historic Hendon outbreak which was investigated by Klein and Power in 1885. Scarlet fever occurred in Marylebone, and was traced to infection conveyed by milk supplied from a farm at Hendon. The infection could not be traced to any human source, and it was therefore concluded that the cows themselves were affected with scarlet fever, and infected the milk. A vesicular eruption was found on the udders and teats of the cows, and this was regarded as the local manifestation of bovine scarlatina. From the vesicles and crusts Klein isolated a streptococcus which, although closely resembling the *Streptococcus pyogenes* (as then known), differed slightly from it; on inoculation into calves it produced death, with lesions of the kidney resembling those of the human disease. Klein also isolated the same streptococcus in five out of eleven cases of the disease in man. Klein

and Power concluded, therefore, that scarlet fever is communicable to, and may exist in cows, the milk thereby becoming infected and conveying the disease to man, and that a streptococcus is the specific infective agent.

The Hendon outbreak was reinvestigated by Axe and Crookshank.[1] Axe found that, so far from there being no source of human infection, cases of scarlet fever had occurred near the dairy within a short time of the outbreak, and the eruptive disease of the cow was shown by Crookshank to be cowpox, while the streptococcus isolated by Klein he regarded as a variety of the *S. pyogenes*.

In 1909 a milk-borne epidemic occurred in certain districts in London and Surrey, and was traced to milk derived from one farm. The outbreak was investigated and reported on by Hamer and Jones, who again traced it to infection of the *cows*. Hunting [2] reviews the evidence and shows how little there is to support this conclusion, as there is no doubt that the family of one of the employees on the farm were suffering from scarlatina.

The existence of bovine scarlet fever is entirely discredited by the veterinary profession, both here and on the Continent.

A streptococcus was also claimed by Gordon to be the causal agent of scarlet fever, and recently Mair [3] has isolated a diplococcus which grows only on serum. On inoculation into apes Döhle's bodies appear in the blood (Döhle's bodies are small bodies in the leucocytes staining blue with Leishman's stain. They are plentiful in scarlet fever, but according to MacEwen are not confined to this disease).

[1] On the Hendon outbreak, see *Trans. Path. Soc. Lond.*, 1888 (Refs.)

[2] *Journ. Roy. Sanitary Inst.*, vol. xxxii, 1911, p. 64.

[3] *Journ. Path. and Bacter.*, vol. xix, 1915, p. 443, and vol. xx, 1916, p. 366.

Gordon [1] found that the *Streptococcus scarlatinæ* or *conglomeratus* of Klein differs distinctly in its cultural characters from other varieties of streptococci, and that it occurs constantly in the mucous secretion on the surface of the tonsils and fauces, and in the nasal, but not in the aural, discharge in scarlatina. It is also present in a somewhat modified form in the blood and tissues post mortem. It was not found in four non-scarlatinal throats examined. Gordon concluded, therefore, that the *S. scarlatinæ* or *conglomeratus* is the " specialised and essential agent " of scarlatina. It is pathogenic to mice.

Cumpston [2] investigated the biological characters of 101 streptococci isolated from scarlet fever, applying Gordon's tests (p. 256). The majority corresponded with the *S. longus* type.

Baginsky and Sommerfeld, Class and Jaques also isolated streptococcoid organisms in scarlatina, but they possessed no very distinctive cultural characters.

Bernhardt ascribed scarlet fever to a filter passer, but his experiments are inconclusive.

Mallory detected small bodies, 2–7 μ in diameter, staining delicately but sharply with methylene-blue, and occurring in and between the epithelial cells of the epidermis and in the lymph-vessels and spaces of the corium. He regards these as protozoa, but others consider them to be degenerated leucocytes (see p. 629).

The blood in the early stages of scarlatina gives the Wassermann reaction.

It is remarkable how frequently diphtheria complicates scarlatina.

Hydrophobia [3]

Hydrophobia attacking man is invariably contracted through the bite of an animal affected with the disease. In the lower animals the disease is termed rabies, and is most frequent in the dog, but the cat, wolf, jackal and deer are

[1] (*a*) *Rep. Med. Off. Loc. Gov. Board* for 1898–99, p. 480 ; (*b*) *ibid.* for 1899–1900, p. 385.

[2] *Journ. of Hyg.*, vol. vii, 1907, p. 599.

[3] See Marie, *La Rage* 1901 ; *Scientific Memoirs Gov. of India*, Nos. 30 and 44 ; Luzzani, *Ann. de l'Inst. Pasteur*, xxvii, 1913, p. 1039 (Bibliog.).

also subject to it, and other animals can be infected by inoculation. The disease may assume two forms—the raging and the paralytic. The latter is not met with in man, unless certain rare forms of acute ascending paralysis (*e.g.* Landry's) be manifestations of it. In the dog either may occur, but in rodents the paralytic form is almost always the one assumed. In man the incubation period is very variable ; it is never less than about twenty days, and possibly may be as long as two years, or even more ; the average seems to be about ten weeks. In the rabbit, after inoculation from the dog, the incubation period is about two to three weeks.

The virus resides in the central nervous system, as was shown by Pasteur. Inoculation with emulsions prepared from the medulla and with the saliva conveys the disease, but the *filtered* emulsions are usually inactive, and the other tissues and fluids of the body, excepting the lacrymals and suprarenals, are non-infective.

Remlinger [1] found that after very complete trituration the virus may pass through a porcelain filter.

No micro-organism has been demonstrated with certainty in rabies. Negri described the constant presence of structures—the Negri bodies—particularly in the grey matter of the hippocampus major, which he regards as protozoa. They are of varying size, apparently encapsuled, taking a homogeneous purplish colour in smears stained with eosin and methylene-blue, the smallest spherical and structureless, larger ones with a central granule or nucleus, the largest, round, ovoid or elongated, containing several (as many as eight) granules (Fig. 65). They occur abundantly in animals suffering from chronic rabies, but in the acute type are scanty, though still to be found ; in " fixed virus " (p. 631) they are very small. So constantly are the Negri bodies present in rabies, and

[1] *Bull. de l'Inst. Pasteur*, iv, 1904, p. 342.

absent in non-rabic conditions, that their presence or absence forms a rapid and simple means of diagnosis.[1]

Inasmuch as the rabies virus is filterable, the view taken by Prowazek of the nature of the Negri bodies is that they represent the *tissue* reaction to invasion by the parasite, the parasite being an extremely minute one contained within the Negri body and belonging to a group of the Protozoa termed the *Chlamydozoa*. In the same category he would place the trachoma bodies, the Mallory

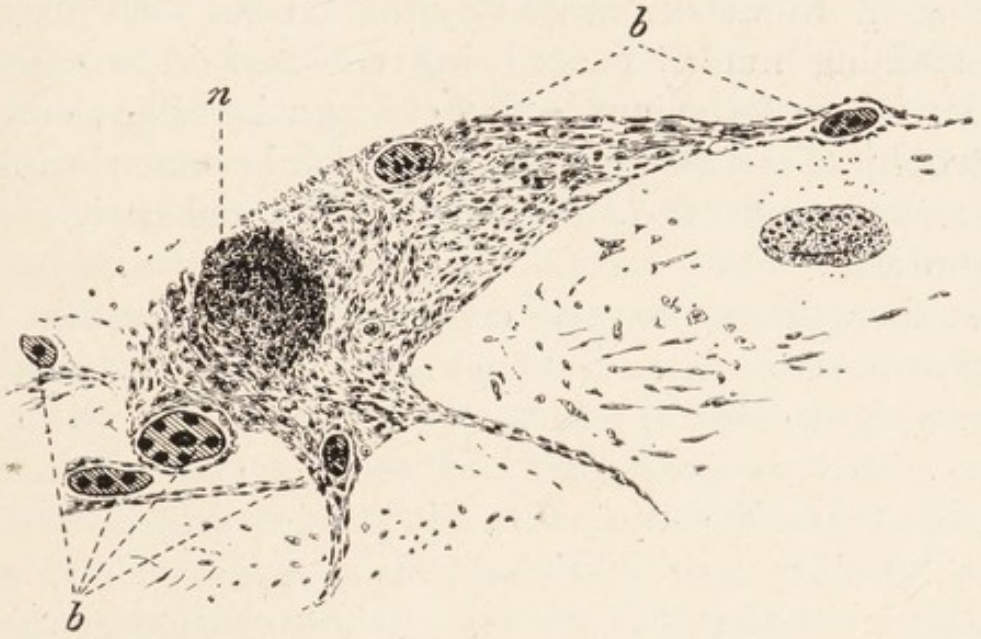

FIG. 65.—Smear from hippocampus major of rabid dog: *n*, nucleus of nerve-cell; *b*, *b*, the Negri bodies (eosin and methylene-blue). (After Williams and Lowden.)

bodies of scarlatina and the Councilman bodies of variola. Noguchi believes that the Negri bodies or derivatives from them can be cultivated in his medium used for the *Trep. pallidum* (p. 569).

Babes states that the virus is destroyed at a temperature of 60° C., but the medulla and other infective material

[1] See Williams and Lowden, *Journ. Infect. Diseases*, vol. iii, 1906, p. 452.

retain their virulence for months in glycerin. He has described certain lesions present in the medulla in cases of rabies, the so-called rabic tubercles. These consist of an invasion of the peri-ganglionic spaces by an accumulation of round-cells, with degeneration of the cells of the bulbar nuclei.

Van Gehuchten has described as pathognomonic of rabies certain lesions in the sympathetic and cerebro-spinal ganglia, especially those of the pneumo-gastric. These ganglia consist normally of a supporting tissue holding in its meshes large ganglionic cells with distinct well-staining nuclei, each being enclosed in a capsule lined with endothelium. The changes in rabies consist in atrophy of the ganglionic cells, which become shrunken and no longer fill the enclosing capsule, and their nuclei at the same time become ill-defined and stain badly. A number of new-formed cells also appear within the ganglionic capsules. Ravenel and McCarthy studied twenty-eight cases of rabies in various animals and consider that these capsular and cellular changes in the ganglia, taken in conjunction with the clinical manifestations, afford a rapid and trustworthy means of diagnosis of rabies, but that the absence of these changes does not necessarily imply that rabies is not present. They also consider that the rabic tubercle of Babes is present sufficiently often to furnish valuable assistance in cases where the central nervous system only is obtainable.[1]

Pasteur showed that the virus can be attenuated by desiccating the infective nerve matter, and in this way was able to prepare a vaccine which protects animals from otherwise fatal doses of the virus. Advancing a step further, he used his vaccines to treat individuals who had been bitten by rabid animals, but in whom the symptoms

[1] See *Journ. Compar. Pathol. and Therapeut.*, vol. xiv, p. i, 1901, p. 37.

had not yet developed, and so inaugurated the present system of anti-rabic inoculation as carried out at the Pasteur and other Institutes.

To prepare the anti-rabic vaccines, a rabbit is inoculated subdurally with an emulsion made from the medulla of a rabid dog. When the animal dies, a second rabbit is similarly inoculated from the first, and the passage through rabbits is continued until a "fixed" virus is obtained, with which the first symptoms appear on the seventh or eighth day, and which kills with certainty in about ten days. This having been attained, two or three rabbits are inoculated subdurally every day, so that there is a daily supply of animals dead of the disease. The spinal cord is removed with aseptic precautions, cut into convenient segments, and suspended in bell jars containing a layer of solid caustic potash at the bottom, which serves to desiccate them. The jars are dated, and preserved in glass cases in a dark room, kept at a constant temperature of about 23° C. In Paris the vaccine fluids are prepared by triturating portions of the dried cords in sterile broth, so as to form an emulsion—1 cm. of cord in 5 c.c. of sterile broth, of which 1 c.c. (*i.e.* 2 mm. of cord) forms a single dose. At the commencement of treatment the cords which have been dried for fourteen days are used, at the end of treatment those which have been dried for only three days; the latter are much more virulent, and would communicate the disease but for the previous treatment. The rabbits employed should all be of the same weight ($2\frac{1}{2}$ kilogrammes in Paris); if the rabbits are small, a slightly shorter period of desiccation of the cords would be necessary. The treatment varies in duration according to the severity of the case, which is gauged by the number and situation of the bites and by the species of animal. Bites on exposed parts are regarded as much more serious than those through clothing, and on the face, where

efficient treatment is difficult, than on the hands, and wolf-bites than dog-bites.

The doses are injected subcutaneously in the flank, and do not produce much constitutional disturbance. At first there is a feeling of lassitude, and considerable muscular tenderness at the seat of inoculation, which later on passes off. At Lille, where there are only a few cases under treatment at a time, the cords, after drying for the requisite period, are placed in pure sterile glycerin. In this they retain their virulence unimpaired for about a month. This method does away with the necessity for the daily inoculation of rabbits, a rabbit being inoculated occasionally as required. The system of dosage employed at the various anti-rabic stations differs somewhat; the following is that employed at Lille, 2 mm. of cord being emulsified in 5 c.c. of sterile broth, or physiological salt solution:

Ordinary Treatment.	
Day of treatment.	Days of desiccation of cord.
1 (two injections)	14 and 13
2 „	12 and 11
3 „	10 and 9
4 „	8 and 7
5	6
6	5
7	4
8	3
9 (two injections)	9 and 8
10 „	7 and 6
11	5
12	4

Ordinary Treatment.	
Day of treatment.	Days of desiccation of cord.
13	3
14 (two in ections)	9 and 8
15 „	7 and 6
16	5
17	4
18	3
For Severe Bites, in Addition	
19 (two injections)	7 and 6
20 „	5 and 4
21	3

At Buda-Pesth a dilution method has been employed; instead of drying the cords, an emulsion is made with the fresh cord, and this emulsion is considerably diluted for the earlier doses, dilutions of 1 in 10,000 to 1 in 6000,

corresponding to cords dried for from fourteen to eight days. Semple [1] has found that a carbolised emulsion of the cord may be employed as the inoculating agent. An 8 per cent. emulsion of the cord in physiological salt solution with 1 per cent. carbolic acid is kept at 37° C. for twenty-four hours. At the end of this time an equal volume of physiological salt solution is added and the emulsion bottled aseptically. This vaccine will keep for months.

Undoubtedly the Pasteur inoculations will protect animals from rabies, the duration of immunity after vaccination in the dog being at least three years. In man the efficacy of the treatment can only be judged by statistics. The mortality after bites by supposed rabid animals is variously stated, the most favourable being about 16 per cent. (Leblanc). At the Pasteur Institute, Paris, among 2730 cases treated in which the animal which inflicted the bites was proved to be rabid by inoculation experiments, nineteen deaths occurred—a mortality of 0·7 per cent. In 1910, 401 cases were treated, with no death ; in 1911, 341 cases, with one death ; in 1912, 395 cases, with no death, and in 1916, 1,388 cases, with three deaths ; mortality 0·21 per cent.

The failure of the treatment may be due to two causes : (1) delay in its commencement, and (2) a short incubation period. The principle of the treatment probably depends upon the long incubation period of the disease, owing to which it is possible to forestall the disease, and to immunise the body by the inoculations before its onset. If, unfortunately, the infective material should be very virulent, and the incubation period thereby reduced to the lower limit, it may be impossible to do this before the onset of the disease, and the same is the case if the commencement of the treatment be delayed. Pasteur's system of

[1] *Sc. Mem. Gov. of India*, No. 44.

inoculation is useless when the disease has declared itself.

By vaccinating animals by the Pasteur method by a long series of injections, and with the most virulent material, the blood-serum acquires "anti-" properties, and this "anti-rabic" serum is said to be of service in the treatment of the declared disease.

Variations from typical rabies have been described both in animals and in man under such names as "chronic rabies," "abortive rabies," etc. Harvey, Carter, and Acton [1] describe a spontaneous disease in dogs due to a general infection with *B. pyocyaneus*, which closely simulates rabies. By subdural inoculation the disease is reproduced in rabbits, with paresis of the hind legs and death in from sixteen to twenty-one days. The Negri bodies are absent, the course of the disease differs somewhat from rabies, and the *B. pyocyaneus* can be isolated from the brain and blood.

Diagnosis of Rabies

In a case of suspected rabies in a dog the animal should *not* be killed immediately, but should be kept under observation until it dies, or for three or four weeks, and then killed.

1. Moderately thin smears on slides are made from (*a*) the cortex in the region of the fissure of Rolando (the crucial sulcus in the dog), (*b*) the hippocampus major, (*c*) the cerebellum. They are dried in the air, fixed for five minutes in methyl alcohol, and then stained in weak Giemsa (1 drop stain, 1 c.c. distilled water; with 1 drop of 1 per cent. potassium carbonate solution to every 10 c.c. of the dilute stain) for three hours. The stained films are then washed in running tap-water for one to three minutes, dried with filter-paper, and examined for the Negri bodies.

Or the moist films may be fixed in methyl alcohol, and without drying stained for one minute in a mixture of 10 c.c. distilled water, 3 drops of a saturated alcoholic solution of basic fuchsin, and 2 c.c. of Löffler's methylene-blue. Eosin-methylene-blue mixtures may also be used.

The cytoplasm of the bodies stains orange, pink, red, or ma-

[1] *Veterinary Record*, July 22, 1911, p. 57.

genta, the central nuclei are granular, and appear bluish or purplish.

Luzzani considers that the Negri bodies can generally be well seen in teased up fresh material unstained. *It is stated that structures resembling the Negri bodies may be present in the brain after death from snake-bite.*

2. If the Negri bodies cannot be detected, inoculation should be performed. The brain should be removed as soon as possible, and if it cannot be manipulated immediately, should be placed in sterile glycerin. From the middle of the floor of the fourth ventricle a small piece about the size of a pea is removed ; this is triturated and thoroughly emulsified in a sterile watch-glass by means of a sterile glass rod with a bulbous end, a little sterile broth being used to make the emulsion, and sufficient being added to measure about 10 c.c. The hair on the head of a good-sized rabbit is cut close, the animal is anæsthetised with ether, the skin on the scalp reflected and a trephine hole made through the skull. The centre of the trephine hole should be in the middle line, and on the line drawn between the posterior corners of the eyes, the diameter of the trephine being about $\frac{3}{16}$ inch. A little of the emulsion is drawn up in a small syringe, having a fine needle, and two or three drops are injected beneath the dura mater. The operation is carried out with antiseptic precautions, the wound closed, and a little wool and collodion dressing applied.

If the material injected be from a rabid animal, the first symptoms will be noticed in from ten to fourteen days. The inoculated animal loses control over its hind legs and throws them about peculiarly when running. This increases, and in another day or so the animal is apt to fall when running, and in another day or two the hinder extremities become paralytic, and the animal is unable to move, and dies shortly. The onset of symptoms is hardly ever delayed beyond twenty-one days.

Van Gehuchten's method.—The ganglion is placed in absolute alcohol for twelve hours. the alcohol being changed once ; it is then embedded, and sections are cut. These are stained for five minutes in Nissl's methylene-blue and mounted. Or the material may be fixed in 10 per cent. formalin before staining. The capsular changes are best shown by staining with hæmatoxylin and eosin.

Babes' method.—A piece of the medulla or cord is hardened in alcohol and stained with anilin red, and sections are prepared.

Infantile Paralysis [1]

Infantile paralysis or acute anterior poliomyelitis occurs sporadically and also in epidemics.

Various organisms have been described in this disease, but Levaditi, Landsteiner, and Flexner, have proved that the virus is a filter-passer, and is present in the central nervous system, the gasserian and some of the other ganglia, in the salivary and some of the lymphatic glands, and sometimes in the blood.

Injection of emulsions of the affected cord into the brain, spinal cord, peritoneal cavity, and blood-stream of monkeys reproduces the disease with the same clinical and pathological features as in man. The disease can be carried on from monkey to monkey by inoculation but does not seem to be transmissible to other animals. Remarkable fluctuations in the virulence of the virus may occur.

Flexner has observed a case of spontaneous infection in the monkey, and found that the naso-pharyngeal mucosa was infective, so that this is probably the channel of infection in man. Flies belonging to the genus *Stomoxys* are stated to be capable of transmitting infection. Human cerebro-spinal fluid was not found infective in some instances, but monkey cerebro-spinal fluid is infective (infectivity in this case may depend on the stage of the disease).

Human ascitic fluid inoculated with the filtered fluid from emulsions of cord became turbid, but no organism could be detected microscopically, and the culture can be carried on from tube to tube (Flexner and Noguchi). Monkeys which have recovered from an attack are refrac-

[1] See Levaditi, *Journ. Roy. Inst. of Public Health*, vol. xix, 1911, pp. 1 and 65 (Bibliog.); Flexner and others, *Studies from the Rockefeller Institute*, 1910 *et seq.*

tory to inoculation. A certain degree of active immunity may be established by subcutaneous injection of the virus. The serum of immunised and recovered animals possesses considerable neutralising power for the virus. Attempts are now being made to prepare a curative serum.

Some cases of the acute ascending paralysis of Landry may be forms of this disease (see also p. 628).

Buzzard, from a case of the latter disease, isolated a coccus which induced a rapidly spreading palsy on subdural inoculation into rabbits.

Typhus Fever

Many organisms have been described in this disease. Nicolle, in Tunis, found that typhus fever of man is communicable to the chimpanzee by inoculation and from the anthropoid to the Chinese bonnet monkey. Nicolle and Conseil have found it possible directly to infect the *Macacus sinicus* and *rhesus* monkeys from human cases.

Nicolle ascertained that the blood is virulent from the commencement of infection and continues so until the day after the temperature becomes normal. The dog and rat are quite refractory. The disease is transmitted by the body-louse (*P. vestimenti*), possibly also by the head-louse.

The blood from a mild case does not produce immunity on injection, nor does a mild attack itself induce any appreciable immunity. On the other hand a severe infection induces considerable immunity.

Researches have also been carried out in America on the typhus of Mexico, known locally as "Tabardillo." Anderson and Goldberger first showed that the *Macacus rhesus* monkey could be directly infected with Mexican typhus. Ricketts and Wilder confirmed this, and found that typhus blood is not infective if passed through a

Berkefeld filter, indicating that the micro-organism is of appreciable size. They also found that the disease is conveyed by the body-louse, and, moreover, that the infection is hereditary in the louse, the second generation of lice derived from infected lice apparently being still infective. Neither bugs nor fleas conveyed the disease.

Many observers have found bacillar, diplobacillar and diplococcal forms (Rabinowitsch, Fuerth, Müller, Hort and Ingram). Topley [1] in Serbian cases obtained a diplococcus, arranged sometimes in clumps, sometimes in short chains, growing well on ordinary media, preferably aërobically. In the coccal form it is Gram-positive. On agar the organism is more bacillar than coccal, and on all culture media pleomorphism is marked and many of the forms fail to stain by Gram's method. Plotz [2] has isolated a small pleomorphic Gram-positive bacillus, which is non-motile and non-sporing. The method of isolation was by culture of 3 c.c. of blood in a 2 per cent. glucose-agar with ascitic fluid. About 20 c.c. of the glucose agar in a tube 2 cm. in diameter are melted and cooled to 40° C.; the blood is injected into it from the syringe, 4 c.c. of ascitic fluid are then added, the whole is well mixed, and the tube is then cooled and solidified. A layer of ordinary agar 2 cm. deep is then poured on the top and the cultures are incubated at 37·5° C. Colonies appear in from 3 to 16 days.

Futaki and co-workers have this year (1917) described the presence of a spirochaete in typhus fever. It is usually 6–8 μ in length, has a general resemblance to the *T. pallidum*, and occurs principally in the kidney and supra-renal. The same organism was found in the kidney of a monkey inoculated with typhus blood. From the kidney the organism may pass into the urine.

[1] *Journ. Roy. Army Med. Corps*, 1915, p. 215 (Bibliog).
[2] *Journ. Infectious Diseases*, xvii, 1915, No. 1.

Ricketts and Wilder discuss the relationship between typhus fever and Rocky Mountain spotted fever. Some years ago Wilson and Chowning made observations on a typhus-like fever occurring in limited areas near the Rocky Mountains and ascribed it to a *Piroplasma*. Subsequent research, however, failed to confirm this, though the disease appears to be conveyed by a tick, and not by fleas, lice, etc. There are clinical differences between typhus and Rocky Mountain spotted fever; moreover, the guinea-pig is susceptible to the spotted fever but not to typhus, and a monkey immunised to typhus is susceptible to spotted fever. Ricketts believes that the spotted fever is due to a bacillus which can be found in the ovary of the tick and is agglutinated by the serum in dilutions of 1–500.

Cathoire has made observations on complement fixation in typhus. Using as an antigen an alcoholic extract of typhus spleen, marked complement fixation was obtained with the serum of typhus cases.

Yellow Fever

As far back as 1889 Sternberg described a bacillus—"*Bacillus X*"—in yellow fever, a facultative anaërobic organism, very pathogenic to rabbits, and in 1897 Sanarelli[1] described his *Bacillus icteroïdes*.

Reed and Carroll[2] critically examined the *B. icteroïdes* and its relation to yellow fever. Their conclusions were that the *Bacillus X* belongs to the colon group, the *B. icteroïdes* to the Gärtner group, and that it is identical with the *B. suicholeræ*. The *B. icteroïdes* is, therefore present in yellow fever as a secondary or terminal infection (see p. 417).

[1] *Ann. de l'Inst. Pasteur*, xi, 1897, pp. 443, 673, and 753.
[2] *Journ. Exper. Med.*, vol. v, pt. iii, p. 215.

Reed, Carroll, and Agramonte,[1] having thus shown the etiological position of the *B. icteroïdes* to be untenable, directed their attention to the transference of yellow fever through the agency of mosquitoes. Finlay, of Havanah, suggested many years ago that yellow fever might be propagated through the intermediary of a mosquito—*Stegomyia calopus* (*fasciata*)—and with this species these investigators worked. They allowed mosquitoes to bite yellow-fever patients at various stages of the disease, and the infected mosquitoes were subsequently allowed to bite eleven individuals, two of whom contracted yellow fever. A further experiment was then devised.[2] Under the same observers a camp was established with several tents, each occupied by one to three non-immune individuals and precautions were taken to prevent the introduction of yellow fever from outside. Five individuals were bitten by infected mosquitoes, and four out of the five contracted yellow fever, no other occupants of the camp being attacked by the disease. Subsequently several non-immune individuals were exposed to yellow fever infection from soiled linen, yellow-fever discharges, etc., in a mosquito-proof hut from which mosquitoes were excluded, with entirely negative results. These experiments proved, therefore, that yellow fever is conveyed by mosquitoes only, and further work by Americans and Cubans, and by French and Brazilian Commissions, has entirely confirmed these researches and conclusions. In order to convey infection, it is necessary for the mosquitoes to bite the patient during the first three or four days of the illness, but they do not become infective until about the twelfth day after feeding, and then retain their infectivity indefinitely. All these facts point to a protozoon as being

[1] *Philad. Med. Journ.*, October 27, 1900, p. 790.
[2] *Journ. Amer. Med. Assoc.*, February 16, 1901, p. 431.

the causative organism, but none has been found with certainty.

The Americans have shown that the blood-serum after filtration through a porcelain filter is still infective ; the organism, therefore, is probably ultra-microscopic, at least at one stage. Seidelin [1] described extremely small rounded bodies with a minute chromatin point and feebly staining protoplasm, without pigment, in the blood corpuscles. Somewhat similar, but larger, bodies may also be present in the organs and free in the plasma. Macfie and Johnston [2] found elements like those described by Seidelin in the red corpuscles in practically every case of yellow fever examined. Other observers have, however, found similar bodies in guinea-pig and other corpuscles, so that their relationship to yellow fever is problematical.

Dengue

No organism, bacterium or protozoon, has been demonstrated in this disease. The intra-venous inoculation of filtered dengue blood into healthy individuals is followed by an attack ; the organism is therefore probably ultra-microscopic. The disease can be transmitted by a mosquito, *Culex fatigans*, and this is probably the common mode of infection.[3]

Phlebotomus Fever

A fever of short duration (three days) occurs in South Austria, the malady being somewhat like dengue. It is known locally as "pappataci," and an apparently identical disease has been described by Birt [4] in Malta under the

[1] *Journ. Pathol. and Bacteriol.*, vol. xv, 1911, p. 282.
[2] *Proc. Roy. Soc. Med.*, vii, No. 3, 1914 (Med. Sec.), p. 49.
[3] Ashburn and Craig, *Philippine Journ. of Science*, vol. ii, 1907, p. 93.
[4] *Journ. Roy. Army Med. Corps*, August 1910.

name of "phlebotomus fever." Investigation has shown that this disease is conveyed by the bite of a dipterous fly, the sand-fly (*Phlebotomus pappatasii*). "Canary fever," "Shanghai fever," "Chitral fever," and the seven days continued and "sand-fly" fevers of India are probably of the same nature. The virus in phlebotomus fever passes through a Berkefeld filter.

Further research must decide whether these and dengue are distinct diseases or whether they are all manifestations of dengue.

Variola and Vaccinia

The specific contagia of these two diseases appear to be filter-passers.

Variola is inoculable on man the calf and the monkey, vaccinia on the rabbit in addition.

A large number of observations have been made on the bacteriology of vaccine lymph. Usually the ordinary pyogenic organisms and many saprophytic forms can alone be isolated. Klein observed the presence of a bacillus in vaccinia, which was subsequently more fully studied by Copeman.[1] It was found in vaccine vesicles at an early stage, but at maturation could no longer be detected. It is a very fine bacillus, and these observers were unable to cultivate it. Subsequently Copeman found a similar organism in variola, and succeeding in cultivating the bacillus from both sources in eggs, and from such egg-cultures was able to inoculate calves. Klein,[2] by storing variola crusts in 50 per cent. glycerin and so getting rid of the saprophytic forms, cultivated an organism which he terms the *Bacillus albus variolæ*. Morphologically it closely resembles the bacillus observed

[1] *Milroy Lectures on Vaccination*, 1898.

[2] *Rep. Med. Off. Loc. Gov. Board* for 1896–97, p. 267.

in vaccine lymph ; it forms small white, opaque, coherent colonies on agar, but grows very feebly on gelatin. Involution forms occur, and it seems to belong to the group of diphtheria and xerosis bacilli. On inoculation into calves some approach to, but not typical, vaccinia was produced. Moreover, the inoculated calves were not immune to subsequent vaccination. Copeman [1] inoculated glycerinated vaccine lymph in which the extraneous organisms had died out into collodion capsules filled with beef broth and inserted them in the peritoneal cavity of rabbits, and observed zoogloœa masses made up of bodies resembling spores which he regards as the resting stage of the specific microbe.

De Korté finds that the vesicles, both in variola and in vaccinia, are sterile before maturation, and regards the bacterial forms that have been isolated as secondary infections.

The failure to isolate a bacterial form has induced many observers to seek for a parasitic protozoon in variola and vaccinia, and various "bodies" have been described. Thus, Guarnieri found small bodies, about half the size of the nucleus, in the epithelial cells of the skin in the pre-pustular stage of variola and in the cornea after inoculation. Somewhat similar bodies have been described by L. Pfeiffer, J. Clarke, Ruffer and Plimmer ; and Councilman, Magarth, Brinkerkoff, Tyzzer, and Calkins,[2] and others.

Much doubt exists as to the nature of these bodies. Prowazek regards the cell inclusions (the Guarnieri and Negri bodies, etc.) in this and other conditions (*e.g.* rabies) not as parasites, but as tissue reactions consisting of plastin and nuclease, and enclosing the parasites, whatever these may be.

[1] *Brit. Med. Journ.*, 1901, vol. i, p. 450.

[2] *Journ. Med. Research*, vol. xi, 1904, p. 173 ; *Philippine Journ. of Science*, vol. i, 1906, p. 239.

Fornet [1] by treating variola or vaccine lymph with ether finds a stage when all the bacteria are killed but the specific virus is uninjured. By inoculating this etherised lymph into nutrient broth and keeping at 37° C., the broth culture inoculated in man produces typical vesicles even after two months' incubation, and moreover the culture can be carried on from tube to tube. In the broth, minute rounded bodies can be detected which may be the specific micro-organism.

The relationship of vaccinia to variola has been a very vexed question. With few exceptions (Ceely, Hime, Simpson, Klein, King, Copeman) attempts to inoculate variola on the calf have failed. In the successful cases the lymph obtained from the calf has, on inoculation upon children, produced typical vaccinia without any untoward results. The positive results obtained by the inoculation of variolous material being so few, a doubt arises whether in these cases there may not have been some fallacy, such as accidental contamination with vaccinia. Simpson, however, performed his experiments within the precincts of a smallpox hospital and away from possible vaccine infection, and Copeman [2] found that variola may be readily inoculated upon monkeys, and after several passages through these animals is easily inoculable upon the calf. He suggests, therefore, that vaccinia in the calf was originally due to infection with *inoculated* smallpox, so prevalent at the time of Jenner's discovery. A somewhat parallel instance of the attenuation of a virus by passage through another animal is recorded by Stickler and Marx in the case of birdpox, which produces an extensive smallpox-like eruption in fowls and pigeons. In fowls and in pigeons the virus retains its pathogenic properties for each bird

[1] *Trans. XVIIth Internat. Cong. Med. Lond.*, 1913, Sect. iv, pt. ii, p. 119.

[2] *Brit. Med. Journ.*, 1901, vol. i, p. 1134, and 1901, vol. ii, p 1736.

unaltered for any number of inoculations, but the pigeon strain, after a few inoculations into fowls, completely loses its virulence for the pigeon. There seems little doubt, therefore, that vaccinia is modified variola, and the rationale of vaccination rests upon a scientific basis.

The preparation of vaccine lymph is fully described by Blaxall.[1] Calves are vaccinated with lymph under aseptic precautions, and five days later the contents of the vesicles are scraped off, the pulp is triturated in a machine, and is then placed in six times its weight of sterilised 50 per cent. pure glycerin in distilled water, and stored for about a month in test-tubes, until agar cultivations show that extraneous bacteria have died out, when it is issued for use, It remains very active for fifty to sixty days, after which it begins to deteriorate.

Green [2] rapidly prepares vaccine lymph by killing off the extraneous organisms with chloroform vapour.

Blaxall [3] has more recently used oil of cloves as a sterilising agent in the preparation of calf lymph.

Malignant Disease

The analogies between carcinoma and sarcoma and many infective diseases have led investigators to search for micro-organisms in these conditions.

Bacteria have been repeatedly looked for, but Shattock was unable to isolate any bacterial form from malignant disease. Doyen isolated a micrococcus (*M. neoformans*, p. 254), but, though frequently present, it is not causative.

A great impetus was given to the study of parasites in malignant disease by the publication of a paper by Russell. He observed, by certain methods of staining, small corpuscles within the epithelial cells. They were spherical in shape, 4 to 10 μ in diameter, occurring singly or in groups, were apparently homogeneous, and surrounded by a capsule. Russell regarded these structures as belonging to the " sprouting fungi " (Blastomycetes), and they have since been known by the name of " fuchsin bodies " or " Russell's corpuscles."

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[1] *Rep. Med. Off. Loc. Gov. Board* for 1898–99, p. 35.
[2] *Rep. Med Off. Loc. Gov. Board* for 1900–01, p. 639
[3] *Ibid* 1911–12, p. 361.

Subsequently structures were observed within the epithelial cells of carcinoma which were regarded by many investigators as parasitic protozoa.[1] These structures are round or ovoid, 2 μ to 10 μ in diameter, with a very distinct outline, as though encapsuled, and clear refractile contents in which is a smaller body of variable size analogous to a nucleus (Fig. 66*a*). Occasionally the refractile contents present a radial striation or a granulation.

These bodies are usually single, but may number as many as eight or ten, and sometimes they invade the epithelial nucleus. The Ruffer's or Plimmer's body, however, is a structure pro-

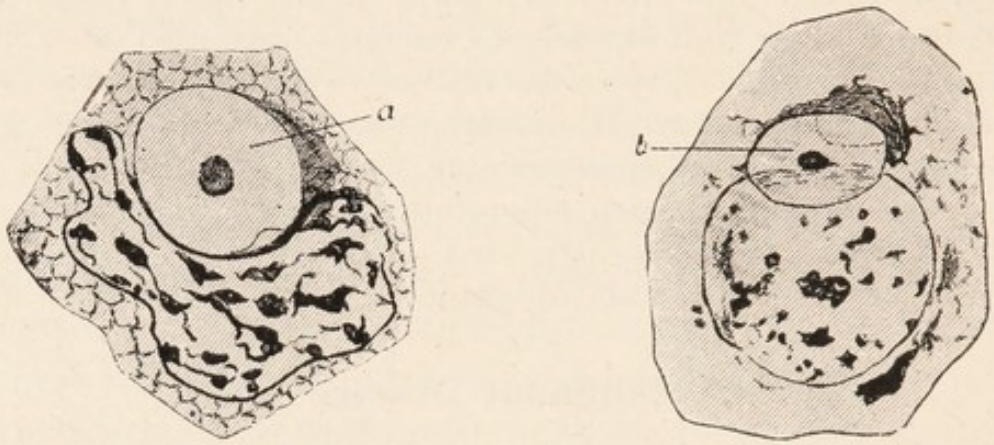

FIG. 66.—*a*, Ruffer's or Plimmer's body in a cancer-cell; *b*, the archoplastic vesicle in spermatid of mouse. (After Farmer, Moore, and Walker.)

bably analogous to the archoplastic vesicle of the cells of reproductive tissue (Fig. 66, *b*). Save for the presence of these structures, there is no proof that protozoa are present in, or are the cause of, carcinoma.

Another hypothesis of the nature of malignant disease is that it is due to a blastomycetic infection (see p. 531) or that it is caused by a myxomycete.

Malignant disease occurs in all classes of vertebrates, and is generally inoculable on an animal of the *same* species as that from which it is derived, but not on other animals. The carcinoma of mice has been the subject of much investigation of late. In the writer's opinion, the trend of recent research is to show that malignant disease is not due to a micro-parasite, but is

[1] See Ruffer and Walker, *Journ. Path. and Bact.*, vol. i, 1893, p. 395.

derived from the irresponsible division of cells of the normal or of embryonic tissues.[1] If there be a parasite, in all probability it is intra-cellular, like the organism of plant cancer (*Bacterium tumefaciens*) described by Erwin Smith.[2]

The molluscum bodies have likewise been regarded as parasitic (coccidial) in nature, but with them also inoculation and cultivation experiments have failed. The virus is stated to be a filter passer, as is also the case with bird molluscum.

Certain malignant-like tumours of birds are also filter-passers, *e.g.* chicken sarcoma.

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[1] For further information consult *Pathology, General and Special*, ed. 4, R. T. Hewlett (Churchill, 1917).

[2] *Trans. XVIIth Internat. Cong. Med. Lond.*, 1913, Sect. iii, pt ii, p. 281.

CHAPTER XX

SOME DISEASES NOT PREVIOUSLY REFERRED TO, WITH A DISCUSSION OF THEIR CAUSATION—MICRO-ORGANISMS OF SKIN AND MUCOUS-MEMBRANES

ABORTION, CONTAGIOUS.—This disease of the cow is caused by the *B. abortus* of Bang. It is a small short Gram-negative bacillus similar to the chicken cholera bacillus, and belonging to the same group. It is difficult to isolate and cultivate. Bang employed melted nutrient agar to which melted gelatin and blood-serum were added. The material introduced into this is well mixed with the medium, the medium allowed to solidify and the tubes are incubated at blood-heat. In three days numerous colonies form a few millimetres below the surface ; the organism is very specialised in its oxygen requirements, being intermediate between fully aërobic and fully anaërobic, though Bang states that it will grow in an atmosphere of *pure* oxygen. The colonies are small and compact. The organism does not curdle milk. *B. abortus* occurs in the vagina of the infected cow and in the heart-blood of the aborted fetus. It causes abortion in the cow and also in guinea-pigs and rabbits and the condition is contagious.

APPENDICITIS.—The following Table shows the usual kinds and relative frequency of the infections in appendicitis :

Micro-organism	Acute Appendicitis.	Chronic Appendicitis.
Bacillus coli in pure culture . . .	70 per cent.	90 per cent.
,, with staphylococci . .	15 ,,	6 ,,
,, ,, streptococci . . .	7 ,,	Very rare.
Staphylococci alone	4 ,,	1 per cent.
Streptococci alone	Very rare.	Very rare.
Other organisms or combinations . .	4 per cent.	3 per cent.

[1] Battle and Corner, *Diseases of the Vermiform Appendix*, 1904.

It is not improbable that in a still greater percentage of cases a mixture of organisms is present at first, the *Bacillus coli* subsequently crowding out the other forms. The *Bacillus proteus*, *B. pyocyaneus*, and *B. Welchii* also occasionally occur.

Castellani [1] describes a bacillus, pathogenic to guinea-pigs, isolated from a case of gangrenous appendicitis. Morphologically it resembled the Shiga-Kruse dysentery bacillus, and was non-motile, produced acid and gas in glucose and maltose and curdled milk, but did not ferment mannite, lactose, and sucrose.

Beri-Beri.—Various observers have attempted to cultivate a micro-organism in this disease. Cocci have been described by Pekelharing and Winkler, Hunter, Okata and Kokubo, a sporing bacillus by Rost, and Hamilton Wright suggests that the disease is due to an intoxication, the result of a gastro-duodenal infection with a large Gram-positive bacillus (unisolated). Daniels suggested that the epidemiology of the disease is best explained on the hypothesis of a protozoan infection conveyed by lice. The writer and De Korté [2] also suggest a protozoan infection, the organism perhaps being eliminated in the urine.

Other views are that beri-beri may be a peripheral neuritis due to arsenical poisoning, or that it is caused by the absence of certain nutritive elements from polished rice. The evidence in favour of the latter view seems to be accumulating, and it has been found that essential nutritive constituents (vitamines ?) are present in the husk of rice which is removed in polishing.

Bronchitis.—Ritchie [3] concludes that acute bronchitis is an infective disease, but is not due to any one specific organism, the most important causal bacteria being the *S. pneumoniæ* and streptococci. In every case of acute bronchitis numerous pathogenic bacteria are present in the bronchi, which are usually sterile in health. The commonest organisms are *B. pneumoniæ*, *B. influenzæ*, and *M. catarrhalis*. Spirochaetes are present in some forms of tropical bronchitis; in others Castellani has described oïdium-like and yeast-like organisms.

Chancre, Soft.—An extremely small bacillus, first described by Ducrey,[4] has been found in the ulcers and buboes. It has not been inoculated successfully on animals, but can be inocu-

[1] *Brit. Med. Journ.*, 1907, vol. i, p. 1513.

[2] *Journ. Trop. Med.*, October 1, 1907, p. 315.

[3] *Journ. Path. and Bact.*, vol. vii, No. 1, p. 1.

[4] *Comp. Rend. Congrès Internat. de Dermatologie* (Paris, 1889), p 229.

lated from a chancre, experimentally, from man to man. The bacillus does not stain by Gram's method, and can be cultivated on blood agar, on which it forms shining greyish colonies 1 mm. in diameter, or in guinea-pig blood.[1]

Conjunctivitis.—Conjunctivitis is of several varieties :

(*a*) *Acute contagious conjunctivitis*, due to the Koch-Weeks bacillus. This is a slender, non-motile organism, 1–1·5 μ in length, occurring singly or in pairs, both free and within the pus-cells. It is decolorised by Gram's method, and is difficult to cultivate, growing best on a serum-agar mixture, on which it forms small, punctiform transparent colonies. It is hardly pathogenic to animals, but in man sets up a typical acute conjunctivitis.

(*b*) *Chronic catarrhal conjunctivitis*, due to the Morax-Axenfeld diplo-bacillus. This organism is 2 μ long by 1 μ broad, is not stained by Gram's method, and can be cultivated on blood-serum, which becomes pitted from liquefaction, or on serum agar, but not on ordinary agar nor on gelatin. Petit described also a closely allied organism distinguished by its ready growth on agar and on gelatin, which is liquefied.

(*c*) *Gonorrhœal conjunctivitis.*

(*d*) *Diphtheritic conjunctivitis.*

(*e*) *Conjunctivitis of streptococcic origin.*

(*f*) *Conjunctivitis of pneumococcic origin.*—Usually in children, and accompanied with coryza and scanty muco-purulent discharge.

(*g*) Micrococci (*aureus* and *albus*) and *B. coli* may also occasionally cause conjunctivitis.

Diarrhœa (Summer) of Infants.—Lesage obtained a bacillus from the "green diarrhœa" of infants which he believed to be the cause of this complaint. It is a small, motile, non-liquefying bacillus, producing on gelatin a whitish expanded growth with crenated margins, and giving rise to a green fluorescence in the medium. The *B. pyocyaneus* may be an occasional cause.

In cases with blood and mucus in the stools, the *B. dysenteriæ* (Shiga-Kruse type) has been found to be present in America but is rare in this country. In London, Morgan has isolated in a number of cases a bacillus of the Gärtner group having particular fermentation reactions (see p. 417). Lewis[2] found

[1] Himmel, *Ann. de l'Inst. Pasteur*, xv, 1901, p. 928.

[2] *Rep. Med. Loc. Gov. Board* for 1911–12, p. 265, and *ibid.* for 1912–13, p. 375.

that non-liquefying and non-lactose-fermenting bacilli are more frequent in the fæces of children suffering from diarrhœa than in normal children, and believes that Morgan's bacillus has a causal relationship in many cases. Alexander [1] also found Morgan's bacillus more frequent in diarrhæa cases than in normal children.

Ralph Vincent ascribes the disease (which he terms "zymotic enteritis") to the ordinary organisms of putrefaction gaining access to milk and multiplying and causing alterations therein.

The stinking motions of the diarrhœa of children have been ascribed to the action of organisms belonging to the *Proteus* group, particularly *B. proteus* (*P. vulgaris*, see p. 715), which occurs in putrefying matter, sewage, and in the intestine. (This organism may also cause abscesses and cystitis, and a form of meat poisoning has been ascribed to its action.) Filtrates of cultures were found by S. Martin to produce a fall of temperature, collapse, and diarrhœa in rabbits.

Distemper, Canine.—According to Galli-Valerio,[2] this is caused by a bacillus (*B. caniculæ*) intermediate in character between the coli-typhoid and hæmorrhagic septicæmic groups of organisms. Torrey and Rahe [3] confirm Ferry and M'Gowan's observations on a bacillus (*B. bronchisepticus*) present in distemper. It does not ferment any sugars and litmus milk becomes markedly alkaline.

Evidence has also been brought forward that distemper is due to a filter passer (Carré). Probably the term "distemper" may include several different diseases.

Dysentery.—Dysentery must be regarded as a term applied to a series of clinical symptoms associated with colitis which is due to different specific agents. There are two principal forms of the disease, the tropical, endemic or amœbic dysentery caused by the *Entamœba histolytica* (p. 553), and the epidemic or bacillary dysentery due to the group of dysentery bacilli (p, 423). The former is met with especially in the East, and is characterised by chronicity, a tendency to relapses, amenability to treatment with ipecacuanha, and the occurrence of the single liver abscess as a sequela; the latter is met with in all parts of the world,

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[1] *Ibid.* 1911–12, p. 288.

[2] *Centr. f. Bakt.* (Ref.), xli, 1908, p. 563. See also M'Gowan, *Journ. Pathol. and Bacteriol.*, vol. xv, 1911, p. 372 (Bibliog.) and xvi, p 257.

[3] *Journ. Med. Research*, xxvii, 1912, p. 291 (Bibliog.).

particularly in times of war and famine, not amenable to ipecacuanha, and not followed by liver abscess. There are also probably other forms occurring in small outbreaks or sporadically.

Coliform bacilli have been isolated from cases of dysentery. Calmette in Tonkin isolated the *B. pyocyaneus*, and this organism seems to have been the cause of a small outbreak in New York State investigated by Lartigau.[1] In Japan, Ogata isolated a fine Gram-staining, liquefying bacillus which does not seem to have been met with by subsequent observers. Spirochaetes have been found in large numbers in a form of dysentery occurring in Bordeaux.

Vedder and Duval,[2] as a result of the study of a number of cases of acute dysentery in the United States, conclude that the disease, whether sporadic, "institutional," or epidemic, is due to the *B. dysenteriæ* of Shiga.

The *B. dysenteriæ* (Shiga type) has been isolated by Eyre, McWeeney, and others from cases of ulcerative colitis or asylums dysentery in the British Isles (see p. 427).

The *Balantidium coli* (p. 598) and certain parasitic worms may also induce a dysenteric condition.

Skin Diseases: *Acne*.—In the acne pustules, the *M. pyogenes* var. *aureus*, with or without var. *albus*, is almost invariably present, and a staphylococcic vaccine generally acts extremely well. In the comedoes a Gram-positive, Hofmann-like bacillus (*B. acnes*) is present in considerable numbers, and may be the cause of the comedo. This organism was cultivated by Fleming on a neutral agar to which glycerin and oleic acid are added. Südmersen and Thompson [3] cultivate it on an acid (+ 40) serum-agar. The organism is anaërobic, at least at first, and will grow in glucose-agar stabs. In culture the organism is diphtheroid. A vaccine prepared with it is of service in the comedo stage.

Eczema is produced by the action of the pyogenic cocci (*M. pyogenes*, var. *aureus* and *albus*). Virulent cultures of these organisms, with or freed from their toxins, seem, however, to produce an impetigo rather than eczema. But the filtered cultures, *i.e.* toxins, are harmful to the skin, and when applied to it for one or two days by means of moist warm pads a typical papular or vesicular eczema ensues. Probably in the human

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[1] *Journ. Exper. Med.*, vol. iii, No. 6, p. 595.

[2] *Ibid.* vol. vi, 1902, No. 2, p. 181.

[3] *Journ. of Pathol. and Bacteriol.*, vol. xiv. 1910, p. 224.

subject in addition to the micro-organisms some peculiarity in the soil is necessary for the disease to develop.[1] In so-called seborrhœic eczema, a non-liquefying micrococcus which forms butyric acid has been isolated.

Impetigo.—The large vesiculo-bullous eruption of impetigo contagiosa is caused by the *Streptococcus pyogenes*: the small pustule in the neighbourhood of hair-follicles, impetigo of Bockhart, is caused by the *M. pyogenes* var. *aureus*. The *B. diphtheriæ* may also cause an impetigo (p. 305).

Pemphigus.—A diplococcus has been isolated in acute pemphigus by Demme, and in the chronic form by Dähnhardt. Bulloch and Russell Wells, in this country, seem to have isolated an identical organism, and the following description of it is taken from their papers. Cocci 0·8 to 1·5 μ in diameter, mostly arranged as diplococci, and staining by Gram's method. On surface agar the organism forms a thick, white, shining growth. In stab agar the growth has a "nail-shaped" appearance. The colonies on agar are at first round, but later, in seven days, they throw out lateral projections and assume a rosette appearance. On gelatin the growth is slow and slight, with some, but not marked, liquefaction. On blood-serum the growth resembles that on agar. On potato a whitish, semi-transparent film forms. Milk is curdled. In broth it causes a general turbidity, with a whitish sediment, and sometimes a pellicle, which soon sinks. Guinea-pigs and mice inoculated or vaccinated with the organism died in four to eight days, fine hæmorrhages occurring in the lungs, and the cocci being obtained from the blood. No bullæ appeared on the skin. The *B. pyocyaneus* may cause dermatitis and bullous eruptions (see p. 262).

The pyogenic cocci or their toxins may produce various bullous eruptions, *e.g.* pemphigus neonatorum and contagiosus and hydroa gestationis.[2]

Herpes zoster.—Pfeffer observed bodies in the cells of the vesicles which he believed to be protozoa. Gilchrist, however, regards these merely as altered nuclei.

Foot and Mouth Disease.—Various organisms have been described in this disease, but a German commission comprising Löffler and Abel [3] stated that they were unable to prove its

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[1] Whitfield, *Practitioner*, February 1904, p. 202.

[2] *Brit. Med. Journ.*, 1902, vol. 1, p. 73.

[3] *Centr. f. Bakt.*, xxiii, 1898, March.

etiological significance. Löffler and Frosch have determined that the organism must be a very minute one, as it passes through the smallest-pored porcelain filter.

Mastoid Disease.—See "Otitis Media."

Measles.—Doehle and Behla described small flagellated bodies which they believed to be protozoa in this disease. Canon and Pielicke found small bacilli in the blood, which Tchaïkovsky confirmed. They are motile, do not stain by Gram's method, and can be cultivated on agar and serum, on which they form delicate colonies. Czajkowski has found a similar organism. Lesage [1] cultivated a small micrococcus from the nasal mucus and blood, which produced a fatal hæmorrhagic septicæmia in animals. The influenza bacillus is present in many cases. The organism is probably a filter-passer.

Meningitis may be caused by *S. pneumoniæ* (60 per cent. of acute cases), *D. intracellularis*, Still's diplococcus, *B. tuberculosis*, gonococcus, and micrococci and streptococci, occasionally *B. influenzæ*. Henry [2] describes influenza-like bacilli causing sporadic meningitis, and also otitis media. They grow only on blood-agar and are more virulent to animals than the *B. influenzæ*.

Mumps (Epidemic Parotitis).—Mecray and Walsh isolated from the parotid and blood in some cases of mumps a coccus resembling that described by Laveran and Catrin. It occurs chiefly as a diplococcus, but also in large groups. The colonies form circular, white, shining points, with slow glowth and gradual liquefaction. On potato a white growth occurs; on blood-serum a plentiful cream-coloured growth; and in litmus milk production of acid with coagulation.

Noma and Cancrum Oris.—Grawitz in 1890 observed bacilli in the affected tissues in this disease, others fusiform bacilli with or without other organisms; Comba considered that there was probably no specific organism; Durante found the *M. pyogenes*, var. *aureus*, with *B. proteus*, and Ravenna the same micrococcus with the typhoid bacillus. Diphtheroid bacilli have also been isolated. Weaver and Tunnicliff [3] in a case of cancrum oris observed the presence of fusiform bacilli and spirilla. Hellesen [4] isolated a diplococcus from a case of noma. The organism is

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[1] *Compt. Rend. Soc. Biol.*, 1900, p. 203.

[2] *Journ. Path. and Bacter.*, vol. xvii, 1912, p. 174.

[3] *Journ. Infectious Diseases*, vol. iv, 1907, p. 8 (Bibliog.).

[4] See *Lancet*, 1908, vol. i, p. 955.

not unlike the pneumococcus, but possesses no capsule, is Gram-positive, gives a general turbidity in broth with acidity, forms no gas from glucose, curdles milk with acid production, and forms punctate, whitish-grey, translucent colonies on surface agar. On inoculation into animals a specific necrosis was produced.

Bishop and Ryan, in two out of three cases, isolated an organism which culturally and morphologically resembled the diphtheria bacillus, but which only produced some local inflammation on inoculation into guinea-pigs. In the third case the *M. pyogenes*, var. *aureus*, and the *Streptococcus pyogenes* were isolated. Guizzetti, and Freymuth and Petruschky have isolated the Klebs-Löffler bacillus in noma.

Oppler-Boas Bacillus.—Met with in the stomach, particularly in cases of carcinoma, and its detection is suggestive of this condition. The bacilli occur in masses, are long and filiform and non-motile, and frequently join one another at an angle. They measure usually 6–8 μ in length, but vary between 3 and 10 μ. The organism is a facultative anaërobe, non-sporing and Gram-positive. It curdles milk and forms lactic acid from various sugars. It is probably the *B. bulgaricus*.

Otitis Media.—The *Streptococcus pneumoniæ* is perhaps the commonest organism met with; next in frequency comes the *Streptococcus pyogenes*, and then the pyogenic cocci. An influenza-like bacillus is sometimes present (Henry; see "Meningitis," p. 654) and occasionally the *S. mucosus*. In scarlatinal otitis media, Blaxall found the *S. pyogenes* to be always present, and generally accompanied by other organisms, pyogenic cocci, etc. In thirty-seven cases of mastoid disease Blake found the following organisms, and remarks that as a rule the same were found in the middle ear:

Streptococcus	12
Staphylococcus	5
Diplococcus (? *pneumoniæ*)	6
Streptococcus and diplococcus	5
Streptococcus and *Bacillus fetidis* (? colon bacillus)	3
Streptococcus and *Bacillus pyocyaneus*	1
Streptococcus and diploccoccus	1
Streptococcus, micrococcus, and diplococcus	2

In two of the cases no organisms could be isolated.

Ozæna (Atrophic Rhinitis).—Löwenberg described in this disease encapsuled bacilli somewhat resembling the pneumobacillus morphologically. Some Italian observers found bacilli

apparently identical with the diphtheria bacillus. Abel [1] described a bacillus somewhat resembling the pneumo-bacillus. It is this organism which produces the atrophy of the mucous membrane, but the fetor is due to the decomposition of the secretions produced by other organisms.

Perez [2] isolated an organism in ozæna (*Cocco-bacillus fetidus ozænæ*) which has the following characters : it is a short bacillus with rounded ends, non-motile, does not stain by Gram's method, does not liquefy gelatin, does not ferment lactose nor curdle milk, but forms indole and ferments urea. Its cultures are foul-smelling, and it is pathogenic for guinea-pigs, mice, rabbits, and pigeons.

Pellagra.—Many hypotheses have been propounded to account for the causation of this disease. It formerly was supposed to be due to the consumption of maize, which contains toxic substances. Lombroso suggested that spoilt maize is the cause, toxic substances being produced by *Penicillium glaucum*. Recent work is discrediting parasitic theories and we are reverting to the former view that the disease is dependent upon food ; it is probably a " deficiency disease " like beri-beri. Of parasitic theories, Ceni and others suggest infection with *Aspergilli*. Tizzoni attributes it to the pleomorphic *Streptobacillus pellagræ* (which may be a pleomorphic form of an actinomycotic organism). Sambon on epidemiological data believes that a protozoan parasite is the agent and is transmitted by small biting flies of the genus *Simulium*. The sun's rays have also been supposed to cause the affection.

Peritonitis.—Treves gives the following Table of the micro-organisms found in peritonitis :

	Fränkel.	Tavel and Tanz.	
	Found alone.	Found alone.	Found in association.
Bacillus coli communis .	11	15	16
Streptococcus . . .	7	3	15
Staphylococcus . . .	1	2	6
Pneumococcus . . .	1	0	2
	20	20	39

[1] *Zeitschr. f. Hyg.*, xxi, p. 89.

[2] *Ann. de. l'Inst. Pasteur*, xiii, 1899, p. 937, and xv, 1901, p. 409.

Dudgeon [1] believes the *B. coli* is frequently a secondary agent and not the primary infection. He finds that the *M. pyogenes*, var. *albus*, is very commonly present from the first, and may exert a protective action by determining the occurrence of phagocytosis.

Psilosis or Sprue.—Bahr regards this disease as being caused by a yeast like form or *Oidium* (*Monilia albicans*).

Puerperal Fever.—This condition may be either a localised infection with intoxication (sapræmia), or a localised infection with general infection (puerperal septicæmia); in both the primary seat of infection may be perinæal or vaginal lacerations, or the contents of the uterus or the placental site. The infecting organisms may be *S. pyogenes*, pure (20 per cent.), or with other organisms (30 per cent.), occasionally the *S. pneumoniæ*, *B. coli*, *M. pyogenes*, var. *albus*, *M. pyogenes*, var. *aureus*, *M. gonorrhœæ*, *B. Welchii*, and diphtheroid bacilli. These are rarely alone, but generally occur with one or other of the organisms named. The *B. diphtheriæ* may exceptionally be met with.[2]

Purpura.—Hæmorrhagic septicæmia may be caused by a number of capsulated bacilli allied to the *B. pneumoniæ* of Friedländer [3] (see pp. 291, 467), as well as by streptococci and pyogenic cocci. Paratyphoid infection may be accompanied with purpura.

Pyorrhœa Alveolaris (Rigg's disease).—Goadby [4] has found the following organisms to be probably causative in this disease: *M. citreus granulatus*, *M. pyogenes*, var. *aureus*, streptococci, *M. catarrhalis*, and diphtheroid bacilli, and has used vaccine treatment with success. Eyre and Payne [5] have found similar organisms. Drew and Griffin [6] find present in pyorrhœa *Entamœba gingivalis* (*E. buccalis*), spirochaetes and treponemata, pyogenic cocci and other bacteria. They consider that mechanical injury starts the condition, the tissues then become invaded by spirochaetes which cause destruction of tissue and the formation of pockets. When once the pockets are formed bacterial invasion occurs. The amœbæ when once established in the pockets appear to aid in the destruction of tissue.

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[1] *Bacteriology of Peritonitis* (Constable, 1905).
[2] See Foulerton, *Practitioner*, March, 1905, p. 387.
[3] See Howard, *Journ. Exp. Med.*, vol. iv, 1899, p. 149 (Bibliog.).
[4] *Proc. Roy. Soc. Med.*, February 1910 (Odontological Section).
[5] *Ibid.*, December 1909.
[6] *Journ. Roy. Microscop. Soc.*, 1917, Pt. 2 (April), p. 185.

Rat-bite Disease.—A disease occasionally met with in England but commoner in Japan, and consequent on the bite of a rat. It is characterised by weekly bouts of severe fever lasting two or three days.[1] A spirochaete was found in the blood, skin and lymph glands of patients by Futaki and co-workers.[2] A similar spirochaete is found in a small percentage of rats. Mice and occasionally guinea-pigs and monkeys can be infected. Salvarsan, etc., is curative. A streptothrix has also been described.

Rheumatism (Acute).—The opinion has gained ground of late years that acute rheumatism is an infective disease. A number of observers have isolated streptococci and micrococci in this disease, and Singer regards the disease as merely an attenuated form of pyæmia. Menzer considers that rheumatic fever is not due to any one organism, but is a particular reaction in predisposed persons to various microbes, especially streptococci. In 1897 Achalme isolated an anaërobic anthrax-like bacillus from several cases. This bacillus agrees in all its characters with the *B. Welchii* as shown by the writer[3]; it is probably a terminal infection or a contamination. Poynton and Paine[4] in 1899 obtained from eight successive cases a diplococcus (*D. rheumaticus*) which in broth develops into a streptococcus. Injected intravenously into rabbits the diplococcus frequently produces enlargement and inflammation of the joints with effusion, and occasionally valvulitis and endocarditis. In man the organism was demonstrated in the vegetations, pericardium, tonsils, and rheumatic nodules, and has been isolated from the blood, pericardial fluid, cardiac vegetations, and tonsils.

Andrewes and Horder found that two strains of the *D. rheumaticus* corresponded with the *S. fæcalis* (p. 257; also p. 162).

Beattie[5] also obtained a streptococcus from the synovial membrane of cases of acute rheumatism, which regularly produced arthritis, and occasionally endocarditis, in rabbits. Beattie and Yates[6] isolated streptococci from all of thirty-two cases giving definite rheumatic histories and nineteen out of thirty-one

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[1] See Hewlett and Rodman, *Practitioner*, July 1913, p. 86.

[2] *Journ. Exper. Med.*, vol. xxv, 1917, p. 33.

[3] *Trans. Path. Soc. Lond.*, vol. lii, pt. ii, 1901, p. 115.

[4] *Lancet*, 1900, vol. ii, p. 861, *et seq.*; *Trans. Path. Soc. Lond.*, vol. lv, 1904, p. 126.

[5] *Journ. Pathol. and Bacteriol.*, vol. xiv, 1910, p. 432.

[6] *Journ. Pathol. and Bacteriol.*, vol. xvii, 1913, p. 538.

strains tested produced arthritis in rabbits. Goadby has observed similar effects wit ı a streptococcus obtained from the mouth.

The manner in which typical acute rheumatism generally reacts to salicylates suggests a protozoan organism, if an organism be the cause.

Rheumatoid Arthritis (Arthritis Deformans).—This disease, which is probably not a single one, may sometimes be caused by an intestinal, urinary, pyorrhœic, or other toxæmia. Blaxall [1] found in the synovial fluid, and occasionally in the blood, a minute bacillus measuring 2 μ in length. It possessed marked polar staining, was decolorised by Gram's method, and could only be stained by prolonged (3–5 days) immersion in anilin methylene blue. The organism can be cultivated on agar, on serum, and in broth. In a clear broth, after three days, minute shining, yellowish particles appear and increase in amount, giving rise on shaking the flask to an appearance of "gold dust." Inoculation experiments on animals failed.

Poynton and Paine [2] isolated a diplococcus (? a form of their *D. rheumaticus*) from an osteo-arthritic joint, which produced arthritis, with osteo-arthritic changes, when injected intravenously into rabbits.

Crowe [3] has found a micrococcus of peculiar type in the urine in many cases. It may be isolated on the neutral-red egg medium (p. 257), and a vaccine prepared with it seems to be of service in treatment. The organism is allied to the *M. epidermidis* and has been named by Crowe *M. deformans*.

Rhinoscleroma.—A bacillus has been described in this disease. It is a short rod, with rounded ends, encapsuled, and frequently linked in pairs. The organism is non-motile, does not stain by Gram's method, and forms on gelatin a whitish growth without liquefaction like that of Friedländer's pneumobacillus. Milk is not coagulated. The organism is slightly pathogenic. It is doubtful if it is the causal agent.

Rinderpest.—Simpson, Koch and Eddington described bacilli in this disease, but Nicolle and Adil-Bey have found that the virus passes through a porcelain filter, and the organism therefore is probably ultra-microscopic.

Trachoma.—Various organisms have been observed in this

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[1] *Lancet*, 1896, vol. i, p. 1120 (Bibliog.).

[2] *Brit. Med. Journ.*, 1902, vol. i, p. 79.

[3] *Lancet*, i, 1913, p. 1377, and ii, 1913, p. 1460.

disease, *e.g.* a diplococcus by Sattler, gonococcal-like organisms by Lindner and others (it is even suggested that the organism, may be an "involuted" gonococcus), the Koch-Weeks bacillus, the Morax-Axenfeld diplobacillus and the pneumococcus. Minute cell-inclusions, which may be demonstrated by the Giemsa method, are present in the epithelial cells, regarded by Halberstaeder and Prowazek as Chlamydozoa. Noguchi has cultivated an extremely minute coccoid form. The disease is inoculable on apes and the virus is stated to be a filter-passer. The causative organism cannot yet be said to be known.

Undulant Fever.[1]—*Synonyms:* Rock, Mediterranean or Malta fever. A disease met with especially on the Mediterranean littoral, but also in South Africa, India, China, the Philippines, and the subtropical countries of America, and clinically often simulating typhoid fever.

A minute micrococcus (*M. melitensis*), first described by Bruce, is the cause of the disease.

Microscopically, the organism from cultures occurs as a coccus, single, in pairs, or in short chains; it is easily stained by the ordinary anilin dyes, but is Gram-negative. In hanging-drop cultures it shows an active Brownian movement, but probably not true motility, though Gordon has described the presence of flagella (other observers have failed to find them). The organism may be isolated from the blood during life and from the spleen of a cadaver.

On agar it grows as minute transparent colonies, which first appear when inoculated from the spleen in 90 to 125 hours. In thirty-six hours more the colonies become amber-coloured, and later still, in four to five days, they become opaque, of a slightly orange colour, and round with granular margins. On gelatin a whitish growth slowly forms without liquefaction, and in broth a diffused cloudiness forms, with a white deposit and without film-formation. Litmus milk becomes alkaline without curdling. Alkali is also produced in glucose media, but galactose, maltose, and saccharose are unchanged (see Table, p. 279). The distribution of the *M. melitensis* in the body corresponds closely with that of the *B. typhosus*; thus it is abundant in the spleen, relatively scanty in the blood, and is excreted in the urine.

The *M. melitensis* maintains its vitality outside the body in

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[1] See *Reports of the Mediterranean Fever Commission* (Royal Society), pts. i.–vii. Harrison & Sons, 1904–1907.

the dry state in dust or on clothing for two to three months, in tap or sea-water for a month. The thermal death-point is about 55° C.

Inoculated into animals no result usually ensues; in the monkey, however, a febrile condition is produced, with enlarged spleen, sometimes terminating in death, the course of the temperature resembling that of the disease in man. By intra-cerebral inoculation Durham found that the organism becomes pathogenic for the rabbit and guinea-pig, otherwise it is without effect. For the diagnosis of the disease the agglutination reaction is most valuable. It may be carried out by the microscopic method, a forty-eight-hours' broth culture being employed, the details of the process being the same as described at p. 211. Dilutions of 1 in 30, 1 in 50, and 1 in 100 should be prepared, as well as controls with normal serum, for old laboratory strains sometimes agglutinate with normal serum in dilution of 1 in 20 or 30 (see p. 211. Neglect of this precaution led Bentley to ascribe kala-azar to a Malta fever infection). The organism being minute, it is necessary to use the $\frac{1}{12}$-inch oil-immersion, the $\frac{1}{6}$-inch with a high eyepiece and draw-tube extended, or better, a $\frac{1}{8}$-inch dry objective. Bassett-Smith [1] for agglutination tests prefers the sedimentation method, for which an emulsion of a forty-eight-hour old agar culture in physiological salt solution should be employed. Three dilutions of the serum are made, 1 in 40, 1 in 100, and 1 in 400, and the tubes are placed in the blood-heat incubator for two hours and the results noted. The tubes should then be allowed to stand at laboratory temperature and the results recorded after a further period of twelve hours. In some two thousand observations, only once was a positive agglutination obtained with a control serum. Complement-fixation tests may also be employed and are satisfactory. Absence of agglutination does not necessarily negative a diagnosis of undulant fever: in cases of long duration it may be absent. Isolation of the organism from the blood is another method that may be used, but similarly may fail in long-standing cases.

The disease may be conveyed to monkeys by contact, by inhalation of infected dust, and by feeding. Mosquitoes and other insects do not seem to convey it.

The investigations of the Mediterranean Fever Commission have shown that the main source of infection of man is by goat's milk.

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[1] *Journ. of Hyg.*, xii, 1912, p. 497.

Goats may be infected (and are largely so in endemic districts, *e.g.* Malta and South Africa) without showing any symptoms, and excrete the organism in large numbers in their milk. Since goat's milk has been boiled the incidence of the disease in Malta has fallen from 663 cases in 1905 to seven cases in 1907 in the Army, and in the Navy there were no cases in 1907 (Bruce).

Toxin, vaccine, and serum therapy.—The *M. melitensis* forms no extra-cellular toxin, but Macfadyen obtained an endotoxin by disintegration. Attempts to prepare an anti-serum have not been successful. A vaccine prepared with cultures killed by heat (see p. 244) has been used in the chronic form of the disease by Bassett-Smith [1] and others with some amount of success (dose 100 to 500 millions).

An organism, the *M. paramelitensis*, has been found by Nègre and Raynaud in certain cases of undulant fever. In such cases, the blood may not agglutinate the *M. melitensis* but does agglutinate the *M. paramelitensis*. A case of this kind is recorded by Bassett-Smith.[2] As regards treatment, yeast or yeast-products have been found of service in the neuritis of the disease. Vaccines (100 to 500 millions) should be given every five to seven days : they are contra-indicated when the pyrexia is continuous or remittent.

Verruga.—A disease occurring in Peru and other parts of S. America. Two forms have been described ; a chronic granulomatous one and an acute form attended with high fever (Oroya fever or Carrion's disease). The latter is probably an acute disease occuring in a verruga patient. Barton found paratyphoid infection sometimes present. Peculiar little bodies, something like piroplasmata, are present in the red corpuscles (*Bartomia*, ? protozoa), The disease may be conveyed by mosquitoes,

Micro-Organisms of the Skin and Mucous Membranes

Skin.—In the normal clean skin micro-organisms are scattered here and there in cracks of the horny layer and in crevices around hairs and glands, but such skin is not swarming with microbes. The *S. pyogenes* and *M. pyogenes*, var. *aureus*, *albus*, and *citreus*,

[1] *Journ. of Hygiene*, vol. vii, 1907, p. 115.
[2] *Journ. Trop. Med. and Hygiene*, February 15, 1913.

and the *M. epidermidis* (*albus*) of Welch, are the commonest (see p. 252). Equally common on the skin and scalp is the scurf micrococcus isolated by Gordon (see Table, p. 252). Sarcinæ, bacilli, and moulds occur also. On the skin of the groin, scrotum, and vulva the smegma bacillus occurs. From sweating feet various organisms have been isolated, which on culture evolve a disagreeable odour, among which is the *Bacterium fetidum* of Thin.

Conjunctivæ.—Some observers have stated that the conjunctiva is generally sterile. A certain number of organisms are, however, usually present, though they are not numerous, the commonest species being the *Micrococcus epidermidis* (*albus*) of Welch, and if artificially inoculated the excess is rapidly eliminated. The *B. xerosis* can often be isolated.

Lawson [1] found the normal conjunctiva to be sterile in 20 per cent. of cases and pyogenic cocci to be rare, and, when present, non-virulent.

Nose.—In the anterior nares crusts and vibrissæ micro-organisms are present in great abundance, but, contrary to the usual opinion, StClair Thomson and the writer [2] showed that the mucous membrane of the interior of the nose is comparatively sterile, and when organisms are present they are very scanty compared with the number of organisms inspired. Moreover, organisms artificially deposited were found to be rapidly disposed of. After two hours, for example, *B. prodigiosus* inoculated on to the inferior turbinate could not be detected by cultivation. Wurtz and Lermoyez asserted that the nasal mucus is germicidal, but StClair Thomson and the writer [3] were unable to confirm this, though it may have an inhibitory action.

Air-passages.—Below the larynx under normal conditions the air-passages are free from micro-organisms. Expired air is also free from organisms, and the air from the naso-pharynx after passing through the nasal cavities is deprived of the majority of its organisms.[4]

Mouth.—Micro-organisms of all kinds are present in the buccal cavity in the greatest abundance—leptothrix, bacilli, pyogenic

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[1] *Trans. Jenner Inst. Prev. Med.*, vol. ii, p. 56 ; also Griffith, *Thompson Yates Lab. Rep.*, vol. iv, pt. i, 1901, p. 99.

[2] *Medico-Chirurg. Trans.*, vol. lxxviii, 1895 (Bibliog.).

[3] "The Fate of Micro-organisms in Inspired Air," *Lancet*, 1896, January 11.

[4] *Ibid.*

cocci, sarcinæ, and spirilla are almost always to be found. The *Streptococcus pyogenes*, *M. pyogenes*, var. *aureus*, and *Streptococcus pneumoniæ* are frequently present. Certain organisms have their normal habitat in the mouth, are difficult to cultivate, and are of considerable importance in the production of dental caries.[1] Well-defined micrococci and streptococci also occur in the saliva (*M. salivarius*, p. 253, and *S. salivarius*, p. 257). The normal saliva is germicidal to some extent. (See also p. 528.)

Stomach and intestine.—Although a vast number of organisms gain access to the stomach, a large number are destroyed by the acid gastric juice. At the same time a considerable proportion are able to survive—sarcinæ, and lactic and butyric acid bacilli. In normal nurslings the mouth and stomach contain few bacteria —a few cocci, and some bacilli of the *B. coli* and *B. lactis aërogenes* groups. The small intestine contains organisms of the same types, but scantily. In the large intestine bacteria are extremely numerous, particularly Gram-positive ones. These are mostly slender, slightly curved bacilli of moderate size, the *B. bifidus* of Tissier, which often has a bifid extremity, also a somewhat similar organism, *B. acidophilus* of Moro, but capable of developing in an acid medium, a few *B. Welchii*, and a diplococcus. The Gram-negative forms are *B. coli*, *B. lactis aërogens*, and cocci. In bottle-fed children the same organisms occur, but the preponderating organisms are Gram-negative of the *B. coli* type, with many cocci and streptococci. In childhood and adolescence organisms of the *bifidus* type become less numerous but putrefactive anaërobes become more so, particularly *B. Welchii* and *B. putrificus* (*coli*) of Bienstock; the latter is a long, slender, Gram-positive bacillus with large terminal spores. During adult life the putrefactive anaërobes tend to become still more numerous, and the putrefactive decompositions they produce were regarded by Metchnikoff as standing in causal relation to old age. In the healthy adult the stomach, duodenum and jejunum contain relatively few organisms, from the lower ileum to the rectum the intestinal contents are crowded with bacteria, and the greatest number of anaërobic organisms occur here and putrefactive changes are most in evidence.[2] Kendall [3] has described the presence of a bacillus (*B. infantilis*) in large numbers in a condition of infantil-

[1] See Goadby, *Mycology of the Mouth*.

[2] See Herter, *Bacterial Infections of the Digestive Tract*, 1907.

[3] *Journ. Biolog. Chemistry*, vol. v, p. 419.

ism, associated, according to Herter, with chronic intestinal infection. The organism is a Gram-positive, motile, sporing bacillus belonging to the *subtilis* group. It is aërobic and facultatively anaërobic, grows readily on the ordinary culture media, and ferments dextrose and saccharose with the production of acid only, but lactose is hardly attacked. In a dog and a monkey diarrhœa was produced by feeding with it.

Urinary and genital organs.—The meatus urinarius and distal portion of the urethra contain a few organisms, which increase in number in inflammatory conditions, and Gram-negative cocci may be found (see p. 279). A few spirochaetes may also be present in the norma urethra. The deeper portion of the urethra, however, is free from organisms, and the bladder is sterile. The genital tract in the female up to the middle zone of the cervix contains organisms, but the uterus and Fallopian tubes are normally sterile. The *B. vaginæ* of Döderlein, a large Gram-positive bacillus capable of growing in an acid medium, is frequently present in considerable numbers in the vagina.

CHAPTER XXI

THE BACTERIOLOGY OF WATER, AIR, AND SOIL, AND THEIR BACTERIOLOGICAL EXAMINATION—SEWAGE—BACTERIOLOGY OF MILK AND FOODS

Some of the Commoner Organisms found in the Air, Water and Soil.

Bacterial Content of Waters and the Factors influencing it. Filtration, etc.

THE bacterial flora of natural waters is a very varied one. The organisms met with in surface waters, such as streams, ponds, and shallow wells, are derived from the air and soil through which the water has passed, and, if not contaminated from human or animal sources, or from the air of towns, or from sewage or manure, consist mainly of non-pathogenic bacilli, the majority of which are chromogenic and non-liquefying, and develop best on culture media at a temperature of 18° to 22° C. or thereabouts, not at blood heat; also of some sarcinæ and a few micrococci; *B. coli* and *B. Welchii* [1] are usually absent. When, however, the water passes through cultivated lands, or receives sewage, the number of organisms is enormously increased; a large proportion of them liquefies gelatin and develops at blood-heat, and *B. coli* and *B. Welchii* appear more or less numerously. Whereas water from shallow wells has

[1] This name is retained in this chapter to indicate a group of closely allied organisms of which the principal members are *B. perfringens* and *B.* (*enteritidis*) *sporogenes* (see pp. 485, 492).

a bacterial content nearly as great as the surrounding surface water, that from deep wells, especially in the chalk is remarkably free from organisms. The following Table illustrates the number of organisms that may be met with in water from different sources :

Source.	Number of organisms per cubic centimetre.
Freshly fallen snow . . .	34–38
Ice	(very variable) 30–1700
Rain water (Paris) . . .	4–5
Rhone, above Lyons . . .	75
Rhone, below Lyons . . .	800
Rhine, at Mühlheim . . .	average about 20,000
Thames, at Hampton (Frankland)	(variable) 2000–90,000
Deep well in the chalk (Kent Company)	3–19
Surface well	1200
Spring water, Reigate (Frankland)	8
Lake of Lucerne . . .	8–50
Loch Katrine (Frankland) . .	74
Filtered water supplied to London (Houston)	average rarely exceeds 100
Sewage (Frankland)	26,000,000

The number of bacteria in a natural water varies considerably with its source, at different seasons, and under different climatic conditions, particularly the rainfall.

As regards seasonal variation the figures for raw Thames water at Hampton may be quoted. For the year April 1915, to March 1916, inclusive, the average gelatin count was 10,315 organisms per c.c., but the numbers varied from a minimum of 1029 in June 1915 to a maximum of 19,526 in March 1916. July, November and December 1915, January and February 1916 all had over 17,000 per c.c., while April, May, August, September and October, 1915 had less than 5,000 per c.c. The high count (17,724 per c.c.) of July 1915, is probably due to the excessive rainfall in that month—4·495 inches at

Oxford. By various methods of treatment of a raw water, the number of organisms may be reduced :

(1) *Storage of unfiltered water.*—A large storage capacity permits of the water being admitted when the source (river, etc.) is in its best condition, so that foul water, in flood time or drought, may be avoided. Moreover, storage alone usually markedly diminishes the number of organisms, partly by subsidence, partly by lack of aëration, and partly probably owing to the struggle for existence going on among them.

(2) *Sand Filtration.*—Efficient sand filtration removes quite 99 per cent. of the organisms originally present. The fine sand only has to be taken into account in estimating the removal of organisms and efficiency of a filter bacteriologically. It probably should form a layer not less than 3 ft. to 3 ft. 6 in. in thickness.

The removal of organisms is less perfect when the rate of filtration is rapid. The rate of filtration should not exceed about 1·5 gallons per square foot per hour.

New, or recently cleaned, filter-beds allow a large number of organisms to pass through. A filter-bed which is not efficient at first becomes so when the surface film forms, composed of sedimented particulate matter, and of a zooglœal mass of bacteria and algæ. The beds must be cleaned from time to time by raking up and clearing away the surface layer of sand, for as time goes on the rate of filtration becomes slower and slower, though the bacterial efficiency of the filter-beds does not appear to be reduced by prolonged use. The normal bacterial efficiency seems to be rapidly regained after cleaning—within two or three days.

(3) *Sedimentation.*—Besides storage and filtration, sedimentation in the presence of fine particles, either naturally present or artificially added, may also effect a marked removal of micro-organisms from water. Thus,

Average Number of Microbes per c.c. in London Raw Waters.

Gelatine at 20°–22° C.; Counted on the third day. Agar and Neutral red bile-salt lactose Agar at 37° C. Counted after twenty to twenty-four hours.

Years. April–March.	River Thames at Hampton.				River Lee at Ponder's End.			
	Routine Samples	Comparable Samples.[1]			Routine Samples	Comparable Samples.[1]		
	Gelatine.	Gelatine.	Agar.	Bile-salt Agar.	Gelatine.	Gelatine.	Agar.	Bile-salt Agar.
1906–16	**5,709**	**6,167**	**343**	**44**	**14,000**	**16,421**	**497**	**52**
1916–17	14,029	15,630	1,591	163	21,761	23,848	483	33

Average Number of Microbes per c.c. in London Filtered Waters.

(Exclusive of Sample containing 100 or more Microbes per c.c).

Gelatine at 20°–22° C.; counted on the third day. Agar and Neutral red bile-salt Agar at 37° C. Counted after twenty to twenty-four hours.

Years. April–March.	New River.	East London (Lee).	Thames.						
			Kempton Park.	East London.	Chelsea.	Grand Junction.	West Middlesex.	Southwark and Vauxhall.	Lambeth.
1906–16[2] Gelatine	**11·5**	**22·6**	**20·3**	**21·4**	**11·0**	**14·2**	**13·5**	**17·0**	**13·3**
1906–16[2] Agar	**3·5**	**4·3**	**4·6**	**3·0**	**1·3**	**1·8**	**2·6**	**3·8**	**3·0**
1906–16[2] Bile-salt Agar	**0·2**	**0·2**	**0·3**	**0·3**	**0·2**	**0·5**	**0·3**	**0·9**	**1·0**
1916–17 Gelatine	23·5	50·8	35·3	30·0	20·0	37·3	20·2	30·9	25·4
1916–17 Agar	3·8	5·4	6·2	4·1	1·6	2·7	2·0	2·3	1·8
1916–17 Bile-salt Agar	0·1	0·3	0·06	0·1	0·06	0·0	0·04	0·03	0·01

[1] All of these samples were tested by each of the three tests (gelatine, agar and bile-salt agar).

[2] Gelatine ten years' average, 1906–16; Agar, six years' average, 1910–16 Bile-salt agar, three years 1913–16.

by the addition of alum, an old method of clarifying turbid water, a large number of the organisms present are carried down in the precipitate.

The Clark process of softening water may also reduce the number of organisms present, but is very uncertain (Moor and Hewlett). By the Porter-Clark rapid process, however, in which the precipitate of calcium carbonate is removed by filtration through canvas bags, very considerable purification is effected.[1]

Houston has introduced an "excess lime" method. Enough lime is added to the water to render it decidedly alkaline and germicidal for the colon bacillus in five to twenty-four hours (for raw Thames water, about 1 part of lime in 5000 parts of water). At the end of this period a sufficiency of water purified by storage is added so as to precipitate the excess of lime. With Thames water, 3 parts of raw water with 1 part of stored water would be the approximate quantities. By this treatment the water is both purified and softened.

The tables on pp. 669 and 671 illustrate the influence of sand filtration and of storage on the bacterial content of a water.

The Bacteriological Examination of Water[2]

The bacteriological analysis of water affords valuable indications as to the purity or otherwise of a water, and, if properly carried out, will indicate a pollution so small in amount as to be incapable of detection by chemical methods.

[1] Nankivell, *Journ. of Hyg.*, xi, 1911, p. 246; Hewlett and Nankivell, *Rep, Med. Off. Loc. Gov.* for 1911–12, p. 350.

[2] See Savage, *Bacteriological Examination of Water Supplies* (Lewis, 1906); Thresh, *Examination of Water and Water Supplies* (Churchill, Ed. 2, 1913); Houston, Gordon and others in *Reps. Med. Off. Loc. Gov. Board*, 1899–1904; Houston, *Reports to the Metropolitan Water Board* and *Studies in Water Supply* (Macmillan & Co., 1913).

Effect of Storage on the Bacterial Content of Water (Houston, 1908)

DESCRIPTION OF THE SAMPLE.	Average Number of Microbes per c.c.			*B. coli* test (typical *B. coli*) (percentage results).							
	Gelatin at 20°–22° C. 3 days.	Agar at 37° C. 2 days.	Bile-salt Agar at 37° C. 2 days.	Negative in 100 c.c. %	+ 100 c.c. %	+ 10 c.c. %	+ 1 c.c. %	+ ·1 c.c. %	+ ·01 c.c. %	+ ·001 c.c. %	+ ·0001 c.c. %
Raw Thames *before* storage — Average of 8 months ended March 31, 1908	**4218** (165 samples)	**308** (59 samples)	**47** (58 samples)	—	**1·8**	**11·5**	**32·7**	**43·0**	**1·30**	**0·6**	—
Staines Stored Water (8 months) 53 samples	230	42	3	24·5	28·3	32·0	15·1	—	—	—	—
Chelsea ,, ,, (8 months) 59 samples	224	52	4	28·8	32·2	22·0	11·8	3·4	1·7	—	—
Lambeth ,, ,, (6 months) 50 samples	391	51	3	6·0	28·0	32·0	22·0	12·0	—	—	—
Raw Lea *before* storage — Average of 8 months ended March 31, 1908	**9442** (165 samples)	**398** (59 samples)	**33** (58 samples)	**0·6**	**0·6**	**5·4**	**41·8**	**38·2**	**9·7**	**3·0**	**0·6**
Lea Stored Water (8 months) 59 samples	56	11	0·5	62·7	32·2	5·1	—	—	—	—	—

The specimen of water should be collected in clean bottles of about 100–200 c.c. capacity, sterilised preferably by heat. If, however, the bottles be thoroughly cleaned and rinsed out with a little strong sulphuric acid, and then thoroughly rinsed several times with the water to be examined before taking the specimen, no error will be introduced. The stopper of the bottle should be tied down with a thin layer of cotton-wool enclosed between two pieces of muslin, and the bottle should be not quite filled. In taking the specimen the following details should be attended to :

(1) If taken from a tap, the water should be allowed to flow for at least five minutes before the specimen is collected.

(2) The water from a cistern is not a representative sample of the water-supply ; to be so the specimen should be taken direct from the main.

(3) If taken from a stream or pond, the bottle should be held about a foot below the surface and away from the edge before the stopper is removed.

(4) If taken from a well the conditions should be noted, *e.g.* whether the well has been recently disturbed or not, whether the pumps have been in operation, etc., for such may markedly influence the number of bacteria found.

The specimen should then be examined with as little delay as possible, for if allowed to stand for any time a large increase in the number of bacteria may take place. Frankland, for example, found that in distilled water, even at the ordinary temperature, organisms multiply enormously :

Hours	Number of organisms in 1 c.c.
0	1,073
6	6,028
24	7,262
48	48,100

In water of good quality the organisms are found to multiply much more rapidly during the first few days, after which time they become less and less numerous; but in impure water multiplication is slower, and the number more persistent, while in very impure water the number may diminish. It is essential, therefore, if reliable results are to be obtained, for the specimen to be examined at once (within three hours). If this cannot be done the specimen should be packed in ice; the cold will then inhibit multiplication to any extent. Special double-chambered metal boxes are made for this purpose: the bottle containing the sample is placed in the inner chamber, the outer chamber (which surrounds the inner) being filled with a mixture of ice and sawdust, and the whole is packed in a wooden box with felt lining. The addition of 10 per cent. of common salt to the sample has been stated to preserve the original bacterial content of the water unaltered up to ninety-six hours after taking the sample, without icing, but Raju and Fox find it unreliable. Besides the sample packed in ice, a "Winchester quart" of the water may also be collected for examination for the spores of the *B. Welchii* group.

The routine bacteriological examination of the specimen may be carried out according to the following scheme (here somewhat modified) drawn up by committees of the Royal Institute of Public Health:[1]

PROCEDURES.—The following procedures should be carried out:

(*a*) Enumeration of the organisms which will develop aërobically in gelatin at 20° C.

(*b*) Enumeration of the organisms which will develop aërobically in agar at 37° C. (Enumeration is carried out by counting the number of colonies which develop in the plates [see below].)

[1] *Journ. State Med.*, vol. xii, 1904, p. 471, and vol. xxii, 1914, p. 558

(*c*) Search for *Bacillus coli*, and identification and enumeration of this organism if present.

(*d*) Search for, and enumeration of, streptococci.

As a *routine* measure it is not necessary to search for the *Bacillus Welchii* group, but in special instances it may be desirable to do so.

The bottle must be well shaken to mix the sample. Before removing the stopper, it and the neck of the bottle should be swabbed with absolute alcohol, which is then ignited and allowed to burn away.

MEDIA, TIME OF INCUBATION, ETC.—For the gelatin count ordinary nutrient gelatin is employed, the period of incubation being seventy-two hours. In hot weather it may be necessary to use 15–20 per cent. gelatin (unless an incubator which can be *cooled* is available), but the development of the colonies is slower. For the agar count ordinary nutrient agar is used, the period of incubation being forty to forty-eight hours.

The media should preferably be recently prepared and be standardised to a reaction of + 10.

In addition to the actual numbers of organisms which develop in the gelatin and in the agar, a comparison of the ratio of the number of organisms developing in gelatin at 20° C. to those developing in agar at 37° C. also gives useful indications. With a pure water this ratio is generally considerably higher than 10 to 1 ; with a polluted water this ratio is approached, and frequently becomes 10 to 2, 10 to 3, or even less. The actual number of organisms growing at blood-heat is of considerable value apart from any question of ratio.

In certain instances it is true that this ratio may be unreliable. Thus with surface waters, especially in the tropics (as pointed out by Horrocks) varieties of the *B. fluorescens liquefaciens* and *non-liquefaciens* and *B. liquefaciens* may be abundant and grow well at blood-heat.

Distilled water gelatin and agar have also been recommended, but since the organisms of polluted water develop better in the ordinary *nutrient* media, the latter are preferable for routine use.

Amounts to be Plated, Size of Dishes, etc. *Gelatin.*—For an ordinary water amounts of 0·1, 0·2 and 0·3 c.c. may be plated in Petri dishes of about 10 cm. diameter, preferably done in duplicate.

Agar.—Two plates may be made with 0·1 and 0·2–0·3 c.c., and are preferably duplicated.

The desired volume of water should be run into the sterile Petri dish by means of a sterile 1 c.c. pipette graduated in hundredths. The tubes of gelatin should be melted in a water-bath at a low temperature (40° C.). A tube is taken from the water-bath, wiped to prevent the adherent water running down into the Petri dish, its mouth is singed in the Bunsen flame to sterilise it, and the contents are then quickly poured into the dish and mixed with the water by tilting the dish several times.

The agar tubes must first be boiled, then cooled to about 45° C., and similarly treated, or surface plates may be made.

If waters are constantly being examined, it saves trouble to have the gelatin and agar in small flasks, 30–60 c.c. of the former and 20–40 c.c. of the latter; a flask of each will then be used for an examination.

In dealing with an unknown water, and in all cases of doubt, additional plates should be prepared with a dilution of the water (made with sterilised tap-water) of ten or hundred fold, according to circumstances.

The amount of the medium in a plate should be 10 c.c.

The counting is done with the naked eye, preferably in daylight, any doubtful colony being determined with the aid of a lens or low power objective. The number of liquefying colonies in the gelatin plates should also be noted. The plates should be inspected daily, in order that the count may be made earlier should liquefaction render this necessary.

In examining an ordinary drinking-water there is no need ever to dilute. As 1000 or 1500 colonies can be counted on a plate, and if the number on a plate should be, owing to crowding, uncountable, *ipso facto* this would be sufficient to condemn without an actual count. Dilution is necessary when dealing with river or other water known to be polluted, and of which an estimate of the number of organisms present is desired. In order to count the colonies if very numerous, ink lines may be drawn across the bottom of the Petri dishes so as to divide them into sectors. Ruled paper discs (Pakes's discs) upon which the dishes are placed can also be obtained. The colonies in the sectors are then much more easily counted: or if the colonies be very numerous and evenly distributed, the number in two or three of the sectors may be counted, and the total number on the plate estimated by calculation.

Search for Bacillus coli, etc.—Various media may be employed for the detection, isolation, and enumeration of *B. coli*. The writer generally employs as a preliminary glucose bile-salt peptone-water, but many other media may be employed, *e.g.* formate or neutral-red broth, or if the organism is abundant, neutral-red bile-salt agar.

As a routine for a purified water, a quantity of 100 c.c. in all should be examined for the presence of the *Bacillus coli*, quantities from a minimum of 0·1 c.c. to a maximum of 100 c.c. being added to the tubes of culture media.

It is preferable to add the water directly to the tubes of culture medium, even with the larger amounts, and not to concentrate the bacteria by any method. The culture media may be diluted with at least an equal volume of the water without interfering with their cultural properties, and large tubes or small flasks are used for the larger amounts.

In the case of glucose or lactose bile-salt peptone-water, the medium may for the larger amounts, down to 10 c.c., be prepared of double strength. The glucose or lactose bile-salt peptone water should be incubated at 37°–40° C. for not less than forty-eight hours.

For composition of glucose formate broth, glucose and lactose bile-salt media, and neutral-red broth, see p. 683, *et seq.* While a lactose medium has the advantage of excluding a number of forms which, though fermenting glucose, do not ferment lactose, and are therefore not typical *B. coli*, Houston has found that a glucose medium is more delicate than a lactose one. For general purposes, quantities of from 0·1 to 100 c.c. may be added to tubes of the medium selected. Houston cultures quantities of 0·1, 1·0, 10 and 100 c.c.; here the gaps are rather wide. Greenwood and Yule consider that from the mathematical standpoint a geometrical series of quantities should be used. In practice it will probably be best to put up two series, one of 100, 50, and 25 c.c., and a second of 40, 20, 10, 5, 2·5, and 1·0 c.c., with an additional one of 0·1 c.c, The first series is available if the water be of good to medium quality, the second if it be medium to bad. For the larger amounts large test-tubes, boiling tubes and flasks must be employed.

If the medium shows changes (acid + gas) suggestive of the presence of *B. coli*, it is only *presumptive* evidence of the presence of this organism. Occasionally other organisms produce a similar change, *e.g. B. lactis aërogenes, B. cloacæ.* Hence the necessity for the isolation and identification of the organism as recommended in the next section.

Isolation of Bacillus coli, if present.—If indications of the presence of the *Bacillus coli* be obtained in the preliminary cultivations (acid + gas), the organism must be isolated and identified. If several tubes show acid + gas, one or two of the tubes with the smallest quantities of the water should be used for this purpose.

This may be done by making *surface* cultures on plates of either (*a*) litmus lactose agar, reaction + 10; (*b*) litmus lactose bile-salt agar; (*c*) Conradi and Drigalski agar, which the writer generally employs; or (*d*) ordinary nutrient gelatin. Agar media, incubated at 37° C., have the advantage of saving time. (For composition of media, see p. 683, *et seq.*)

Identification of, and Tests for, the Bacillus coli.—Having obtained coli-like colonies on the plates

made from the preliminary cultivations of the water, various tests must be used for identification. The organism should conform in morphology, motility and staining reactions with the characters of the typical *B. coli* as given at pp. 434–438, and must be subjected to various cultural tests, *e.g.* the "flaginac" reactions of Houston (p. 437). The writer generally employs these, with the addition of the fermentation reactions given by dulcitol, mannitol, and adonitol litmus peptone water, and gelatin for absence of liquefaction. If atypical *Bacilli coli* (see pp. 434 and 441) are met with, the fact should be noted, but their significance is not yet fully determined. Experience shows that if a water yields glucose-fermenters, sooner or later it will contain lactose-fermenters.

Streptococci.—It is a distinct advantage to search for streptococci. They may be looked for by making hanging-drop preparations of the fluid media employed for the preliminary cultivation of the *B. coli* (glucose or lactose bile-salt peptone water, etc. Glucose formate broth or glucose neutral red broth incubated for 40–48 hours are the best). The presence or absence of streptococci in these tubes gives also a quantitative value to the examination, just as in the case of *B. coli*, and the result obtained should be stated. The streptococci can be readily isolated on Conradi-agar plates.

According to Houston (*loc. cit.*), fæces contain *at least* 100,000 streptococci per gramme. The type of streptococcus generally present is one forming short chains, producing a uniform turbidity in broth, acid and clot in litmus milk within five days at 37° C., and non-pathogenic for mice. (See Table, p. 257.) All but lactose-fermenters should be neglected.

Bacillus Welchii Group.—As already stated, it is not essential as a routine procedure to search for the *Bacillus Welchii group*, though in certain instances it may

be of advantage to do so. A negative result in such cases is probably of more value than a positive one.

For the isolation of *B. Welchii*, 500 c.c. of the water may be filtered through a Pasteur-Chamberland filter, the deposit is suspended in 5 to 6 c.c. of sterile water, and 1 c.c. of the suspension added to each of five to six tubes of sterile milk, which are then heated to 80° C. for ten minutes in a water-bath, and incubated anaërobically at 37° C. for forty-eight hours (filter-brushing method). A better method [1] is to employ large boiling tubes or small Erlenmeyer flasks, each containing 25 to 50 c.c. of sterile milk. To each tube a quantity of water equal to that of the milk is added, the tubes are then heated in a water-bath to 80° C. for fifteen to twenty minutes, some sterilised liquid paraffin, oil or melted vaseline is poured on the surface to exclude air, the tubes are cooled in water to 37° C or thereabouts, and incubated for forty-eight hours at 37° C. Not less than 200 c.c. of the water should be used. The typical change in the milk (see p. 486) indicates the probable presence of the organism. To make sure that the change is due to the *B. Welchii* group and not to the *C. butyricum*, 1 c.c. of the whey per 100 grm. of body-weight should kill a guinea-pig in forty hours when injected subcutaneously.

The virulence of a peptone-water culture has been suggested as an index of contamination, but in the writer's hands has not given reliable results. If sufficient peptone and salt be added to a measured volume of the water to form a 1 per cent. solution of the former and a $\frac{1}{2}$ per cent. solution of the latter, the mixture incubated at 37° C. for twenty-four hours and injected intraperitoneally into a guinea-pig, a bad water is stated to kill, whereas a good one does not. The amount to be injected is 2 c.c. and death should ensue within forty-eight hours.

Interpretation of Results.—The interpretation of the results of the bacterioscopic examination of water is a difficult matter, for which experience is necessary. Just as in chemical analysis, it is not possible to lay down an *absolute* standard, a knowledge of the source and surrounding conditions being of the greatest importance in forming an opinion. The ultimate aim is, of course, the

[1] R. T. Hewlett, *Trans. Path. Soc. Lond.*, vol. lv, 1904, p. 123.

detection of sewage or fæcal pollution ; the bacterioscopic analysis does not give any information as to the suitability of the water for household, trade, or factory purposes.

Number of colonies on the gelatin plates.—The number of colonies represents approximately the number of organisms in the original sample capable of development aërobically at 20° C. in gelatin. This number in a good water rarely exceeds 100 or 150 ; in pure waters, particularly those coming from deep chalk-wells, there may be only a few—5 to 10 per c.c. (the results are always expressed in numbers per cubic centimetre of the original water). In waters of poorer quality the number may approach 500 per c.c. Anything over this casts suspicion on the water, and 1000 per c.c. or more should probably condemn the sample, always supposing, of course, that multiplication *in vitro* has been excluded by the proper storage of the sample bottle in ice. As a rule in water of good quality liquefying organisms are scanty, while in a polluted water they are numerous.

Number of colonies on the agar plates.—As mentioned before (see p. 674), it is the ratio of the number of organisms developing on the agar plates to the number of those developing on the gelatin plates that is of importance.

Number of B. coli.—The detection and enumeration of *B. coli* are regarded by all as perhaps the most important part of water examination. The number of *B. coli* is estimated from the amounts of water that have been added to the tubes of media, which, however, assumes that the organism is regularly distributed throughout the sample, and this must so far as possible be ensured by thorough mixing. The results generally come out fairly concordantly, though irregularities exceptionally occur ; Greenwood and Yule[1] give some data by which

[1] *Journ. of Hygiene*, vol. xvi, 1917, p. 36.

approximation may be made if this happen. It is better to state the result as "*B. coli* present in, or absent from, ... c.c. of water" rather than to say that so many *B. coli* are present, though as a matter of fact the latter statement is probably approximately correct.

If nothing is known about the water, the following standards may be adopted :

(*a*) *Waters of first-rate quality.*—*B. coli* absent from 100 c.c.

(*b*) *Waters of good quality.*—*B. coli* present in 100 c.c., but absent in 50 c.c. of the water.

(*c*) *Waters of medium quality.*—*B. coli* present in 50 c.c., but absent in 25 c.c.

(*d*) *Waters of poor quality.*—*B. coli* present in 50 c.c. and 25 c.c., but absent in 10 c.c.

(*e*) *Waters of suspicious quality.*—*B. coli* present in 50 c.c., 25 c.c., and 10 c.c., but absent in 1 c.c.

(*f*) *Waters unfit for drinking.*—*B. coli* present in 1 c.c. or less.

Waters which show no *B. coli* in 50 c.c. are of a high degree of purity, and therefore the proved absence of this organism in this amount, and still better in larger quantities, is of great value.

B. coli should be absent from at least 50 c.c. of spring or deep well water, possibly from greater amounts.

In upland surface waters the presence of *B. coli* in 40, 10, or even 2 or 1 c.c. means contamination, but not necessarily a contamination which it is essential to prevent. It may be from contamination with the excreta of animals grazing on the gathering areas, and is by no means necessarily from sewage or other material containing specific organisms of infection. If *B. coli* are present in numbers greater than, say, 500 per litre (or even in that amount), such a water is suspicious, as it is rare to get so many *B. coli* in a water from the kind of animal contamination indicated, and further investigation is desirable. In filtered samples the number of *B. coli* is, as a rule, considerably reduced.

In surface wells *B. coli* in large numbers indicate surface or

other contamination, generally very undesirable if not actually dangerous.

It must clearly be understood that the presence of the *B. coli* in water is used as an *index* of pollution, just as the organic ammonia is in a chemical analysis. This organism is not necessarily harmful in itself; it is what it indicates, viz. *pollution*, probably with human excremental matters, which may contain the organisms of specific disease, *e.g.* typhoid, dysentery, and cholera. As a *routine*, the typhoid bacillus is never looked for, and the statement sometimes seen in the report on the bacteriological examination of a sample of water that "no typhoid bacilli have been detected" is of little value. It is on the *general* results of the examination, as detailed in preceding pages, that a conclusion is arrived at respecting the purity or otherwise of a water.

Bacillus Welchii Group.—This group of organisms being abundantly present in fæces and sewage, its presence in water has been suggested as an indication of pollution. The spores, however, are very resistant, and might, therefore, gain access to the water in ways other than by direct pollution—*e.g.* in dust—and for this reason the Committee did not recommend the search for this organism as a routine procedure. On the other hand, Thresh [1] lays a good deal of stress on it. If spores of *B. Welchii* are present but no *B. coli* it may indicate old pollution or that there is drainage from manured soil. In gross polution both *B. coli* and spores of *B. Welchii* will be present.

Streptococci.—Streptococci are abundant in fæces and sewage, but are extremely rare, if ever present, in unpolluted natural waters; hence the value of their detection. Streptococci as a class are delicate organisms, and it was supposed that their presence indicates *recent* pollution.[2] Horrocks, on the other hand, believes that they maintain their vitality longer even than *B. coli*, and this is rather

[1] *Public Health*, 1904.

[2] Houston, *Rep. M d. Off. Loc. Gov. Board* for 1898–99.

the opinion at present. We need further data before we can exactly estimate the value of streptococci as indicators of pollution. There can be no question, however, that the detection of many streptococci, together with *B. coli*, indicates serious pollution.

There can be no doubt of the value of the bacteriological examination of water, but it cannot entirely supplant chemical analysis, which on account of its rapidity and the valuable data it yields will probably always remain an integral part of the examination of potable waters. If the water be pure and uncontaminated, the bacteriological examination will occupy three days; but if contamination be present, though it may be *presumed* in the same time, ten days or a fortnight may be required to convert this presumption into a *certainty*, owing to the length of time necessary for determining the characters of the organisms present.

Media Employed for the Isolation of B. Coli

(1) *Carbolised gelatin.*—Ordinary nutrient gelatin with the addition of 0·05 per cent. of phenol. (Hardly used now.)

(2) *Bile-salt peptone water* (MacConkey and Hill).—The composition of this medium is as follows: Sodium taurocholate 0·5 grm., glucose or lactose 1·0 grm., peptone 2·0 grm., water 100 c.c. The constituents are dissolved by heating; the mixture is filtered, and after filtration sufficient neutral litmus solution is added to give a distinct colour. The medium is then distributed into Durham's fermentation-tubes and sterilised by steaming for twenty minutes on three successive days. The medium may be put up in various sized tubes, a measured volume in each—*e.g.* 10 c.c., 20 c.c., 25 c.c., etc., according to the quantity of water which is to be added. For the larger quantities the medium may be made double the above strength. The inoculated tubes are incubated at 37°–40° C. for forty-eight hours. The *B. coli* reddens and ferments both the glucose and lactose media, so that gas collects in the fermentation tube.

(3) *Neutral-red broth* (Hunter, Makgill, Savage).—The dye known as neutral-red (Grübler's) is reduced by the action of the *B. coli*, the colour changing to a canary yellow, accompanied by a green fluorescence. The *B. enteritidis* (Gärtner) also reduces neutral-red, but the *B. typhosus* does not do so, nor do strepto-

cocci, *B. pyocyaneus*, and *Vibrio choleræ*. Some anaërobes also possess a reducing action. Glucose agar or broth (0·5 per cent. of glucose) is employed, and to every 10 c.c. of the medium 0·1 c.c. of a 0·5 per cent. aqueous solution of neutral-red is added. Savage recommends the following procedure: 10 c.c. of the water are added to a 10 c.c. tube of neutral-red broth; also to 40, c.c. of the water contained in a bottle or flask a 10 c.c. tube of the broth of *quadruple strength* is added. Both are incubated at 37° C., and examined daily up to eight days. If reduction occurs, *B. coli* is almost certainly present in the water; if reduction does not occur its presence is highly improbable.

(4) *Glucose formate broth* (Pakes).—To ordinary meat infusion 1 per cent. peptone, 0·5 per cent. sodium chloride, 2 per cent. glucose, and 0·4 per cent. sodium formate are added. When these have been dissolved by heating, the medium is neutralised (indicator, litmus), and after neutralisation 2 c.c. of normal caustic soda solution per litre are added; the broth is then steamed for twenty minutes, filtered, and distributed into test-tubes, 10 c.c. in each, which are steamed for twenty minutes on each of three successive days. These tubes are inoculated with the water, and incubated anaërobically at 42° C. for twenty-four to seventy-two hours. Tubes showing any growth at the end of twenty-four, forty-eight, or seventy-two hours are removed and examined microscopically and by plating.

(5) *Bile-salt lactose agar* (MacConkey).—This medium is prepared by adding to 1000 c.c. of tap-water in a flask 2 per cent. of peptone, 0·5 per cent. of sodium taurocholate, and 1·5 per cent. of agar. The mixture is autoclaved at 105° to 110° C. for 1½ hours, cleared with a small addition of white of egg, and filtered. To the filtrate 1 per cent. of lactose is added. The medium is then distributed into test-tubes, 10 c.c. in each, and sterilised by fifteen minutes' steaming on three successive days. Plates are made and incubated at 42° C. for forty-eight hours. The colonies of organisms which ferment lactose with the formation of acid are surrounded with a cloudiness or haze owing to the precipitation of the taurocholate. Neutral-red or krystal violet or litmus may be added (proportions, see Nos. 3 and 6).

(6) *Conradi-Drigalski agar. Mixture A.*—To 1 litre of acid beef broth (p. 62) add:

Witte's peptone	10 grm.
Nutrose	10 ,,
Sodium chloride	5 ,,

Steam for one hour, and add 25 grm. of powdered agar. Steam for three hours, bring to a reaction of + 10, and filter through "papier Chardin."

Nutrose is now unobtainable. As a substitute Mackenzie Wallis recommends the following:

Pea-nut flour	94 parts
Casein	5 parts
Sodium carbonate	1 part

The mixture is used in the same quantity and manner as nutrose.

Mixture B.—Boil for a few minutes 100 c.c. of Kubel-Tiemann's litmus solution, add 15 grm. of pure powdered lactose, and boil again for a few minutes.

Add *B* to *A*, and to this mixture add 2 c.c. of a hot 10 per cent. solution of anhydrous sodium carbonate and 10 c.c. of a 0·1 per cent. solution of krystal violet. The medium is then tubed, 10 c.c. being placed in each test-tube, and sterilised.

In using the medium it should be employed as *surface* plates. The required number of tubes are melted in a water-bath, and their contents poured out into sterile Petri dishes and allowed to set. These sterile plates are then placed in the warm incubator for an hour or so with the lids slightly tilted at one edge, so that the surface of the medium may dry somewhat. The matter to be plated is sufficiently diluted, and from a few drops to 0·5 c.c. are run on to the surface and spread by means of a glass rod bent into a flattened hook, and sterilised by boiling. On this medium in forty-eight hours *B. coli* form large red colonies, *B. typhosus* and *B. dysenteriæ* small blue colonies, and streptococci small delicate red colonies. Other organisms are to a large extent inhibited from developing.

(7) *S.D.S. rebipelagar* (Houston).—"Rebipelagar" has been much used by Houston [1] for the isolation of *B. coli*. It has the following composition: Agar 20 grm., taurocholate of soda 5 grm., lactose 10 grm., neutral-red 4 c.c. of a 1 per cent. solution, peptone 20 grm., water 1 litre. The S.D.S. rebipelagar has the following composition: Agar 20 grm., taurocholate of soda 5 grm., lactose 2·5 grm., neutral-red 4 c.c. of a 1 per cent. solution, peptone 20 grm., saccharose 2·5 grm., dulcitol 2·5 grm., salicin 2·5 grm.

[1] *First Rep. on Research Work*, Met. Water Board, 1908.

The Isolation of Specific Organisms from Water

The principal disease-producing organisms conveyed by water are the *B. typhosus*, *B. paratyphosus*, *B. dysenteriæ*, and *Vibrio choleræ*.

THE ISOLATION OF B. TYPHOSUS, B. PARATYPHOSUS, AND B. DYSENTERIÆ FROM WATER.—There is great difficulty in isolating the *B. typhosus* from water that has been very copiously contaminated with specifically polluted sewage, there is, therefore, far greater difficulty when the specific pollution has been small in amount. The earlier records of the isolation of the *B. typhosus* must be accepted with much scepticism, as the methods of identification were formerly incomplete and unsatisfactory. It is necessary to bear in mind that usually, when drinking-water has suffered sewage-pollution, the amount of the pollution is relatively very minute when compared with the great bulk of the water-supply. Moreover, allowing ten days as the average incubation period of typhoid fever, another week before the disease comes under notice, and another week before the fact that an epidemic is in progress is recognised, at least a month will have elapsed between the date of infection of the water-supply (supposing this to have occurred on one occasion only, as may be the case) and the taking of the samples for examination, a period during which all the typhoid bacilli may have died out. The contamination of water may, however, be of an intermittent nature.

Numerous methods [1] have been devised for the isolation of the typhoid bacillus from an infected water. With rare exceptions, it is impossible to detect the organism by direct plating ; it is too scanty and too mixed with other organisms to admit of this, and therefore concentration of the bacterial content of the water must be attempted. The following are some of the methods which have been suggested for this purpose ; they serve equally well for *B. paratyphosus* and *B. dysenteriæ*.

1. *Filtration through a porcelain filter.*—By passing one to two litres of the water through a sterile Pasteur-Chamberland filter, the whole of the organisms present may theoretically be collected in a few c.c.s. Practically, however, a large proportion of the organisms are lost in the process : perhaps they get carried into

[1] See H. S. Willson, *Journal of Hygiene*, vol. v, 1905, p. 429 ; McWeeney, *Brit. Med. Journ.*, 1909, vol. ii, p. 866.

and remain in the superficial layers of the filter-candle, and for this reason, though sometimes employed, this method has been largely given up.

2. *Concentration.*—W. J Wilson [1] has devised the following method: The water is placed in one or two Winchester quart bottles, and 10 c.c. of nutrient broth are added for every litre. The bottles are placed in a water-bath maintained at 37°–40° C., and are connected by rubber corks and tubing with a condenser (at a lower level) through which cold water continuously passes, and the tube of the condenser is connected to a large bottle (at a still lower level). This bottle is kept partially exhausted by means of a filter-pump. The water evaporates and is thus concentrated, the evaporated water being condensed and collected in the exhausted bottle. It requires twenty-one to twenty-two hours to evaporate a litre of water. The water remaining in the bottles, now concentrated to a few c.c.s., is then plated on Conradi-Drigalski or malachite-green agar.

3. *Chemical precipitation.*—These methods depend on the formation in the water of a fine, inert precipitate, which entangles, and carries down with it a large proportion of the bacteria present. Thus in the Vallet-Schüder [2] method, to 2 litres of the water are added 20 c.c. of a 7·75 per cent. solution of sodium hyposulphite and 20 c.c. of a 10 per cent. solution of lead nitrate. The precipitate is allowed to settle or is centrifuged off, is dissolved in a small volume of a saturated solution of the hyposulphite, from which plates are made in suitable media. Ficker [3] uses ferrous sulphate after making the water faintly alkaline with caustic soda; the ferrous hydrate formed carries down the micro-organisms (this must be a risky procedure, as the typhoid bacillus is very sensitive to caustic alkalies). Iron oxychloride may also be used as the precipitant. H. S. Willson (*loc. cit.*) employs alum. A stock solution of alum is prepared, containing 10 grm. per 100 c.c., and of this sufficient is added to the water to obtain 0·5 grm. to the litre. After the precipitate of aluminium hydrate has formed, the vessel is well shaken to mix its contents, and the mixture is centrifuged for fifteen minutes at 2000 revolutions per minute. The clear, supernatant fluid is then syphoned or poured carefully off from the precipitate, and

[1] *Brit. Med. Journ.*, 1907, vol. i, p. 1176.
[2] *Zeitschr. f. Hyg.*, xlii, No. 2, p. 317.
[3] *Hyg. Rundschau*, xiv, No. 1, 1904, p. 7.

the mass of precipitate in the conical extremity of the tube stirred up with the little fluid (0·5 to 1 c.c.) remaining. The suspension is then plated out on Conradi-Drigalski, malachite-green or brilliant-green, agar. This seems to be a very promising method.

4. *Serum agglutination.*—An anti-typhoid serum—the serum of an animal which has been inoculated several times with the typhoid bacillus, having the power of agglutinating typhoid bacilli—if added to a water would presumably agglutinate any typhoid bacilli into masses which will sediment or may be centrifuged off. The method has been used by Schepilewsky [1] who adds 10 to 20 c.c. of the water to flasks containing 50 c.c. of nutrient broth, to which after three or four days' incubation at 37° C. an addition of the typhoid serum is made, and after standing for some hours and centrifuging, the deposit is plated out.

5. *Method of enrichment.*—The principle of this method is to devise a medium which will allow of the multiplication of the typhoid bacillus and at the same time prevent, or at least retard, the growth of *B. coli* and allied forms. Almost all the methods which have been introduced for this purpose fail, inasmuch as though they inhibit the growth of a great many organisms, they do not inhibit the growth of the *B. coli*, or, if they do, inhibit the *B. typhosus* to a still greater degree. Roth [2] found that caffeine in broth would retard *B. coli*, but allow *B. typhosus* to multiply. The method has been further elaborated by Hoffmann and Ficker,[3] who convert the water itself into a nutrient medium by the addition of 1 per cent. of nutrose, 0·5 per cent. caffeine, and 0·001 per cent. of krystal violet. The mixture is incubated at 37° C. for not more than twelve to thirteen hours, at the end of which time the typhoid bacilli should have multiplied to such an extent as to permit of direct isolation by plating, the *B. coli* being inhibited. Many observers have shown, however, that while caffeine may materially help, it cannot be entirely relied on to eliminate *B. coli* and allied forms.

6. *Process of Cambier.*—Cambier [4] has devised a process based on the idea that an actively motile organism will find its way through the pores of a porcelain filter more quickly than feebly or non-motile forms. His procedure is to make use of a special

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[1] *Centr. f. Bakt., Orig.,* xxiii, No. 5, 1903.
[2] *Hyg. Rundschau,* xiii, 1903, p. 489.
[3] *Ibid.* xiv, 1904, p. 1.
[4] *Rev. d'Hyg.,* 1902, p. 64.

alkaline peptone medium, which is placed in a glass jar. In this is immersed a Pasteur-Chamberland filter-candle half filled with the same solution, to which is added a little of the fluid to be examined, and the whole is incubated at 37° C. Sooner or later growth appears in the fluid outside the candle, and Cambier states that if typhoid bacilli be present they will make their appearance before *B. coli*. In hands other than those of Cambier, however, the method has not proved successful.

7. *Fuchsin agar* (Endo).—One litre of 3 per cent. nutrient agar is made alkaline with 10 c.c. of 10 per cent. NaOH solution after neutralisation. Pure lactose 10 grm. and saturated alcoholic fuchsin solution 5 c.c. are added, and after mixing, 25 c.c. of fresh 10 per cent. solution of sodium sulphite are added. The medium when cold should be colourless. The medium is used as surface plates, and on it typhoid and paratyphoid colonies are colourless, coli colonies are red.

8. *Malachite-green media.*—Löffler has found that malachite green (No. 120 Hoechst) in the proportion of about 1 in 5000 in media inhibits the growth of *B. coli* while still permitting the growth of *B. typhosus*. The dye may be added either to liquid or to solid media. The medium recommended by Löffler [1] is composed of 3 per cent. agar made with meat infusion, with 1 per cent. nutrose, and containing in every 100 c.c. 2–2·5 c.c. of a 1 per cent. solution of malachite green. On this medium the *B. typhosus* grows in twenty-four hours as delicate, slightly crinkled colonies, surrounded by a colourless zone (due to alkali formed by the bacilli). Thus it is possible to detect one colony of *B. typhosus* among 300 to 600 colonies of other bacteria. As a medium for "enriching"—*i.e.* for specially advancing the growth of the *B. typhosus*—Löffler recommends a 15 per cent. gelatin, prepared with beef-juice and peptone, and containing per 100 c.c. 3 c.c. of doubly normal phosphoric acid and 2 c.c. of 2 per cent. malachite-green solution. With the suspected matter, firstly, one series of malachite-gelatin plates is prepared and incubated at 25° C. for twenty to twenty-four hours; secondly, a tube of malachite gelatin is inoculated and incubated at 37° C. for twelve to twenty-four hours; from this a second tube is inoculated and incubated at 37° C., and then plated out on malachite gelatin and incubated at 25° C. The colonies of *B. typhosus* are well marked after twenty to twenty-four hours, as large as a pin's head,

[1] *Deutsch. med. Woch.*, 1906, No. 8.

transparent, highly refractile, light grey and granular. Their shape is circular or oval, and they show characteristic offshoots resembling a bone-corpuscle or the body of an acarus. By using this 15 per cent. gelatin, which can be incubated at 25° C., there is the double advantage of speedy growth and formation of very characteristic colonies.

Houston recommends S.D.S. rebipelagar (p. 685) with the addition of malachite-green to the extent of 1 in 5000 (0·2 grm. to the litre). On this medium *B. typhosus* forms colourless colonies; most other bacteria do not grow, or appear as blue-black colonies.

9. *Werbitzki's China green agar.*—For this 3 per cent. nutrient agar (reaction + 13) is used, and to every 100 c.c. of the agar 1·4–1·5 c.c. of a 0·2 per cent. aqueous solution of china green (Grübler's) are added.

10. *Brilliant green agar.*—Conradi devised an agar containing brilliant green and picric acid, and this has been modified by Fawcus [1] as follows: To 900 c.c. of tap-water are added sodium taurocholate, 5 grm.; powdered agar, 30 grm.; peptone, 20 grm.; and sodium chloride, 5 grm. Dissolve the constituents by steaming for three hours, filter through wool, and bring to a reaction of + 15 (by means of lactic acid or NaOH, as the case may be). In 100 c.c. of distilled water dissolve 10 grm. lactose and add this to the former, filter, distribute in flasks (100 c.c. in each), and sterilise. At time of using, melt and add to each 100 c.c., 2 c.c. of a 1–1000 aqueous solution of brilliant green and 2 c.c. of a 1–100 aqueous picric acid (extra-pure, Grübler's). Typhoid forms round, transparent refractile colonies of a light pale green colour by transmitted light, *B. coli* dark green colonies with an opaque spot at the centre.

Houston found malachite green agar a better medium for the isolation of the typhoid bacillus than a liquid brilliant green fluid medium (see p. 422).

Conclusion.—The writer would suggest for the isolation of *B. typhosus* from water: (1) Concentration of the organism by precipitation with alum (Willson's method) or iron oxychloride, followed by plating of the precipitate on Conradi-Drigalski agar, or, better, on malachite green agar (Löffler's or Houston's, No. 8 above), or brilliant-

[1] *Journ. Roy. Army Med. Corps*, February 1906, p. 147.

green agar (No. 10 above); (2) enrichment by Löffler's method and subsequent plating. In all cases the organism isolated must be examined as to its morphological, cultural, and biological characters, and should have its agglutination reaction tested with a high-grade typhoid serum. Two organisms which are likely to be mistaken for the *B. typhosus*, unless *all* tests are applied to them, are the *B.* (*fæcalis*) *alkaligenes* and *B.* (*aquatilis*) *sulcatus*. Both occur in the dejecta and in polluted water, and are very like the *B. typhosus* in morphology, motility, staining, and cultural reactions, but neither agglutinates with typhoid serum. The *B. alkaligenes* may form a brownish growth on potato, and renders litmus milk alkaline and produces alkali, but no gas, in glucose, lactose, dulcitol, mannitol, saccharose, and salicin. It occasionally causes a general infection in man, with intermittent or continuous fever. The *B. sulcatus* hardly grows at 37° C. andis almost a strict aërobe. Some varieties of typical and of a typical *B. coli* tend to agglutinate with typhoid serum, so the agglutination reaction must be carried out quantitatively.

The Isolation of the Cholera Bacillus from Water.—The detection of Koch's comma bacillus (*Vibrio choleræ*) in water, as in the case of the typhoid bacillus, is a matter of some difficulty, as this organism is rapidly overgrown by the ordinary water bacteria. In the examination of suspected water supplies, the best method to employ for the detection of this organism is to take advantage of the fact, first noted by Dunham, that the cholera vibrio multiplies with great rapidity in alkaline saline peptone solution. The suspected water is examined as follows: To 300–500 c.c. of the water are added 1 per cent. each of pure peptone and of common salt; the mixture is made faintly alkaline with sodium carbonate, distributed in a dozen small Erlenmeyer flasks having a layer not more than an inch deep in each, the flasks are loosely capped with caps of filter-paper, and incubated at 37° C. At intervals of ten, fifteen and twenty hours respectively, hanging-drop and cover-glass preparations are made from the top of the liquid, on which there is

often a surface film, and care must be taken not to disturb this ; these are then examined microscopically for vibrios and spirilla. At the same time agar (3 per cent.), or, better, blood alkali agar plates are prepared and incubated at blood-heat. Any colonies that appear which resemble the cholera spirillum are examined microscopically ; if the organisms are comma-shaped, they are at once subcultured into peptone water and other media. The original peptone water cultures are tested for the indole reaction with pure hydrochloric acid, withdrawing some of the contents of the flasks with a sterile pipette. Any likely vibrio isolated must have its cultural and biological reactions investigated and be tested for the agglutination and Pfeiffer reactions with a high-grade cholera serum.

On the survival of the typhoid and cholera organisms in water, see pp. 400 and 502 respectively.

Ice and *ice-creams* may be examined by methods similar to those used for water, the material being first melted at a low temperature. Some of the fluid should also be centrifuged and the deposit examined microscopically for gross contamination.

The infection in typhoid fever and cholera, and perhaps also in bacillary dysentery, is perhaps more frequently water-borne than conveyed in any other way. It might be supposed that the acid gastric juice would prevent this, and it may do so in many instances. Experiments by Macfadyen [1] showed that, whereas in fasting animals, to which suspensions in water of the cholera vibrio were administered, living vibrios pass into the intestine, when the vehicle is milk none could be detected in the intestines. The inference is that when there is no food there is no gastric juice secreted and the organisms are able to pass into the intestine, but when food is present the gastric juice is secreted and the organisms are destroyed.

Sterilisation of Water.—This may be done on the small scale by heat, by the use of germicidal agents, or by filtration through a filter (see p. 694). Heat may be applied by simple boiling, or by the use of apparatus in which the water is heated

[1] *Journ. of Anat. and Physiol.*, vol. xxi.

to 65°–90° C., and the outgoing hot water is cooled by the ingoing cold water, which itself is thus warmed, thereby effecting economy in fuel (Griffiths' and other sterilisers). The chemical germicides that have been employed are (1) sodium bisulphate, 15 grains to the pint ; (2) Potassium permanganate, sufficient to tinge the water deeply for at least half an hour ; (3) chlorine gas or iodine tablets,[1] in both cases the taste of the agent being destroyed by the addition of sodium sulphite ; (4) copper and copper sulphate. Sufficient metal is dissolved from bright copper in twenty-four hours to destroy typhoid and cholera. Copper sulphate 1 in 100,000 or less is similarly germicidal, and in still smaller quantities (1 in 1,000,000) destroys algæ, and has been used for the purification of reservoirs overgrown with algæ On the large (also small) scale, chlorine derived from hypochlorites is one of the simplest and most efficient agents. Chloride of lime or sodium hypochlorite obtained by the electrolysis of sea-water may be used. Moor and Hewlett [2] showed that 0·25 part of chlorine (equivalent to about 0·75 part of good chloride of lime) per million parts of chalk water is sufficient to kill *B. coli* in half an hour. The taste disappears quickly in bright sunlight and on standing, or may be removed by an addition of sodium sulphite. If the water is organically polluted, more chlorine must be used.

Ozone produced by high-tension electric discharge is also employed on the large scale for the sterilisation of water-supplies, *e.g.* at Chartres (see also p. 732).

Examination of Shell-Fish.—Shell-fish may come from sewage-polluted layings (see p. 401). The following method may be employed for their examination (after Houston) :

The outsides of the shells are cleansed by thorough scrubbing and rinsing in tap-water, and a final rinse in sterile water. The fish after cleansing are laid on a sterile towel. The operator then cleanses his hands and opens the shells aseptically with a sterile oyster-knife, care being taken to avoid loss of their contained liquor. The liquor as each fish is opened is poured into a sterile litre cylinder, and the fish is cut up with sterile scissors and added to the liquor in the cylinder. Ten fish should be treated, the volume of fish + liquor noted, and sterile water is then added to make up to 1 litre ; 100 c.c. liquid therefore corre-

[1] Nesfield, *Journ. Prev. Med.*, vol. xiii, 1905, p. 623.

[2] *Rep. Med. Off. Loc. Gov. Board* for 1909–10, p. 559.

sponds to one fish. In addition, four dilutions of the liquid are prepared—1 in 10, 1 in 100, 1 in 1000, and 1 in 10,000. With the liquid and dilutions gelatin and agar plate cultivations are prepared for the enumerations of the organisms present. Cultures are also made in litmus lactose bile-salt peptone water and in milk for the enumeration and isolation of *B. coli* and *B. Welchii* respectively, taking 100 c.c., 10 c.c., and 1 c.c. of the liquid, and 1 c.c. of each of the four dilutions; in this way the contents of the fish, ranging from one fish to $\frac{1}{1000000}$ of a fish, are examined. The process and principles involved correspond to those described for water. Houston has suggested for oysters as a lenient standard less than 1000, and as a stringent standard less than 100, *B. coli* per oyster. Even ten *B. coli* per fish should be viewed with suspicion, for Hewlett and others have shown that oysters from pure layings contain *no B. coli.*

Watercress, etc., may be examined in a similar manner, 100 grm being weighed out and transferred bit by bit with sterilised forceps and scissors to a flask containing 900 c.c. of sterile water. The flask is shaken vigorously, and the washings examined in a manner similar to that employed for shell-fish.

Filters.—Reference has already been made to the removal of organisms in water by sand filtration. With regard to filters for domestic use, few of those in the market are capable of doing more than removing particles of suspended matter, while they allow from 5 to 50 per cent., or even more, of the bacteria present in the water to be filtered, to pass through. Such filters are, of course, useless for the prevention of disease—in fact, rather favour it, by engendering a false sense of security; and when in use for some time without cleaning, the water after filtration may be worse, bacteriologically and chemically, than before filtration. The only efficient filters are those composed of unglazed porcelain or some such material, *e.g.* the Pasteur-Chamberland, Doulton and Berkefeld.

Woodhead and Wood [1] found that the only filters capable of completely removing organisms are the

[1] *Brit. Med. Journ.*, 1894, vol. ii, p. 1053 *et seq.*

Pasteur-Chamberland, Berkefeld, and Porcelaine d'Amiant. The Berkefeld, while more rapid in action than the other two, after being in use for a few days may allow some organisms to appear in the filtrate. This, perhaps, is due to a growth of organisms through the pores of the filter-candle rather than to a direct passage. Lunt [1] found that while the ordinary water bacteria, such as the *B. fluorescens liquefaciens*, appeared in the filtrate from a Berkefeld filter within a few days of the infection of the sample, the typhoid bacillus and the comma bacillus similarly introduced had not passed through the filter four or five weeks after infection.

Horrocks,[2] however, found that when sterile water is inoculated with typhoid bacilli and run daily through a Berkefeld filter, the bacilli appear in the filtrate in one or two weeks, whereas this is not the case with the Pasteur-Chamberland. The writer has made some similar experiments, which partially, but not entirely, support Horrocks's conclusions. Much evidently depends upon the chemical composition of the water.

All porcelain filters should be cleaned weekly by well scrubbing with a nail-brush and boiling in water containing some sodium carbonate.

The Bacteriological Examination of Water-Filters

The large majority of water-filters at present in use are incapable of preventing organisms from being washed through into the filtrate. In order to ascertain whether this is the case with any particular filter, it should be sterilised in the steam steriliser, and water containing organisms of known species (*B. prodigiosus, B. violaceus*, and *M. agilis* are very suitable) should be passed through it for twenty-four hours. This water and the filter should during this period of the examination be maintained, if conveniently possible, at a temperature below 5° C. This will almost invariably prevent any growth or multiplication of the organisms. Samples

[1] *Trans. Brit. Inst. of Prev. Med.*, vol. i, 1897.
[2] *Brit. Med. Journ.*, 1901, vol. i, p. 1471.

should be taken immediately after the filtration has begun, and at intervals during the day, and again at the end of twenty-four hours. If they are all sterile, the filter is capable of preventing organisms from being directly washed through. In the case of filters of very great density or depth of filtering medium, it may be necessary to prolong the period of examination beyond the first day; but most ordinary filters which permit organisms to be washed through do so within the first few hours.

Protozoa and Algæ in Water

The examination of water for the minute forms of life other than bacteria, and their enumeration, can be carried out by the Sedgwick-Rafter method.[1] A 6-inch glass funnel is plugged at the bottom of the stem with a perforated rubber cork, over the upper end of which a disc of fine silk bolting cloth, cut by a wad-cutter, is laid. Sharp, clean, dry quartz sand is then poured into the stem of the funnel to the depth of half an inch above the plug. The sand should be of such a size that the grains will pass through a sieve of 60 meshes to the inch, but not through one of 120 meshes. The sand is washed into place and well moistened with a little distilled water free from organisms.

The water to be examined is thoroughly shaken and 500 c.c. are poured into the funnel; it runs through the sand, which detains any organisms it may contain. After the water has all passed through, the rubber plug is carefully removed and the sand washed down into a test-tube with 5 c.c. of distilled water. The contents of the test-tube are agitated and the tube is allowed to rest until the sand has deposited. Immediately this is the case the supernatant fluid is decanted into a second test-tube, carrying with it the organisms. One cubic centimetre of this is withdrawn by a pipette from midway between the top and bottom and transferred to the counting plate. This consists of an ordinary glass slide on which a rectangular brass cell (20 × 50 mm.) is cemented, so enclosing exactly 1000 square mm. The brass cell is 1 mm. thick, so that the cell contains exactly 1 c.c. The preparation is covered with a cover-glass and examined with a low power.[2]

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[1] Calkin, *Twenty-third Ann. Rep. State Board of Health, Massachusetts*, 1891.

[2] On the microscopy of water, see Whipple, *Microscopy of Drinking Water*.

The Bacteriology of Air

Just as in water, the bacteria in the air vary considerably at different times and seasons, under different conditions, and in various localities. The species met with are mostly saprophytes, consisting largely of chromogenic forms. A number of moulds occur (as spores), and, in fact, ordinarily are in large excess, together with yeasts and torulæ.

It is not easy for micro-organisms to become diffused through the atmosphere; they are incapable of a voluntary rising, and cannot be torn from a fluid or moist solid medium by a strong current of air. The medium on which they are growing must dry up completely and crumble into fine dust before they can be distributed through the agency of air-currents (but see p. 404).

The number of organisms in the air varies with the season, with rain, with altitude, with movement, etc. At Montsouris, Miquel found in one cubic metre of air 49 organisms in winter, 85 in spring, 105 in summer, and 142 in autumn. After heavy rain the air is largely freed from organisms. Frankland found at Norwich Cathedral at an altitude of 300 feet 7 organisms in two gallons, while on the ground 18 were found; at the Golden Gallery at St. Paul's two gallons of air contained 11 organisms; in St. Paul's churchyard the number was 70. On high mountains organisms are nearly absent from the air, and the same is the case at sea at a distance from land exceeding about 100 miles. Organisms are much fewer in the air of the country than in that of towns. At the entrance-hall, Natural History Museum, South Kensington, Frankland found in the morning 30 organisms; in the afternoon, when many visitors were present, the number had risen to 292, showing the influence of movement. By keeping a volume of air absolutely still, enclosed in a box

the walls of which were smeared with glycerin, Tyndall was able to free it completely from particles and organisms. The writer found from 43 to 150 organisms per 10 litres of air in some of the principal streets of London during the daytime.

Gordon,[1] by exposing dishes of neutral-red broth to the air, or by aspirating air through neutral-red broth (p. 683) has been able to detect the presence of the *S. salivarius*, *M. epidermidis*, and scurf micrococcus (p. 253) in air subjected to human contamination. By these tests and by the use of *B. prodigiosus* as an indicator he concludes that particles of saliva are disseminated as far as 40 feet in the act of loud speaking, indicating the possibility of the wide distribution of such pathogenic organisms as the tubercle, plague, and influenza bacilli and the pneumococcus by speaking, and still more so by coughing.

The number of *dust* particles in the air may be enormous. In London Macfadyen and Lunt observed as extremes from 20,000 to nearly 600,000 per c.c. The ratio of micro-organisms to dust particles is therefore a very small one.

Bacteriological Examination of Air

A number of methods have been devised for the estimation of the number of micro-organisms in the air, of which the following are the principal ones :

(1) *Plate method.*—Melted sterile nutrient gelatin is poured into a sterilised Petri dish, and allowed to set. The plate is then exposed to the air, by removing the lid, for a given time—one, five, ten, or fifteen minutes, etc.—the lid is replaced, and the plate incubated at 22° C. for some days. The number of colonies of moulds, bacteria, yeasts, etc., is counted, and, having estimated the area of the gelatin plate,[2] the result is expressed as the number of organisms falling per square foot per minute. The results

[1] *Reps. Med. Off. Loc. Gov. Board* for 1902–1904.

[2] The area of a circular dish is calculated by multiplying the square of the diameter by 0·785.

obtained by this method are roughly comparative, but no estimate can be formed from it of the number of organisms contained in a given volume of the air.

(2) *Hesse's method.*—This is a quantitative method for estimating the number of organisms contained in a given volume of air. The apparatus consists of a glass tube 30 in. long by 1½ to 2 in. in diameter. One end of this tube is plugged with a rubber cork through which a glass tube passes, the other end is covered with a piece of sheet rubber perforated with a hole ¼ to ½ in in diameter; over this is placed another sheet of rubber, unperforated. The small tube being plugged with cotton-wool, the whole is sterilised for an hour in the steam steriliser. Just before use 40 to 50 c.c. of melted sterile nutrient gelatin are poured into the tube, and its walls coated with the medium. The tube is then strapped horizontally on to a tripod stand, and the small tube connected by means of a piece of rubber tubing to an aspirator consisting of two flasks arranged so as to form a reversible syphon. A litre of water is poured into the flask connected with the tube, and the outer sheet of rubber having been removed from the end of the tube, the water is syphoned over to the second flask, placed at a lower level, and an equal volume of air is thus aspirated through the tube. The second flask is then connected with the tube, and the position of the flasks being reversed the water is again syphoned over and a second litre of air passes through the tube, and this process is repeated until 5, 10, 15, or 20 litres of air have been drawn through the tube. The rate of flow is controlled by a screw-clamp on the rubber connecting-tube; it should not exceed half a litre per minute. With this rate of flow all the organisms are deposited on the gelatin-coated tube. The aspiration being completed the rubber tube is disconnected, and the sheet of rubber replaced over the end of the tube, which is then incubated, and the colonies are counted when they have developed.

(3) *Petri's method.*—Petri aspirates the air through a glass tube containing sterilised sand, kept in place by fine wire-gauze wads. When the sample has been taken the sand is distributed in Petri dishes, and melted sterile gelatin is poured over it and allowed to solidify, plate cultures being thus prepared. The objection to this method is the presence of the opaque particles of sand in the culture medium.

(4) *Frankland's method.*—The air to be examined is aspirated through a tube 5 in. in length and ¼ in. in diameter (Fig. 67).

One end of the tube is open, the other (C) is plugged with cotton-wool. At a distance of 1 in. from the open end the tube is slightly constricted to support a plug of glass wool (A). At a distance of 2½ in. from this plug the tube is again constricted to support a second plug (B), consisting of glass-wool and finely powdered cane-sugar, supported in front and behind by plugs of glass-wool. Several such tubes having been prepared, they are placed in a tin box and sterilised at 130° C. for three hours, and can then be easily transported without risk of contamination. When required for use, a tube is quickly removed from the box, being handled by the plugged end, which is connected by stout rubber tubing to aspirating flasks such as are used in Hesse's apparatus. The tube is clamped horizontally to a retort stand, and by attaching the second flask to a small hand exhaust-pump, the water can be syphoned over from the first flask, a corresponding volume of air passing through the tube. When the desired volume of air has been aspirated through the tube, it is disconnected and placed in another sterile tin box. As many tubes

FIG. 67.—Frankland's tube for air analysis.

as desired can be employed to control one another or to examine the air in different localities and under different conditions. All the samples having been taken, the tubes are manipulated on returning to the laboratory. The tubes, as before, being handled by the ends only, a file-mark is made across the centre of each tube, which is then broken in half and the plugs of glass-wool and sugar are shaken, or pushed by means of sterile wire, into a sterile flask of about 250 c.c. capacity. Into this 10 or 15 c.c. of liquefied sterile nutrient gelatin are then introduced; the sugar dissolves, the glass-wool becomes disintegrated, and a roll-culture is made on the walls of the flask, which is incubated at 22° C., and the colonies are counted when they have developed.

(5) *Sedgwick and Tucker's method.*—One of the best and more convenient methods for the bacteriological examination of air. A glass tube of special form is employed (Fig. 68); this consists of an expanded portion (A) about 15 cm. long and 4.5 cm. in diameter; one end of this is contracted so as to form a neck 2·5 cm. in diameter and in length; to the other end is fused a glass

tube (B C) 15 cm. long and 0·5 cm. in diameter. The neck of the tube is plugged with cotton-wool, and two cotton-wool—or better, glass-wool—plugs are inserted in the narrow tube, one at its open end, the other (C) about 6 to 8 cm. from the wide part. The whole is then sterilised. When cool, the narrow part of the tube, from its origin at the wide part down to the first plug (C), is filled with powdered cane-sugar (No. 50, B.P. gauge) which has been carefully dried and sterilised at 120°–130° C. The tube is again sterilised at 120°–130° for two or three hours, the greatest care being taken not to melt the sugar. After sterilisation the tube is ready for use. The wool plug is removed from the mouth and a measured volume of air is aspirated through the layer of powdered sugar by means of a small hand air-pump, the volume of air being measured by the displacement of water in a flask. Having taken the sample (5 to 20 litres), the wool plug is replaced in the neck. The powdered sugar is then shaken down into the wide part of the tube (A), and 15 c.c. of melted sterile nutrient gelatin are poured in. The powdered sugar readily dissolves in the melted gelatin, and when solution is complete a roll-culture is made in the tube, just as in Esmarch's method (p. 91). The tube is then placed in an incubator at 20° C., and the colonies are allowed to develop.

FIG. 68.—Sedgwick and Tucker's tube for air analysis.

In both Frankland's and Sedgwick and Tucker's methods the sugar, after powdering and sifting and before introducing into the tubes, should be thoroughly dried by keeping in the warm incubator for several days with occasional stirring. Unless this be done, the sugar is apt to cake and discolour during sterilisation.

Soil

The upper layers of soil contain large numbers of organisms, chiefly bacilli. The species are very varied; among pathogenic ones may be named the bacillus of tetanus and of malignant œdema. The *B. mycoides* is very abundant, and the varieties

of *Proteus*, the hay and potato bacilli, are common, while the nitrifying forms are of course present, but do not develop on ordinary media.

Below five or six feet aërobic organisms become scanty, but the anaërobic and thermophilic ones are still met with. The number of organisms present in soil is variable, from 200,000 to 45,000,000 in ordinary earth, while in dirty and busy streets there may be as many as 1,000,000,000 per grm. According to Houston, uncultivated sandy soil averages 100,000, garden soil, 1,500,000, and sewage polluted soil 115,000,000 per grm.

Houston [1] found that in virgin soils the *B. coli*, *B. Welchii*, and streptococci are practically absent, but that in soils polluted with animal excrement by manuring or otherwise the spores of *B. Welchii* are present in great abundance, also *B. coli* and streptococci if the pollution be of *recent* date.

The length of time pathogenic bacteria retain their vitality in buried corpses has been the subject of experiment by Lösener,[2] who injected cultures into the bodies of pigs, which were then wrapped in linen, placed in wooden coffins, and buried. The conclusions he arrived at were that, provided the soil has good filtering properties, there is practically no chance of the dissemination of a virus.

Klein,[3] experimenting with the bacilli of diphtheria, cholera, plague, typhoid fever, etc., also found that the vitality and infective power of these organisms passed away in a comparatively short time, in most cases within a month.

On the survival of the typhoid and cholera organisms in soil see also pp. 402 and 502 respectively.

Examination of Soil

The bacteria in the soil may be examined by adding traces of the soil to sterile nutrient broth, thoroughly crushing and soaking it, and then making plate or roll cultures, aërobic and anaërobic.

To make anything like an accurate quantitative examination is almost impossible. Weighed amounts of the soil, after thorough pulverisation in an agate mortar, may be introduced into sterile test-tubes and thoroughly exhausted by repeated washing with

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[1] *Rep. Med. Off. Loc. Gov. Board for* 1889–1900.

[2] *Centr. f. Bakt.* (I^te^ Abt.), xx, 1896, p. 454.

[3] *Rep. Med. Off. Loc. Gov. Board* for 1898–99, p. 344.

sterile water or broth, plate cultivations being made with the washings.

Various forms of boring apparatus have been devised for withdrawing soil from different depths.

Sewage [1]

Sewage is exceptionally rich in organisms, but the numbers present are variable. Jordan in Massachusetts found an average of 708,000 per cubic centimetre. Laws and Andrewes found from 905,000 to 11,216,000, the latter being the highest number obtained. The number of organisms naturally varies at different seasons and with the amount of dilution. The organisms present are very varied, but moulds, yeasts, and sarcinæ only occasionally occur. A few micrococci are met with and streptococci are present in considerable numbers, at least 1000 per c.c., but bacilli, especially liquefying forms, largely predominate. The commonest species are the *B. fluorescens liquefaciens* and varieties, several varieties of *Proteus*, the *B. filamentosus*, varieties of the *B. mesentericus*, *B. mycoides*, *B. subtilis*, *B. cloacæ*, and the colon bacillus. The latter numbers from 20,000 to 2,000,000 per c.c., and the other bacilli mentioned number 200,000 to 2,500,000 per c.c. Many anaërobic sporing bacilli are also found, especially the *B. Welchii*, the spores of which number from 30 to 2000 per c.c., averaging 500–600. Foreign bacteria introduced into sewage are probably soon suppressed by the predominant species of the sewage.

The air of well-ventilated sewers differs but little from that of the external air, and the organisms in it contrast with those of sewage by the abundance of moulds. Specific organisms may, however, gain access to it (p. 404).

The powerful liquefying and solvent actions of the bacteria present in sewage have suggested a means of dealing with sewage so as to make use of these properties, and many bacterial systems of sewage disposal have been devised. The principle most widely adopted is to run the sewage into large covered reservoirs (septic tanks), where it remains at rest for twenty-four to forty-eight hours. Here it is under practically anaërobic conditions, and

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[1] See various *Reports to the London County Council* by Clowes, Houston, Laws and Andrewes; Klein, Houston, *Reps. Med. Off. Loc. Gov. Board* for 1897–1904; *Rep. of the Sewage Commission.*

anaërobic bacteria exert their action on the solids, partly dissolving them, partly disintegrating them, with the formation of a sludge which has to be cleared out from time to time. From the septic tanks the sewage passes on to beds composed of broken brick, coke, or some similar material, through which it slowly percolates, and here it is subjected to the action of aërobic organisms, which complete the decomposition to such an extent that the effluent does not affect fish life nor putrefy, so that it may be run into a stream without causing a nuisance. Four sets of these aërobic bacterial beds are usually provided, each set being worked in turn for six hours and resting for eighteen hours during the twenty-four hours. The effluent from such bacterial beds may contain as many bacteria as, or more than, the sewage itself. Pathogenic organisms may be present in it, for Houston found that the *B. pyocyaneus* added to the beds soon appeared in the effluent.

On the survival of the typhoid and cholera organisms in sewage see pp. 402 and 503 respectively.

Examination of Sewage and Sewage Effluents

To ensure a fair average sample, the sewage or effluent should be collected in small portions at intervals. The portions are mixed, strained through muslin, and dilutions of 1 in 10, 1 in 100, 1 in 1000, and 1 in 10,000 made with sterile tap-water. These are then examined according to the following scheme (see table on opposite page).

Milk[1]

Milk is an admirable nutrient soil for the development and multiplication of micro-organisms, and, though sterile in the udder,[2] as delivered to the consumer may contain an appalling number of bacteria. In milk as ordinarily supplied there are from one to five million bacteria per c.c.,

[1] See Houston, *Rep. to the London County Council*, No. 933, 1905; MacConkey, *Journ. of Hygiene*, vol. v, 1905, p. 333; Hewlett and Barton, *ibid.* vol. vii, 1907, p. 22; Savage, *Rep. Med. Off. Loc. Gov. Board* for 1909–10, p. 474, and *Milk and the Public Health* (Macmillan, 1912); Swithenbank and Newman, *Bacteriology of Milk.*

[2] The "fore" milk may contain organisms which have lodged in the milk-ducts, and it is extremely difficult to obtain completely sterile milk.

Examination of Sewage and Effluents.

Tests.	Procedure.	Amount of sewage in c.c.
1. Total number of bacteria	Gelatin and agar plate cultivations	0·001, 0·0001, 0·00001
2. Number of spores of aërobes	Gelatin plate cultures with material previously heated to 80° C. for ten minutes.	1·0, 0·1, 0·01
3. Number of spores of anaërobes	Agar plate cultures with material previously heated to 80° C. for ten minutes and incubated anaërobically	1·0, 0·1, 0·01
4. Number of organisms liquefying gelatin	*Surface* gelatin plates	0·001, 0·0001, 0·00001
5. Spores of *B. Welchii*	Milk cultures heated to 80° C. for ten minutes and incubated anaërobically	0·1, 0·01, 0·001
6. Number of *B. coli*	*Surface*-plates of Conradi-Drigalski, or bile-salt media, etc., as described for water (p. 684)	0·001, 0·0001, 0·00001
7. Number of streptococci	*Surface*-plates of Conradi-Drigalski medium (p. 685)	0·01, 0·001, 0·0001

EFFLUENTS ONLY.

8. Incubate some of the effluent in beakers at 22° C. and 37° C. for some days. A good effluent should yield little or no unpleasant odour (an unpleasant odour indicates the presence of decomposable organic matter, and such an effluent might give rise to a nuisance).
9. Place a gold-fish or two in a bowl of the effluent. The fish will live in, and be unaffected by, a satisfactory effluent. (This may be done only by a licencee under the Vivisection Act.)

and it frequently contains ten to fifteen millions, with an average of about three to four millions. Hewlett and Barton found an average bacterial content of about 1,500,000 in London milk *as delivered at the railway termini* (the range was from a minimum of 20,000 to a maximum

of 8,390,000), but this does not represent the condition of the milk *as delivered to the consumer*, for the bacteria present rapidly multiply in warm weather. Eyre [1] in the middle of summer found the following rate of multiplication :

	Microbes per c.c.
Initial content . . .	56,000
After 12 hours . . .	526,000
After 24 hours . . .	20,366,000
After 30 hours . . .	clotted

A similar specimen in the middle of winter gave the following results :

	Microbes per c.c.
Initial content . . .	20,000
After 12 hours . . .	24,000
After 24 hours . . .	43,000
After 30 hours . . .	280,000

In New York, Park estimated the average bacterial content of milk as supplied to the consumer at 1,000,000 per c.c. in winter and 5,000,000 per c.c. during the hot months. Eyre (*loc. cit.*) states that, as the result of his observations, the numbers are in London about 3,000,000 to 5,000,000 in December, January, and February, and 20,000,000 to 30,000,000 in June to September, smaller numbers than these always being associated with the presence of boric acid or formaldehyde. Even in so-called sterilised milks bacteria are rarely completely absent.

Cream is even richer in bacteria than milk, and averages about 8,000,000, and may contain as many as 30,000,000 organisms per c.c. Although all the ordinary species may be met with, milk has a bacterial flora largely its own, comprising many forms producing lactic and butyric acid fermentations. Organisms also occur having more or

[1] *Journal of State Medicine*, vol. xii, 1904, p. 728.

less specific effects, and giving rise to bitter milk, viscid milk, etc. The lactic ferments are mostly non-sporing, the butyric chiefly sporing, species. The commonest of the lactic ferments are *Streptococcus lacticus* (non-gas-forming) and *B. acidi lactici* (gas-forming), which has some similarity to the colon bacillus (see Table, p. 428). Another common lactic organism is the *Oïdium lactis*, a mycelial form, the colonies of which appear as little fluffy tufts. In addition to the organisms named, pathogenic species may be met with—viz. the tubercle, diphtheria, typhoid, paratyphoid, Gärtner, dysentery, and comma bacilli, the *M. melitensis*, *M. pyogenes*, and the *Streptococcus pyogenes* (lactic-acid-forming streptococci are also common). The *B. coli* and *B. Welchii* are generally present in milk, and the *B. lactis aërogenes* is sometimes found (p. 443). Scarlatina (see "Scarlatina") and foot-and-mouth disease may likewise be conveyed by milk, and the diarrhœa of infants is largely due to the use of milk swarming with microbes, some of which in themselves may be harmful, and which also by the products they form tend to set up gastro-enteritis. The percentage of samples infected with tubercle bacilli varies much: Barton and Hewlett found only one out of 26 samples taken at London railway termini. The supply of the large dairy firms is also comparatively free from tuberculous infection, as considerable precautions are taken to exclude tuberculous animals. For the quarter ending March 31, 1911, of 760 samples examined for the London County Council, 106, or 13·9 per cent., were found to be tuberculous, and since 1907 of 5698 samples, 640, or 11·2 per cent., proved tuberculous (see also p. 367). A poisonous body, tyrotoxicon (p. 40) has been isolated from milk and milk products. Sources of contamination and infection are derived from the insanitary conditions of many farms and dairies and the dirty methods of those handling the

milk. In order to render milk wholesome for infants, and free from infective organisms when the conditions of supply are not above suspicion, two methods may be adopted —sterilisation and pasteurisation. To ensure sterilisation it is necessary to heat the milk to boiling point for six hours, or to expose it for a shorter period to steam under pressure. Such treatment, however, markedly alters the flavour of the milk, and is said to diminish its nutritive value. If the milk be heated to a temperature not exceeding 70° C., the flavour and nutritive qualities are far less altered, while the pathogenic species are all destroyed. This method is termed "pasteurisation," and consists in heating the milk to about 60°–65° C. for twenty to thirty minutes by the "bulk" system, or to 68°–70° C. for half-a-minute or so by the "flash" system. Pasteurisation destroys 92–99 per cent. of the total organisms present. The objections to pasteurised milk are that the natural enzymes present in fresh milk are destroyed and such heated milk is stated to induce scurvy rickets,[1] the lactic-acid-forming organisms are killed, and if the treated milk be kept, the residuum of resistant putrefactive, etc., bacteria multiply enormously, without obvious change in the milk, and "returned" milk can be utilised again and again. Pasteurised milk should be rapidly cooled and be consumed within twenty-four hours of treatment. Behring has advocated the addition of formaldehyde to all milk used for the feeding of children. Another method for sterilising milk is the Budde process,[2] in which the milk, after the addition of hydrogen peroxide, is heated for three hours to 52°–53° C. All non-sporing organisms are destroyed, and the added hydrogen peroxide is decomposed into H_2O and O.

[1] Dr. Lane-Claypon denies this, and considers that the enzymes in milk are derived from the bacteria in it (*Rep. to Loc. Gov. Board*, 1913).

[2] Hewlett, *Lancet*, 1906, vol. i, January 27.

The ideal system is for all milk to be distributed in closed bottles.

The thermal death-point of pathogenic organisms in milk is as follows :

Organism.	Temperature.	Period of Exposure.
B. tuberculosis	60° C.	20 min.
B. typhosus	60° C.	2 min.
B. diphtheriæ	60° C.	1 min.
Spir. choleræ	60° C.	1 min.
B. dysenteriæ	60° C.	10 min.
M. melitensis	60° C.	20 min.

The thermal death-point of the tubercle bacillus, especially in milk, has been the subject of some controversy (see also p. 355). De Man found that an exposure of fifteen minutes at 65° C. was necessary to destroy the infective properties of tuberculous milk. Bang, of Copenhagen, considers that pasteurisation cannot always be relied upon, and recommends that milk should be heated to 85° C. The writer found that the vitality of the ordinary non-virulent laboratory cultures was destroyed by a temperature of 60° C. acting for ten minutes, and that the infective properties of tuberculous sputum, tested on guinea-pigs, were destroyed by a temperature of 65° C. acting for fifteen minutes in five out of six instances. Woodhead's experiments (First Royal Commission on Tuberculosis) gave irregular results which seem to be explained by Theobald Smith's careful work.[2] This showed that tuberculous milk was rendered non-infective by heating to 60° C. for ten to fifteen minutes, *provided there was no formation of a surface scum* ; the latter seems to protect the bacilli. Russell and Hastings[3] confirmed Smith's experiments, and assert that it is sufficient to heat milk to 60° C. (140° F.) *in a closed receptacle* for a period of not less than twenty minutes in order to destroy the tubercle bacillus. The surface scum forms on milk only when it is heated in contact with air ; all pasteurisers, therefore, should be closed vessels. The writer has devised a

[1] Rosenau, Hygienic Lab., Washington, *Bull.* 42, 1908.
[2] *Journ. Exper. Med.*, vol. iv, 1899, p. 217.
[3] *17th Ann. Rep. Wisconsin Agricult. Exp. Station.*

simple form of domestic pasteuriser, which is made by Messrs. Allen and Hanbury.

The occurrence of so-called leucocytes and pus-cells in milk must be considered. A certain number of cells resembling polymorphonuclear leucocytes are always present in milk, more numerous during the first week of lactation and then accompanied by colostrum corpuscles. An excess of these cells *may* indicate some local inflammatory affection of the udder, or, if streptococci and blood are present in addition, suppuration, but not necessarily, for Russell and Hoffman, and Revis have shown that a very large cell count (500,000–1,000,000, or even 10,000,000, per c.c.) may often be obtained from quite healthy cows. The nature of these cells has been the subject of an extended investigation by Hewlett, Villar, and Revis.[1] Their conclusion is that the majority of these cells are not leucocytes, but are germinal cells of the secreting epithelium of the udder. Blood may also be present transitorily in health (Revis); a few red corpuscles are probably present in all milk. The presence of squamous epithelial cells indicates desquamation from the teat or udder or from the hand of the milker—*i.e.* want of cleanliness.

There is no doubt that micro-organisms are far more abundant in milk as supplied to the consumer than should be. This arises from the ignorance and carelessness of those charged with the duty of providing and distributing this important article of diet. The udder and teats of the cow and the hands of the milker (who should wear a special dress) should be wiped before milking, and all vessels should be clean and steamed or scalded before use. The milk should be cooled at once, the newer types of milk churn adopted, and the milk not stored, but forwarded without delay by the railway companies in

[1] *Journ. of Hygiene*, vols. ix, x, xi, and xiii.

special refrigerator vans. Distribution in bottles would be a great improvement.

The following might be suggested as a bacteriological standard for milk :[1] (*a*) Number of organisms not to exceed 1,000,000 per c.c. ; (*b*) absence of excess of leucocytes or of pus-cells ; (*c*) *B. coli*, *B. Welchii*, and streptococci should not be present in 1 c.c. or less ; (*d*) the sediment after centrifuging should be less than 100 parts per million ; (*e*) the milk as delivered should not have a temperature above 10° C. ; (*f*) absence of pathogenic organisms.

Soured milk.—Soured milk is used as an article of diet in many parts of the world, *e.g.* Bulgaria. In these soured milks a particular micro-organism or a variety of it, the *B. bulgaricus* or "bacillus of Massol," is generally present in association with lactic streptococci. It is a large, pleomorphic, Gram-positive, aërobic bacillus, non-motile, non-sporing, growing best at about 40° C., but only in milk or in culture media made with milk or whey. It has been much employed for the preparation of a soured milk which is of considerable service in the treatment of certain disorders.[2]

The Boas-Oppler bacillus, met with in the stomach in cases of carcinoma of that organ, appears to be identical with the *B. bulgaricus*.

Examination of Milk

Number of organisms per c.c.—This is carried out by diluting the milk to 1 in 1000–1 in 1,000,000 with sterile water, or preferably nutrient broth, as a better mixture is obtained. Plates are then made either in gelatin or in distilled water agar (1½ grm. powdered agar, distilled water 1 litre, Eastes), or preferably in both media.

Breed and Brew count the organisms directly. With a special pipette, 0·01 c.c. of milk is deposited on a slide, the droplet of

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[1] See "Rep. of a Committee on Milk Supply," *Philad. Med. Journ.* October 1900, p. 758 ; Park, *Journ. of Hygiene*, vol. i, 1901, p. 391 ; Houston, *loc. cit.*

[2] See Hewlett and others, *Brit. Med. Journ.*, 1910, vol. ii (Bibliog.).

milk is spread evenly with a stiff needle over an area of one square centimetre, and the slide is dried quickly in a warm place. When dry, it is treated with xylol for one to a few minutes, then treated with 80 per cent. alcohol for one to a few minutes and is then transferred to a fresh saturated aqueous solution of methylene blue in which the slide remains for from five to sixty seconds. The film is then rinsed with water and decolorised with alcohol to the required degree. The slide is finally dried and the film is examined directly with the oil-immersion lens. The organisms and cells in 100 fields are then counted, and knowing the area of the field (which must be ascertained) the total number of organisms in 1 c.c. of the milk can be calculated. The pipette is of the hæmocytometer pattern and should be calibrated with mercury to contain 0·0104 c.c. to allow for the loss of milk due to adhesion to the glass (0·0104 c.c. of mercury = 0·1423 grm.).

B. coli, B Welchii, and streptococci.—These are searched for quantitatively by the methods detailed for "Water" (pp. 676, 678). Amounts of milk in decreasing decimal order from 100 c.c. to 0·000001 c.c. should be examined. The *B. coli* must be differentiated from *B. lactis aërogenes* and *B. acidi lactici* (see pp. 442, 428).

Pathogenic organisms.—The detection of these, with the exception of the tubercle bacillus, is difficult and uncertain. In all cases the milk should be centrifuged and the deposit examined.

1. For the detection of the tubercle bacillus [1] staining methods are almost useless (except in cases of advanced tuberculosis of the udder or when the milk of a single cow is examined) and inoculation must be performed. At least 250 c.c. of the milk should be centrifuged at 2000 to 2500 revolutions per minute for an hour. As many organisms become entangled in the cream, it is advisable to stop the machine after half an hour, stir in the cream, and again centrifuge. The fluid is poured or pipetted off carefully, so as not to disturb the sediment, leaving about 3 c.c. in the tube. The sediment and the remaining fluid are then well mixed and about 1 c.c. is inoculated subcutaneously and intraperitoneally into two guinea-pigs respectively (see also p. 376). For staining, a process of solution of the milk may be employed, 20 c.c. of the milk being mixed with 1 c.c. of a 50 per cent. potash solution, and heated in a water-bath until the solution turns brownish; 20 c.c. of acetic acid are then added. The

[1] See Delépine, *Rep. Med. Off. Loc. Gov. Board* for 1908–09, p. 134.

mixture is shaken, heated in a water-bath for three minutes, and centrifuged for ten minutes. The fluid is poured off, 30 c.c. of hot water are added to the sediment, and the mixture is again centrifuged. Films are then prepared from the sediment, and stained for the tubercle bacillus (see also p 371), the films being always treated with alcohol as well as with acid.

Non-pathogenic acid-fast bacilli occur in milk (p. 388).

2. The diphtheria bacillus is searched for by making serum cultures from, and inoculating guinea-pigs with, the sediment. If a diphtheroid organism is detected it must be isolated and examined by culture tests and animal inoculation.

In milk and cheese a bacillus is frequently met with closely resembling the diphtheria bacillus in its morphological and cultural characters ; it is, however, quite non-pathogenic.[1]

3. The typhoid, paratyphoid, Gärtner, and dysentery bacilli and cholera vibrio may be searched for by the methods given for "Water."

(4) The *M. pyogenes* and the *Streptococcus pyogenes* may be searched for by means of plate cultures on glycerin agar.

(5) *Examination of sediment.*—Houston and Savage (*loc. cit.*) have devised methods for the quantitative estimation of the sediment by centrifuging in special graduated tubes. For the microscopical examination of the sediment the milk is centrifuged for twenty minutes at 1500 revolutions per minute, and the upper fluid is pipetted or syphoned off. Some of the sediment should be examined with the $\frac{2}{3}$ in. and $\frac{1}{6}$ in. objectives for the presence of "dirt," *e.g.* hairs, straw, etc. Three smear preparations are then made, each with four drops of the sediment, which are spread evenly over three-fourths of the slide. The slides are air-dried, and may be treated with a mixture of absolute alcohol and ether for ten minutes. One slide is stained with Löffler's blue, another by Gram's method for streptococci, and a third by the tubercle method. The Löffler's blue specimen gives a general idea of the number of bacteria present, and of the presence of cells.

From what has been said above (p. 710), considerable caution must be exercised in stating the presence of pus-cells. Streptococci present are not necessarily pathogenic, as non-pathogenic lactic-acid-forming streptococci are common. For counting the

[1] See *Scientific Bull.* No. 2, Health Dept., City of New York, 1895, p. 10.

number of cells present, Revis [1] employs a centrifuge tube of 10 c.c. capacity, the lower third of which is contracted to 0·8 cm. in diameter, and contains 1 c.c. The procedure is as follows :

In the tube are placed 5 c.c. of the well-mixed milk, diluted to the 10 c.c. mark with 0·8 per cent. salt solution. After inserting a rubber stopper the contents are well mixed. The tube is then centrifuged at about 2000 revolutions per minute for two minutes, the cream is broken up by violently shaking the upper part of the tube, and the rotation continued for four minutes longer. A glass rod, fitting roughly the narrow neck of the tube, is inserted, and the major part of the milk poured off, and the upper part of the tube well rinsed with water to remove cream, etc. ; the contents of the narrow end down to within $\frac{1}{4}$ in. of the deposit are sucked out with a fine glass pipette, the upper part of the tube is wiped clean and the tube is then filled to the 10 c.c. mark with salt solution The tube, having been violently shaken till all the deposit is distributed through the liquid, is then rotated for four minutes, and the liquid down to within $\frac{1}{4}$ in. of the deposit again removed. In the case of small deposits, two to three drops of saturated aqueous solution of methylene-blue are added, and the deposit is stirred up by blowing through a fine glass capillary pipette (which is afterwards used for filling the counting chamber). After fifteen minutes, water is added to the 1 c.c. mark, and counting done in the usual way with a Thoma-Zeiss blood counter. Counting should not be restricted to the ruled spaces, but the field should be so arranged that a definite number of squares is included, and fields are counted all over the chamber. At least two different preparations should be made of the same deposit for counting.

Food Poisoning.[2]—Apart from the presence of the ordinary poisons, food may be poisonous on eating—(*a*) naturally, *e.g.* certain fish, (*b*) from the results of the activity of micro-organisms with the formation of toxic products, the ordinary "ptomine poisoning" (see p. 40), in which case the poison is pre-formed and is ingested, (*c*) from *infection* with certain organisms, particularly *B. enteritidis*, which generally induce gastro-enteritis. In the last named, symptoms do not usually ensue until a lapse of twelve to forty-eight hours after the consumption of the food. Mayer and Mandel describe an outbreak following the consump-

[1] *Journ. of Hygiene*, vol. x, 1910, p. 58.

[2] See Savage, *Rep. to the Loc. Gov. Board*, No. 77, 1913.

Common Organisms of Air, Water, and Soil

Organism and its size.	Morphology.	Motility.	Spore formation.	Gram-staining.	Growth on Agar.	Growth on Gelatin.	Liquefaction of gelatin.	Growth on Potato.	Litmus milk.	Glucose.	Habitat.	Other characters, etc.
1 *Micrococcus agilis*, 0·7–1·0 μ	Coccus diplococcus, tetracoccus, chains and masses	+	—	+	Coral-pink creamy layer at 20° C.	Coral-pink	+	Coral-pink	—	—	Air, water	Does not grow at 37° C. General turbidity in broth, no film.
2. *Micrococcus candicans*, 0·8–1·0 μ	Cocci	—	—	+	White shining creamy layer	White porcelain-like	—	White porcelain-like	A	—	Air, water, milk	General turbidity in broth. Stated by MacConkey to produce acid and gas in bile-salt lactose media.
3. *B. filamentosus*, 3–5 μ	Anthrax-like	—	+	+	Wavy feathery greyish layer	Grey	+ +	Grey	AC	A	Sewage, water	Does not grow at 37° C. Strict aërobe. Broth clear with sediment.
4. *B. mycoides*! 3–5 μ	Somewhat anthrax-like	+	+	+	Grey creamy layer	Grey	+ +	Grey slimy	*Ca*	A	Soil, water	Broth turbid. Colonies on agar plate woolly, tufted, and mould-like.
5. *B. megaterium* 3–5 μ	Large rods and filaments	+	+	+	Grey creamy layer	Grey, thin, yellowish	+ +	Grey creamy	*Ca*	A	Water	Broth turbid.
6. *B. mesentericus* (*vulgatus*), 2–4 μ	Slender rods and filaments	+	+	+	Dry grey wrinkled film	Grey with film	+ +	Grey or dry pinkish, crinkled, abundant	*Ca*	A	Water, soil	The potato bacillus. Strict aërobe. Broth turbid with film. Varieties produce pigment (*fuscus*, brown; *ruter*, red; *niger*, black).
7. *B. subtilis*, 2–4 μ	Slender rods and filaments	+	+	+	Moist, grey, sometimes wrinkled	Grey	+ +	Grey, dull, thickish	*Ca*	A	Hay, dust, soil	The hay bacillus. Strict aërobe. Spores germinate equatorially. Broth turbid with film.
8. *B. proteus* (*P. vulgaris*) 2–4 μ	Slender rods, filaments and threads	+	—	∓	Thin, moist, whitish	Grey	+ +	Slight grey	AC	AG	Soil, sewage bowel, putrid matter	The *B. termo*. Occurs in putrefying matter. Colonies on gelatin wavy and motile. Varieties: *miratilis*, slow liquefier: *Zenkeri*, non-liquefier.
9. *B. prodigiosus* 1–2 μ	Short rod, almost coccoid	+	—	—	Thick creamy, brilliant red	Red or pink	+	Red creamy	AC	AG	Air, water	Grows well at 37° C., but produces no pigment. Broth turbid.
10. *B. fluorescens liquefaciens* 2–4 μ	Slender rod	+	—	—	Thin creamy, fluorescent, greenish-yellow	Fluorescent, greenish-yellow	+ +	Brownish	*Ca*	—	Water, sewage	Stated by Lehmann and Neumann to be identical with *B. pyocyaneus*, but non-pathogenic (p. 263). *fluorescens non-liquefaciens* similar but non-liquefying.

+ = positive; — = negative, or no change; C = curdling; A = acid; *a* = alkaline; G = gas.

All the above forms are practically non-pathogenic except *B. proteus* (cystitis, abscesses ? diarrhœa). *B. prodigiosus* is pathogenic to guinea-pigs by intra peritoneal inoculation. Chromogenic sarcinæ, *e.g. S. aurantiaca* and *flava*, torulæ, *e.g.* pink torula, and numerous other bacilli occur in air, water, and soil.

tion of broiled fish, in which *B. proteus* was isolated from the stools and was agglutinated by the patients' serum.

Meat is not likely to convey any infective disease with the exception of tuberculosis and anthrax. It may be examined by cultures and plate cultivations, and by inoculation and feeding experiments. *Tinned meats, etc.*, frequently contain sporing organisms of the *B. subtilis* and *mesentericus* groups. They may be examined by aërobic and anaërobic cultures, and by feeding mice. Poisonous ptomines are occasionally present. The *B. enteritidis* occurs in meat, and causes a form of poisoning (see p. 415).[1] In certain intoxications due to bad meat, known as "botulism," Van Ermengen isolated the *B. botulinus* (see p. 494).

Bread.—Troitzki states that new bread contains no micro-organisms, but Waldo and Walsh found that such organisms as the comma bacillus are not destroyed by passing through the ordeal of the baker's oven. Cut bread forms a good nidus for the development of pathogenic organisms. Bread becomes glutinous or "ropy" through the action of certain organisms belonging to the *B. mesentericus* group, viz. *B. m. panis viscosi* and *B. m. fuscus panis viscosi*.

The *Bacillus prodigiosus* may grow upon various food-stuffs, and give rise to suspicion of foul play. L. Parkes [2] describes cases of diarrhœa which he suggests were caused by this organism.

Butter contains from two to forty-seven millions of micro-organisms per gramme. Tubercle bacilli have been found in butter, and the comma bacillus artificially introduced survives for over a month. "Acid-fast" non-pathogenic forms also occur (p. 388).

For the isolation of the tubercle bacillus from butter and cheese the only certain method is by inoculation. Butter may be melted and allowed to stand in the incubator at 37° C. for some days, and the sediment inoculated. As this involves the multiplication of septic organisms, it is preferable to centrifuge the melted butter, keeping it melted during the process, and to inoculate the sediment immediately.

Clothing, etc.—Attempts have been made to examine clothing, bedding, flock, etc., by bacteriological methods for filth contamination, but without much success.

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[1] See Savage, *Rep. Med. Off. Loc. Gov. Board* for 1909–10, p. 446.

[2] *Brit. Med. Journ.*, 1905, vol. ii, 1330.

CHAPTER XXII

HEAT—STEAM DISINFECTION—CHEMICAL DISINFECTANTS —THEORY OF DISINFECTION—METHODS OF DETERMINING DISINFECTANT POWER

Disinfection [1]

NATURAL agencies restrain the multiplication of disease organisms, but enough survive to determine the persistence of infective diseases, and to call for measures by which communities attempt to cope with them. These measures are broadly isolation, prophylactic inoculation, general improvement in sanitation and nutrition, and disinfection. In the present chapter the methods by which the fourth means of protection may be applied are considered. Disinfection implies the removal or the destruction of infective properties, but, for practical purposes, should be understood to mean the killing of the infective organisms to which those properties are due. For this purpose, the two agencies ordinarily used are heat and chemical action, though, in addition, other methods can occasionally be employed for destroying or excluding micro-organisms. Such are light, desiccation, and filtration.

HEAT.—*Fire* is the simplest and most efficient agent for destroying infective matter. Burning should always be employed where possible, as for rags, old clothing or bedding, native huts, etc.

For surfaces which would not be unduly injured, such

[1] See Hewlett, "Milroy Lectures," *Lancet*, 1909, vol. i.

as stables, pens, yards, etc., a torch-fire generated by means of the cyclone burner described by Forbush and Fernald has been favourably reported on by Stiles. The apparatus consists of a portable tank, from which paraffin gas oil is driven by a pump through a hose (such as is used for the delivery of oil) to which is attached a pole, consisting of an iron pipe 12 ft. long, which is protected by a covering of wood, and to the end of which is attached a cyclone nozzle. The fine spray from the nozzle is ignited, and the resulting fierce flame passed over the surfaces to be disinfected. The thorough wetting with water of all such surfaces would practically abolish danger from fire, and by proper adjustment of the power of the flame, and experience on the part of the operator, the method is an efficient one. An ordinary plumber's blow lamp might be used on a small scale.

Dry heat may also be used, and forms the basis of some disinfectors (Ransome's), but is not nearly such an efficient means as moist heat. The objections to dry heat are, that to ensure the destruction of bacteria and spores the temperature must be high and the heating prolonged. Koch and Wolfhügel found that two hours at 150° C. did not always ensure sterilisation, and Gaffky and Löffler state that the spores of some organisms are killed only by exposure to hot air at 140° C. for three hours. Moreover, dry heat has little power of penetration, and it requires many hours for the centre of a mass of bedding, or the like, to attain the temperature requisite for sterilisation, while some articles and fabrics are distinctly injured by the prolonged heating. The highest temperature which can be safely adopted for a dry-heat disinfector is about 120° C., and then if large masses have to be treated the heating has to be continued for from eight to ten hours. A rise of 5° C. above this temperature is sufficient to damage many woollen goods, which enhances the objec-

tions to a dry-heat disinfector, as it is difficult to keep the temperature of a large chamber constant.

For the reasons given above, disinfection by dry heat is often impracticable ; on the other hand, *moist heat* is more effective, is found to work well in practice, and is now generally adopted. In the household, for articles which cannot be burnt, brisk boiling for an hour or so will suffice.

Steam disinfection.—For public disinfectors, steam under pressure—*i.e.* at a pressure greater than that of the atmosphere—is employed. Steam under pressure has not such a deleterious action on articles, with the exception of leather, as dry heat, while its penetrating powers are far greater. By " saturated steam " is meant steam at the temperature at which it can condense, and the temperature of the condensation point rises as the pressure increases. By " superheated steam " is meant steam at a temperature higher than that at which it can condense ; therefore superheated steam has to be cooled down into the state of saturated steam before condensation ensues. If superheated steam is used for disinfection, it loses heat by conduction, and the rise in temperature of the articles treated approximately corresponds to the fall in temperature of the steam. With saturated steam, on the other hand, immediately it is cooled an enormous amount of latent heat is set free by the change in state from the gaseous to the liquid condition, therefore saturated steam is a far more efficient disinfectant than superheated steam. These considerations should always influence the choice of a steam disinfecting apparatus for efficient working.

The Equifex disinfector is worked with saturated steam at 10 lb. pressure (239° F.). The chamber consists of a cylinder of mild steel, made without steam jacket, so as to avoid risk of superheating. The cylinder is lagged with non-conducting composition and wood, to reduce loss of

heat by radiation, and, as usually supplied, is furnished with separate doors for infected and disinfected articles respectively. An arrangement can be supplied to prevent both doors being opened simultaneously. The Washington-Lyons apparatus, or its modifications, is an elongated cylindrical boiler with double walls, forming a jacket, and a door at each end. The chamber is of sufficient size to admit bedding, and is built into the partition wall between two rooms, so that each door opens into a different room. Into one of the rooms the infected articles are conveyed, and are placed in the disinfector as lightly packed as possible; when disinfected they are removed by the opposite door into the other room, thereby avoiding all chance of reinfection. Steam at a pressure of about 20 lb. is admitted into the jacket and then passes to the inner chamber, the object of the jacket being to warm the chamber, and so prevent condensation. For the same purpose hot air is sometimes injected beforehand to warm the chamber and articles, and after the steam disinfection, can again be injected for drying. The length of time required for disinfection does not exceed a half to one hour.

In Thresh's disinfector the steam is generated from a saline solution (calcium chloride), which has a boiling-point (105° C.) higher than that of water.

The thermal death-point of a number of organisms in pure culture has been determined by many investigators. Eyre suggests the following as "standard conditions" for determining thermal death-points:

1. Length of "time exposure" to be ten minutes.
2. Emulsion to be prepared from "optimum cultivation."
3. The vehicle in which culture is suspended to be sterile salt solution or sterile distilled water.
4. Strength of emulsion to correspond to about 1 milligramme of culture per cubic centimetre.
5. Bulk of emulsion to be not less than 3 c.c.
6. Emulsion to be contained in test-tube of 1·5 cm. diameter with walls 1 mm. thick.

7. Emulsion to be exposed to moist heat in a water-bath regulated by a delicate and accurate thermo-regulator.

8. Broth cultivations and agar plates both to be used in determining the death of the bacteria, and the period of observation of these cultures to be extended, when necessary, to seven or fourteen days. The experiments to be repeated at least once.

9. Thermal death-point to be first roughly determined to within 5° C.

10. Thermal death-point to be finally determined to within 1° C., and to be defined as that temperature which causes the death of *all* micro-organisms exposed to it, within the ten minutes in these standard conditions.

LIGHT is not used directly for disinfection, but indirectly in nature and in our homes may not be an unimportant factor. It has previously been referred to at p. 24. Sunlight, and artificial light rich in violet and ultra-violet radiations, such as that emitted by a *quartz* mercury vapour lamp, are efficient germicides. The latter has been tested by Barnard and the writer with excellent results, but, unfortunately, the germicidal rays have practically no power of penetration and are stopped even by thin glass.

DESICCATION, although one of Nature's methods of disinfection, is not made use of to any extent by man except as an inhibitory agent for the preservation of many articles of food. Shattock and Dudgeon found that many bacteria, *e.g. B. coli* and *B. typhosus*, rapidly succumb to complete desiccation, but *B. pyocyaneus* maintained its vitality for two years under these conditions.

FILTRATION is a method of disinfection by exclusion, and in the form of sand filtration and filtration through a porous candle, as in the Berkefeld and Pasteur-Chamberland filters, is made use of for the sterilisation of water and other fluids.

CHEMICAL DISINFECTANTS.—A large number of chemical substances variously known as germicides, antiseptics,

disinfectants, deodorants, etc., have the power of interfering with, or masking the results of, the vital activities of micro-organisms. Germicides are substances which kill bacteria or germs ; antiseptics, by inhibiting bacterial development, prevent sepsis or putrefaction ; and by " disinfectant " is meant a substance which prevents the action of, or destroys, infective matters, while deodorants destroy or absorb foul-smelling gases the result of putrefactive and similar processes. All germicides are disinfectant and antiseptic, but many antiseptics, though preventing or inhibiting the development of bacteria, are not necessarily germicidal.

Many *deodorants* act largely mechanically, and although often not germicidal, and hence not ideal disinfectants, are of some value in preventing the deleterious and depressing effects of the emanations from decomposing organic matter. Such are charcoal, ashes, dry mould, and peat (peat has also a germicidal action). Other deodorants, such as quicklime and chloride of lime, act chemically.

The germicides and antiseptics may be considered together, for although many antiseptics are not germicidal, all the germicides in small amounts act as antiseptics. The principal germicides and antiseptics are the halogen elements, the mineral acids, a large number of metallic salts, phenol and many coal-tar derivatives, and various organic bodies and essential oils.

Theory of chemical disinfection.—The theory of chemical disinfection is not yet fully understood. It is probable, as suggested by Paul and Krönig, that the degree of ionisation of a solution may have an important bearing on its disinfecting efficiency.

Paul and Krönig [1] made a number of experiments on the *M. pyogenes*, and spores of anthrax, with a view of determining the effects of various acids, bases, oxidising

[1] *Zeitschr. f. physikal. Chem.*, 1896, xxi, p. 414.

agents, and metallic salts on bacteria. The salts of mercury, gold and silver exert a marked germicidal action, strongest in the case of mercury, while the platinum salts are almost inactive. The efficiency of mercuric chloride is markedly lessened by the presence of sodium chloride or other chlorides. Of the oxidising agents, nitric, chromic, chloric, and permanganic acids act in the order stated; chlorine has the most powerful action of the halogens. Phenol acts better in a 5 per cent. solution than in higher concentrations, and the efficiency is increased by the addition of sodium chloride, but diminished by the presence of alcohol, and under the most favourable conditions it is not such a powerful germicide as mercuric chloride. Mercuric chloride dissolved in absolute alcohol has little or no efficiency, and the addition of sodium chloride reduces its activity. Organisms in masses are less readily acted upon by antiseptics than when they are isolated.

The efficiency of a germicidal salt in solution seems to vary with its dissociation. It is believed that the molecules of a salt in solution are more or less dissociated into constituent electrified atoms or "ions," and the greater the dissociation the more active will the substance be as a germicide. Taking mercuric chloride, bromide and cyanide, the ionisation of the chloride is greatest, and that of the cyanide is least, and the following results show that the germicidal power of the three is in this order: [1]

Solution.	Number of colonies which developed. After 20 minutes' treatment.	After 85 minutes' treatment.
1 mole $HgCl_2$ in 64 litres	7	0
1 ,, $HgBr_2$,, ,,	34	0
1 ,, $Hg(CN)_2$ in 16 litres	8	33

[1] Findlay, *Physical Chemistry*, 1905.

Since the amount of this dissociation may be greatly influenced by the presence of other substances, much caution should be exercised in adding salts, etc., to increase solubility or prevent precipitation, as the addition may seriously impair germicidal or antiseptic power (see pp. 731, 734).

The disinfection process is a gradual one. In the early stages of disinfection large numbers of organisms are killed, but the rate of killing becomes slower and slower as time elapses. Madsen and Nyman and Miss Chick [1] have found that if the results be plotted, ordinates representing the numbers of surviving bacteria, and abscissæ the corresponding times, the points lie on a logarithmic curve. The curve so obtained, in fact, appears to be similar in form to that of a " unimolecular reaction," and may be expressed by the formula $\frac{1}{t_2 - t_1} \log \frac{n_1}{n_2} = \mathrm{K}$, where n_1 and n_2 are the numbers of bacteria surviving after times t_1 and t_2 respectively, and K is a constant. In the case of disinfection of anthrax spores with phenol, Miss Chick found the mean value of K to be 0·44. In the case of *B. paratyphosus*, however, the course of the disinfection is different unless the culture is very young, and Miss Chick concluded that the older individuals are less resistant than the younger. The progress of heat disinfection apparently follows the same course. Miss Chick asserts that the act of disinfection is a unimolecular reaction, but it is difficult to accept this view. Disinfectants in emulsion tend to be more efficient than when in solution.

Factors modifying disinfectant action.[2]—The efficiency of a disinfectant liquid partly depends on its concentration. The rate of penetration into bacterial cells decreases as

[1] *Journ. of Hygiene*, vol. viii, 1908, p. 92 (Summary and Bibliog.).

[2] This section is largely taken from *Applied Bacteriology*, Moor and Hewlett, 1907.

the concentration increases above a certain limit. Most disinfectants yield, therefore, a greater amount of disinfectant energy per gramme-hour in dilute than in strong solutions. In oil, glycerin, or alcohol, disinfectants lose some or most of their activity. Spores in anhydrous glycerin, oil, or vaseline, are not killed at a temperature lower than 170° C. acting for half an hour.[1] Of fats, lanolin alone seems compatible with disinfectant efficiency. Some disinfectants form an emulsion on the addition of water, and their efficiency for a given amount of active material may vary within wide limits according to the manner in which they are emulsified. The temperature at which the organism is exposed to the disinfectant has a considerable influence on the extent or rate of disinfection. Up to the optimum temperature at which the organism to be disinfected grows on the medium in which it is exposed the activity of a disinfectant may fall off as the temperature rises, owing to the increased vigour which the organism derives from the improvement in its conditions in respect of temperature. A relatively small difference of temperature—two or three degrees—may make an appreciable difference in the activity of the disinfectant, and in the examination of disinfectants the failure to remember this fact has led to serious error. Above the optimum a rise of temperature increases the activity of the disinfectant, sometimes to an enormous extent. The same is sometimes the case, even at temperatures below the optimum, when the organism is in unfavourable conditions for growth. A mixture of disinfectants in many cases has a more powerful effect than can be produced by either separately (Chamberland). The resistance of bacteria to disinfection by chemical agencies is extremely variable and is also selective. Bacteria of one class may be many times more sensitive to one

[1] Bullock, *Journ. of Hygiene*, xiii, 1913, p. 168.

disinfectant than to another when both substances exert an equal effect on bacteria of another class. The presence of organic matter may profoundly modify the action of chemical disinfectants, particularly those acting by oxidation, considerably reducing their efficiency.

Requirements for an efficient disinfectant.—The conditions which should be satisfied by an efficient disinfectant for general use are simple, but not easy to obtain. Because a disinfectant effect depends on the strength of the solution, the substance should have an approximately definite efficiency for particular organisms in given conditions, and for the same reason it should be permanently homogeneous. In practice disinfectants must be used with water or in an aqueous solvent ; it should, therefore, yield a stable solution or uniform emulsion in all proportions. Because bacteria as presented for practical disinfection usually have some organic coating, it should be stable in the presence of organic matter ; and as this coating is often of a greasy character, it should, especially if intended for use on dirty or greasy surfaces, have high solvent power for grease. For use when heat can also be applied, whereby its activity is enhanced, unless it breaks up, it should be stable at all reasonable temperatures. These conditions may be considered to be indispensable. It is further desirable that it should have a sufficiently high specific efficiency to allow of its being used in a readily diffusible dilution ; that it should yield a cheap solution or emulsion, not act on metals, and be neither caustic nor toxic. Some disinfectant substances may now be considered more in detail.

Acids.—All acids have disinfectant action, and their relative values are interesting in the respect that for them a general law has been fairly well established by Von Lingelsheim, and confirmed by Boer—namely, that the efficiency varies with the degree of acidity. Solutions of

acids not of equal percentage concentration, but of *equal acidity*, have approximately the same disinfectant efficiency whatever may be the acid, and whether it be inorganic or organic.

The acids have no great practical application in disinfection. That which has been most commonly used is sulphurous acid, applied either direct from burning of sulphur (in which case it will also contain SO_3 if there is sufficient moisture to hold the sulphur dioxide in solution) or by the use of the liquefied gas. It produces a slow superficial disinfection of a weak and uncertain character even under laboratory conditions. Such experiments avoid, however, to a far greater extent than is possible in practice the difficulty of diffusion, and the unequal diffusion of sulphurous acid in air and its small power of penetration make it less efficient in practice. To obtain even the poor efficiency which is its maximum possible it is necessary for the air to be damp and the room most carefully sealed, and in these conditions it is often more injurious to the objects under treatment than to the bacteria against which it is directed. One of the most efficient methods of applying sulphurous acid disinfection is by means of the Clayton apparatus. The gas is generated by burning sulphur in a current of air at a high temperature, and contains, in addition to SO_2, traces of higher oxides of sulphur. It is also a very efficient vermin-killer, destroying rats, cockroaches, bugs, fleas, flies, etc.

Alkalies and soaps.—The degree of alkalinity of a solution affects, but does not by itself altogether determine, its germicidal power, which is also dependent on the nature of its metal. The hydrates of thallium, lithium, barium, calcium, potassium, sodium, and ammonium have widely different efficiencies, roughly in the order named. For practical purposes only those of potassium, sodium, and

calcium need be considered.[1] They exhibit notably the characteristic of all disinfectants in that they work much more vigorously in hot than in cold solution. It is to the hydrates or alkaline carbonates of potassium and sodium that the soaps owe such power as they possess against naked organisms. The relative efficiency of soaps in practical disinfection may be understated by the results of comparative experiment on laboratory cultures because the resistance of the microbe itself to disinfection by chemical substances, and, indeed, by other agencies, may be small compared with the resistance offered by the envelope of grease or greasy dirt, derived from perspiration, pus, fat, and the oily grime which pervades cities and is everywhere caused by handling. A disinfectant of greater efficiency than soap on a laboratory culture may, therefore, be of much less efficiency on an infection in actual practice. Soaps are incompatible with most disinfectant substances, but not with all. Biniodide of mercury can be prepared with soap, and for surgical purposes is a disinfectant of high value. The "carbolic soaps" of commerce are, for the most part, worthless.

Caustic lime, used generally as a 20 per cent. milk, has considerable disinfectant power, and has been applied to the disinfection of fæces. For this purpose care has to be taken to break up any lumps of excreta, and whenever practicable a heat process, of which the efficiency and rapidity may be greatly increased by an alkaline disinfectant, is much to be preferred. Lime is inefficient against the more resistant organisms, and lime-washing cannot be considered a sufficient precaution against them or against infections, such as those of scarlet fever and small-pox, of which the exciting organism is unknown.

Halogens.—The disinfectant values of dry chlorine,

[1] See Forrest and Hewlett, *Journ. Roy. Army Med. Corps*, February 1904.

iodine, and bromine are low. Both in a dry and a damp state chlorine is inconvenient, and the others are costly ; and the use of halogens is therefore practically confined to solutions, notably "chloride of lime" (a mixture of calcium hypochlorite, hydrate, and chloride) and hypochlorite of soda (chloros). These have a powerful effect on laboratory cultures, but in practice need to be used in excess proportionate to the amount of organic matter which may be present. Thus, for instance, a 1 per cent. solution of hypochlorite of soda mixed with an equal volume of urine loses the whole of its available chlorine almost immediately, and becomes inert as a germicide. Where the amount of organic matter is small, and the objects are not likely to be injured, the hypochlorites are among the best of known disinfectants, provided they are used fresh. The slow addition of hydrochloric acid, yielding nascent chlorine, increases the activity of a hypochlorite considerably. A solution of iodine is now used for skin disinfection in surgical practice. Iodine trichloride is a powerful disinfectant, of which the use has been suggested, among other purposes, for the sterilisation of water. Nessfield suggested the use of chlorine for sterilising water on the large scale, and iodine for the same purpose on the small scale (p. 693). Chloride of lime or other hypochlorite is now being extensively used for sterilising water on the large scale (p. 693).

Hypochlorites, *e.g.*, Eusol and Dakin's solution, have been much used of late for the treatment of septic gunshot and shrapnel wounds, as in the Carrel-Dakin system. Dakin's solution which is used contains 0·5–0·6 per cent, of sodium hypochlorite. It is prepared as follows :

One hundred and forty grams of *dry* sodium carbonate (Na_2CO_3), or 400 grams of the crystallized salt (washing soda), are dissolved in 10 litres of tap water, and 200 grams

of chloride of lime (chlorinated lime) of good quality are added. The mixture is well shaken, and, after half an hour, the clear liquid is siphoned off from the precipitate of calcium carbonate and filtered through a plug of cotton ; 40 grams of boric acid are added to the clear filtrate, and the resulting solution is ready for use. A slight additional precipitate of calcium salts may slowly occur, but it is of no significance. The solution should not be kept longer than one week. *The boric acid must not be added to the mixture before filtering, but afterwards.*

When a hypochlorite acts upon an organic substance containing the $=NH$ group, the first reaction consists in the replacement of hydrogen by chlorine and the formation of substances of the group known as chloramines, thus :

$$\begin{matrix} R_1 \\ R_2 \end{matrix}\!\!\rangle NH + HClO = \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!\rangle NCl + H_2O$$

This $=NCl$ group is strongly antiseptic.

The Chloramines themselves have therefore been introduced as antiseptics. The best known is Chloramine-T which is Benzene-sodium-sulphochloramide ($C_6H_5.SO_2$-$NaNCl$).

Other inorganic substances.—Solutions of salts of mercury exercise a powerful disinfectant action in proportion to the amount of dissolved metal which they contain. The most commonly used is the perchloride (corrosive sublimate). Apart from its extremely poisonous character, it has the disadvantage of forming with albuminoid substances both insoluble and soluble compounds of little or no germicidal value, sulphuretted hydrogen converts it into the insoluble and inert sulphide, and it acts on some metals. The addition of acids or salts (*e.g.* hydrochloric or tartaric acid or sodium or ammonium chloride) prevents or largely reduces the formation of insoluble com-

pounds ; but it does not prevent the reactions resulting in soluble substances, it may reduce the germicidal power, and the action of perchloride in the presence of albuminoids is therefore very variable. The reduction in germicidal power by addition of sodium chloride is well seen from the following results (Finlay, *loc. cit.*) :

16 litres of solution contained.	Number of colonies developing after treatment for 6 minutes.
1 mole $HgCl_2$	8
1 ,, $HgCl_2$ + 1 mole NaCl . . .	32
1 ,, $HgCl_2$ + 2 moles NaCl . . .	124
1 ,, $HgCl_2$ + 4 ,, NaCl . . .	382
1 ,, $HgCl_2$ + 10 ,, NaCl . . .	1087

Extremely high values were at one time given for the germicidal efficiency of corrosive sublimate. This is now known to have been due to its powerful *inhibitory* action, traces of the substance carried over into the subcultures preventing growth (see p. 740).

The Local Government Board recommended the following solution of corrosive sublimate for disinfecting purposes :

Corrosive sublimate . . .	½ oz.
Hydrochloric acid	1 oz. fl.
Anilin blue	5 gr.
Water	3 gals.

This forms a solution of 1–900 nearly ; it would be preferable to use 1 oz. of corrosive sublimate.

The biniodide is also a powerful disinfectant when dissolved in potassium iodide. It is not affected by albuminoids nearly as much as is perchloride, and may be incorporated with soap.

Soluble silver salts are powerful disinfectants, weaker than mercuric chloride, but far less sensitive to albuminoids; in blood-serum, for instance, silver nitrate is several times as powerful as corrosive sublimate. They are incompatible with chlorides, except in certain organic combinations, from which silver chloride is only partially precipitated. Silver salts are poisonous, though less so than those of mercury.

Iron and zinc salts have been credited with useful disinfectant action; but, in fact, their value is very small, and no practical account need be taken of them. A very strong antiseptic power has been attributed to copper salts, which, according to some experiments, exercise a sufficient disinfectant action on sporeless organisms, such as the *B. typhosus*, to enable drinking water to be sterilised from such infections by the small quantity of copper which it dissolves (p. 693).

There is some ground for connecting the disinfectant action of metallic salts with a reducing action on some forms of protoplasm, as pointed out by Loew.

The permanganates have considerable germicidal power when in strongly acid or alkaline solution, but the readiness with which they are affected by organic substances makes them unsuitable for practical use. Peroxides and ozone are open to the same objection, and have less disinfectant power. Hydrogen peroxide is used in the Budde process for sterilising milk (p. 708), and ozone has been practically applied in the sterilisation of water-supplies (p. 693), and attempts have been made to utilise it for the disinfection of wounds. Acetozone also slowly liberates ozone.

Organic substances.—The methane and the aromatic series furnish the disinfectants which are most important in practice.

Alcohol itself possesses some disinfectant power for

sporeless organisms, but only when absolute or in very strong solution.

Formaldehyde is by far the most important of the methane group. It can be applied either as a solution (formalin) or as gas. The gas can be produced by the incomplete combustion or oxidation of methyl alcohol, by the evaporation, with or without pressure, or spraying, of formalin, either alone or mixed with calcium chloride or glycerine, by the depolymerisation by heat of the solid polymer paraformaldehyde, or by mixing this substance with potassium permanganate. Many forms of apparatus have been designed for the production of formaldehyde gas for disinfection. In any form the gas seems to give little more than superficial disinfection, and to require precautions to ensure diffusion throughout the atmosphere of a room. The conditions desirable for disinfection by formaldehyde gas are saturation of the air with moisture, maintenance of a good room temperature, sealing of the room, the use of at least 60 grm. of formaldehyde per 1000 cubic feet (preferably more, up to 120 grm.), and in the case of large rooms mixture of the gas with the air of the room, either mechanically or by the provision of a multiplicity of inlets for the gas into the atmosphere. By the use of a vacuum formaldehyde can be evaporated in a closed chamber at temperatures indifferent to many substances which will not stand steam at 100°, and considerable penetration can be obtained (Defries process). As a spray formalin can be used in any ordinary apparatus. Formalin seems to have a very slow germicidal action, for tested by the Rideal-Walker method, its carbolic coefficient is only about 0·7 for the *B. typhosus*. Yet 2 per cent. formalin kills anthrax spores in two or three days and gaseous formaldehyde is similarly active.

Bacterol is a proprietary formalin-containing disinfectant. The vaporising form vaporised in the special

Bacterol cabinet is extremely efficient for the destruction of both bacteria and vermin and it has no deleterious action on any articles.

Of the aromatic series, the number of substances and preparations is extraordinarily large. The standardisation of methods of examination will, it is to be hoped, eliminate the less efficient.

The best known is phenol (carbolic acid). Its saturated solution contains about 9 per cent. It is only slightly affected by albuminoids, and generally is stable in the presence of organic matter at ordinary temperatures. Its compounds, when it forms any, have themselves some disinfectant action. With acids this action is usually greater than that of pure phenol, with alkalies less. Light tends to decompose it, but the efficiency is not affected. It is poisonous and caustic. For practical uses its chief value is as a standard, as its disinfectant value is comparatively low, and for spore-bearing organisms it is practically useless. Like the cresols, its efficiency is greatly increased by the addition up to saturation of common salt or hydrochloric acid. The following results well demonstrate the increased germicidal power of phenol by additions of sodium chloride (Findlay, *loc. cit.*) :

Solution.	Anthrax spores treated. Number of colonies developing after treatment (days).			
	0	1	3	7
3 per cent. phenol	6300	1390	1260	950
3 „ „ + 1 per cent. NaCl .	5720	1450	1320	360
3 „ „ + 8 per cent. NaCl .	1940	150	50	0

Probably the addition of salt alters the distribution of the phenol between the water and the cells, the salt increasing the concentration of the phenol in the bacterial cells.

"Crude carbolic acid" consists mainly of cresols and higher phenols in proportions largely dependent on the source of the tar from which they are prepared ; phenol

is nearly absent from it. By themselves the cresols are extremely insoluble in water ; in oil or alcohol they have little or no disinfectant value. Cresols are much reduced in efficiency by albuminoids. In saturated salt solution the disinfectant value of crude carbolic acid is greatly increased.

Ordinarily neutral tar oils with no appreciable disinfectant value are left in, or mixed with, tar distillate, and the saponified product produces an emulsion with water. Innumerable products of this type are made. Their efficiency varies not only with their active ingredients, but also with the character of the emulsions which they form, from about the same as that of phenol to about three times as much. Commercially they are known as soluble carbolic acid, soluble creosote, etc. Creolin is a type of numerous preparations of the same character. They are all poisonous and sensitive to albuminoids. If naphthalene is present in excess it is deposited in cold weather on standing. Lysol is mainly a solution of the cresols in fat or linseed oil, saponified, with addition of alcohol. It gives a clear solution with water, having slightly less efficiency on naked bacteria than cresol, much superior solvency for grease, and equal sensitiveness to albuminoids. A number of proprietary disinfectants of high germicidal power are now to be obtained. Such are cyllin, McDougall's M.O.H. fluid, izal, kerol, etc. The active agents appear to be oxidised hydrocarbons without phenol and cresol, in emulsion in glue, soaps, oils, etc., and they are comparatively non-toxic. The active principle of cyllin is an oxidised hydrocarbon, having a diphenyl nucleus in place of the single phenyl present in carbolic acid ; it is insoluble in water, hence for the purpose of even distribution in water it is emulsified with a neutral hydrocarbon oil. The finished product contains 50 per cent. of the active principle, and is free from

carbolic acid and its homologues. The active principle of kerol consists of oxidised hydrocarbons with a diphenyl nucleus and it contains no phenol or cresol. The germicidal efficiency, expressed as the carbolic-acid coefficient (p. 741), of a number of substances is given in the Table on page 737.

Some of the *anilin dyes* have been claimed to be powerfully antiseptic. Of these the best known are Brilliant Green, Malachite Green, Crystal Violet and Flavine. Browning and co-workers [1] state that these have the following germicidal potency on the *M. pyogenes aureus* and *B. coli*:

	M. aureus.		*B. coli.*	
	In 0·7 % Pep. Water.	In Serum.	In 0·7 % Pep. Water.	In Serum.
Brilliant Green Sulphate	$1 : 10 \times 10^6$	1 : 30,000	1 : 130,000	1 : 3,500
Brilliant Green Oxalate	$1 : 10 \times 10^6$	1 : 100,000	1 : 200,000	1 : 3,500
Malachite Green, Oxalate and Sulphate	$1 : 10 \times 10^6$	1 : 40,000	1 : 20,000	1 : 1,000
Crystal Violet	$1 : 4 \times 10^6$	1 : 400,000	1 : 8,000	1 : 8,000
Flavine	1 : 20,000	1 : 200,000	1 : 1,300	1 : 100,000

It will be seen that Flavine is the only one of these which is more active in serum, and it does not inhibit phagocytosis until a concentration of 1 : 500 is attained. On these grounds its use has been strongly urged for surgical practice. Browning's results with Flavine have, however, been severely criticised by Fleming and by Hewlett; moreover, its action is exerted extremely slowly.

Chloroform is a powerful antiseptic, but at least 1 per cent. must be present to act as a germicide; it is costly, and not much used as a practical disinfectant, but in

[1] *Brit. Med. Journ.*, 1917, vol. i, p. 73.

bacteriological and physiological chemistry is a useful antiseptic for preserving solutions which putrefy easily.

Iodoform is valuable for dusting wounds, though its penetrating odour is objectionable, and has led to the introduction of many substitutes. Its value as an anti-

Carbolic Acid Coefficients obtained by the Rideal-Walker Method [1] (*p.* 740)

Disinfectant.	Observer.	Date of experiment.	Organism.	Carbolic Acid Coefficient (carbolic acid = 1).
Absolute alcohol .	Fowler	8·05	*B. typhosus*	0·03
Boric acid . . .	Walker	10·04	,,	0 (?)
Chinosol	Fowler	11·03	,,	0·15
Chloros	,,	1·04	,,	21·0
,, (with 50 per cent. urine) . .	Walker	7·06	,,	8·0
Copper sulphate. .	,,	6·04	,,	0·04
Cyllin*	Fowler	11·06	,,	14·0
,, (with 50 per cent. urine) . .	,,	5·06	,,	11·0
Cyllin	Klein	5·05	*M. pyogenes*	9·3
,, ...	Simpson and Hewlett	6·06	*B. pestis*	34·0
Formalin. . . .	Fowler	3·05	*B. typhosus*	0·7
Hydrochloric acid .	Walker	2·05	,,	11·0
Izal*	Fowler	3·06	,,	11·0
Kerol*	,,	9·06	,,	12·0
,, (with 50 per cent. urine) . .	,,	8·06	,,	8·5
Little's phenyle .	,,	5·04	,,	2·0
Lysol	,,	2·06	,,	2·5
Mercuric chloride .	,,	8·05	,,	1000·0
,, ,, .	Walker	8·05	,,	400·0
Potass permanganate	Fowler	8·05	,,	42·0
,, ,, (with 3 per cent. organic matter) . . .	Walker	1·07	,,	1·0
Zinc chloride . .	,,	1·06	,,	0·15

* The germicidal efficiency of these substances has been increased since the date of the experiments recorded, and they now have a carbolic-acid coefficient of from 16 to 20–22.

[1] Fowler, *Journ. Roy. Army Med. Corps*, July 1907.

septic has been greatly discussed; micro-organisms will develop in nutrient media containing a considerable proportion, but probably when in contact with living cells a decomposition is effected, free iodine being liberated, hence its value. A paste, known as B.I.P., composed of iodoform, bismuth subnitrate and liquid paraffin, has been used in the treatment of wounds.

The essential oils, *peppermint*, *mustard*, *cloves*, *thymol*, and *menthol*, are powerfully antiseptic.

Disinfectant powders at best exert but a superficial action. They act chiefly as deodorants, but may be useful in preventing the breeding of flies in garbage, etc.

It is useless to add a small quantity of disinfectant to a large volume of fluid or solid; the disinfectant must be added in sufficient amount so that the mixture contains the minimum percentage which has been found by experiment to be efficient. For this reason the attempt to disinfect sewers, sewage, streets, etc., by relatively small quantities of disinfectants is useless, and the money so wasted would be far better employed in providing more water for flushing purposes.

In medical practice, while antiseptics can be applied locally with success and, to some extent, for disinfecting the alimentary tract,[1] no substance has yet been discovered which can be administered with safety to such a degree as to saturate the body, and so exert a general germicidal action in bacterial infective diseases. Salvarsan, perhaps, to some extent possesses this power and has been used with success in certain general infections, *e.g.* anthrax. Protozoa are attacked selectively by many substances, *e.g.* the malaria parasite by quinine, spirochaetes by salvarsan, trypanosomes by atoxyl, trypan red, etc., *Piroplasma canis* by methylene-blue, etc.

In surgical practice no unbiased observer can doubt the efficacy of antiseptic treatment, but many so-called "antiseptic operations" are marred by faults of omission and commission which render them far from being perfectly antiseptic. There has been some controversy between the advocates of "antiseptic"

[1] See F. E. Taylor, "Intestinal Disinfection in Alimentary Toxæmia," *Medical Press*, January 14, 1914.

and of " aseptic " surgery. Undoubtedly antiseptics do diminish the vitality and therefore the reparative power of the tissues and aseptic methods should so far as possible replace antiseptic ones. The skin of the patient and the hands of the operator having been disinfected as far as possible, no antiseptic should be permitted to come into contact with the wound, which may be irrigated with *warm* sterile physiological salt solution. A *dry* wound is an important element to success, and a dry, sterile, unirritating dressing should be employed. Instruments, sponges, etc., may be kept in sterile salt solution after the preliminary disinfection —by heat (not sponges) or chemicals. But the aseptic system requires more care to ensure success than the antiseptic one, and unless the assistants can be trusted and the details rigorously carried out, the latter seems preferable.

With regard to septic wounds Wright maintains that no antiseptic can be applied to a wound in sufficient concentration to destroy micro-organisms without causing inhibition of phagocytosis and other natural defensive mechanisms and that antiseptics therefore do more harm than good. To increase the flow of germicidal lymph he recommends salt packs and hypertonic salt solution. On the other hand Carrel flushes out the wound every two hours with Dakin's hypochlorite solution by an arrangement of tubes maintained in position and claims that this is the most satisfactory treatment.

The Determination of the Germicidal Power

For determining germicidal power on sporing organisms anthrax spores are generally used, on non-sporing organisms cultures of the *B. typhosus* are usually employed.

(1) *Thread method.*—Sterilised silk threads are impregnated with sporing and non-sporing organisms, lightly dried, and then exposed to the action of the antiseptic solution of a known strength for a given time. After treatment the threads are thoroughly washed with distilled water to remove the antiseptic, and sown on the surface of agar or other suitable culture medium. If no growth occurs the organisms are assumed to have been destroyed. As a matter of fact, however, it is extremely difficult to get rid of the last traces of the antiseptic, which may inhibit growth although the organisms may yet be alive, a fallacy which caused an exaggerated value to be assigned to many substances—for

example, corrosive sublimate. If pathogenic organisms be the subject of experiment, the threads may be inoculated into a susceptible animal. The writer finds that in disinfection experiments with anthrax spores, surface agar is a much better medium than broth.

In experiments with corrosive sublimate, by whatever method, the last traces of this substance must be converted into the inert sulphide by treatment with hydrogen or ammonium sulphide.

(2) *Garnet method.*—Small garnets the size of a pea are sterilised, soaked in a suspension or a broth culture of the organism, removed and dried. The garnets with the organisms attached are then soaked in solutions of the disinfectant of known strengths for various periods of time ; they are then removed from the solution,

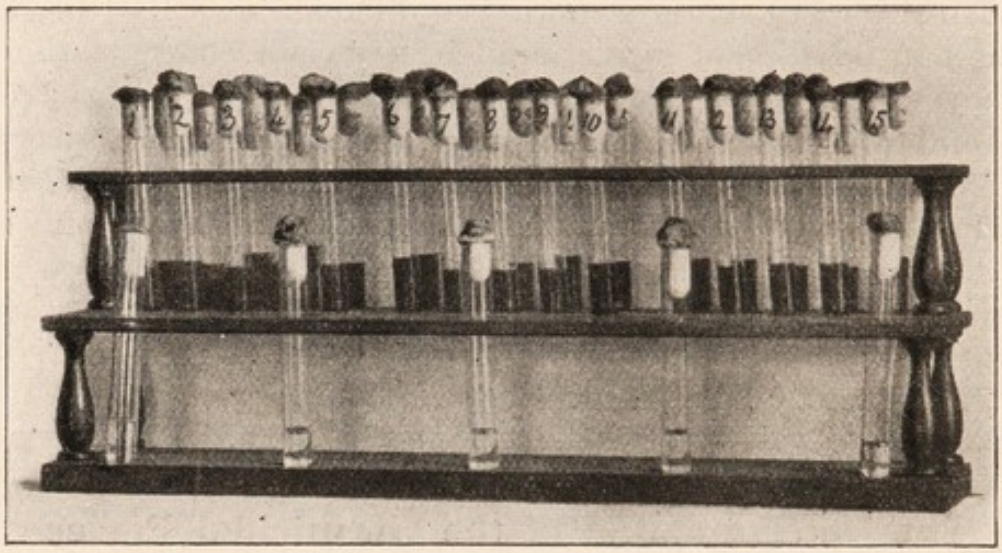

FIG. 69.—Test-tube rack with test-tubes arranged for the Rideal-Walker method of testing disinfectants.

well washed with sterile water, and finally placed in tubes of broth.

(3) *Rideal-Walker or drop-method.*—Moor first suggested that the germicidal efficiency of a disinfectant might be compared with that of a standard solution of carbolic acid, which has a definite composition, is stable, and can be accurately standardised, and Rideal and Walker devised an ingenious and simple method for carrying this out. A special test-tube rack is very convenient (Fig. 69), in which the lower tier has five holes which hold three or four tubes containing the solutions of decreasing strengths of the disinfectant to be tested, and two tubes or one tube containing standard carbolic acid solution of known strength

for comparison. The upper tier has thirty holes in two rows spaced into six sets of five holes each. These hold tubes of sterile nutrient broth which are numbered from 1 to 30. The test is usually made with a broth culture of *B. typhosus*, but other organisms may be employed. The process is as follows: The five tubes in the lower tier each contain 5 c.c. of the disinfectant and carbolic solutions. Into each in succession, at intervals of half a minute, 0·2 c.c. of the typhoid broth culture is added with a pipette. Half a minute after the *last* tube has been inseminated, a loopful is taken from the *first* tube and inseminated into the first broth tube, and this process is repeated at half-minute intervals until all the broth tubes have been inoculated. The inoculated broth tubes are then incubated at 37° C. for three days, and the occurrence or not of growth is taken as indicating the killing or non-killing of the organism respectively. It will be seen that the first set of five broth tubes inoculated are sub-cultures in which the organism has been acted upon by the disinfectant and carbolic solutions for two and a half minutes, the second set for five minutes, and so on. The results (taken from an actual test) may be charted as follows:

B. typhosus, 24-hour *broth culture at* 37° *C.*

Room-temperature 60° F.

Disinfectant.	Dilution.	Time culture exposed to action of disinfectant (in minutes).						Subcultures.	
		2½	5	7½	10	12½	15	Period of Incubation.	Temperature.
X	1–1400	+	*	*	*	*	*	3 days	37° C.
X	1–1500	+	+	*	*	*	*		
X	1–1600	+	+	+	*	*	*		
X	1–1700	+	+	+	+	*	*		
Carbolic	1–100	+	+	+	*	*	*		

+ = growth in the subcultures. * = no growth in the subcultures.

From this it will be seen that the disinfectant X in a solution of 1 in 1600 kills in the same time (7½ minutes) as carbolic 1 in 100. This result is expressed as a coefficient obtained by dividing the strength of disinfectant by the strength of carbolic which kills each in the same time; in the present instance the coefficient

is $\frac{1600}{100} = 16{\cdot}0$, and this figure is known as the "carbolic acid coefficient."

If nothing is known concerning the germicidal strength of the disinfectant, some preliminary experiments should be performed with dilutions at wide intervals as regards strength (*e.g.* 1–100, 1–500, 1–1000, 1–1500, 1–2000, etc.), and when the limit has thus been approximately ascertained, the test is performed as above.

Precautions to be taken in carrying out the test.—(*a*) The *culture* should be a broth one about twenty to twenty-four hours old, and should be free from clumps; this may be attained by filtration through paper. The culture is added by means of a small graduated pipette or by means of a standardised dropping pipette. Four drops of culture added with a pipette the point of which is No. 27, Stubbs Wire gauge, is equivalent to 0·2 c.c.

(*b*) The *carbolic acid* (the crystals of which should have a melting-point of not less than 40·5° C.) should be kept in the form of a 5 per cent. aqueous solution standardised by the bromine method. Failing this, the solutions may be made with the *acidum carbolicum liquefactum* of the Pharmacopœia, which contains 100 parts of phenol in 110, but is not absolutely constant in composition.

(*c*) All *measures*, *pipettes*, and *test-tubes* used for making dilutions should be sterile.

(*d*) The *dilutions* of the disinfectant and carbolic should be made with sterile distilled water.

(*e*) The *broth* used for culturing and subculturing should have the following composition:

Lemco	20 grm.
Peptone	20 grm.
Salt	10 grm.
Water	1000 c.c.

The medium should be standardised to a reaction of + 10 (Eyre's scale).

(*f*) The *loop* used for subculturing should have an internal diameter of 3 mm., and be made with platinum wire of 27–28 B.W.G.

(*g*) *Growths* in the subcultures should be obtained in those taken at not less than two and preferably at three of the time intervals ($2\frac{1}{2}$, 5, and $7\frac{1}{2}$ minutes) from both the disinfectant and the carbolic solutions which correspond.

(*h*) The *temperature* at which the determination is made should be noted, and the strength of carbolic varied accordingly (*e.g.* 1–100 for 56°–62° F., 1–110 for 62°–67° F., and 1–120 for 67°–73° F. for *B. typhosus*), or the determination may be made at a standard temperature (*e.g.* 20° C.) by warming (or cooling) the disinfectant and carbolic tubes in a water-bath.

(*i*) When the organism does not form a uniform culture in broth, a suspension of an agar or other culture must be made in water and filtered. Subculturing in some cases (*e.g.* with *B. pestis* and *B. anthracis*) must be made on agar or other suitable culture medium.

The method is an admirable one for determining the relative efficiencies of disinfectants on *naked* organisms in the *absence* of organic matter. But in practice disinfection is almost always carried out in the presence of organic matter, and various suggestions have been made with a view of introducing this factor into the test, for the presence of organic matter may reduce the carbolic-acid coefficient of many disinfectants (see pp. 726, 732, and Table, p. 737). Among the substances suggested are urine, fæces, 2 per cent. suspension of dried and sterilised fæces (Martin and Chick), and milk. Kenwood and Hewlett found that the presence of urine or fæces reduced the carbolic acid coefficient of some proprietary disinfectants to a greater relative extent than that of carbolic.

The method is also sometimes somewhat erratic in practice, and a number of determinations may be needed before the strengths of disinfectant and carbolic which coincide are found. Occasionally also two strains of *B. typhosus* may differ widely as regards the germicidal action of the disinfectant on them, while they are practically identical as regards the germicidal action of the carbolic.

Woodhead and Ponder proposed a modification of the method. In this, *B. coli* is used as the test-organism and bile-salt peptone water as the culture medium, a platinum spoon being used for culturing, and more cultures at shorter intervals up to half an hour are made.

4. Volatile disinfectants may be tested by moistening the wool plug of an agar tube, inoculating the agar, and capping with a rubber cap, and observing whether any growth occurs.

5. Volatile disinfectants may also be tested by exposing silk threads, pieces of paper or fabrics, splinters of wood, etc., impregnated with organisms, some free, others done up in packets

of cotton-wool, in a room or chamber of known cubic capacity, to the action of the gas, a known amount of which is present in the chamber. After exposure for a given time, the threads are sown in broth or agar tubes, and the tubes incubated.

The Rideal-Walker method is particularly applicable to the coal-tar disinfectants which act rapidly and are used for sanitary disinfection. It is unsuitable for the determination of the germicidal value of the more slowly acting disinfectants. The conditions of the test also render it unsuitable for the determination of the germicidal value of disinfectants which are to be used in the treatment of wounds where there is much organic matter and the temperature approximates to 37° C. For the latter Emery's method may be applied.

6. *Emery's method.*—"Re-constituted" blood is prepared as follows: Some blood is collected aseptically, allowed to coagulate, and the serum is pipetted off. Some blood is collected in citrate solution, centrifuged, and the deposited corpuscles are washed two or three times with saline. Equal volumes of the serum and corpuscles are mixed. Nine parts of this reconstituted blood are mixed with one part of an eighteen-hour old broth culture of the *Strep. fæcalis*, which is chosen as the test-organism, though other organisms may be substituted. One volume of this infected blood is then taken in a Wright's pipette, having a unit mark about 2 in. from the end, and one volume of the antiseptic solution, the two are well mixed and then half the total is sucked up into the same pipette and the other half into a second pipette. The two pipettes are sealed at the point in the flame and incubated at 37° C. in an opsonic incubator or in a water-bath. At the end of fifteen minutes, one pipette is taken, the point is sterilised in the flame and broken off and a loopful of the mixture is spread over as wide an area as possible on the surface of an agar plate. The second pipette is treated in the same way at the end of an hour. The plates are incubated for twenty-four hours, and the results noted. In this way the germicidal value of the disinfectant is ascertained after it has acted for periods of fifteen and sixty minutes.

The following is a table of the results obtained by Emery by this method:

Antiseptic.	15 Minutes.		60 Minutes.	
	Does not Kill*	Kills.	Does not Kill.*	Kills.
Carbolic acid . .	1 in 70	1 in 60	1 in 60	1 in 50
Eusol	Undiluted	Inert	Undiluted	Inert
Dakin	Undiluted	Inert	Undiluted	Inert
Perchloride . . .	1 in 100	1 in 80	1 in 100	1 in 80
Biniodide . . .	1 in 60	1 in 40	1 in 60	1 in 40
Iodine	1 in 100	?	1 in 100	?
Lysol	1 in 40	1 in 30	1 in 150	1 in 120
Malachite Green .	1 in 250	1 in 200	1 in 250	1 in 200

* Causes diminution in number of organisms but does not kill all of them.

On the Rideal-Walker method, etc., see Rideal and Walker, *Journ. Sanitary Inst.*, vol. xxiv, 1903, p. 424; Kenwood and Hewlett, *ibid.* vol. xxvii, 1906, p. 1; Firth and Macfadyen, *ibid.* p. 17; Kenwood, *Public Health*, 1908; Fowler, *Journ. Roy. Army Med. Corps*, July 1907; Partridge, *Bacteriological Examination of Disinfectants*: Woodhead and Ponder, *Lancet*, 1909, vol. ii; Emery, *Lancet*, vol. i, 1916, p. 817; Watson Cheyne, *Lancet*, vol. i, 1915, February 27.

FRENCH WEIGHTS AND MEASURES AND THEIR ENGLISH EQUIVALENTS

1μ (micron)	=	0·001 millimetre ($\frac{1}{25000}$ inch, nearly).
1 millimetre	=	0·04 ($\frac{1}{25}$) inch.
25 millimetres	=	1 inch.
1 centimetre	=	0·39 inch.
2·5 centimetres	=	1 inch.
5 centimetres	=	2 inches.
1 gramme	=	15½ (15·432) grains.
4 grammes	=	1 drachm (apothecaries'), nearly.
28 grammes	=	1 ounce (avoirdupois), nearly.
1 kilogramme	=	2·2 pounds (avoirdupois).
0·5 kilogramme	=	1 pound (avoirdupois), nearly.
1 cubic centimetre	=	16 minims, nearly (16·23 minims).
3½ cubic centimetres	=	1 fluid drachm, nearly.
28 cubic centimetres	=	1 fluid ounce, nearly.
568 cubic centimetres	=	1 pint ($\frac{4}{7}$ litre).
1 litre	=	1¾ pints, or 35 fluid ounces, nearly.

SOLUBILITIES

Amount of Substance contained in 10 c.c. of a Saturated Solution

Alcoholic solution of methylene-blue	0·068 grm.
Aqueous solution of methylene-blue	0·646 grm.
Alcoholic solution of gentian violet	0·442 grm.
Aqueous solution of gentian violet	0·175 grm.
Alcoholic solution of fuchsin	0·292 grm.
Aqueous solution of fuchsin	0·066 grm.
Aqueous solution of corrosive sublimate	0·507 grm.

STEAM TEMPERATURE-PRESSURE

	Mm. of Hg.	Pounds per sq. in. Absolute Pressure.	Atmospheres.
100° C.	760	14·7	1·00
105° C.	906·4	17·5	1·19
110° C.	1075·3	20·8	1·41
115° C.	1269·4	24·5	1·67
120° C.	1491·2	28·8	1·96
125° C.	1743·8	33·7	2·29

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The principal reference to a subject is given in italics.

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