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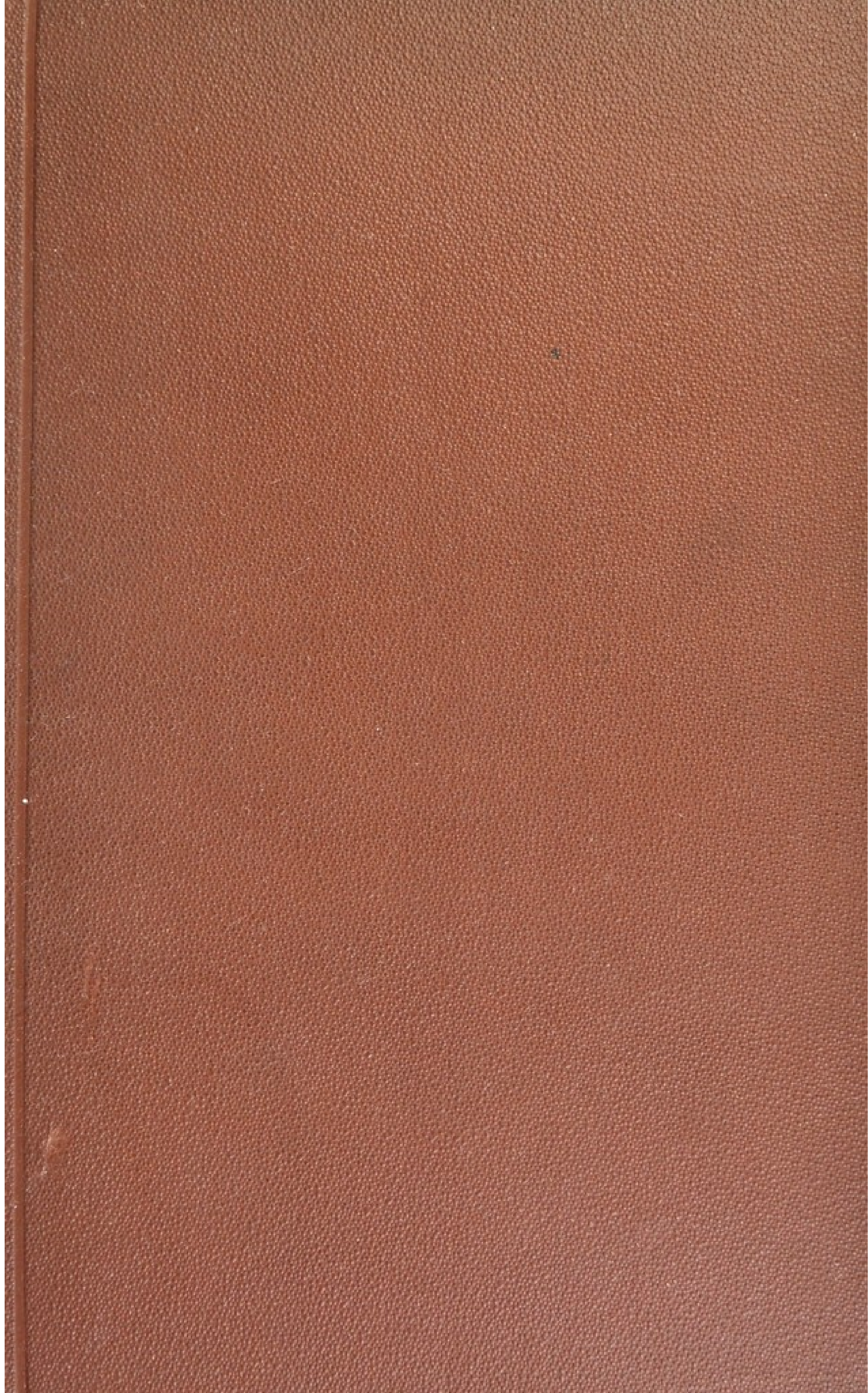

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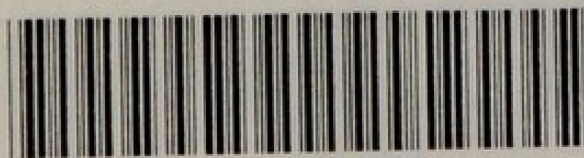
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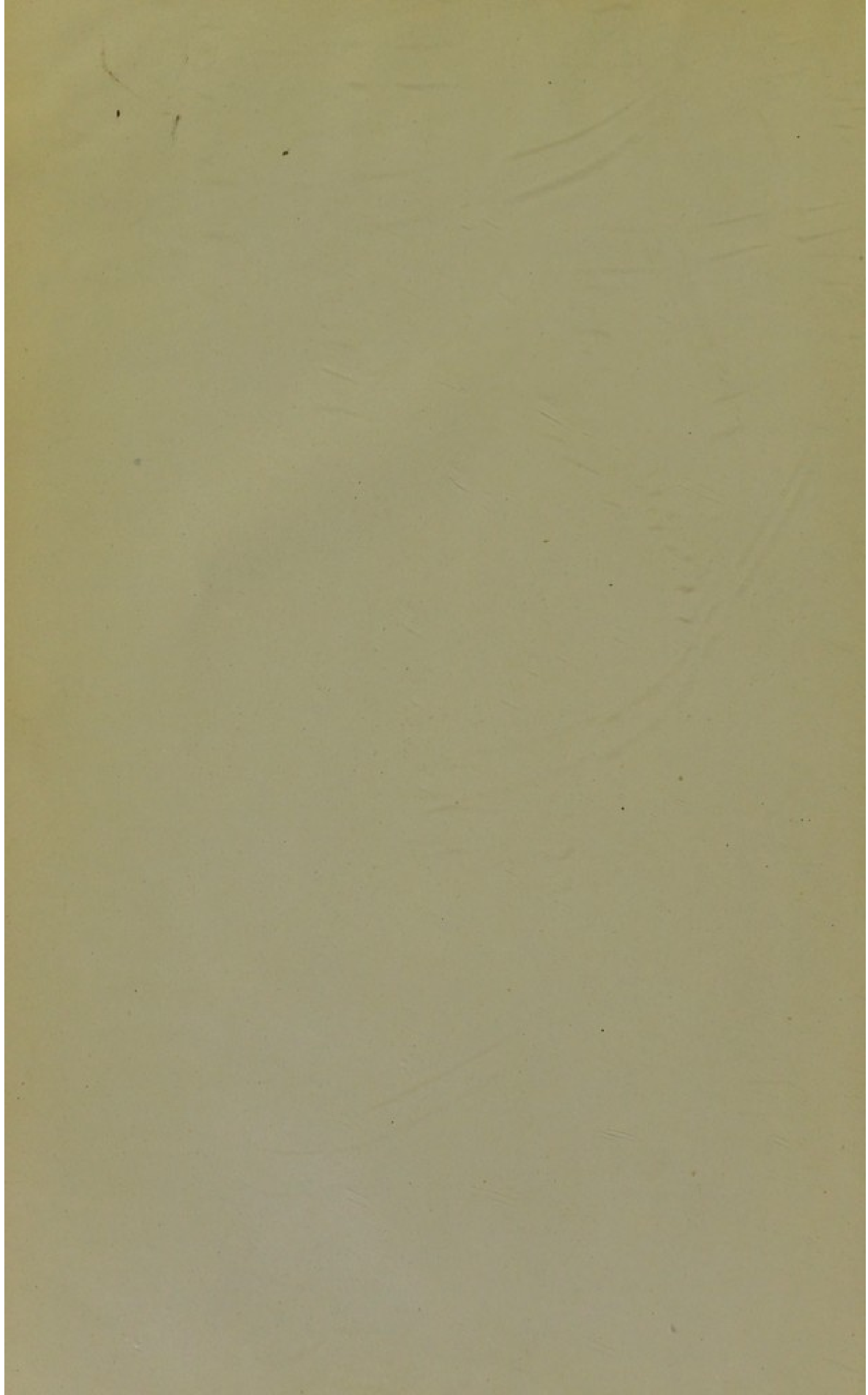
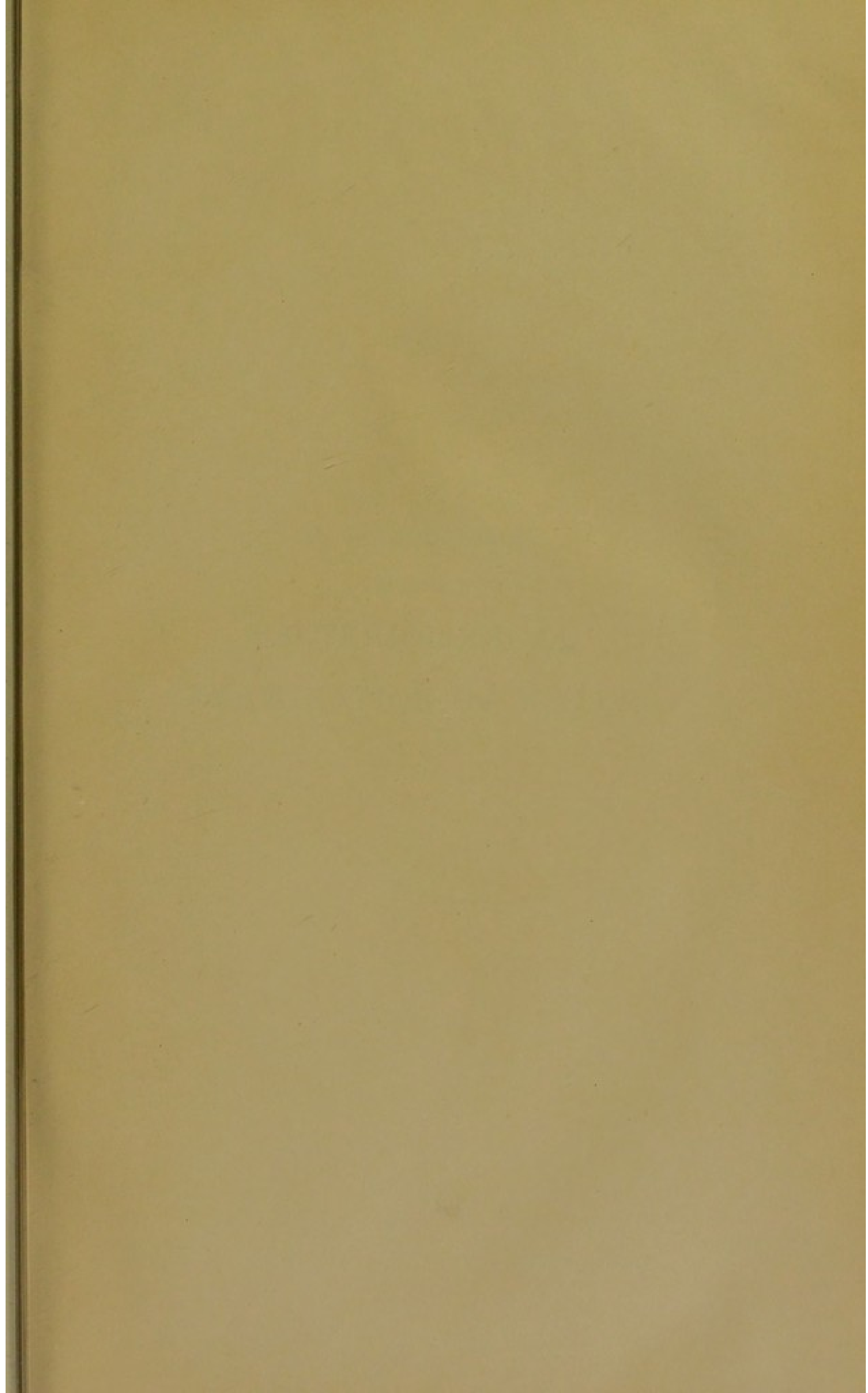
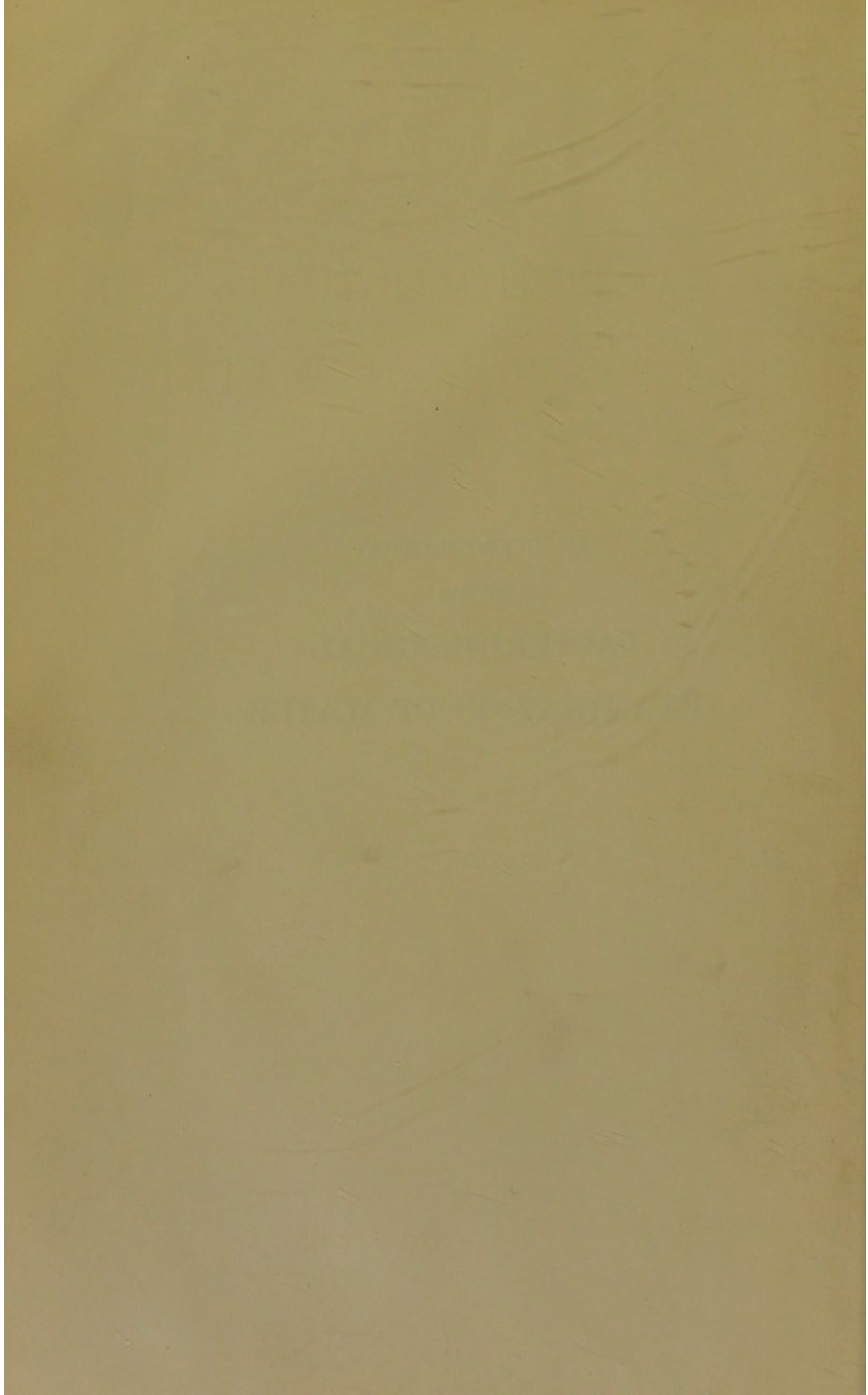




AN INTRODUCTION

TO THE

BACTERIOLOGICAL EXAMINATION OF WATER



AN INTRODUCTION

TO THE

BACTERIOLOGICAL EXAMINATION OF WATER

BY

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ASSISTANT PROFESSOR OF MILITARY HYGIENE IN THE ARMY MEDICAL SCHOOL, NETLEY; ASSISTANT EXAMINER IN HYGIENE UNDER THE BOARD OF EDUCATION, SOUTH KENSINGTON; MAJOR, ROYAL ARMY MEDICAL CORPS

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PREFACE.

In this volume I have endeavoured to present in a simple form the methods and results of a bacteriological examination of water. The object of such an examination being to discover whether a water is likely to be prejudicial to health, it is obviously necessary to have clear ideas as to the hygienic value of the various micro-organisms which have been isolated from water. Great difficulties have been introduced into the subject owing to the extreme variability of water bacteria. The microscopical appearances of these micro-organisms are largely influenced by the media on which they are growing, and the cultural characteristics are greatly dependent on whether the micro-organisms are enfeebled or not. It is also well known that bacteria, which normally inhabit the human body, find in water very unfavourable conditions for their existence; consequently, after a comparatively brief existence in this medium, they often exhibit weakened forms when isolated on ordinary bacteriological media. In order to simplify matters as much as possible, the bacteria which occur in water have been arranged in three classes, viz., (1) bacteria which are found in pure water; (2) bacteria which are common in sewage but rarely met with in pure water; and (3) bacteria which give rise to specific disease in human beings. The micro-organisms in the first class are very numerous, but many of them have been so imperfectly described that it has been very difficult to group them in a satisfactory manner. As the easiest solution of the difficulty, and to facilitate reference, certain well-defined types have been selected, and under each type have been described all those bacteria which are probably varieties, the variations being caused by differences in food, habitat, &c. I have examined nearly all the bacteria mentioned, and the descriptions are taken from

observations of pure cultures obtained either from Kral's laboratory or from the original discoverers of the bacteria.

The subject of the bacterial flora of sewage is yet in its infancy, but enough work has been done to enable certain bacteria to be classed as the normal inhabitants of sewage, and I hope this section will assist in the solution of the difficult problems which are often placed before the water bacteriologist.

Of the bacteria which give rise to specific disease in human beings, only the B. typhosus and Sp. Choleræ Asiaticæ occur sufficiently often in water to merit description in a small work. The differential diagnosis of the B. typhosus by the serum test has lately received much attention, and an attempt has been made to summarise the most recent work on the subject.

The plates have been drawn from my own sketches, and the colonies depicted are believed to represent the forms most commonly seen during routine examinations of "water-plates." I have to thank Major Leishman, R.A.M.C., for kindly reading the proofs, and for many valuable suggestions. I have further to express my indebtedness to Dr. A. C. Houston, who has allowed me to reproduce the descriptions of the sewage bacteria given in his report to the London County Council; and to Dr. Mervyn Gordon for the use of his valuable paper on B. coli.

W. H. Horrocks.

Sholing, Southampton.
September 1901.

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PLATE I.

FIG.

1. B. Fluorescens Liquefaciens.—Surface colony on a gelatine-plate × 100; forty-eight hours growth at 22° C. Liquefaction has commenced.

2. B. Fluorescens Liquefaciens.—Surface colony on a gelatine-plate × 100; twenty-four hours growth at 22° C.

3. B. Arborescens.—Surface colony on a gelatine-plate × 100; forty-eight hours growth at 22° C.

4. B. Arborescens.—Early stage of colonies; of tenseen in the depth of gelatine-plates.

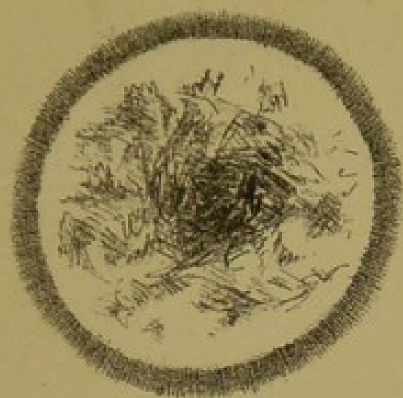
5. B. Fluorescens non-Liquefaciens.—Surface colony on a gelatine-plate × 100; forty-eight hours growth at 22° C.

6. B. Aquatilis.—Surface colony on a gelatine-plate × 100; forty-eight hours growth at 22° C.

7. B. Erythrosporus.—Surface colony on a gelatine-plate × 100; seventy-two hours growth at 22° C.

8. B. Mesentericus Vulgatus.—Surface colony on a gelatine-plate × 100; forty-eight hours growth at 22° C.

Plate I.



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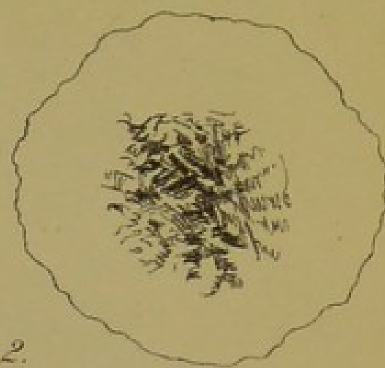

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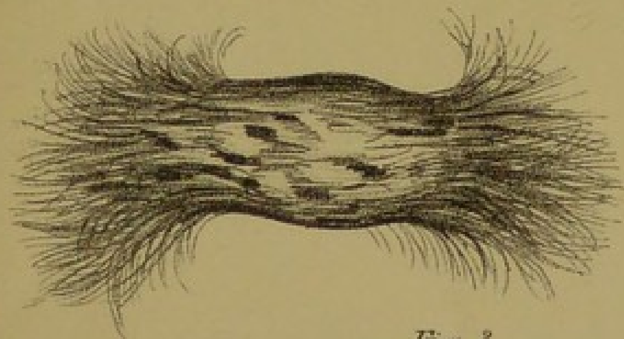

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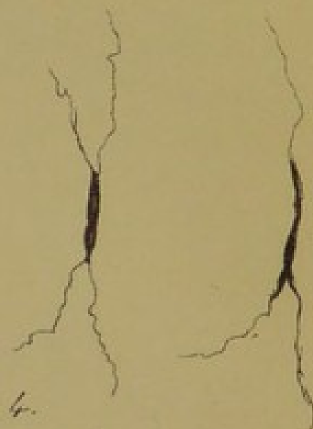

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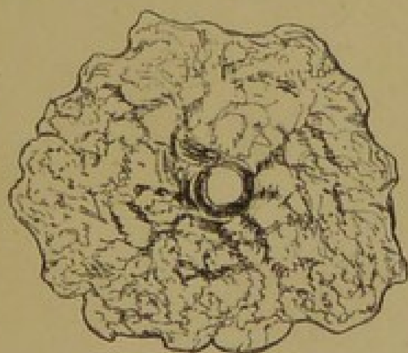

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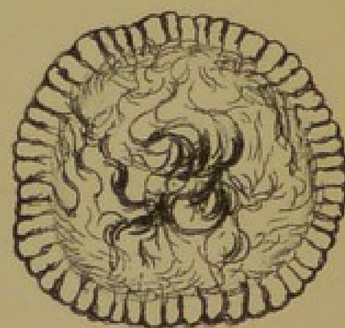

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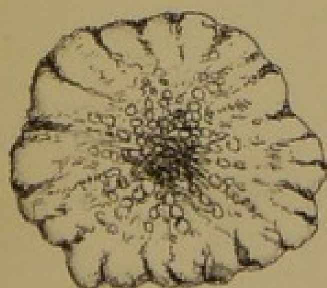

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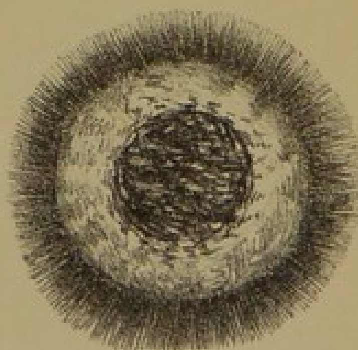

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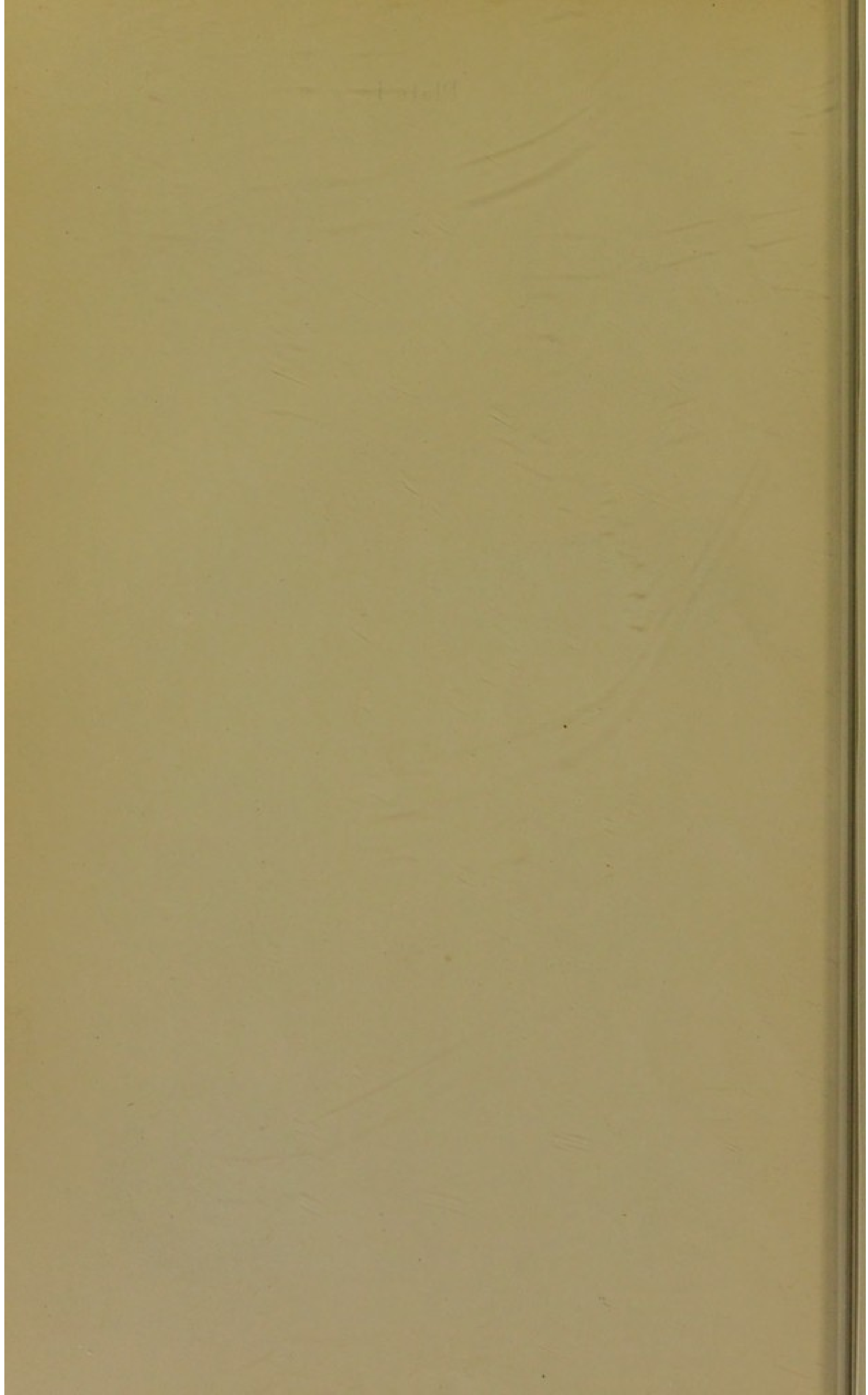


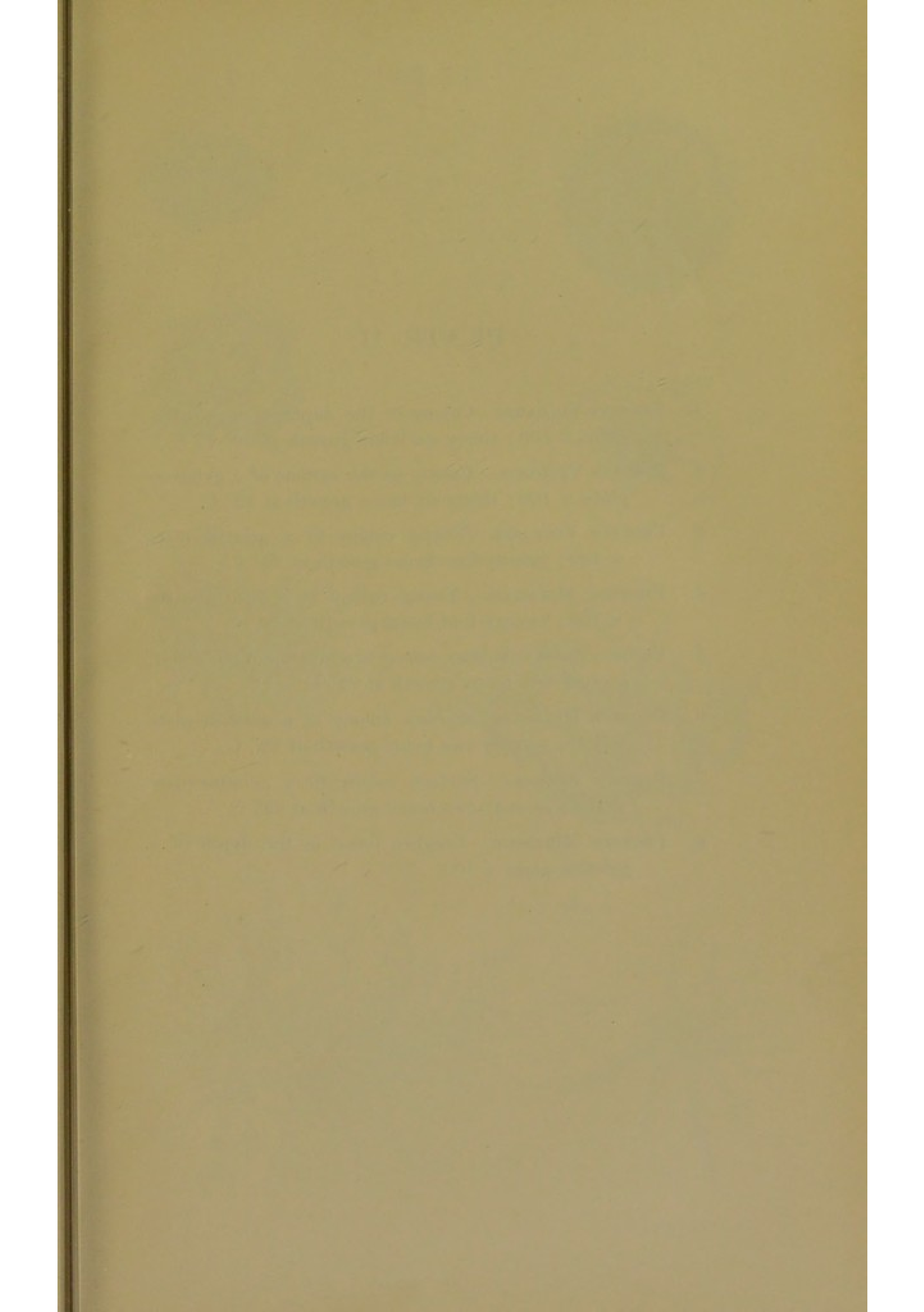
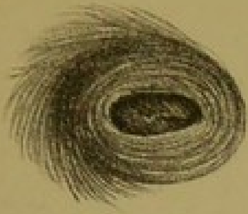


PLATE II.

FIG.

1. Proteus Vulgaris.—Colony in the depth of a gelatine-plate × 100; thirty-six hours growth at 22° C.
2. Proteus Vulgaris.—Colony on the surface of a gelatine-plate × 100; thirty-six hours growth at 22° C.
3. Proteus Vulgaris.—Young colony in a gelatine-plate × 100; twenty-four hours growth at 22° C.
4. Proteus Mirabilis.—Young colony in a gelatine-plate × 100; twenty-four hours growth at 22° C.
5. Proteus Zopfi.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C.
6. Proteus Mirabilis.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C.
7. Proteus Zenkeri.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C.
8. Proteus Mirabilis.—Zooglœa forms in the depth of a gelatine-plate × 100.

Plate II.



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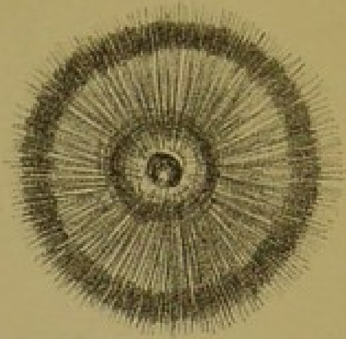

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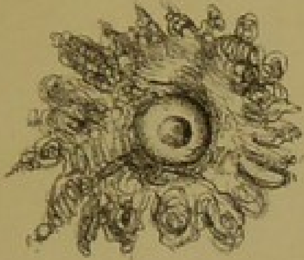

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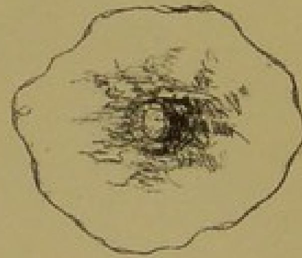

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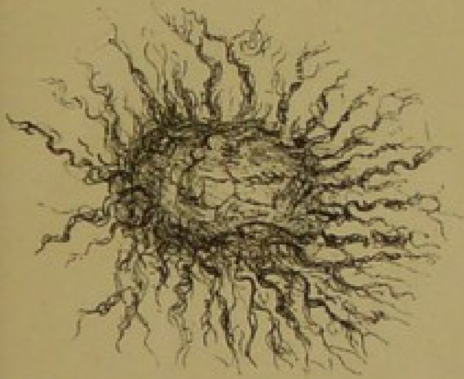

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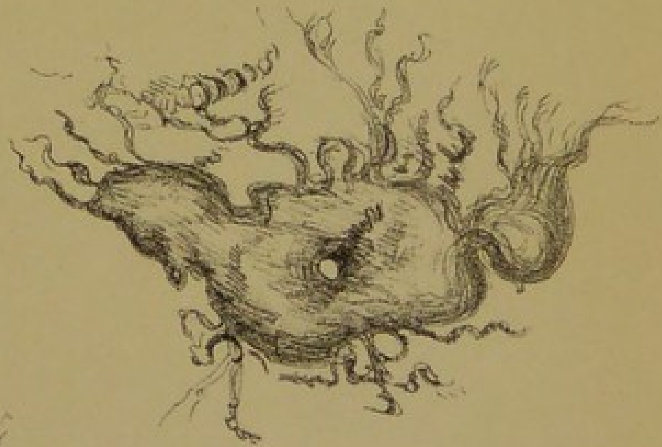

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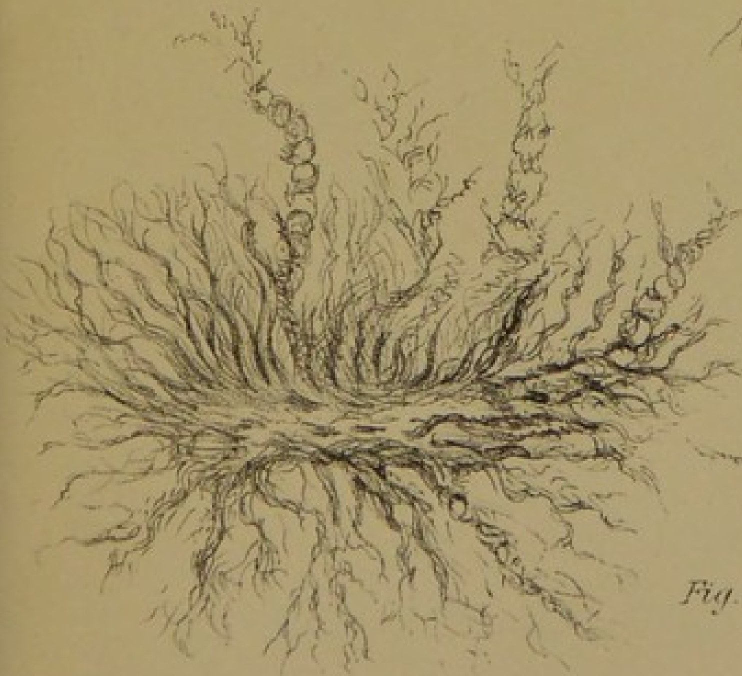

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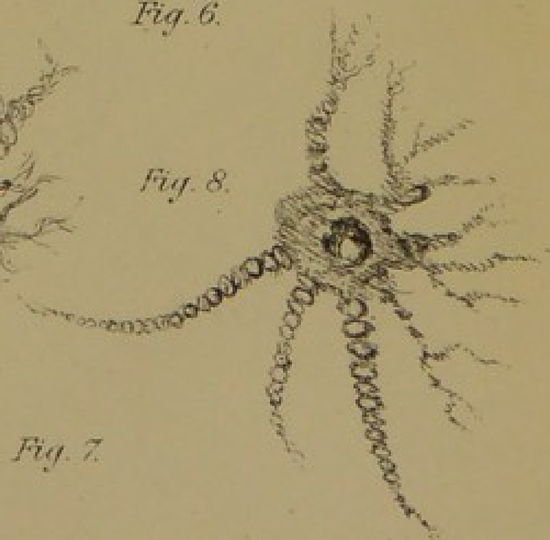

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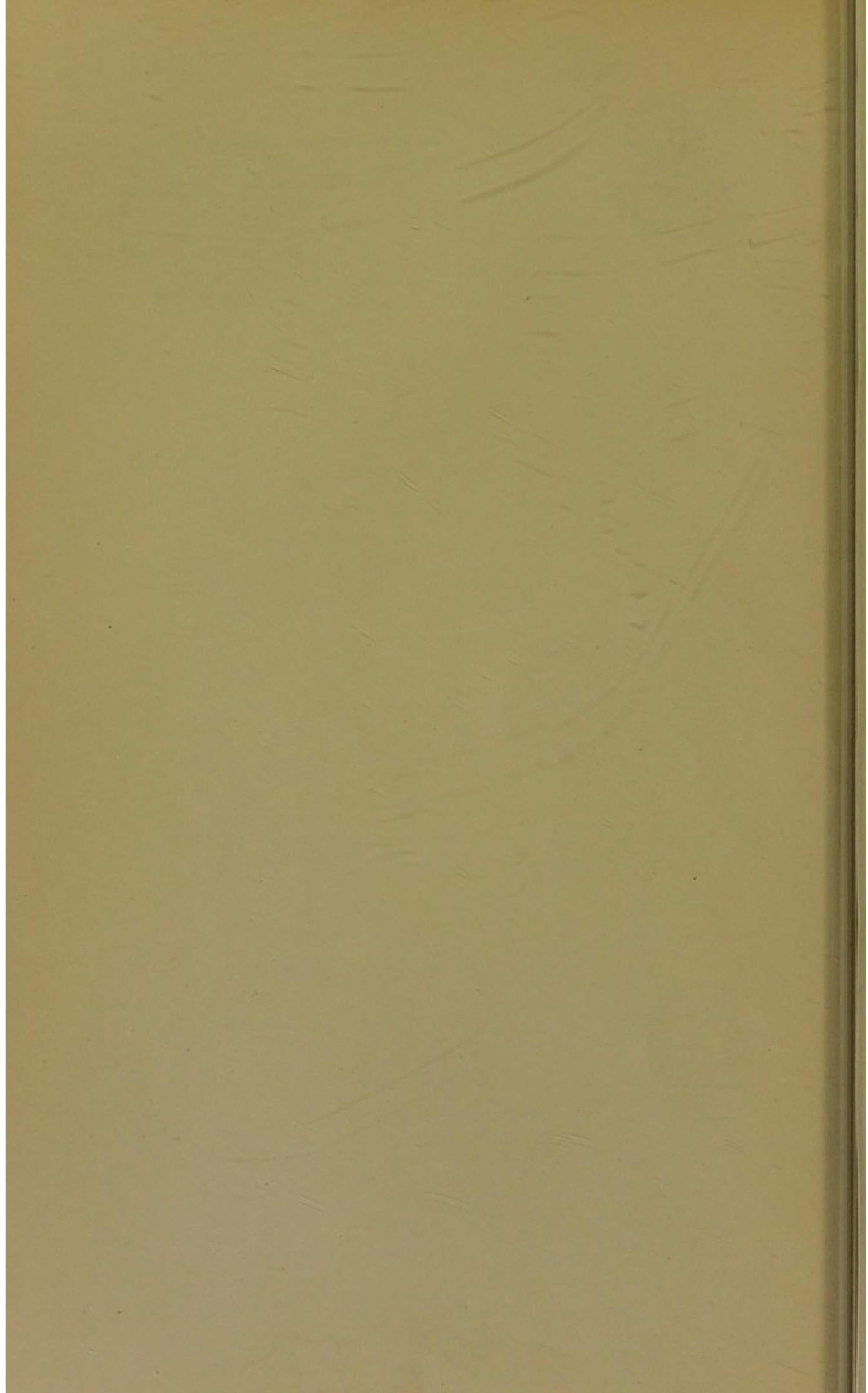


PLATE III.

FIG.

1. B. Subtilis.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

2. B. Megaterium.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

3. B. Mycoides.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

4. B. Megaterium.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C. A later stage than fig. 2.

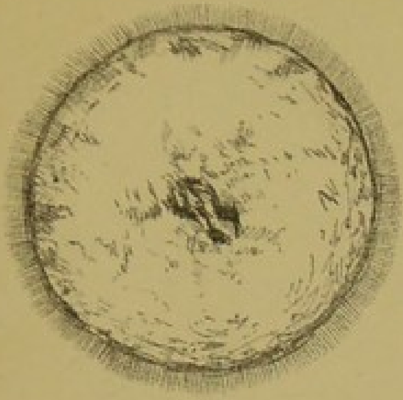
5. B. Aureus.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C. The colony has a golden-yellow colour.

6. B. Ochraceus.—Surface colony in a gelatine plate × 100; thirty-six hours growth at 22° C. Liquefaction is just commencing.

7. B. Liquefaciens.—A young colony in a gelatine-plate × 100. Liquefaction has not commenced.

8. B. Fuscus.—Surface colony in a gelatine-plate × 100. The colony has a dark brown colour in the centre and a thin filmy margin.

Plate III.



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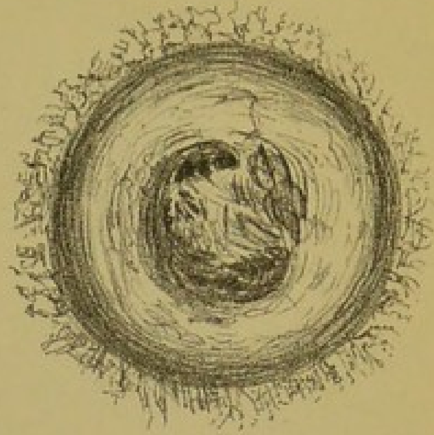

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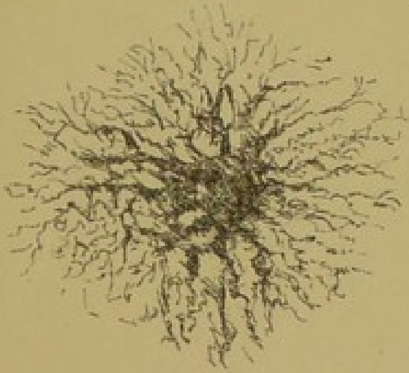

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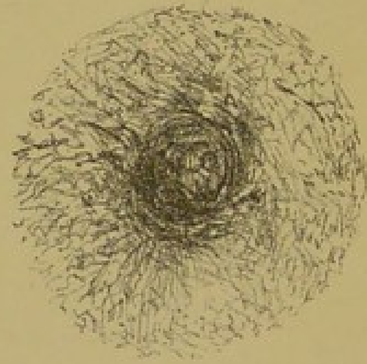

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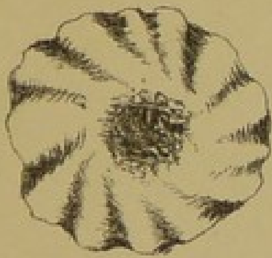

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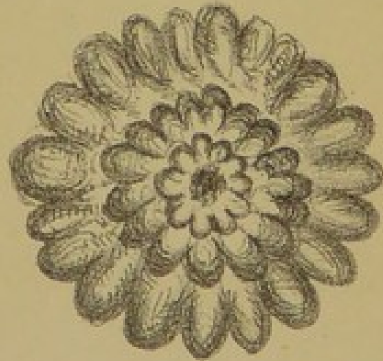

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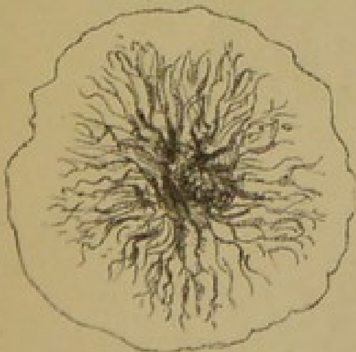

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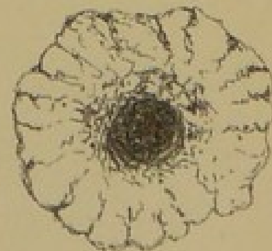

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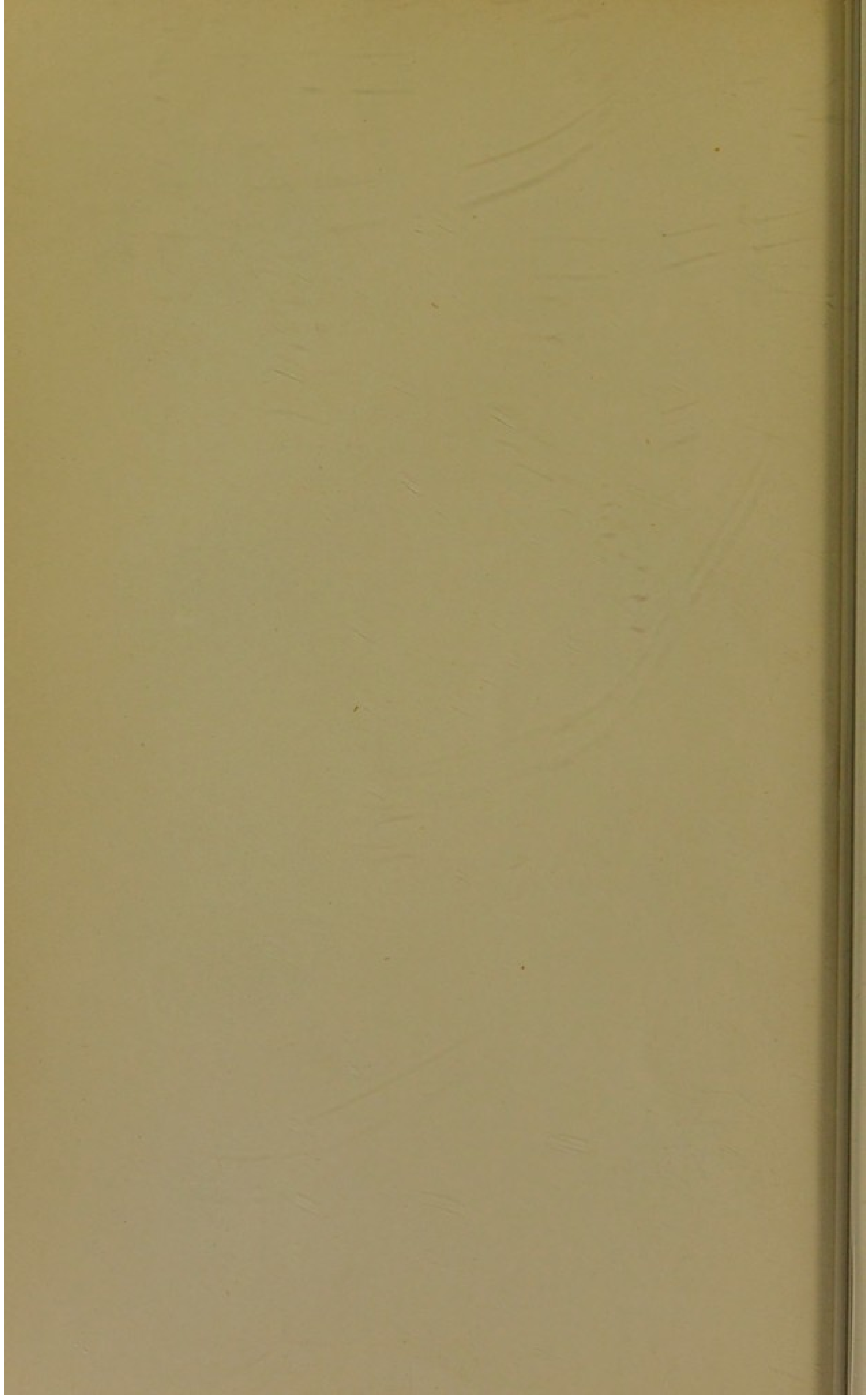


PLATE IV.

FIG.

1. B. Enteritidis, Gärtner.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C.

2. B. Coli Communis.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

3. B. Typhosus.—Surface colony in a gelatine-plate × 100; four days growth at 22° C. "Ridges and valleys" well marked.

4. B. Acidi Lactici.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C.

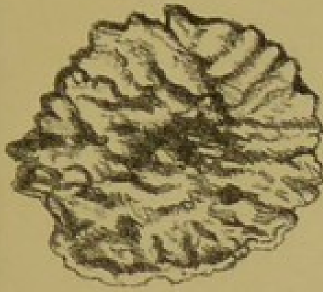
5. B. Typhosus.—Surface colony in a gelatine-plate × 100; four days growth at 22° C. "Glass pellicle" variety of colony.

6. B. Cavicida.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

7. B. Pyocyaneus.—Surface colony in a gelatine-plate × 100; thirty-six hours growth at 22° C. Liquefaction just commencing.

8. B. Violaceus.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C. The colony has a violet colour. Liquefaction has not commenced.

Plate IV.



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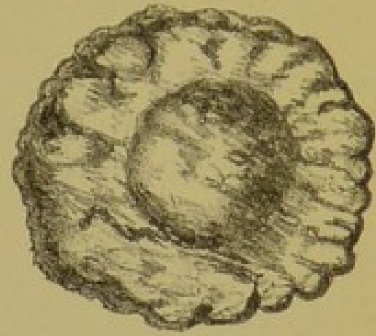

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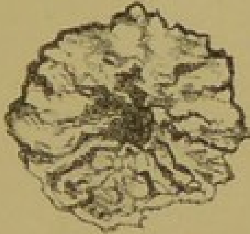

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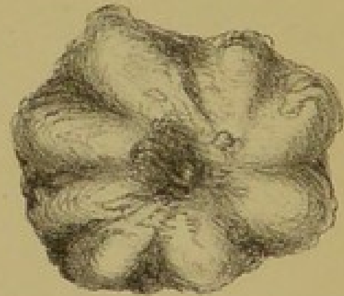

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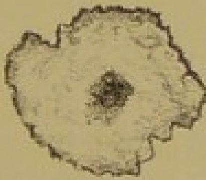

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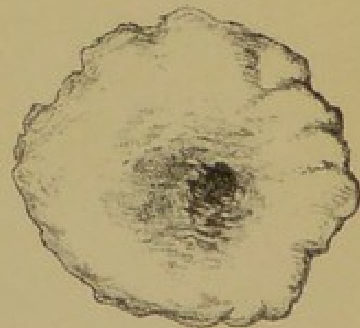

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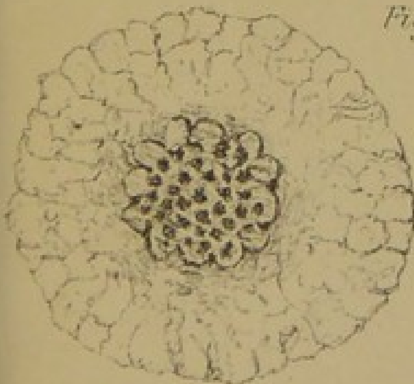

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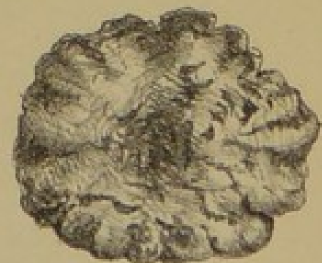

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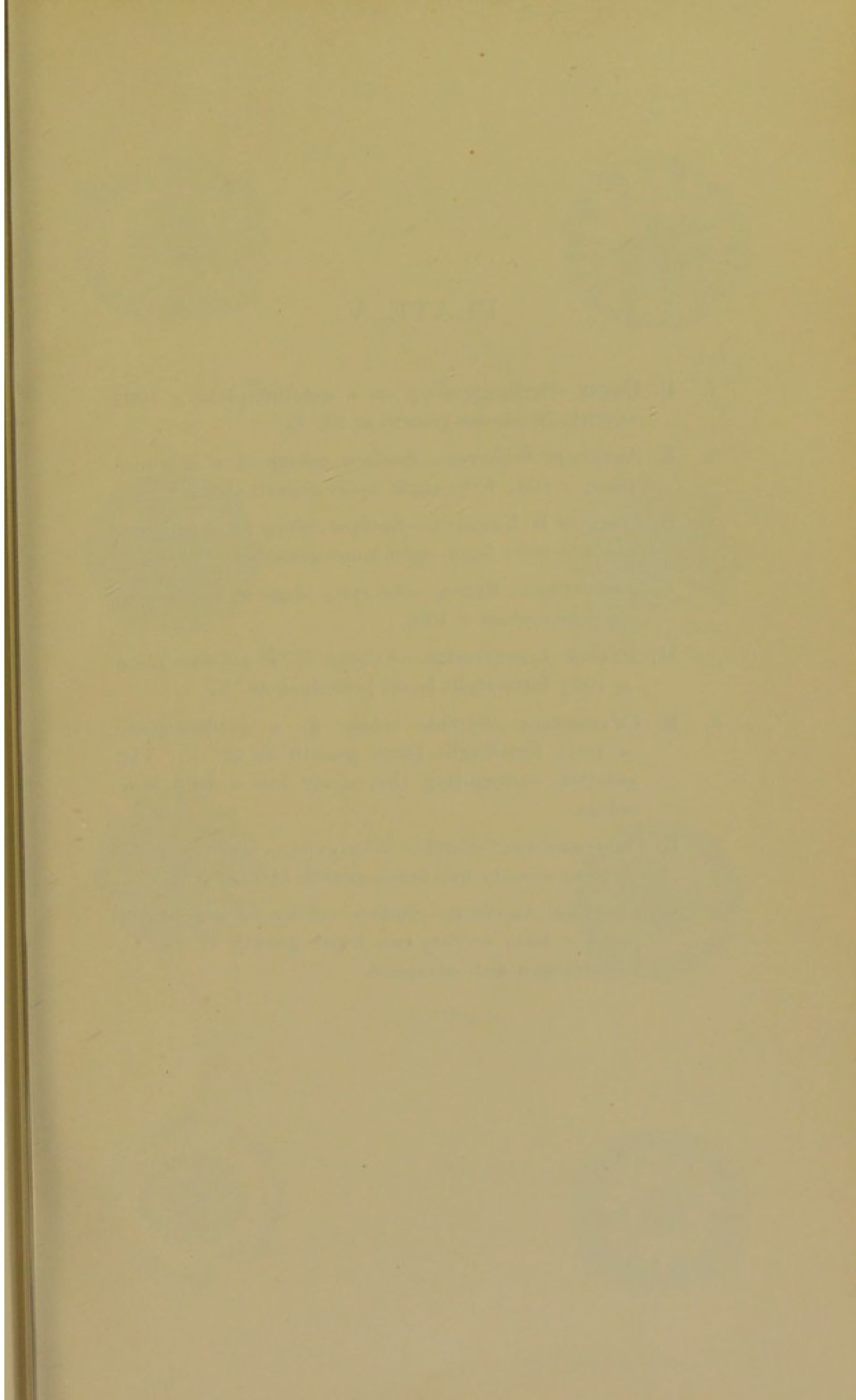


PLATE V.

FIG.

1. B. UREÆ.—Surface colony on a gelatine-plate × 100; seventy-two hours growth at 22° C.

2. B. AQUATILIS SULCATUS.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

3. B. ALBUS or B. UBIQITUS.—Surface colony on a gelatine-plate × 100; forty-eight hours growth at 22° C.

4. B. MESENTERICUS RUBER.—An early stage of a colony in a gelatine-plate × 100.

5. M. FLAVUS LIQUEFACIENS.—Colony in a gelatine-plate × 100; forty-eight hours growth at 22° C.

6. B. CYANOGENUS.—Surface colony in a gelatine-plate × 100; forty-eight hours growth at 22° C. The gelatine surrounding the colony has a deep blue colour.

7. B. DENITRIFICANS.—Surface colony on a gelatine-plate × 100; seventy-two hours growth at 22° C.

8. SP. CHOLERÆ ASIATICÆ.—Surface colony in a gelatine-plate × 100; seventy-two hours growth at 22° C. Liquefaction well advanced.

Plate V.

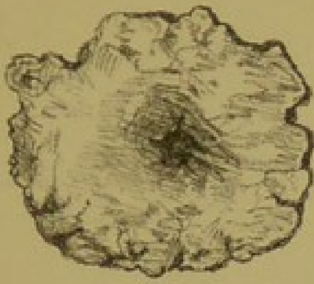


Fig. 1.

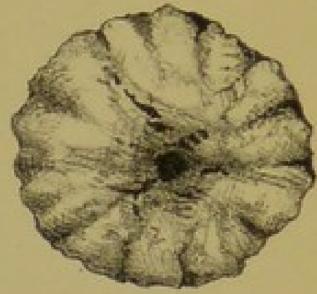


Fig. 2.

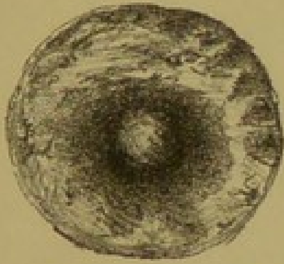


Fig. 3.

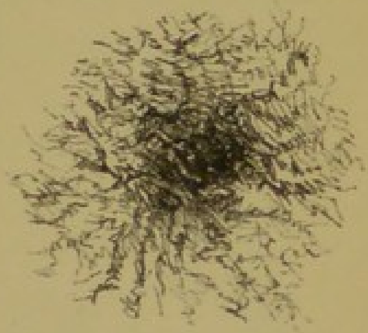


Fig. 4.

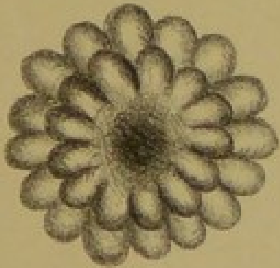


Fig. 5.

Fig. 6.

Fig. 7.

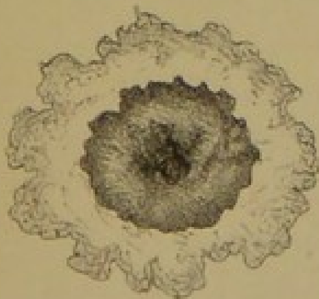
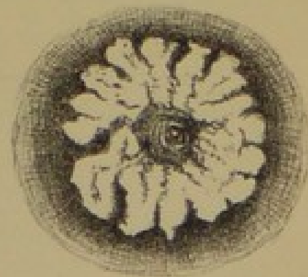
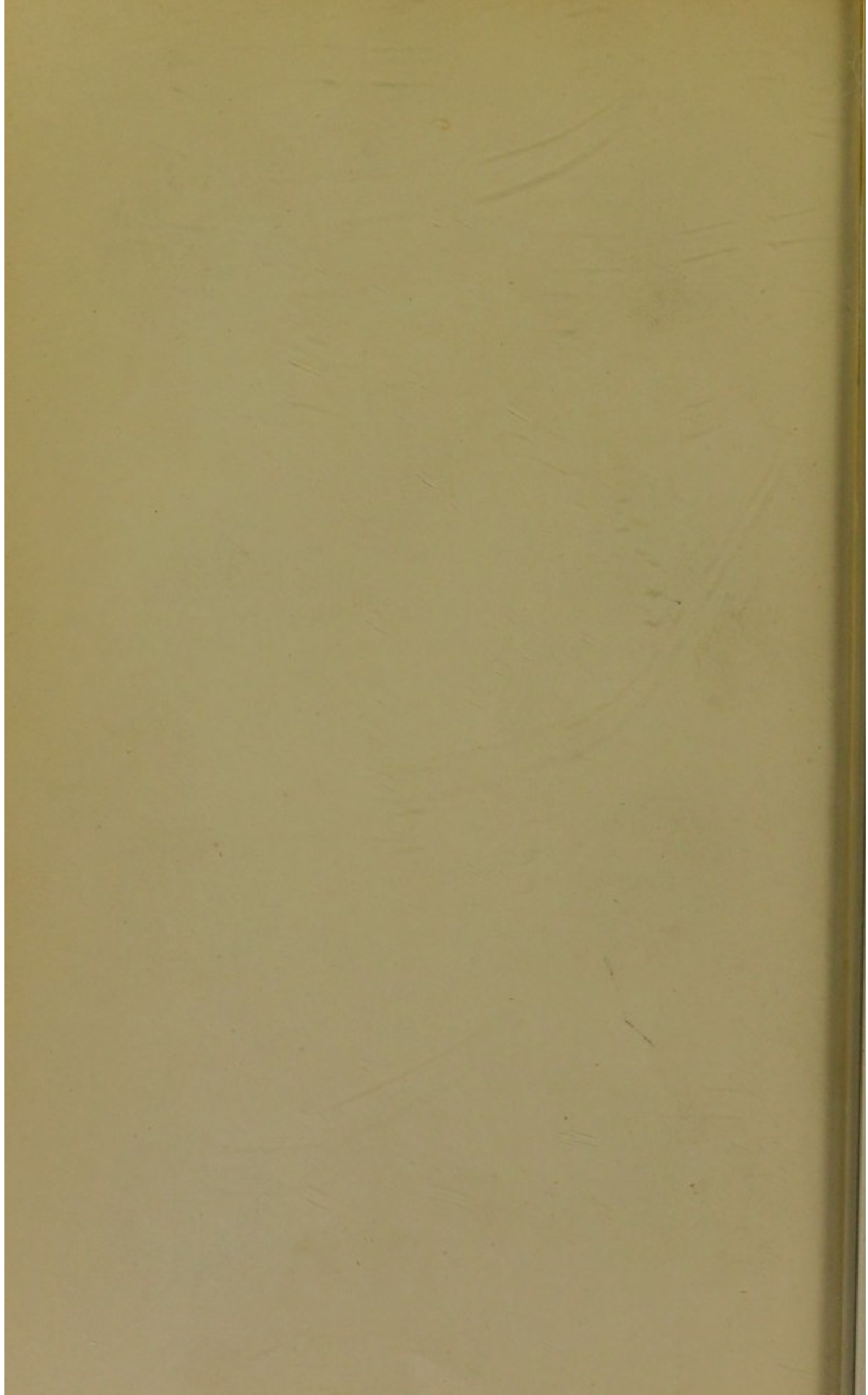


Fig. 8.



A. S. Huth. Lith. London.



CHAPTER I.

COLLECTION OF WATER.

Opinion as to the value of a bacteriological examination of water has passed through many phases. The elaboration of Koch's gelatine plate method gave a great impetus to the bacteriological study of water supplies; it enabled an estimate to be made of the number of micro-organisms present in a given quantity of water, and so permitted a comparative study of various sources of supply. It was at first assumed that from the number of bacteria found in a water distinct evidence of purity or contamination could be at once deduced. Further investigations, however, soon showed that water organisms were capable of multiplying enormously within a very short time, and waters of great organic purity, under certain conditions, might be found teeming with bacterial life. It thus became evident that, though a paucity of organisms might indicate a condition of purity, it did not necessarily follow that a large number of micro-organisms pointed to contamination. Realising that the mere enumeration of the bacteria present in a sample of water was surrounded by many fallacies and, unless performed under the strictest conditions, might give rise to false conclusions, bacteriologists turned their attention to the study of the kinds or species of bacteria, and attempts were made to distinguish between the microbes normally present in a water and those derived from sewage—*i.e.*, to distinguish between water-organisms and sewage-organisms.

The qualitative bacteriological examination, though undoubtedly of the first importance and likely in the long run to be of the greatest practical value to hygienists, has been found to be attended with exceptional difficulties. In the early days of the study of bacteriology, and when modern methods of

distinguishing bacteria were unknown or imperfectly practised, eager investigators of the bacterial flora of waters isolated a large number of water-organisms. Many micro-organisms were described as distinct species which were really only varieties, the variation being caused by differences in food, habitat, temperature, &c. The confusion thus originated gradually became so great that many hygienists lost faith in the bacteriological examination of water and were inclined to believe that a reliable opinion as to the purity of a supply could be given only from the results of a chemical examination. But the indications given by chemical means are merely relative, the results so obtained cannot be correctly interpreted except by reference to local standards. There is no general standard which can be fixed for all waters; the source and immediate surroundings of the supply must be known before a reliable opinion as to the quality of the water can be given. Also it is a matter of common knowledge how quickly some waters change after collection, and this is especially the case when the sample is obtained from a polluted source. It is now well known that in most waters there are two groups of micro-organisms which may largely influence the chemical composition of the water. In the first group are included the micro-organisms which break up organic matter into its chemical elements and reduce nitrates and nitrites to ammonia; to the second group belong the oxidising micro-organisms which convert ammonia into nitrites and nitrates. The exact conditions under which these two groups of micro-organisms operate are not worked out at the present time. The presence of oxygen is supposed to have an important influence, but there must be other conditions, as yet unknown, which influence the final result. It appears from the researches of Heræus that one species of bacteria in the absence of nitrates oxidises a part of the ammonium salts to nitrites; but if nitrates are present they are reduced to nitrites and ammonia notwithstanding an abundant supply of air. Another species also appears to oxidise ammonia to nitric acid in the presence of air, but in the absence of oxygen reduces nitric acid to ammonia. Winogradsky doubts the possibility of the same species possessing both oxidising and reducing characteristics. It seems probable that Heræus' cultures were impure, and that the

reducing and oxidising powers really belong to distinct groups of micro-organisms. Still, the fact that a potable water may undergo entirely opposite chemical changes according as one group or the other gains the ascendency shows how frail a basis there is for the belief that the determination of ammonia, nitrites and nitrates will enable a reliable opinion to be given as to "previous sewage contamination." At the same time, it is certain that in surface waters ammonia and nitrites are scarcely ever present to any extent without sewage contamination. Consequently, if a chemical examination is made immediately after collection of the sample it will assist us in arriving at a proper judgment; this is especially the case when the pollution is solely from urine, under which condition bacteriology is often at fault. Much patient work on sewage organisms has shown that a recent contamination of a water supply by sewage can easily be detected even when the pollution is so slight as one part per million. Chemistry is quite unable to indicate such a slight contamination as this. If, however, a considerable time has elapsed since the pollution occurred it becomes more and more difficult to detect it by bacteriological processes; and this is especially the case when waters of great original purity are under examination. It is therefore evident that a bacteriological examination has its limits of usefulness, and a slavish adherence to it under all conditions, combined with neglect of the hints to be obtained by chemical means, may lead to a perfectly erroneous judgment. Still, there is one branch of hygienic study in which bacteriology must always reign supreme; it is now acknowledged on all sides that the working of sand filters for public water supplies cannot be properly kept under control except by appealing to bacteriological methods of examination.

From what has been said it is obvious that a bacteriological examination of water consists essentially of two parts—viz., (*a*) the quantitative analysis or determination of the number of bacteria in a known volume of water, and (*b*) the qualitative analysis or determination of the species of bacteria present in the sample.

Before proceeding to the consideration of these analyses it will be advisable to mention a few points in relation to the

collection of the sample of water which is to be subjected to a bacteriological examination.

The collection of water for bacteriological examination.—Water from a river, well, lake or service-pipe should be collected in glass bottles closed with glass stoppers, previously sterilised by heating in the hot-air chamber to 150° C. for three hours; if sterilisation cannot be effected, the bottles may be cleaned sufficiently for all practical purposes by washing with a little pure sulphuric acid, all traces of acidity being finally removed by thoroughly rinsing the bottle with some of the water to be examined. In the case of a river or lake the bottle should be plunged below the surface before the stopper is removed; in this way a sample of the main body of the water will be obtained. In some cases, as when investigating an outbreak of cholera, it is desirable to examine the surface water. This specimen should be collected in a separate bottle and labelled accordingly. If the source of supply is a "service-water," the tap should be opened and the water allowed to run to waste for a few minutes before the specimen is collected; in this way local impurities will be washed out of the tap and the water which has been standing in the service-pipes will be removed. It is always desirable in the case of a "service-water" to obtain a specimen direct from the mains. In the case of a well not in constant use it is generally found that the sample obtained on first pumping contains a very large number of micro-organisms; so that, to gain an idea of the water which enters the well from the surrounding strata, it is advisable to pump continuously for several hours before collecting the example for examination. After the specimen has been obtained the glass stopper should be covered with oiled silk and carefully tied down. When the source of supply is a shallow stream, such as the feeder of an upland surface water, it is often impossible to use a bottle without disturbing the sediment. Under these conditions a test-tube with stout walls is drawn out just below the open end; the contained air is then removed by prolonged heating, or more simply by allowing a little water to enter the tube, which is then heated until steam escapes, when the point is rapidly sealed. The fine point of such a tube should be placed under the surface of the stream, and then the tube opened by breaking the

point with a pair of sterile forceps. The water will run in and fill about two-thirds of the tube; the point is then sealed up by heating with a spirit lamp. When examining lakes and rivers it is often desirable to take specimens from various depths. This may be done by using Miquel's apparatus, which consists of a glass bulb weighted below and suspended by a wire marked in feet or metres. The neck of the bulb is drawn out to a fine point and bent like a swan's neck. A second wire, running through eyes on the suspension wire, is bent at the end into a loop which is placed round the neck of the bulb. When the required depth is shown on the suspension wire, the second wire is pulled sharply so as to break the neck and allow the water to enter the bulb. The same result may be obtained by weighting the ordinary glass bottle and supporting it by means of a string knotted at intervals of a foot. A second string is tied round the glass stopper, which should be partially unscrewed so as to be easily pulled out when the required depth is reached. The samples should be examined immediately after collection; if this cannot be done they must be packed in ice for transmission to the laboratory. It has been found that when the temperature is kept below 5° C. there is practically no increase in the number of micro-organisms in the water. This effect appears to be due, not to an immobilising of the bacteria, but rather to the fact that cold acts differently on certain species, so that the deaths of some organisms are balanced by the births of others. In order to keep the temperature of the sample below 5° C. during its conveyance to the laboratory, Miquel recommends the following procedure to be adopted. The glass bottle containing the sample is placed in a cylindrical metal box just large enough to hold the bottle firmly. The metal box is placed inside a second slightly larger metal box and the space between the two boxes is filled with sawdust. The whole is then placed in a much larger metal box, which is filled with from six to eight pounds of ice broken up into pieces about the size of a walnut. The third metal box is placed in a wooden box and the space between them is filled with sawdust. The lid of the wooden box, which is provided with a handle, is then clamped down. Pakes has suggested a very useful box for the same purpose. The box is made of wood and lined with

felt; it contains two metal cylinders placed one inside the other; the inner cylinder contains the sample, which is packed round with wool; the outer cylinder is much larger, and is divided by partitions into sections, which are filled with pounded ice. A metal lid is placed over, and securely clamped to, the everted lips of both the inner and outer metal cylinders. A thick layer of felt is then placed over the metal lid. The wooden box is finally closed with a strong well-fitting lid, on which a handle is placed.

CHAPTER II.

THE QUANTITATIVE BACTERIOLOGICAL ANALYSIS.

The determination of the number of micro-organisms in a known volume of water is mainly effected at the present day by cultures on solid media. Miquel, however, has suggested a dilution method, in which a known volume of water is diluted amongst a series of broth tubes, the object being to obtain ultimately a series of broth tubes, each of which shall not receive more than one microbe. This method is exceedingly laborious, and quite unsuitable for hygienic work. Most workers now employ what is known as Koch's method of plate cultivation. In this method a small quantity of water is intimately mixed with fluid gelatine or agar-agar, which is then spread out in a thin layer on a glass plate, or preferably in a Petri dish. Sometimes the mixture of water and fluid gelatine is simply rolled round the sides of a test-tube and solidified, producing what is known as an Esmarch roll culture. The frequent presence in water of micro-organisms which rapidly liquefy gelatine renders the Esmarch tube less serviceable than Petri dishes. Much difference of opinion exists as to the method of preparation of the solid media. The committee of American bacteriologists recommended that gelatine and agar-agar should have such a reaction as would be obtained by adding 1·5 per cent. N acid to the media after they had been rendered neutral to phenol-phthalein. Sedgwick and Prescott stated that the best results were obtained by adding 0·2 of $\frac{N}{20}$ acid to each cubic centimetre of nutritive gelatine. They also found that more colonies developed on nutritive gelatine containing 15 to 20 grammes of peptone per litre than on gelatine containing 5 grammes of peptone per litre. Reinsch's experiments with

water from the Elbe, taken below Hamburg and Altona, showed that the greatest number of bacteria was obtained when 0·1 per cent. of sodium carbonate was added to ordinary slightly alkaline gelatine-peptone. Dahmen, working with water from the Rhine, found that the addition of 0·15 per cent. of sodium carbonate to ordinary gelatine caused the development of the greatest number of micro-organisms. My own experiments with the South Hants water supply showed that more bacteria were obtained on alkaline than on acid media. Two batches of ordinary gelatine-peptone were prepared, one rendered just alkaline to phenol-phthalein, and the other to litmus. To the first batch N acid was added to the extent of 1·5 per cent., as recommended by the American committee, and to the second 0·1 per cent. of sodium carbonate as suggested by Reinsch. Gelatine plates were then made with 0·5 c.c. of the South Hants water and incubated at 22° C. The following average results were obtained:

1 c.c. South Hants water.	Acidified Gelatine. (1·5 per cent. N acid.)	Alkaline Gelatine. (0·1 per cent. sodium carbonate.)
After 48 hours incubation at 22° C. ...	59 Colonies. ...	81 Colonies.
„ 72 „ „ „ ...	88 „ ...	96 „

On the fourth day the plates were usually destroyed by the liquefying organisms present in the water. All the water-organisms usually found in the alkaline media appeared to grow in the acid media, only a little more slowly. Hesse and Niedner recommend the following medium for the bacteriological examination of water:

Agar-agar	1·25 per cent.
Albumose (Heyden)	0·75 „
Distilled water	98·00 „

This medium requires no correction for acidity or alkalinity, and permits the development of the greatest number of water-organisms. The authors consider that water-plates for the simple enumeration of colonies should be kept under observation for three weeks, as numerous experiments have shown them that at 20° C. in the first three days 30 per cent., in the first five days about 70 per cent., and in the first ten days about 90 per cent.

of the colonies are able to develop in the plates. Gelatine plates, however, can rarely be kept under observation beyond the fourth or fifth day, owing to liquefying organisms destroying the plates. Up to the present time the quantitative analyses of most sources of water supply have been made by means of faintly alkaline gelatine-peptone plates, consequently, if it is desired to compare the results with previous analyses, this medium must be used.

The Preparation of "Water Plates."

The water to be examined should be gently shaken for a few minutes; in this way all clumps of bacteria will be broken up and each organism will produce only one colony when it develops in the Petri dish. Forcible shaking of the sample must be avoided as it may cause the death of some of the micro-organisms. The next step is to determine the amount of water which should be used for each plate, as it is necessary that each organism shall have room to develop without touching its neighbours. The simplest method of arriving at an idea of the number of bacteria present in the water is to remove $\frac{1}{20}$ c.c. of the water from the bottle and deposit it on a cover-glass; then by staining and counting the microbes on the cover-glass preparation it is possible to determine very roughly the number of micro-organisms present in a c.c. of the water. When working with Petri dishes having a diameter of four inches, it is best to use such an amount of water as will give not more than 300 colonies in each plate. If the water contains a very large number of bacteria it must be diluted with sterile tap-water. Usually three plates are made from each sample, containing respectively $\frac{1}{10}$ c.c., $\frac{1}{4}$ c.c. and $\frac{1}{2}$ c.c. of the water diluted or not as required. In order to measure the calculated amount of water required for each plate, accurately graduated pipettes must be used. One c.c. pipettes graduated in $\frac{1}{100}$ths are most useful. Each pipette must be thoroughly cleaned, the upper end plugged with cotton-wool, and the lower end placed in a test-tube, the mouth of which is firmly plugged with cotton-wool. The test-tubes and pipettes are then sterilised at 150° C. for three hours and kept in metal boxes until required for use. The Petri dishes, wrapped in thin paper, are also sterilised at the same temperature.

The gelatine plates should be made in the following manner: Melt three gelatine tubes in a water-bath at a temperature of about 30° C., sterilise the cotton-wool plugs in a Bunsen flame and twist the plugs round so as to loosen them. Draw up the required amount of water in a sterile pipette; then holding the pipette almost horizontally with the right hand, take the tube containing the melted gelatine in the left hand between the thumb and first finger and again sterilise the plug. Now hold the tube as nearly horizontal as possible, without permitting the gelatine to touch the wool; remove the plug with the third and fourth fingers of the right hand, introduce the water, again sterilise the neck of the test-tube, and replace the plug. Place the pipette on a sterile metal stand and roll the test-tube, held vertically between the palms of the hands, so as to thoroughly mix the water and gelatine. The test-tube must not be shaken up and down or air-bubbles will be introduced into the gelatine. Having thoroughly mixed the water and gelatine, hold the test-tube in the right hand, sterilise the wool and neck of the tube in the flame, and then withdraw the plug with the inner margin of the left hand. Next raise the lid of the Petri dish with the thumb, first and second fingers of the left hand, introduce the neck of the test-tube into the aperture, pour out the mixture of water and gelatine, replace the lid and gently diffuse the contents into a thin layer over the bottom of the dish. When making the plates it will be noticed that it is almost impossible to pour out all the gelatine from the test-tubes without opening the Petri dishes to a dangerous extent, and as the portion of gelatine still remaining at the bottom of the test-tube may contain a few organisms, it is best, after the plug has been replaced, to distribute the gelatine over the walls of the tube in as thin a layer as possible—in fact, to convert the tube into an Esmarch roll. The plates and test-tubes are usually incubated at 20° to 22° C. and the colonies which develop are counted from day to day. As a rule it will be found that there is no very material increase in the number of colonies after the fifth day, so if possible the plates should be kept for this period. If many liquefying organisms are present the plates will usually be destroyed by the third or fourth day.

The simplest method of counting the number of colonies is to

divide up the outer surface of the bottom of the Petri dish into sections by straight lines running from the centre to the periphery; this can easily be done with a wax pencil. A small hand magnifying glass will be found useful in differentiating masses of granules in the gelatine from true colonies. The result of the enumeration should be stated as so many aerobic colonies or organisms which have developed at 20° to 22° C. after from forty-eight hours to five days incubation, as the case may be. Neisser recommended that the plates should be counted under the microscope, and showed that the number obtained by this method after twenty-four hours incubation would equal the number obtained by the ordinary microscopic method after seventy-two hours incubation. In tropical climates it is often impossible to use gelatine, for during many months of the year the air temperature is above the melting-point of this medium, consequently the plates remain fluid and the bacteria are not kept apart so as to permit a proper isolation of the colonies. Under these conditions agar-agar must be used as the nutrient medium. Agar tubes are melted and seeded with the water after they have been cooled down to 40° C.; the mixture of water and agar is rapidly poured out, as before described, into Petri dishes and allowed to incubate at the prevailing temperature. A simpler method is to pour out the melted agar into a Petri dish and allow it to set; the calculated amount of water is then introduced by means of a pipette and the fluid evenly distributed over the surface of the agar by means of a platinum spreader. The high temperatures which obtain in tropical climates are prejudicial to the development of water-organisms, so that the counts obtained on the agar plates are smaller than those obtained from the same water seeded in gelatine during the colder months.

The interpretation of the results of a quantitative bacteriological analysis is a matter of considerable difficulty. In order to give a reliable opinion on the results obtained, it is necessary to study the causes of the multiplication of micro-organisms and the way in which they are influenced by light, temperature, movement, and the chemical composition of the water. The bacterial contents of the various sources of supply must also be examined to see if they maintain, under natural conditions, a fairly constant numerical composition.

CHAPTER III.

QUANTITATIVE ANALYSIS—*continued.*

The Multiplication of Micro-Organisms in Water.

Miquel's researches showed that in surface waters, such as rivers containing a large number of micro-organisms, the multiplication is slow and persistent, whilst in deep well waters and springs, containing only a few bacteria, the multiplication is rapid but soon followed by a marked and steady decrease.

Frankland's experiments with the rivers Thames and Lea gave the following results:

	Day after collection.	After 2 days in the dark at 20° C.	After 4 days in the dark at 20° C.
	Organisms per c.c.	Organisms per c.c.	Organisms per c.c.
River Thames at Hampton ...	12,250 ...	4,386 ...	2,018
River Lea at Chingford ...	7,300 ...	2,148 ...	1,286

The number of micro-organisms in the river water underwent a marked diminution after storage for from two to four days in stoppered bottles.

An examination of a deep well water gave results analogous to those obtained by Miquel.

	Day after collection.	After 3 days in the dark at 20° C.	After 16 days in the dark at 20° C.
	Bacteria per c.c.	Bacteria per c c.	Bacteria per c.c.
Kent Well ...	96 ...	178,379 ...	51,843

Cramer's experiments on filtered water from Lake Zurich also strongly supported Miquel's observations as to the changes occurring in pure waters. He filled a sterilised flask with the

filtered water from Lake Zurich and examined specimens from time to time with the following results :

	Colonies per c.c.
Immediately after filling the flask	143
After 24 hours	12,457
" 3 days	328,543
" 8 "	233,452
" 17 "	17,436
" 70 "	2,500

A low temperature appears to arrest the multiplication of micro-organisms. Miquel showed that when a water was kept below 5° C. the number of bacteria did not appreciably alter. The number was kept relatively about the same, not because the organisms were unable to increase but because they were unequally affected by temperature, the deaths of some species being balanced by the births of others.

Wolffhügel and Reidel stated that there was a difference in the multiplication of micro-organisms in water according as the samples were exposed to the air or not. It was generally found that in the vessels closed with india-rubber stoppers somewhat less multiplication took place than in those which were closed with cotton-wool plugs. The apparently more flourishing condition of the micro-organisms in the latter case seemed to be due to the interchange of air being less restricted.

Frankland obtained the same results when working with water from the Thames and Loch Katrine.

The diminution in the number of bacteria in a sample of water kept for many days, appears to be due partly to some one or other of the food materials necessary for the growth of certain varieties being used up, and partly to the products of the bacteria being harmful so that the water becomes both "impoverished and poisoned."

Sirotinin made some experiments on the impoverishment of media. He planted out the Bacillus typhosus on a thin layer of gelatine and obtained a good growth ; on removing the first crop, however, he was unable to obtain a second until the gelatine already used had been melted and mixed with 1 per cent. peptone and 0·1 per cent. meat extract. Similar results were

obtained with B. pyogenes fœtidus, B. flavescens putidus and B. murisepticus.

The influence of the products of one species on the growth of another is well shown by Garré's experiments. B. fluorescens putidus was grown on gelatine slopes and then carefully scraped off so as to injure the medium as little as possible. B. typhosus was then planted out on one of the scraped tubes and Sp. choleræ on the other. The tube containing the B. typhosus remained sterile, but the Sp. choleræ grew well. B. typhosus was then grown on gelatine and, after the growth had been carefully removed, B. fluorescens putidus was planted out on the scraped surface. A good growth occurred, showing that, whereas the products of the B. fluorescens putidus were harmful to the B. typhosus, the converse was not the case. Working with B. fluorescens liquefaciens I found that B. typhosus would not grow on gelatine which had already yielded a growth of the B. fluorescens liquefaciens, but gelatine which had already served as a medium for the B. typhosus was still capable of nourishing the B. fluorescens liquefaciens.

Zagari also found that anthrax bacilli would not grow in broth which had served as nutrient material for the Sp. choleræ.

Sirotinin appeared to believe that the restraining influence was due either to a strong acidity or strong alkalinity produced by the development of the organisms previously planted out on the media, as he found that when the media were neutralised the inhibiting influence was removed. A change in the reaction of the medium cannot be the cause of the prejudicial influence in all cases. In my experiments with the B. fluorescens liquefaciens and the B. typhosus I found that after scraping off the former organism from a gelatine slope, melting the medium and rendering it exactly neutral had no influence on the development of the B. typhosus: the organism still refused to grow.

The Influence of Light on Micro-Organisms.

In the year 1877 Downes and Blunt published a paper on the action of light on micro-organisms. These observers showed that, under favourable circumstances, exposure to light entirely

prevented the growth of bacteria in Pasteur's solution; direct sunlight had the greatest influence, but diffused daylight also appeared to have a restraining effect. Experiments were also made by them which showed that the blue and violet rays of the spectrum entirely prevented the growth of bacteria, while the red and orange-red rays merely delayed development.

Downes and Blunt also found that spores were uninjured by exposure to light in a vacuum, and came to the conclusion that the action of light was due to a gradual process of oxidation set up by the sun's rays in the presence of oxygen.

Duclaux experimented with Tyrothrix scaber and Micrococci, and demonstrated that the power of resistance to light varied with the species, the kind of nutrient medium and the intensity of the light.

Arloing showed that anthrax spores were killed more quickly than anthrax bacilli. Roux confirmed Arloing's results, and pointed out that the action of light might be due to the production of oxygen.

Engelmann showed that all bacteria are not unfavourably influenced by light, the Bacterium photo-metricum being motionless in the dark but mobile when exposed to light.

Janowski studied the influence of light on the B. typhosus, and found that diffused daylight delayed its growth both on gelatine and in broth. The same cultures were killed in four to ten hours by exposure to sunlight, although the temperature of the media had not reached 40° C. Further investigations showed that the chemical rays were especially harmful.

Buchner introduced B. typhosus, B. coli and B. pyocyaneus into water containing a little Liebig's extract and exposed the cultures to direct sunlight and diffused daylight. His results are shown in the following table:

	B. Typhosus.		B. Coli.		
	Exposed to light.	Kept in the dark.	Exposed to light.	Kept in the dark.	Conditions.
Number of bacteria at the commencement.	3,000	4,600	4,600	4,800	Direct sunlight.
Number of bacteria after two days	0	7,600	0	12,600	
Number at the commencement	1,400	1,200	5,800	5,200	Direct sunlight.
After three hours	0	5,000	0	9,800	

	B. Typhosus.		B. Coli.		
	Exposed to light.	Kept in the dark.	Exposed to light.	Kept in the dark.	Conditions.
At the commencement.	2,048	2,200	1,472	9,856	Diffused daylight.
After four hours.	0	1,024	3,328	11,776	

	B. Pyocyaneus.		
	Exposed to light.	Kept in the dark.	Conditions.
At the commencement	50,200	38,400	Direct sunlight.
After two days	9,600	269,000	
At the commencement	22,600	21,000	Direct sunlight.
After three hours	0	21,000	
At the commencement	3,328	2,048	Diffused daylight.
After four hours	0	2,432	

Buchner also arranged a series of experiments to determine the effect of sunlight on cultures placed at different depths in water. He prepared agar and gelatine capsules containing B. typhosus, B. pyocyaneus and Sp. choleræ, and closed them with india-rubber. On the upper surface of the capsules a dark paper cross was fastened. The capsules were then placed in water exposed to light with the following results:

Distance of the plate from the surface of the water.	Culture.	Development of Colonies. In the covered part of the plate.	In the part exposed to light.
0·1 metre.	Sp. choleræ.	Very good.	Nil.
1·1 ,,	B. pyocyaneus.	,,	,,
1·6 ,,	B. typhosus.	,,	,,
2·6 ,,	B. pyocyaneus.	Better growth than on the part exposed to light.	Good.
3·1 ,,	B. typhosus.	Slightly better than the part exposed to light.	,,

It appears therefore that in clear water the influence of light on bacteria extends to a depth of two metres.

Dieudonné worked with B. prodigiosus and B. fluorescens, and showed that it was the light and not the heat rays which had a destroying effect. The most powerful rays were the ultra-violet, violet and blue rays; the green rays had less power, and the yellow and red rays appeared to have no action. Culture media also appeared to be affected, hydrogen peroxide being developed from the water present.

Frankland and Ward investigated the influence of light on

anthrax spores. They found that the sun's rays in December killed the spores in gelatine plates after a few hours exposure, although the temperature was so low that the plates were not liquefied. The spores also seemed to be more resistant to the action of sunshine when diffused in water than when sown in ordinary culture materials. Ward showed that the spores were killed by the direct action of sunlight and not through changes in the media.

The following conclusions seem to be justified by the results obtained up to the present time:

(1) There is no doubt that light, and especially sunlight, has an injurious action on micro-organisms, and that it acts directly on bacteria as well as by producing changes in the culture media.

(2) The action of light is greatly increased by the presence of air and moisture, the process being largely an oxidation attended by the development of hydrogen peroxide.

(3) The precise duration of exposure required to destroy micro-organisms must necessarily vary with the intensity of the light and the inherent vitality of the various species.

(4) The influence of sunlight probably extends through water to a distance of about two metres, but undue reliance must not be placed on its bactericidal powers even in shallow streams, as the micro-organisms may be attached to suspended particles, and so protected to a great extent from the action of light.

The Influence of Movement and Rest on Micro-Organisms.

Cramer investigated the action of movement by shaking a specimen of water for four hours. He found 80 bacteria per c.c. in the water which had been standing for the same time without shaking, and 87 bacteria per c.c. in the water which had been shaken.

Leone found no difference in the number of bacteria in a water before and after shaking.

Miquel obtained similar results when working with the water from the Varne and Dhuis. Tiemann and Gärtner found such slight differences that shaking was considered to have very little influence. Schmidt could not find any difference in the growth

of B. prodigiosus, M. candicans, M. staphylococcus aureus, M. staphylococcus albus, or B. typhosus, after shaking by hand or in an apparatus. The growth of B. violaceus, however, seemed to be restrained, and the liquefaction produced by Sp. Finkler Prior appeared to be slower. Meltzer concluded from his researches that many bacteria were killed by strong shaking in a machine, while others remained alive and multiplied.

From these observations it is apparent that the movement which micro-organisms in water sustain under ordinary circumstances has no appreciable influence on their vitality.

On the Sedimentation of Micro-Organisms.

Sedimentation plays a very important part in changing the bacterial contents of water supplies. The effect of subsidence has been investigated by many workers.

Bolton allowed a tall flask to stand at a low temperature, and then took specimens from the surface, the centre, and the bottom of the flask. The following results were obtained :

	From the surface. No. of bacteria per c.c.	From the centre. No. of bacteria per c.c.	From the bottom. No of bacteria per c.c.
First specimen after twenty hours.	2,120	—	48,460
	2,240	—	44,980
Second specimen after twenty-four hours.	23,760	7,320	13,060
	24,000	7,500	14,000
Third specimen after two days.	11,740	—	25,940
	11,140	—	27,520
Fourth specimen after three days.	1,720	—	1,920
	2,040	—	1,200
Fifth specimen after four days.	2,280	—	9,580
	3,840	—	10,549

Three other specimens were examined after seven months, no bacteria were found on the surface in any of the flasks, but after shaking, 540 to 760 organisms per c.c. were counted. Hueppe examined a service supply which contained only 16 bacteria when drawn from the pipes. After allowing the water to stand for two months, 11,280 bacteria per c.c. were found at the surface and 123,750 bacteria per c.c. at the bottom. In these researches the observers worked with a mixture of organisms, but there is no doubt that different species behave differently as regards sedimentation.

Tiemann and Gärtner made many experiments with pure cultures. Their results are given in the following tables :

Organism.	Place from which the specimen was taken.	Number of bacteria in 1 c.c. after 1 day.	2 days.	3 days.	4 days.
A motile bacillus producing green pigment. The column of fluid was 20 c.m. high and maintained at 18° to 20° C.	1 c.m. under the surface.	164,400	226,000	288,000	346,000
	From the bottom.	173,000	232,000	286,000	384,000
				(after shaking)	
				288,000	368,000
Non-motile coccus obtained from the air. Column of fluid 20 c.m. high.	1 c.m. under the surface.	25,700 28,200			
	From the bottom.	25,200 28,000 (after shaking, 22,000).			

In order to test the effect of a greater depth of fluid, a glass tube 60 c.m. high and 2·5 c.m. wide was filled with sterilised service water containing 11,000 motile bacilli per c.c. and kept at 50° C. for ten days. The following results were obtained :

1 c.m. under the surface	910,000 per c.c. 960,000 „
Direct from the bottom	850,000 „ 900,000 „

Next a glass tube 54 c.m. high and 2·5 c.m. wide was filled with sterilised water containing 13,000 white micrococci per c.c. ; the results were as follows :

	After 10 days at 5° C.	After 20 days at 12° C.	After 30 days at 10° C.
1 c.m. under the surface, bacteria per c.c.	2,200	510,000	400,000
From the bottom, bacteria per c.c.	4,040	500,000	295,000

These experiments appeared to show that neither motile nor non-motile micro-organisms sank to the bottom.

Scheurlen placed in a tube, 17 c.m. high, suspensions of B. typhosus, B. megaterium, Proteus vulgaris, Proteus mirabilis, and Sp. Choleræ Asiaticæ. At the end of four days he found that the three first microbes had in great part sunk to the bottom, whilst the last two appeared to remain equally distributed through the water.

It is possible that oxygen, being necessary for the maintenance of vitality of ærobic organisms, exercises an influence on the

sedimentation of different species. Very few observations have been made on the sedimentation of bacteria in large masses of water. Large lakes, away from the shore, appear to contain very few micro-organisms; yet during rain large numbers of bacteria are brought down by rivers, and the mud at the bottom of lakes is found to be very rich in micro-organisms.

Frankland's experiments with Thames water at the works of the West Middlesex Company show the effects produced by storage extremely well. Samples were collected (*a*) from the Thames water at Hampton; (*b*) from the same water after passing through one storage reservoir; and (*c*) after passing through two storage reservoirs. The following results were obtained:

	No. of organisms per c.c.
(*a*) Thames water at Hampton	1,437
(*b*) „ after passing through one reservoir .	318
(*c*) „ „ „ two reservoirs .	177

It thus appears probable that a water after receiving a large number of micro-organisms will, in a very short time, show a great diminution in its bacterial contents. But sedimentation is not the only factor in the change; currents, oxygen requirements, inorganic and organic matters sinking to the bottom, all exercise great influence. Moreover many of the bacteria are not natural inhabitants of water but are derived from earth, house-washings, &c., which are rich in nutritive material. Consequently, with the sinking of suspended material and the loss of food material, these foreign bacteria may quickly die out.

The self-purification of water may be due to the following causes:

(1) To the death of those bacteria which either do not belong to the so-called water-bacteria or are sensitive to the influence of light.

(2) To a sedimentation of non-motile microbes and the spores of motile bacteria.

(3) To a spontaneous sinking of bacteria with solid materials which form nutritive centres.

(4) To a mechanical sinking of bacteria with the subsidence of heavy materials.

The Duration of Life of Micro-Organisms.

The life of micro-organisms may extend over a long period; this is especially the case when they possess the power of forming spores. Duclaux found living spores of Tyrothrix after five years existence in a fluid medium. He also showed that 15 out of 65 vessels which Pasteur used in his experiments on spontaneous generation were capable of giving rise to new cultures after being closed for from twenty to twenty-five years. The reaction of the fluid in the fertile flasks was slightly alkaline whilst the reaction in the sterile flasks was either strongly acid or strongly alkaline. Miquel found after nine years only 220 living organisms in a flask which had originally contained 4,800 organisms. Another flask containing Vanne water which originally showed 66 microbes per c.c. was found quite sterile after being closed for ten years.

Roth placed water, from a well, containing 5000 bacteria per c.c. in sterile flasks and found

After	2 days	closure	80,000	organisms	per c.c.
,,	3 ,,	,,	120,000	,,	,,
,,	5 ,,	,,	60,000	,,	,,
,,	10 ,,	,,	43,000	,,	,,
,,	4 weeks	,,	2,200	,,	,,

The presence of carbonic acid gas in a water has a great influence on the duration of life of micro-organisms. Liborius in his researches on anærobes, showed that the development of colonies was arrested if the air present in the nutritive gelatine was driven out by carbonic acid gas. Leone found in a water containing carbonic acid gas that out of 786 bacteria originally present, only 87 could be detected after five days.

Hochstetter's researches showed that carbonic acid gas acted differently according to the species exposed to its influence; some organisms were killed, others merely restrained in their growth. In Seltzer water anthrax bacilli only lived for one hour, but anthrax spores lived for several days. In water containing carbonic acid gas the B. typhosus lived for five days, but the Spirillum choleræ could not be detected after twenty-four hours. The effect was not due to chemical changes nor to pressure, but simply to the presence of carbonic acid gas.

The Influence of the Chemical Composition of a Water on the Number of Micro-Organisms.

The relation of the chemical composition of a water supply to the number of micro-organisms has been investigated by many observers, especially in Germany.

Proskauer examined the unfiltered water from Stralaw and Tegel Works, and the filtered supply delivered to the houses. His results were as follows:

Time of the investigation.	Stralaw.			Tegel.		
	Organisms per c.c.	Mgms. of $KMnO_4$.	Chlorine.	Organisms per c.c.	Mgms. of $KMnO_4$.	Chlorine.
		Parts per 100,000.			Parts per 100,000.	
April, 1888	9,400	2·27	1·775	620	1·83	1·59
May ,,	1,400	3·06	1·775	88	2·14	1·59
June ,,	110,000	2·85	2·130	48	1·46	1·77
July ,,	10,200	2·13	2·307	13	1·58	1·77
Aug. ,,	1,632	3·15	2·791	23	2·14	1·57
Sept. ,,	10,400	2·93	2·928	52	1·89	1·49
Oct. ,,	8,400	2·32	3·195	14	1·62	1·59
Nov. ,,	144,000	2·86	3·017	14	1·79	1·42
Dec. ,,	220	2·92	2·662	25	1·79	1·59
Jan. 1889	18,500	1·98	2·041	56	1·70	1·50
Feb. ,,	12,500	2·10	2·485	160	1·58	1·42
March ,,	190,000	2·10	2·488	68	1.24	0·88

Time of the investigation.	Filtered Water.		
	Organisms per c.c.	Mgms. of $KMnO_4$.	Chlorine.
April, 1888	18	1·64	1·77
May ,,	66	1·67	1·59
June ,,	22	1·49	1·77
July ,,	65	1·16	1·59
Aug. ,,	43	1·77	1·59
Sept. ,,	131	1·53	1·49
Oct. ,,	12	1·39	1·59
Nov. ,,	6	1·49	1·42
Dec. ,,	12	1·53	1·42
Jan. 1889	21	1·53	1·59
Feb. ,,	7	1·17	1·52
March ,,	110	1·19	1·77

G. Frank obtained similar results when examining the Spree water from its entrance into Berlin up to Sacrow. From these experiments it is apparent that there is no constant direct

relation between the number of organisms and the chemical composition of the water.

Bolton's work pointed in the same direction; he distinctly said that the chemical composition of a water had no relation to the number of micro-organisms.

Tiemann and Gärtner believe that, in relation to nutritive material, bacteria may be arranged in two groups. The first group includes the water bacteria, which have very slight dependence on the nutritive material present, and under favourable conditions multiply rapidly. The second group embraces those organisms which are greatly dependent on the nutritive material present, and are derived from the earth and living beings.

With regard to the organic matter the difficulties are very great; we are not able to determine exactly which of the constituents form suitable food for bacteria, indeed Duclaux has said that the quality of the organic matter is far more important than the quantity. Using the oxygen derived from potassium permanganate as a test, Fischer found in one case 7·4 parts per 100,000 of oxygen absorbed with 800 bacteria present, and in another case only 0·5 part per 100,000 of oxygen absorbed with 360,000 bacteria present. Tiemann and Gärtner investigated the effect of inorganic substances, especially lime salts and total solids on the bacterial contents; the results obtained were as follows:

Number of wells.	Hardness.	Organisms per c.c.
1	0–10	536
30	10–20	1,695
14	20–30	1,910
1	30–40	720

Total solids.	Organisms per c.c.
0–50	81
50–100	261
100–150	250
200–500	46

These results show that often a high bacterial count is associated with high total solids, but with an excess of solids the organisms may diminish again.

A review of all the experiments appears to justify the

statement that there is no direct and constant relation between the chemical composition and the number of micro-organisms; but, speaking generally, it may be said that those waters which contain much organic matter usually show a high bacterial count.

The Action of Electricity on Bacteria.

The action of electricity on bacteria was first investigated by Schiel, who judged the action of the electric current by an examination of its effects on the motility of micro-organisms. Cohn and Benno Mendelsohn used a purely mineral solution containing 5 grammes of potassium phosphate, 5 grammes of sulphate of magnesia, 10 grammes of neutral tartrate of ammonia, and 0·5 gramme of calcium chloride per litre. The nutritive fluid was placed in a U-tube, then inoculated with the micro-organism to be studied, and a current from several elements of Marié-Davy pile passed through it. If a growth occurred the current was deemed to have had no action. If no growth took place this might have been due to the death of the bacteria or to changes having taken place in the fluid which rendered it an unfavourable medium. To judge these effects a loopful was withdrawn from the U-tube and planted out on some fresh medium, and a new culture was then inoculated into the tube. Cohn and Mendelsohn found that when the action of the current was short and feeble its effect on bacteria was *nil.* When the current from two powerful elements acted for twenty-four hours the bacteria were not killed, but the fluid was so altered that bacteria would not grow in it. When the current from five elements acted for twenty-four hours the bacteria were killed at both poles, and the fluid was rendered sterile and profoundly modified. In order to prevent the decomposition of the fluid by the current, induction currents were tried but without any appreciable result. Experiments were then made by passing an electric current through a potáto sown with B. prodigiosus. Chemical effects were also produced in the potato, just as in the nutritive fluid, so that though the bacillus was killed, its death could not be ascribed to the physical action of the current. Prochownick and Spaeth covered the plates of the battery with nutritive agar, which were then infected with

the bacteria to be studied. When development had taken place, the plates were placed in salt solution and the current allowed to pass. It was found that the positive pole was more bactericidal than the negative pole, and that the effect depended on the intensity and duration of the current. A current of 50 milliampères passing for a quarter of an hour did not kill the Staphylococcus pyogenes aureus, but a current of 60 milliampères killed it at once. In order to kill a spore-bearing bacillus a cnrrent of 200 to 230 milliampères was required for two hours. The bactericidal power of the positive pole appeared to be due to the chlorine liberated from the salt solution. The effect of the current, as in the previous experiments, was due to a chemical and not to a physical action. According to Fischer strong electric currents are fatal to bacteria, their protoplasm being killed, although some of the deleterious effect may be due to the electric dissociation of the media and to the increase of temperature caused by the current.

CHAPTER IV.

QUANTITATIVE ANALYSIS—*continued.*

The Bacterial Contents of the Various Sources of Supply.

Snow.—Janowski examined snow which had been lying on the ground for some time; the superficial layers were removed in order to avoid organisms which might have been derived from the air. The sample was then taken, placed in a test-tube and melted. One c.c. of snow-water being used for each plate the following results were obtained:

Sample.	24 hours old. Air temperature 6° C.	48 hours old. Air temperature 6·5° C.	72 hours old. Air temperature 10° C.	96 hours old. Air temperature 11·6° C.
I.	2	18	228	145
II.	4	20	—	212

Schmelck found in the melted water from snow on an iceberg in Norway 2 bacteria and 2 moulds per c.c. Janowski also examined freshly-fallen snow, and from one c.c. of the melted snow-water obtained the number of micro-organisms given in the following table:

Sample.	Taken on Feb. 2, 1888. Air temperature 7·20° C.	Taken on Feb. 20, 1888. Air temperature 11° C.	Taken on Feb. 28, 1888. Air temperature 12° C.	Taken on Feb. 29, 1888. Air temperature 3·4° C.
I.	34	203	140	134
II.	38	384	168	463

Thus, even at a low temperature, snow has been found to contain micro-organisms, and the number appears to increase with the length of time the snow has been lying on the ground.

Ice.—Heyroth examined ice obtained from various places in Berlin. Blocks of ice were broken and portions removed from the centre, were then melted in sterile test-tubes and the ice-

water plated out in gelatine. The following results were obtained:

Plotzen Lake on September 19, 1885	490	organisms	per c.c.
„ „ October 5, 1885	4,900	„	„
River Spree on May 17, 1886	171	„	„
„ „ „	1,780	„	„
Weïssen Lake	735	„	„
Pond water	14,400	„	„

Hail.—Bujwid and Foutin investigated the bacterial contents of hailstones. In a very large stone 21,000 organisms per c.c. were found, but in a smaller one, about the size of a walnut, only 729 bacteria per c.c. were counted.

Rain.—Very few bacteriological examinations of rain have been made. Miquel, at Mountsouris, found the bacteria to average 4·3 per c.c. during the years 1883–1886. In the middle of Paris 19 bacteria per c.c. were found.

Rivers.—The Thames and Lea were examined by Frankland for each month during the years 1886, 1887, and 1888. The following table gives the number of bacteria found in Thames water collected at Hampton.

Month.	Micro-organisms per c.c		
	1886.	1887.	1888.
January	45,000	30,800	92,000
February	15,800	6,700	40,000
March	11,415	30,900	66,000
April	12,250	52,100	13,000
May	4,800	2,100	1,900
June	8,300	2,200	3,500
July	3,000	2,500	1,070
August	6,100	7,200	3,000
September	8,400	16,700	1,740
October	8,600	6,700	1,130
November	56,000	81,000	11,700
December	63,000	19,000	10,600

Similar results were obtained with water from the Lea. Both rivers contained fewer bacteria during the summer months. This is due to the fact that in the summer the rivers are chiefly dependent on springs for supply, while in the winter they receive surface washings from cultivated land.

Miquel examined the Seine, Marne, and Ourcq, and found the

least number of micro-organisms during the months of June, July, and August.

Fränkel and Dietrich examined the water at Marburg both chemically and bacteriologically. The first specimen was taken above the town, the second and third specimens from a place where many drains existed, the fourth one kilometre below the town, and the fifth 7·5 kilometres below the town. The results are shown in the following table:

Specimen.	Total solids.	Fixed solids.	Volatile solids.	NH_3.	NO_2.	Cl.	$KMnO_4$.	Bacteria per c.c.
(1)	126	107	20	nil	nil	7.1	5.3	1,200
(2)	126	105	20	trace	,,	7.8	5.9	14,000
(3)	123	101	21	,,	,,	8.5	5.3	4,320
(4)	127	106	21	,,	,,	8.5	5.3	1,575
(5)	124	103	21	,,	,,	8.5	5.9	2,300

Rosenburg studied the Main above and below Würzburg:

	Above the town, bacteria per c.c.	Below the town, bacteria per c.c.
February	520	15,500
,,	355	2,950
,,	680	16,000
,,	780	6,600
,,	640	6,400
,,	720	18,000
,,	565	17,200
,,	1,020	14,000
,,	680	22,000

The River Ure was examined by Frankland; a specimen taken above Ripon yielded 1800 bacteria per c.c., but a second specimen taken below the town was found to contain 33,400 bacteria per c.c. In a report to the Corporation of Aberdeen, Frankland stated that the River Dee above Braemar contained only 88 bacteria per c.c., but below Old Mar Castle, having received sewage from Braemar, it contained 2829 bacteria per c.c.

Lakes.—Water from lakes usually contains fewer micro-organisms than river water. Cramer examined fifty specimens of water obtained from Lake Zurich during October and December 1884, and January 1885. During this period the average count was 184 organisms per c.c. From the 13th to the 24th of June 1885, he examined forty-two specimens, and obtained an average result of only 71 organisms per c.c.

Karlinski studied a lake in Herzegovinia and found near the shore 16,000 bacteria per c.c. At the centre of the lake, however, 3000 organisms per c.c. were found at the surface; at a depth of five metres 1000; at ten metres 600; and at fifteen metres only about 200 ærobic organisms were detected.

Wells.—Great differences have been found in the number of bacteria present in shallow wells. Egger examined a large number of wells in Mainz with the following results:

No. of wells.	No. of bacteria.	No. of wells.	No. of bacteria.
1	0	3	0–5
4	5–10	10	10–20
16	20–100	9	100–200
7	200–300	2	300–400
1	400–500	4	500–1000
3	1000–2000		

Maschek obtained the following results in Leitmeritz:

No. of wells.	No. of bacteria.	No. of wells.	No. of bacteria.
1	'10–20	2	30–50
1	100–300	8	300–500
15	500–1000	21	1000–2000

B. Fischer's examinations in Kiel gave the following counts:

51 wells .	Up to 500	64 wells .	1,000–10,000
34 „ .	500–1,000	22 „ .	10,000–20,000

Deep wells as a rule contain few bacteria. Frankland found in a Kent well, sunk in the chalk, between 6 and 26 organisms per c.c.; and in his report to the Local Government Board on the monthly bacteriological examination of the London Water Supply for the years 1886, 1887, 1888, he gave the results shown in the following table:

Wells.	Number of bacteria per c.c.											
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.
						1886.						
New wells .	—	5	44	7	8	4	12	9	5	3	—	11
						1887.						
Bath well .	9	19	80	26	27	12	14	5	5	7	3	6
Garden well .	48	20	4	4	—	24	18	—	8	—	5	12
New well .	12	10	5	12	20	14	8	59	27	30	65	67
						1888.						
Bath well .	6	47	6	33	7	17	8	—	8	—	4	34
Garden well .	5	19	8	4	27	71	5	—	10	9	18	—
New well .	12	4	5	7	8	20	4	3	—	96	19	—

Egger counted only four organisms per c.c. in the artesian wells in Mainz, and Hueppe found only 4 bacteria per c.c. in the deep wells in Wiesbaden. Heraeus, C. Fränkel, and Maschek made numerous studies of wells after they had been at rest for some time and after pumping. Heraeus found 5000 organisms in a well which had been at rest for 36 hours, but, after the well had been emptied by continuous pumping, a sample collected showed only 35 bacteria per c.c. Fränkel and Maschek obtained similar results.

Springs are closely allied to deep wells as regards their bacterial contents. Cramer found in Zurich springs 9, 17, 17, 31, 36, 182, 15, 25 and 45 bacteria per c.c. Frommelt counted in the water supply of the town of Altenburg, which was derived from springs, only 28, 35, and 25 bacteria per c.c. Buchner found the springs in a garden at Brunnthal to contain only from 4 to 35 colonies per c.c.

A brief consideration of these results shows that, with the exception of deep wells and springs, it is impossible to decide from the quantitative examination alone whether a water supply has been polluted or not. This is due to the fact that the great variations in the results are attributable not only to possible sewage contamination, but also to the influence of the physical and chemical conditions of the supply, which operate sometimes so as to increase the number of micro-organisms present, and at other times lead to a rapid disappearance of the microbes. It is also evident that the general standards given by many observers cannot be used for all waters. Macé certainly says that the number of micro-organisms present does not give any accurate information as to the value of a water; at the same time he suggests the following classification, which gives the mean results of a long series of examinations:

Very good waters contain from			0–50	micro-organisms per c c.			
Good	,,	,,	,,	50–500	,,	,,	,,
Mediocre	,,	,,	,,	500–3,000	,,	,,	,,
Bad	,,	,,	,,	3,000–10,000	,,	,,	,,
Very bad	,,	,,	,,	10,000–100,000	,,	,,	,,

The only sound way to judge a supply is to examine each source separately at different times of the year, especially before and after rain, in summer and in winter; in this way

local standards for comparison will be obtained which will have great practical value. For general reference water-supplies may be arranged in the following groups: (*a*) spring and deep well waters; (*b*) upland surface waters; (*c*) shallow well waters; (d) river waters.

(*a*) The supply is obtained from main springs and deep wells. These two sources are classed together because they both represent water which has percolated through a considerable depth of ground; they are really filtered supplies containing, as a rule, very few bacteria. Tiemann and Gärtner consider that water derived from these sources should never contain more than 50 micro-organisms per c.c., and the analyses previously given show that this is a reasonable limit; as the water has percolated through a considerable depth of ground it keeps a fairly uniform temperature throughout the year, and is very little influenced by climatic conditions. Consequently if deep wells and springs are kept free from surface pollution, and the outcrops of the water-bearing strata are above suspicion, there is every reason to expect a constant bacterial composition of these supplies.

(*b*) The supply is derived from an upland surface. The water is usually collected in an impounding reservoir and may be distributed without filtration. Such a supply must be studied locally by determining the bacterial contents of unpolluted feeders at different times of the year. If to this study there be added an examination of the reservoir at its upper end where the various feeders enter, and at the lower end where the water is discharged after sedimentation has helped to reduce the number of micro-organisms, a very good idea of the true bacterial contents of the supply will be obtained. Tables can then be made for reference which will form an effective control of the supply. It often happens, however, that the upland surface grounds are obviously open to pollution from human habitations, &c. In such a condition of things it will be of little use to determine the average bacterial contents of the feeders, because even daily examinations would not enable the supply to be effectively safe-guarded. The water must then be subjected to the artificial conditions of filtration.

(*c*) The water is derived from shallow wells. The analyses

already given show how impossible it is to fix any standard for shallow wells. Unless properly covered, imperviously steined to a sufficient depth and surrounded by at least an acre of virgin ground, shallow wells must be considered dangerous sources of supply. The number of micro-organisms in water from such a source is also liable to extreme variation from climatic conditions independently of pollution, so that practically local standards are of little value.

(*d*) The supply is derived from a river. The bacterial contents of rivers vary within very wide limits, depending upon seasons of the year and proximity to towns, &c. As a rule, they are dangerous sources of supply, for it appears that there are few, if any, rivers which by natural processes can purify themselves sufficiently to justify their consumption without artificial treatment.

CHAPTER V.

QUANTITATIVE ANALYSIS—*continued.*

The Relation of a Quantitative Bacteriological Analysis to Filtration of Water through Sand.

The purification of water by filtration through sand is chiefly a biological process, the object being to remove micro-organisms from the supply. Piefke was the first to show that sand alone could not remove micro-organisms from water; filters made of sterilised sand were found to increase the number of bacteria in the filtrate during the first few days of working, but, with the formation of a gelatinous layer on the surface, the true filtering action commenced. This gelatinous layer consists of zooglœa of bacteria combined with suspended materials in the water; it is extremely friable and readily broken by excessive pressure on the surface or disturbance of the body of the filter bed. The degree of fineness and the uniformity of the sand grains are also of importance in securing a good filtrate. By using fine sand the current of water passing through the bed is rendered more uniform, and the walls of the lacunar spaces are approximated, permitting molecular action to take place, and giving greater firmness to the gelatinous layer. Hence the work of a sand filter is partly mechanical and partly vital. By the growth of bacteria in the bed food material is used up, and products of bacterial life, which have a powerful effect in arresting the growth of bacteria through the filter, are eliminated. It must not be supposed that a sand filter will arrest all the micro-organisms contained in a water applied to its surface. At one time it was considered that a filter bed consisted of two superimposed systems, viz., an upper system which arrested all bacteria, and a lower system formed by the inferior layers of the bed which contained a few bacteria clinging to the materials. It

was thought that the bacteria which appeared in the filtrate were derived solely from the inferior system and had no relation to the bacteria applied to the surface of the bed. Fränkel's experiments with B. violaceus and the Lawrence experiments with B. prodigiosus showed conclusively that applied bacteria do find their way into the filtrate. Fränkel poured water charged with B. violaceus on to a ripe filter, and found colonies of the organism in the filtrate. In order to avoid the objection that the B. violaceus might have gradually grown through the filter owing to the nutriment supplied by the broth culture, Fränkel made his cultures in broth so dilute that their addition increased the nutritive powers of the water only in a very infinitesimal degree. The State Board of Health, Massachusetts, has carefully investigated the working of sand filters by means of experimental beds at Lawrence. The Board employed the terms "bacterial efficiency," "bacterial purification," and "hygienic efficiency" to express the results of the action of sand filters. "Bacterial efficiency" is the percentage of the number of bacteria in the applied water which fails to appear in the effluent. The number of bacteria in the effluent, however, includes those which have their origin in the lower portions of the filter and its under-drains, as well as those which have passed directly through the filter from top to bottom. At the present time there are no well-defined methods by which the two classes of bacteria can be readily separated. "Bacterial purification" is the percentage removal by filtration of the applied bacteria. The expression bears no relation to those bacteria in the effluent which have their origin within the filter or under-drains; consequently to gain an idea of the bacterial purification produced by a filter it is necessary to employ a special micro-organism. The Board employed the B. prodigiosus because it appeared incapable of growth within the filters and their under-drains and could be readily detected in the effluents. The following table shows some of the results obtained at Massachusetts:

Depth of sand.	Form of filtration.	Rate per acre daily.	Percentage of bacteria which appeared in the filtrate.	
			B. Prodigiosus.	Water bacteria.
5 feet	continuous	7,660,000	0·171	1·48
,,	,,	7,700,000	0·148	1·19
12 inches	,,	3,700,000	0·337	2·34
5 feet	intermittent	3,660,000	0·463	3·10
,,	continuous	5,550,000	0·183	1·04

The Board employed the term "hygienic efficiency" to indicate the percentage removal by filtration of the applied bacteria capable of producing disease. The hygienic efficiency of the Lawrence filter beds, tested with the B. typhosus, was as follows:

Material of filter.		Size of sand.	Filtration per acre daily.	Form of filtration.	Percentage of B. tyhosus germs removed.
Sand.	Loam.				
5·25 feet	—	0·48	980,000	Intermittent	99·95
,,	—	,,	1,000,000	,,	99·00
5 feet	2 inches	0·26	460 000	Continuous	100·00
,,	,,	,,	400,000	,,	100·00
,,	1 inch	0·19	1,560,000	Intermittent	100·00
,,	—	0·19	2,100,000	Continuous	97·20
,,	—	0·19	1,540,000	,,	100·00
2 feet	—	0·19	1.500 000	,,	99·16
1 foot	—	0·19	1,540,000	,,	99·00

Bacterial efficiency appears to depend largely on the rate of filtration. Low rates are safer than high rates; but up to a certain limit the rate does not exert much influence. At Altona Kümmel obtained the following results:

Rate of filtration.	Bacteria per c.c. in the effluent.
2 inches per hour (4 feet per day) . .	11–97
4 ,, ,, (8 ,, ,,) . .	5–79
8 ,, ,, (16 ,, ,,) . .	7–72

Kümmel did not regard four inches per hour as beyond doubt the maximum rate of safe filtration, but considered that the danger of passing pathogenic organisms was more unlikely at the lower than at the higher rates, and that the best velocity was not the same for all waters. Differences in the mineral, vegetable, and animal admixtures were of great importance in this question.

The Massachusetts Board of Health believed that the factor which caused the rate of filtration to become practically *nil* was the age of the filter. Increasing the rate of filtration ten to twenty per cent. might cause a temporary increase in the number of bacteria if the filter were new and composed of coarse grains; but with ordinary ripe filters no increase in the bacteria was noticed. Stopping the filtration for thirty-six hours, and then increasing it, had no effect on old filters, but on new filters with coarse grains it had a temporary effect. Lowering the rate and then

suddenly increasing it had no effect except on new coarse filters. With gradually increasing age and consequent thickening of the gelatinous layer in the filter, the rate of filtration, without a dangerous head, became so low that cleaning of the surface became inevitable. The depth of sand removed from the surface, and the mode in which the filter was again put into operation after cleaning, were found to be the principal factors influencing bacterial efficiency.

In Germany the gelatinous layer on the surface appears to be regarded as essential, and the main body of the filter is simply looked upon as an under-drain and support for the surface film, having a steadying influence on the process of filtration during times of abnormal stress. The Lawrence experiments also showed that the surface film removed more bacteria than any other layer in the filter. Still, in many cases, filters were found to remove bacteria in the absence of any surface coating. Also 0·1 to 0·3 inch could be scraped off a continuous filter without affecting its action, and coarse filters did not give perfect results when the coating was so thick as to almost completely clog the filter.

From a practical point of view the influence of the removal of clogging on bacterial efficiency depends on (*a*) the depth of the material which is removed from the surface by scraping, and on (*b*) the mechanical disturbance of the main body of the sand which may be caused by the scraping and the process of refilling. Until the scraping reaches about one inch the influence of the removal is slight, but when more than one inch is removed the change in the bacterial contents of the effluent becomes marked. As the depth of material removed increases, not only do the bacteria in the effluent increase in number, but also the period of lessened efficiency of the filter becomes longer. The most efficient method of putting filters into operation after treatment to relieve clogging, has been carefully studied at Lawrence. Filling from the top did not appear to affect small experimental filters; but on the large scale, in order to get the upper layers of the filter dry enough to walk on, it was found necessary to lower the water to a greater depth below the surface, thereby furnishing an opportunity for mechanical disturbance of the main body of the sand when its pores are

again filled with water. The best plan is to fill the bed from below with filtered water to the top of the sand and then run on raw unfiltered water, which is allowed to stand for ten to twelve hours before filtration is started. Kümmel found that when a filter had new sand placed on it the numbers of bacteria in the effluent were as follows:

Before cleaning	42	bacteria	per c.c.
One day after cleaning	1,880	,,	,,
Two days after cleaning	752	,,	,,
Three days after cleaning	208	,,	,,
Four days after cleaning	156	,,	,,
Five days after cleaning	102	,,	,,
Six days after cleaning	84	,,	,,

He therefore insists on wasting the water for several days after filling. The Massachusetts Board, however, considers that it is best to have the fine sand in a filter of such a depth that it can be cleaned and used for a year without fresh sand being put on it, otherwise there is a tendency for clogging to occur at the junction of the new and old sand. The Board found in their experiments that it was not necessary to waste the water, provided there was no disturbance in the main body of the filter. Piefke, however, considers that it is best to waste the water for twenty-four hours after scraping.

As regards the influence of the depth of the filtering material upon bacterial efficiency, it has been found that filters containing two feet, and even one foot, of sand yield satisfactory results so long as there are no disturbing influences. But if these occur, their effects on the efficiency of the filtration are more marked and of longer duration in shallow than in deep filters. The depth of fine sand in a filter should never be reduced below twelve inches. The size of the sand grains has a powerful influence on bacterial efficiency; this is shown by differences in the length of time required by new filters to yield effluents of normal bacterial contents. As the size of the sand grains increases the effect of deep scraping and unequal rates of filtration become more marked even in filters which have been some years in operation. Good results may be obtained with grains ranging from 0·14 to 0·38 mm.; but the size must be uniform

throughout the filter. Sand as coarse as 0·48 mm. requires a longer period of operation to yield normal results.

The question as to whether a filter should be used continuously or intermittently is considered by the Massachusetts Board to depend on the relation of the dissolved oxygen to the organic matter in the water. Some waters are saturated with oxygen and contain only a small quantity of organic matter; these may be filtered continuously with satisfactory results. Successful filtration by the ordinary continuous method is out of the question when the water contains much organic matter and very little or no free oxygen. Whether the beds are worked continuously or intermittently it is acknowledged that an effluent under satisfactory conditions will not contain more than 100 bacteria per c.c. At the Altona works the filtered water usually showed counts under 30 per c.c.; 50 to 70 per c.c. were rarely found. The London water companies have not been so successful, those companies which derive their water from the Thames and Lea being hampered by insufficient storage. The impurity of the water in the rivers is so largely increased during periods of flood as to render it a matter of vital importance that there shall be sufficient provision of subsidence reservoirs to enable the necessary daily supplies being taken while keeping the intakes closed. A certain amount of suspended organic material is required to assist in forming the gelatinous film in the filter bed, but an excess soon blocks up the bed and necessitates frequent cleaning and consequent disturbance of the body of the filter. It was at one time thought that double filtration might improve river supplies; but in the process of double filtration the organic matter will be almost entirely arrested on the first filter leaving only a small amount of material to form the film in the second filter, and as a result the partially purified water from the first bed passes through the second bed with only a very slight improvement. The exact conditions which will give the best results must be worked out more or less experimentally for each supply. It is also of the first importance that there shall be means of making a daily bacteriological examination of the filtered water from each bed. Filtered water containing more than 100 organisms per c.c. must not be passed into the reservoir for pure water; the beds must be so

constructed that imperfectly filtered water cannot mix with the pure water.

Reinsch and Kümmel have devised an apparatus by which samples of water for bacteriological examination can be taken from filter-beds of old construction. The importance of being able to examine each bed is well shown by the following results of one of their investigations of filter-beds Nos. 2 and 3 at the Altona water-works; No. 2 bed had been cleaned and a fresh layer of sand 60 c.m. thick put on the bed:

Date.	Bacteria per c.c. in water from filter. No. 2.		Bacteria per c.c. in water from filter. No. 3.
June 26	520	...	10
„ 27	394	...	6
„ 28	224	...	10
„ 29	96	...	10
„ 30	56	...	6
July 1	34	...	4

When examining the filtered water at least three gelatine plates should be made and counts taken after forty-eight hours incubation at 20° C.

During the last two or three years the Massachusetts State Board of Health has made experiments in order to determine whether B. coli was present in the filtered water or not. "From the studies of B. coli in filtered water it was thought probable that a rate which would be entirely safe, judging only from the determinations of the total number of bacteria present, might really be too high to produce safe water, judging from the B. coli determinations." The Board considers that the typhoid bacillus cannot be present naturally in water without the occurrence of large numbers of B. coli communis, and regards the absence of this bacillus from the filtered water as conclusive evidence of the absence of the typhoid germ. But as the Merrimack river, which receives the sewage from several large cities, only contains fifty colon bacilli per cubic centimetre at the intake of the Lawrence city filter, it is evident that the typhoid bacilli contained in the river water must be very few in number as compared with B. coli, even when the cities have typhoid fever in an epidemic form. Consequently the volume of water which must be declared free from B. coli in order to

establish the absence of B. typhosus becomes a very important question. In view of the above considerations the work of the Lawrence city filter is of interest. During the year 1899, 180 samples of the river water at the intake of the filter were examined, and B. coli were found in all of them, the number per cubic centimetre averaging 47 for the entire year. During the same period 189 samples of the filtered water collected at the pumping-station were also examined for B. coli, and it was found in 1 c.c. of 45 of these samples. Twenty-seven of the samples in which it was found, however, were collected during the months of January and February. "Probably never more than one colony per c.c. was present in any of these samples of effluent from the city filter." It is evident from the above results that seasonal variations of temperature affect the work of sand filters in eliminating B. coli communis, the poorer work being done during the colder months of the year. The experiments of the State Board of Health are not yet completed; their additional studies gave somewhat contradictory results, so the very important questions as to whether B. coli must be completely absent from, or whether it may be allowed in, a certain volume of the filtered water in order to make sure of the absence of the B. typhosus, must be considered still unsettled.

CHAPTER VI.

THE QUALITATIVE BACTERIOLOGICAL ANALYSIS.

The most important part of the bacteriological examination of water supplies consists in the isolation and recognition of the various kinds of bacteria present; unfortunately, much confusion exists as to the species of micro-organisms which normally inhabit waters from various sources. Many organisms have been described as different species which are really only varieties, the variation being caused by differences in food, habitat, temperature, &c. Marshall Ward believes that many of the bacteria isolated from impure waters behave as weakened forms, and considers that the description of these enfeebled forms explains many of the species found in the various works on water-bacteria. Of late years, however, there has been a great increase in the number of tests to which an organism must be submitted in order to determine the species to which it belongs. Consequently it is only quite lately that bacteria have been separated into distinct species, characterised by well-defined biological and chemical reactions. Following on the elaboration of these differential tests, there has been a marked diminution in the reports of the successful isolation of pathogenic organisms, such as the B. typhosus, from water supplies supposed to have been infected with the germs of specific disease.

Bacteriologists now realise that the isolation of such an organism as the B. typhosus is a matter of considerable difficulty, and that it can only be accomplished under exceptionally favourable circumstances. Admitting this to be the case, and considering from a hygienic point of view that it is highly desirable, in the absence of the discovery of a specific pathogenic organism, to be able to state that a suspected water has been polluted with sewage, many bacteriologists have recently studied

the flora of sewage with great care. As a result of their work a number of organisms have been described which appear to be so characteristic of sewage as to justify the term sewage-bacteria in contradistinction to water-bacteria. In the present state of our knowledge, therefore, it appears most advantageous, from a hygienic point of view, to arrange the micro-organisms found in water in the following three classes:

Class I.—Micro-organisms which are usually found in pure waters—viz., the so-called water-bacteria.

Class II.—Micro-organisms which are common in sewage, but are rarely found in pure waters.

Class III.—Micro-organisms which are the cause of specific disease in human beings, and which have been isolated from water supplies.

CLASS I.

MICRO-ORGANISMS WHICH ARE FOUND IN PURE WATERS.

A very large number of water-bacteria have been isolated, but the biological characteristics of many of them are so vague that it will serve no useful purpose to attempt to describe them. For hygienic work it seems better to try and arrange the water-bacteria into groups of certain well-defined types, and then to mention the variations from the type which have been described.

GROUP I.

In this group are included the microbes which produce a green colour in nutrient media; they may be again sub-divided into those which liquefy and those which do not liquefy gelatine.

Bacilli which liquefy Gelatine and produce a Green Fluorescence in Nutrient Media.

B. Fluorescens Liquefaciens.

This microbe is exceedingly common in water from all sources, and has the following characteristics:

Colonies on Gelatine Plates.—After twenty-four hours at 20° C.

fine films appear on the surface with granular contents; after forty-eight hours the films become converted into shallow cups surrounded by a green fluorescence. Under a low power the colonies appear to have a margin set with very fine rays, and in the centre a membrane may be seen; later the membrane disappears, and as the liquefaction extends the fine rays are replaced by a hazy granular appearance. The colonies in the depth of the gelatine are circular or oval in shape, and by transmitted light appear to have a greenish-brown tinge.

Stab-gelatine.—The gelatine is liquefied in the form of a funnel, and the medium acquires a green colour.

Agar-slope.—A smooth white growth, the agar assumes a green colour.

Broth.—There is a diffuse growth, a slight pellicle forms on the surface and a thick deposit appears at the bottom of the tube; the broth acquires a green colour.

Potato.—A yellowish-brown growth.

Glucose-gelatine.—No gas formation.

Nitrate-broth.—After seven days incubation at 22° C., large quantities of ammonia are produced.

Milk.—A clot is formed which is slowly digested, the whey assumes a green colour.

Peptone-water.—No indol reaction is obtained.

Litmus-whey.—There is no formation of acid. The blue colour is changed to green; after seven days incubation the medium has a faintly alkaline reaction.

Microscopical Appearance.—A very short bacillus, generally found in pairs. It may form chains. No spores observed. It does not stain with Gram's method, and is very motile.

The above description gives the characteristics of the type of this organism which is most commonly found in water. Varieties, however, are frequently found which vary considerably from the type; some liquefy the gelatine in forty-eight hours, others do not produce this result until the expiration of fourteen days. The optimum temperature is usually 20°-25° C.; many varieties will not grow at 37° C.; others grow well at the latter temperature and produce a copious growth on agar. The oxygen requirements vary greatly. Sometimes liquefaction of gelatine takes place rapidly in the form of a deep funnel; at other times it is

horizontal and limited to the surface layers. The colour of the pigment produced in the various media may be bright green or only greenish-yellow. The growth on potato usually shows a yellowish-brown colour, but at times it may have a rose tint or deep brown colour. Also some varieties which I have isolated do not coagulate or digest milk. The B. cloacæ fluorescens, the B. fluorescens stercoralis (Laws and Andrewes), the B. fluorescens Schuylkilliensis, B. fluorescens mutabilis (Wright), B. butyri fluorescens (Lafar), B. fluorescens nivalis (Schmolck), and B. viscosus (Frankland) are merely varieties of the B. fluorescens liquefaciens. The B. viridans, described by Zimmermann, is a small non-motile bacillus, which produces a steel-green colour in media, but no fluorescence is observed. It grows well at 30° C., is ærobic, and does not stain with Gram. It rapidly liquefies gelatine, but in gelatine-stab the liquefaction only occurs horizontally at the surface; there is no growth along the line of inoculation.

Bacilli which produce a Green Fluorescence in Nutrient Media, but do not Liquefy Gelatine.

These bacilli are also extremely common in water, and, when the green colour of the media is slowly produced, there may be difficulties in diagnosing this group from other species.

B. Fluorescens Non-Liquefaciens.

Colonies on Gelatine Plates.—After forty-eight hours at 22° C., the surface colonies are thin, with an irregular margin, and when held up to the light have a bluish, almost transparent appearance; the surrounding gelatine shows a green fluorescence. Under a low power the colonies show a beautiful venation resembling that of a leaf. The colonies in the depth of the gelatine are round, and by transmitted light appear greenish-brown in colour.

Gelatine-stab.—There is a growth on the surface resembling a colony, and a thin white growth along the line of inoculation; the gelatine is not liquefied and acquires a bright green colour.

Agar-slope.—A white growth; the medium assumes a green colour.

Broth.—A diffused growth; a pellicle appears on the surface in forty-eight hours. The broth assumes a green colour.

Potato.—A greenish-yellow growth.

Glucose-gelatine.—No gas formation.

Nitrate-broth.—No reduction of nitrates.

Milk.—Unchanged.

Peptone-water.—No indol reaction.

Litmus-whey.—No formation of acid takes place. The medium often becomes slightly alkaline.

Microscopical Appearance.—Short thin bacilli with rounded ends; as a rule not motile. It does not form spores. Optimum temperature 21°–25° C.; it does not develop at 37° C. It does not stain by Gram's method. The type above described corresponds to the B. fluorescens non-liquefaciens of Eisenberg, B. fluorescens albus of Zimmermann and B. aquatilis fluorescens of Lustig. The Bacillus viridis pallescens isolated by Frick form green sputum, and found by Tils in the Freiburg pipe-water, differs from the type only in that the bacillus is highly motile, and the growth on potato has a nut-brown colour.

B. Fluorescens Longus.

This variety of the B. fluorescens non-liquefaciens was isolated by Zimmermann from the Chemnitz water-supply. Its characteristics are as follows:

Colonies on Gelatine Plates.—The young colonies in the depth are white dots with a greenish tinge. On the surface at first they are thin, almost round, and have a mother-of-pearl shimmer. They grow rapidly, have a greenish-yellow colour, and look as if yellowish-white threads had been drawn through them. Under a low power, the deep colonies are yellow, sharply outlined with a convoluted interior; the surface colonies show a similar appearance, but the convolutions are broader and resemble the convolutions of the intestine in a small animal.

Gelatine-stab.—Forms a thin expansion at first, but later becomes thicker in the centre and soon reaches the margin of the gelatine. At first the colour is blue, but later blue-green.

Agar.—A thin growth, and the agar becomes greenish-yellow in colour.

Potato.—Forms a yellowish moist growth which rapidly spreads over nearly the whole surface.

Gas-production.—Not observed.

Microscopical Appearance.—Short and long bacilli observed; the short forms are motile but the long ones are stationary. Unstained spots appear in the rod, but they are probably not spores. It grows best at room-temperature, and produces a greyish-yellow pigment. A culture of this organism from Kral's laboratory produced colonies closely resembling the B. fluorescens non-liquefaciens. Its cultural reactions in the various media also were identical with those of the latter organism. The colour of the media, however, was more yellowish in the case of the longus. The bacilli stained with Gram.

B. Fluorescens Tenuis.

This micro-organism was isolated by Zimmermann from the Chemnitz water supply. It has the following biological characteristics.

Colonies on Gelatine Plates.—The surface colonies are thin, irregularly round with a denticulated edge; the surrounding gelatine acquires a green colour, and is not liquefied.

Gelatine-stab.—A thin greyish-white expansion appears on the surface, which in about four days reaches the margin of the tube. The stab is clearly defined, but no further growth takes place.

Gelatine-streak.—After three days a leaf-like expansion is produced.

Agar-slope.—A greyish growth is produced; the agar assumes a green colour.

Potato.—A thin, greyish-yellow, smooth layer is produced, which only after some time grows out from the line of inoculation. Later, the colour of the growth becomes reddish-brown.

Gas-production.—Not observed.

Microscopical Appearance.—Short thick bacilli with rounded ends, often arranged in groups; in old cultures the rod consists of five or six links. It has only rotatory movement and is decolorised by Gram's method.

A culture from Kral's laboratory produced very characteristic colonies, which had the denticulated edge well marked. The appearance of a surface colony strongly resembled a small shell.

The other cultural reactions corresponded to those already given under the B. fluorescens non-liquefaciens. The colour produced in gelatine, broth and agar was bright green.

B. Fluorescens Aureus.

This bacillus was also isolated by Zimmermann from the Chemnitz water supply. It has the following cultural characteristics.

Colonies in Gelatine Plates.—The colonies in the depth appear as small yellowish-white dots; the surface colonies are yellowish-grey, smooth, not sharply outlined expansions; they are darker in the centre and delicately streaked at the margin. The gelatine is not liquefied.

Gelatine-stab.—A thin yellowish layer, which later becomes thicker in the centre, appears on the surface, and in eight to ten days reaches the margin of the gelatine. In the stab there is very little growth, which later becomes brownish in colour.

Gelatine-streak.—It produces a thick ochre-yellow growth, under which the gelatine acquires a marked brown colour. In transmitted light the gelatine appears greenish-yellow.

Agar-slope.—A rich ochre or golden-yellow growth; the agar becomes darker but does not fluoresce.

Potato.—The growth is the same as on agar.

Gas-production.—Not observed.

Microscopical Appearance.—A short bacillus with rounded ends, about twice as long as broad, found in pairs, rarely in greater numbers. It is very motile. It produces a yellow-ochre pigment, and stains faintly with Gram.

A culture of this organism from Kral's laboratory produced colonies exactly like those described above. On potato and agar the growth had a yellow-ochre colour. The growth in gelatine was yellowish, and the gelatine acquired a green fluorescence. The microbe did not coagulate milk, produce indol, nor form gas in glucose-gelatine.

B. Fluorescens Crassus.

This microbe is described by Flügge in "Die Mikroorganismen." The colonies are very granular in the depth of the gelatine; on the surface they are round, slightly opaque, and have a

nail-head appearance. The bacillus is not motile. It is said to be identical with the B. iris of Frick, and to be often found in air and water.

I have frequently isolated this organism from water. The nail-headed colonies when fished and planted out in gelatine produce a marked green fluorescence of the medium; the growth on the surface sometimes remains thick, at other times it produces a thin layer like the stab of B. fluorescens non-liquefaciens. The organism noes not coagulate milk, produce indol, nor form gas in glucose-gelatine. The growth on potato is greenish-yellow. It does not reduce nitrates appreciably. In nearly all its cultural reactions it resembles B. fluorescens non-liquefaciens, and according to Flügge it is only a variety of this organism.

The bacilli above described form the most important varieties of the B. fluorescens non-liquefaciens which are found in pure water. They are important because in the early days of bacteriological study they were sometimes mistaken for the B. typhosus. When the green fluorescence is not present, which happens if the organism is enfeebled, the colonies have a superficial resemblance to those of B. typhosus, and as the bacilli do not coagulate milk, produce indol, nor form gas in glucose media, and some varieties are decolorised by Gram's method of staining, it is easy to understand that they might be mistaken for typhoid bacilli if the further tests given under B. typhosus were not carried out.

The B. fluorescens putridus described by Flügge is also closely allied to the B. fluorescens non-liquefaciens, but as it is considered by many authorities (Macé, &c.) to be characteristic of foul waters, it has been included under the sewage bacteria.

The B. erythrosporous and the B. pyocyaneus also produce pigment which gives rise to a greenish fluorescence in media; these organisms are rarely, if ever, found in pure waters, and have therefore been included in the sewage group.

GROUP II.

This group includes the varieties of the B. aquatilis sulcatus described by Weichselbaum. These bacilli are frequently found in water derived from wells, springs, and rivers. The cultural characteristics of the most common variety are as follows:

B. Aquatilis Sulcatus.

Colonies on Gelatine Plates.—After forty-eight hours incubation at 22° C. the colonies appear on the surface as bluish-white films with an irregular margin; the centre is thicker than the periphery. Under a low power numerous fine lines are seen passing from the centre to the periphery, producing an appearance closely resembling the ridges and valleys on the colonies of B. typhosus. Later, the centre of the colony acquires a yellow colour. The gelatine is not liquefied.

Gelatine-stab.—The surface growth exactly resembles a colony; there is a scanty growth along the stab.

Agar-slope.—A white smooth growth; later, becomes yellow in colour.

Glucose-gelatine (shake).—No gas formation.

Peptone-water.—No indol reaction obtained.

Milk.—Unchanged.

Potato.—A thin yellow film appears.

Nitrate-broth.—No reduction of nitrates.

Broth.—Diffuse growth, no pellicle appears on the surface.

Microscopical Appearance.—A small motile bacillus with rounded ends, closely resembling B. typhosus. It does not form spores and is decolorised by Gram. It will grow at 5° C., but develops very feebly at 37° C.

The above description corresponds with B. Aquatilis sulcatus V. Four other varieties were described by Weichselbaum. No. I. differs from the type in that at 37° C. it produces a thick white expansion on agar, and on potato it produces at first only a moist appearance, which later becomes cream-coloured. No. II. shows colonies which are thicker than the above, and the system of lines is hardly visible; the growth on potato has a yellow-blue colour which later becomes yellowish-grey or yellowish-brown, and is often very luxuriant. No. III. produces colonies resembling the type; but the growth on potato has a bright yellow colour. Bacillus No. V. shows typical colonies, but does not produce any growth on potato.

The B. sulcatus is an important organism to recognise, owing to its strong resemblance to the typhoid bacillus; it is, however, easily differentiated by the colonies acquiring a yellow colour

its quicker growth on gelatine plates, its power of developing at 5° C., and the absence of all traces of agglutination with anti-typhoid serum.

Two other organisms have been described which have received the name sulcatus; they are easily differentiated from the bacilli isolated by Weichselbaum.

B. Sulcatus Liquefaciens.

This organism is described by Flügge, and said to be frequently found in water. It is a medium-sized motile bacillus which does not form spores. The colonies in the depth are small, round, slightly granular and yellowish in colour. The surface colonies are larger, thin and transparent, and have an irregular margin; the surface of each colony also shows a fine system of ridges and furrows like the typhoid bacillus. Liquefaction slowly sets in and the colonies sink in the gelatine. On agar there is a thin grey transparent growth. On potato a yellowish-brown layer appears.

B. Aquatilis Sulcatus.

This bacillus is often found in water supplies and in many respects resembles the organisms in Group III. It is a rather large bacillus, which forms spores, and has a slow waddling movement like B. subtilis. It does not stain with Gram. The colonies in gelatine plates closely resemble those of B. subtilis, but the liquefaction is not so rapid. On agar it produces a dry wrinkled growth. In broth there is a diffused growth, and later a wrinkled film forms on the surface. Milk is coagulated and slowly digested. On potato there is a white, dry, slightly folded layer. In glucose media there is no gas formation. Indol is not produced in peptone and salt solution, and the reaction of litmus-whey remains unchanged.

GROUP III.

This group includes the three varieties of the "potato bacillus," viz.: the B. mesentericus vulgatus, B. mesentericus fuscus, and the B. mesentericus ruber. Flügge includes in the group of the "hay bacilli" the above organisms and also the following: B. subtilis, B. liodermus, B. mycoides, B. ramosus,

B. megaterium. They have many points in common, and varieties which seem intermediate between the various members of the group are constantly isolated from water supplies. It therefore appears best to place all these bacilli in one large group.

B. Mesentericus Vulgatus.

This micro-organism has the following cultural characteristics:

Colonies on Gelatine Plates.—At the end of twenty-four hours the colonies develop on the surface as small opaque white spots, which at the end of forty-eight to seventy-two hours appear sunk in small cups of liquefied gelatine. Examined at this stage under a low power an opaque membrane is seen in the centre surrounded by a clear circular zone; the periphery of the colony, outside the clear zone, is set with fine rays running out into the gelatine. Sometimes this peripheral zone of hairs is not seen.

Stab-gelatine.—The gelatine is liquefied in the form of a funnel.

Glucose-gelatine (shake).—No gas formation.

Agar-slope.—A thin, white, dry wrinkled growth.

Potato.—A thick white growth which becomes wrinkled and furrowed.

Peptone-water.—No indol reaction obtained.

Broth.—There is at first a diffuse growth; later, a wrinkled pellicle appears on the surface and the broth clears below it.

Nitrate-broth.—Nitrates are reduced to nitrites.

Milk.—At 37° C. milk is coagulated; the casein is digested after several days incubation.

Litmus-whey.—After seven days incubation, the blue colour disappears and the medium is rendered faintly alkaline.

Microscopical Appearance.—A small motile bacillus with rounded ends. It forms short ellipsoidal spores. This bacillus is smaller than the hay bacillus, but often has the same waggling movement. It is stained by Gram's method.

B. Mesentericus Fuscus.

This bacillus resembles the vulgatus in most of its cultures. The growths on agar and potato, however, are thinner and have

a brown colour. It reduces nitrates more vigorously, and is smaller and more motile than the vulgatus.

B. Mesentericus Ruber.

This bacillus was described by Globig. The colonies on the surface and in the depth show quite early numerous irregular processes running in all directions from the periphery; later, on the surface colony a clear zone appears between the membranous centre and the peripheral rays, and the colony in this stage closely resembles the above varieties. The agar growth in the early stage shows processes running out from the margin; later, a dry wrinkled film is produced. On potato at 37° C. a wrinkled growth appears and the potato acquires a red colour. The red colour is not formed at 22° C. In other media the cultures resemble those of the vulgatus. The bacillus is thinner and more motile than the vulgatus, and possesses a powerful reducing action on nitrates, large quantities of ammonia being produced after seven days incubation.

B. Liodermus.

Flügge described this organism, which is closely related to the potato bacillus. On potato it produces a gummy layer, which later is thrown into folds. The gummy material is soluble in water and precipitated by alcohol like gum arabic. Milk is coagulated and peptonised.

B. Subtilis.

This bacillus was first isolated by Ehrenberg from an infusion of hay, hence it is often called the hay bacillus.

Colonies on Gelatine Plates.—The colonies at first are small white points, which under a low power appear as irregularly round, yellowish-brown membranes, from which fine hair-like processes pass off into the gelatine. Later, the colonies appear as small cups of liquefied gelatine, with little or no central membrane; from the margin of the colony hair-like processes are still seen passing into the gelatine. In the depth the colonies often appear as granular centres from which many long and curved processes spread out into the gelatine; sometimes very

irregular forms are seen in which the bacilli appear packed in parallel rows.

Gelatine-stab.—The gelatine is liquefied in the form of a funnel; a flocculent deposit appears at the bottom and a tough pellicle on the surface.

Agar-slope.—A thick white growth appears, which later becomes wrinkled.

Glucose-gelatine.—No gas formation.

Nitrate-broth.—No reduction of nitrates.

Broth.—A pellicle forms on the surface, which later becomes wrinkled and furrowed.

Potato.—A thick white growth, which is often thrown into large transverse folds; later, dry white granules may appear on the surface.

Peptone-water.—No indol reaction is obtained.

Litmus-whey.—After seven days incubation the blue colour disappears and the medium is rendered faintly alkaline.

Milk.—At 37° C. milk is peptonised and partially digested.

Microscopical Appearance.—A large bacillus with rounded ends, often in pairs and chains. It has a slow waggling movement, and forms oval spores. It is strongly ærobic, and stains with Gram. Sewage varieties of B. subtilis and B. mesentericus have been described by Houston.

B. Mycoides.

This bacillus is said to be identical with the B. ramosus in all its cultures. It is found in sewage and also in surface waters.

Colonies on Gelatine Plates.—The colonies appear as white patches, from which numerous irregular processes extend on all sides into the gelatine, producing an almost mould-like growth. The gelatine becomes liquefied after about seventy-two hours incubation.

Gelatine-stab.—A white growth occurs along the stab, from which processes extend horizontally into the gelatine, producing a tree-like growth; later, liquefaction occurs.

Agar.—A white, rather dry growth, from which mould-like processes extend at the margin.

Glucose-gelatine (shake).—No gas formation.

Peptone-water.—No indol reaction.

Milk.—Unchanged.

Broth.—Diffuse growth; a deposit forms at the bottom, and later a pellicle appears on the surface.

Nitrate-broth.—Powerfully reduced to nitrites and ammonia.

Potato.—A moist, white growth.

Litmus-whey.—The reaction of the medium remains unchanged.

Microscopical Appearance.—A large bacillus, with rounded ends; forms oval spores. It is motile; movement sluggish. It is stained by Gram's method.

The B. megaterium (De Bary) is described with the sewage organisms.

The B. filiformis described by Tils also belongs to this group. A culture of this organism produced colonies on gelatine exactly resembling moulds. In stab-gelatine a beautiful tree-like growth was obtained. On agar there was a thick growth with fibres passing out from the margin. The growths in peptone-water, broth and glucose-gelatine exactly resembled those of B. mycoides. It formed long threads, and divided into segments, each containing a spore.

GROUP IV.

This group contains a large number of bacilli which are very common in unfiltered water supplies. It includes the B. liquefaciens of Eisenberg, the B. liquefaciens of Lustig, the B. liquidus of the Franklands, the B. punctatus of Zimmermann, the B. aquatilis communis of Flügge, B. liquefaciens communis of Sternberg, &c. The B. liquefaciens which occurs in unpolluted waters has the following characteristics:

Colonies on Gelatine Plates.—The surface colonies appear in twenty-four hours as small white points, which under a low power have a regular margin and granular contents; often there is an appearance of convoluted threads passing from the centre to the periphery. In forty-eight hours the colonies appear as cup-shaped depressions, which rapidly extend; under a low power the margin is now seen to be smooth, and each cup contains flocculent masses made up of clumps of bacteria; later the margin of the colony often shows indistinctly marked spinous processes.

Stab-gelatine.—The gelatine is rapidly liquefied in the form of a funnel.

Glucose-gelatine.—There is no gas formation.

Milk.—Remains unchanged.

Broth.—There is a diffuse growth, and an abundant deposit at the bottom of the tube.

Nitrate-broth.—Nitrates are reduced to nitrites and ammonia.

Potato.—The growth on potato varies; sometimes it has a light yellow colour, at other times it has a flesh-coloured tint changing to reddish-brown.

Peptone-water.—No indol reaction is obtained.

Agar-slope.—A white expansion.

Microscopical Appearance.—A short motile bacillus, often in pairs. It does not form spores. It does not grow well at 37° C.

The B. liquefaciens of Eisenberg corresponds to the above type, but does *not* grow at 37° C. The B. liquidus, described by the Franklands, forms large cup-shaped depressions, and produces a thick, moist, flesh-coloured growth on potato.

The B. punctatus of Zimmermann forms cup-shaped liquefying colonies; in the bluish-grey liquid whitish dotted groups of bacteria occur, which frequently appear joined to one another by white streaks. In stab-gelatine the gelatine is liquefied in the form of a stocking. On potato there is a brownish flesh-coloured expansion. It grows better at 30° C. than at the ordinary temperature.

The B. aquatilis communis is said by Flügge to be one of the commonest organisms in river-water and well-water, and probably identical with the B. punctatus and B. liquidus. It is a plump, medium sized very motile bacillus. It does not form spores and it is not stained by Gram's method. It liquefies gelatine very rapidly, and in forty-eight hours a stocking-shaped liquefaction is produced. The colonies in the depth are round plates, and on the surface form round cups which are filled with masses of bacteria without any special arrangement; the margin is finely granular but not rayed. On agar at 37° C. it produces a transparent grey expansion. On potato it produces a yellowish-brown or reddish growth. The B. liquefaciens communis of Sternberg is found in fæces; it resembles the B. aquatilis communis both morphologically and in cultures.

The B. aquatilis radiatus (B. radiatus aquatilis of Zimmermann) is also very common in water. It liquefies a gelatine-stab in the form of a stocking. The colonies have a rayed margin. The growth on agar is grey and transparent and on potato there is a yellowish-brown layer. Gas producing varieties have been observed. The bacillus is morphologically like the above, but somewhat longer forms are seen; it is not motile.

Closely allied to this group is an organism which I have described as the B. liquefaciens sewage variety. It differs from the type in that it coagulates milk and produces intense gas formation in glucose-gelatine shake. I have found this organism very frequently in sewage and polluted waters; it is apparently identical with the sewage proteus of Houston, which is fully described under the sewage organisms.

Between this sewage variety and the type there are many connecting links formed by bacteria which resemble the type in not coagulating milk, but approach the sewage organism by giving rise to intense gas formation in glucose-gelatine. The Bacillus gasoformans, described by Eisenberg, appears to belong to this group and to be identical with the intermediate forms just mentioned. It is a small, very motile bacillus, which does not form spores. In gelatine plates it produces cup-shaped liquefaction, and in gelatine-stab the medium is rapidly liquefied in the form of a stocking. In glucose-gelatine it produces marked gas formation. It does not grow at 37° C.

The members of this group vary extremely in their reaction to temperature; some of the varieties will not grow at 37° C.; others grow better at blood-heat than room temperature; but even when the cultural characteristics are identical the relation to temperature may not be the same; this statement also applies to the sewage varieties.

The B. hyalinus, B. cloacæ, and B. delicatulus, isolated by Jordan from Lawrence sewage, probably belong to this group and appear to be varieties of B. liquefaciens (sewage proteus, Houston) sewage variety. The characteristics of these organisms as described by Jordan will be found in the section devoted to sewage organisms.

The B. devorans, isolated by Zimmermann from a much-used well-water, produces colonies which are characterised by forming

conical depressions in the gelatine, containing an irregular white mass at the bottom. Under a low power the margin of the colony shows threads of varying length passing off from the periphery. In stab-gelatine the gelatine becomes cleft in the form of an air-bubble, without any liquefaction; sometimes, however, true liquefaction occurs. There is no growth on potato. On agar there is a uniform grey expansion. It is a small, very motile bacillus, which usually occurs singly or in pairs. It does not form spores. It is not stained by Gram's method and grows at 37° C.

GROUP V.

This group comprises a number of organisms which produce a red pigment. The type of the group is the B. prodigiosus of Ehrenberg, which is often found in waters from various sources.

B. Prodigiosus.

The organism has the following characteristics:

Colonies on Gelatine Plates.—The colonies in the depth of the gelatine are round or oval, sharply outlined, and of a clear red colour. The surface colonies, after twenty-four hours, are seen as round granular films, which at the end of forty-eight hours appear as circular depressions with a red deposit in the centre. Under a low power the colonies show a red granular centre, surrounded by a circular clear zone and a narrow granular edge at the periphery.

Gelatine-stab.—The gelatine is liquefied in the form of a stocking and with a red coloured deposit at the bottom.

Agar-slope.—At first there is a colourless growth which gradually acquires a red colour.

Agar-stab.—In the line of inoculation the growth is colourless, but on the surface it has a red colour; the absence of colour in the stab is due to the deficiency of oxygen.

Potato.—It forms a brilliant red growth.

Milk.—Red pigment is produced, and the milk is coagulated through the simultaneous production of acid and lab-ferment.

Broth.—There is a diffuse growth with a red deposit.

Glucose-gelatine.—No gas formation.

Peptone-water.—No indol reaction is obtained.

Litmus-whey.—After seven days at 22° C. the medium has a markedly acid reaction.

Microscopical Appearance.—A small non-motile bacillus, often described as a micrococcus. In broth containing an acid or antiseptics rods and chains are well marked; these bacilli are also motile. Scheurlen has described flagella at the sides of the bacillus. It does not form spores, but resists drying for a long time, when involution forms are seen. The pigment is best produced at 20°–24° C. When cultured continuously at 37° C., forms are obtained which cannot produce the red pigment; old cultures at room temperature also lose the power of producing pigment, which, however, can be restored by cultivating the organism on potato. It is not stained by Gram's method.

B. Ruber Indicus.

Described by Koch and also found by Pasquale at Massowah. It is a motile, small, thin bacillus, which does not form spores. The colonies in the depth are golden-yellow with sharply-defined edges; the superficial colonies quickly liquefy the gelatine. In gelatine-stab there is a stocking-shaped liquefaction; on the surface there is a brick-red membrane and a white deposit at the bottom. On agar and potato at 35° C. it produces a brick-red growth.

B. Ruber Balticus.

This organism was found by Breunig in the Kiel water. It is a slender motile bacillus, which does not form spores. The colonies in the depth are round and clear yellow in colour; the surface colonies are thin layers, which gradually liquefy and have a rose-red colour. In stab-gelatine there is liquefaction at the surface and gas development in the depth. It grows and produces colour at 35° C.; at this temperature the growth on potato is purple or carmine red; at 20° C. it is orange red; the deeper layers are always reddish-violet. Milk at 20° C. is coloured and eventually coagulated through acid production; at 37° C. it is quickly coagulated but there is no coloration.

B. Ruber Berolinensis.

The red water bacillus of C. Fränkel is probably a modification of the above, which it closely resembles in morphology and cultures. The colour in gelatine is yellowish-red, on agar yellow, and on potato rust-red or orange-yellow.

B. Ruber Aquatilis.

The red water bacillus of Lustig is a small motile bacillus, two or three times as long as broad. It does not form spores, but it is not killed by heating to 60° C. for twenty-four hours. In the body of the rods fuchsia-red pigment granules are seen. It forms colonies with a serrated margin ; the centre of each colony has a raspberry-red colour. The gelatine is quickly liquefied and the red colour extends in all directions. In gelatine-stab there is a funnel-shaped liquefaction at the surface, in the centre of which pigment is found. In the depth of the stab there is a growth but no pigment formation. On agar, potato, and in broth there is a growth and formation of a raspberry-red colour at ordinary temperatures, but at 37° C. there is no pigment formation.

B. Carneus.

This bacillus was isolated by Tils from the Freiburg water supply. It is a slender, very motile bacillus and does not form spores. On gelatine it forms white colonies which liquefy the gelatine producing cup-shaped depressions. In gelatine-stab it causes a funnel-shaped liquefaction, the lower part of which becomes filled with pink-coloured masses. On potato it grows slowly producing a dark flesh-coloured expansion. The same organism is described by Zimmermann as the B. carnosus.

B. Lactis Erythrogenes.

Found by Hueppe in red-coloured milk and probably identical with the B. rosa fluorescens, isolated by Tataroff from the Dorpat water. It is a small bacillus which is not motile, and does not form spores. Its colonies are round, greyish-yellow in colour, and slowly liquefy the gelatine which round the colonies acquires a reddish colour. In gelatine-stab it grows slowly, and

liquefies the gelatine; the upper part in the dark has a red colour, but in the light is colourless. On agar and potato it produces a yellowish layer, and around this there is a slightly yellowish-red colour. Milk is coagulated and peptonised by lab-ferment; it is at first reddish-brown in colour and finally blood-red.

B. Rubefaciens.

Found by Zimmermann in water. It is a slender very motile bacillus. The colonies in the depth are small, round, and have a yellowish or brown colour; on the surface they form flat expansions which do not liquefy the gelatine, and have a suggestion of a red colour. In gelatine-stab it forms a greyish-white layer on the surface; the gelatine itself has a bluish-white colour which later becomes a light wine-red. On agar it forms a thick bluish-grey expansion. On potato there is a yellowish or brown layer, and the potato assumes a flesh colour.

B. Lactericius.

Described by Adametz as the "ziegelrother" bacillus which he isolated from water. It is a bacillus three to five times as long as broad. It is not motile and does not form spores. It does not liquefy gelatine, and on the surface forms a thick brick-red layer; there is very little growth in the stab. On potato it forms a brick-red layer.

B. Rubescens.

Found in canal-water by Jordan. It is a slightly motile large bacillus, which does not form spores. The colonies on the surface are thick porcelain-white drops, which later become brownish in colour; in the depth the colonies are small. The gelatine is not liquefied. In gelatine-stab there is a porcelain-white nail-head projection on the surface, but only slight growth along the stab. On agar there is a white growth, which later becomes wrinkled, and in about three weeks a pinkish tinge is seen. On potato there is a rapid growth, at first brown, but slowly changing to pink; in three weeks there is a luxuriant flesh-coloured growth. Milk is not coagulated, and eventually assumes a pinkish tinge at the surface. It does not reduce nitrates.

B. Rubidus.

Found in water by Eisenberg. It is a medium-sized, motile bacillus. It does not form spores and will only grow at room temperature. The colonies in gelatine plates are round, finely granular, and have a brownish-red colour in the centre. The gelatine is gradually liquefied. On agar and potato it forms a brownish-red growth.

GROUP VI.

In this group are contained the bacilli which produce a yellowish or orange coloured pigment; some of them liquefy gelatine, others do not.

B. Arborescens.

This organism is common both in surface and deep well-waters. It has the following cultural characteristics.

Colonies on Gelatine Plates.—The colonies have a golden yellow colour, under a low power they resemble a wheat-sheaf; some small growths are also seen consisting of a rod branching at the ends. Sometimes the wheat-sheaf is surrounded by a thin film with fine processes projecting from the margin. Very often the colonies consist simply of a mass of threads spreading out in every direction through the gelatine.

Gelatine-stab.—The gelatine is liquefied in the form of a cup with an accumulation of orange pigment at the bottom.

Agar-slope.—A thick golden-yellow growth.

Glucose-gelatine.—No gas formation.

Peptone.—No indol produced.

Potato.—A thick coarsely granular deep orange-coloured growth.

Nitrate-broth.—Nitrates are reduced to ammonia.

Milk.—After seven days incubation at 22° C. the medium is unchanged.

Litmus-whey.—After seven days incubation at 22° C. the medium is distinctly alkaline.

Microscopical Appearance.—A thin slender bacillus which does not form spores and possesses only rotatory movement. It grows feebly at 37° C. It is decolorised by Gram. The bacillus

described above differs from Frankland's organism by reducing nitrates to ammonia. Some varieties of this organism send out long wandering convoluted processes on the surface of gelatine plates, and in this respect resemble the Proteus species.

B. Nubilus.

This organism occurs very frequently in water derived from wells, rivers, and upland surfaces. It is a slender bacillus, which possesses only rotatory movement and does not form spores. It does not stain with Gram, and cannot develop at 37° C. or under anærobic conditions. The colonies in gelatine appear as cloudy patches, with an ill-defined margin, which grow chiefly in the depth; under a low power each colony is seen to be made up of interlacing threads of bacilli; the gelatine is rapidly liquefied. In gelatine stab there is a thick white growth, but under the surface and along the line of inoculation delicate cloudy growths appear, the gelatine is liquefied horizontally at the surface, and the growth in the liquefied gelatine acquires a faint yellow colour. On agar there is a white growth with a thin filmy margin; later, the growth acquires a faint yellow tint. On potato there is a marked yellow growth and the potato becomes discoloured. Milk is not affected by the growth of this organism, and litmus-whey acquires a faint alkalinity. There is no gas formation in glucose and lactose media. There is a diffused growth in broth with a slight deposit, but no pellicle forms on the surface. Nitrate broth is powerfully reduced, ammonia being produced. In peptone and salt solution there is no formation of indol.

The organism above described, which I have often found in waters, appears to closely resemble the B. nubilus described by P. and G. Frankland. The colonies are identical; but the growths in gelatine and on agar have a yellow colour, which is not seen in the growths of the Franklands' bacillus; also the latter produces a film in broth, gives a very slight growth on potato, and reduce nitrates feebly, only a small proportion of nitrites being found. The B. gracilis, described by Zimmermann, appears closely related to the B. nubilus. This organism, however, liquefies gelatine very slowly, and on potato produces only a slight moist appearance. It is also said to produce elliptical spores.

B. Aquatilis (Percy Frankland).

This organism was found by Percy and G. C. Frankland in water obtained from deep wells sunk in the chalk by the Kent Company. It is very similar in appearance to the B. arborescens, forming long wavy threads sometimes as long as 17 μ; spores are not observed and the movement is only vibratory. In gelatine plates the colonies in the depth at first appear smooth-rimmed, but the contour gradually becomes more and more irregular; on reaching the surface slow liquefaction of the gelatine commences and convoluted bands of threads extend from the centre to the periphery. In gelatine tubes it grows extremely slowly; a yellow expansion forms on the surface but hardly any growth appears in the depth; later slight liquefaction takes place. On agar there is a small, shining, yellow growth. Broth is rendered turbid; there is a white deposit, but no pellicle forms on the surface. On potato there is hardly any growth. In nitrate broth there is a good growth but the nitrates are not reduced.

B. Aquatilis.

In water supplied by the Kent company, I have frequently found a bacillus which appears to be closely allied to the B. aquatilis of Frankland. The colonies of this organism in gelatine plates appear at first as delicate circular films, which under a low power show a series of concentric rings; later liquefaction of the gelatine slowly sets in and under a lower power the concentric rings are not so marked, but a mass of convoluted bands, passing outwards from the centre, are now seen, and the margin appears set with wedge-shaped processes. In gelatine-stab there is a cup-shaped liquefaction, the surface being covered with a folded membrane. On agar there is yellowish, very sticky, growth. Milk is not coagulated. In glucose and lactose media, there is no gas formation. In peptone solution, after incubation for seven days at 37° C., there is a slight indol reaction on adding potassium nitrite and a few drops of pure sulphuric acid. Litmus-whey, after twenty-four hours at 37° C, is rendered strongly alkaline. Nitrate-broth is not reduced. Potato shows a yellow, limited growth. In broth there is a

diffused growth. It is a small, highly motile bacillus, which is not stained by Gram. It does not form spores.

B. Ochraceus.

This organism was first described by Zimmermann.

In the depth of gelatine plates the colonies are small and round and have a pale yellow colour; on the surface they show a deep yellow-ochre coloured centre surrounded by a thin film of a light yellow colour. Later, processes grow out from the centre into the margin and the colony becomes surrounded by a zone of liquefaction. On agar and potato it forms a yellow-ochre coloured growth. It liquefies a gelatine-stab horizontally with a deposit of yellow ochre pigment. It also powerfully reduces nitrates. In glucose-gelatine there is no gas formation. It is a thin, slowly motile bacillus; grows best at 20° C. and does not form spores. It is not stained by Gram's method.

B. Tremelloides.

Found by Tils in the Freiburg water supply. The colonies in the depth of gelatine are smooth-rimmed yellow dots; on the surface they form thin yellow expansions with an irregular margin. In gelatine-stab there is a growth on the surface resembling a colony, and along the stab a thin yellow growth consisting of isolated colonies. On agar and potato it forms a yellow growth. There is no gas formation in glucose-gelatine. Indol is not produced in peptone, and milk is not coagulated. It is a small, thin, motile bacillus. Gelatine is slowly liquefied.

Yellow Bacillus.

Described by Lustig. The colonies in gelatine plates are small round grey dots, which under a low power have a golden-yellow colour and a serrated margin; from the periphery of the colony delicate extensions pass into the surrounding gelatine; in about ten days the colony rests in a cup of liquefied gelatine. In gelatine-stab it forms a yellow slimy growth on the surface, along the stab there is a yellow growth with short lateral

extensions into the gelatine; about the fourth day there is a cup-shaped liquefaction at the surface containing a golden-yellow slimy deposit. On agar there is a straw-coloured growth. On potato there is a coffee-coloured layer. It is a short, thin, non-motile bacillus, occurring usually in long spiral or bent threads containing many individual bacilli.

B. Helvolus.

Described by Zimmermann. The colonies in the depth of gelatine plates are small, circular, bright yellow dots; on the surface there is a pale yellow expansion with an irregular margin which in a short time rests in a depression of liquefied gelatine. In gelatine-slab it forms a button-like growth of a Naples-yellow colour; the growth extends to the margin of the tube and a saucer-like depression is produced; there is very little growth along the stab. On agar it produces a thick Naples-yellow growth. On potato there is an abundant yellow expansion. It is a thin bacillus with only rotatory movement. It stains with Gram.

The following organisms do not liquefy gelatine:

B. Aureus.

Found in water by Adametz. In gelatine plates the surface colonies have a golden yellow centre, the margin, however, is lighter in colour and crinkled; growth is slow and the gelatine is not liquefied. In gelatine-stab the growth on the surface resembles a colony; along the stab there is a fine growth of a golden-yellow colour. On potato a golden-yellow, raised, coarsely granular growth appears. In broth there is a diffused growth with a formation of the same golden-yellow pigment. There is no gas formation in glucose gelatine. Nitrates are reduced to ammonia. It is a fine bacillus of variable length. Movement is feeble, and no spores have been observed.

B. Aurantiacus.

This bacillus was isolated by Frankland from deep wells in the chalk. On gelatine plates it produces bright orange pin-heads. Under the microscope the colonies in the depth are seen to be smooth-rimmed. Growth is slow, and no liquefaction of the

gelatine takes place. In gelatine-tubes a shining orange-coloured expansion forms on the surface, whilst hardly any growth is visible in the depth. On agar there is a bright orange expansion which does not extend much beyond the point of inoculation. In broth there is a slightly orange-coloured deposit, the fluid above is clear and a thin pellicle forms on the surface. On potato it produces a thick and magnificent brilliant red orange pigment, which is, however, restricted to the point of inoculation. It reduces nitrates to nitrites very slightly. It is a short, fat bacillus, forms pairs and short chains. The individual bacilli are motile.

B. Chrysogloia.

This organism was first described by Zopf. It was isolated from polluted water by Zimmermann. It is a thin, very motile bacillus of variable length, and often found in chains. It does not form spores. In gelatine plates the colonies, to the naked eye, appear as small golden yellow drops. Under a low power they are seen to be brownish-yellow, granular, round discs. In gelatine-stab, on the surface, there is a circular yellow-ochre growth, which later shows concentric zones and becomes folded; there is no coloured growth along the stab. In gelatine-streak and on agar a yellow-ochre growth develops. On potato a thick growth gradually appears, which at first has a yellow-ochre colour, but later this changes to an orange-yellow tint. In broth a pellicle forms on the surface and an orange-yellow deposit appears at the bottom. The bacillus grows equally well at blood and room temperatures. It is ærobic and stains slightly with Gram.

B. Subflavus.

Found by Zimmermann in the Chemnitz water supply. It forms yellowish-white dots in the depths of gelatine-plates; on the surface it appears as a yellowish-white shining drop, which forms a flat expansion with an irregular and lobular periphery. Under a low power the surface appears arranged in scales. The gelatine is not liquefied. In gelatine tubes it forms a delicate, greyish-yellow expansion on the surface. On agar it produces a pale yellow growth, which later becomes chrome-yellow or yellow-ochre. It does not grow well on potato, and produces a

faint, loamy yellow colour. It is a thin, slowly motile, small bacillus, grows best at room-temperature, and is not stained by Gram's method.

The Orange-red Water Bacillus.

Described by Adametz-Wichmann. In gelatine plates it grows very slowly, forming orange-red centres, which under a low power are finely granular and brown in colour. The colonies in the depth appear to be colourless. In gelatine tubes it grows slowly on the surface, forming a moist, slimy layer of an orange-red colour. The gelatine is not liquefied. It is a long, thin, non-motile bacillus. Its cultures appear to be identical with those of the B. aurantiacus.

B. Luteum.

Described by List and Adametz. In gelatine plates it forms irregular, slimy, orange-yellow centres which gradually grow out into flat expansions; under a low power they appear to consist of club-shaped, coarsely granular, zooglœa masses, each of which is made up of several pieces. No liquefaction occurs. In gelatine tubes it forms an orange yellow expansion on the surface, but grows slowly in the depth. It coagulates milk. It forms elliptical cells which are not motile.

The B. fulvus described by Zimmermann appears to stand between the liquefying and non-liquefying varieties. It is a small non-motile bacillus. Media are coloured a gamboge-yellow. Gelatine is liquefied after some weeks.

GROUP VII.

This group includes the micro-organisms which produce a violet blue or indigo colour in the nutrient media.

B. Janthinus (Zopf) or B. Violaceus.

This bacillus was first isolated by Zopf; it has also been found by Zimmermann in the Chemnitz water supply and by Proskauer in the Spree. In gelatine plates the colonies at first are milk-white; later, they grow out, and the margin is irregular and thinner than the centre; each colony acquires a violet colour

and the gelatine is slowly liquefied. In gelatine-stab there is a white growth on the surface, which later assumes a violet colour; the gelatine is slowly liquefied; there is very little growth along the line of inoculation. On agar there is a yellowish-white growth, which later acquires a violet colour. On potato there is a marked violet-coloured growth. Milk is not coagulated. Nitrates are rapidly reduced to nitrites. It is a motile, medium sized bacillus, which does not form spores.

B. Violaceus Laurentius.

Isolated by Jordan from the Lawrence. In gelatine plates the colonies in the depth are round and coarsely granular; on coming to the surface they grow out into thin irregular expansions and the gelatine is almost immediately liquefied; a round violet spot appears in the centre surrounded by cloudy, liquefied gelatine. In gelatine-stab the medium is rapidly liquefied along the inoculation line; the liquefied gelatine has a violet colour, and there is a dark violet precipitate at the bottom of the tube. On agar and potato there is an abundant growth of a dark violet colour, which soon becomes jet black. In milk there is a rapid growth, the milk acquires a violet colour and is coagulated. In broth there is only a very poor growth, but in nitrate-broth there is a luxuriant growth with formation of a rich violet colour. It reduces nitrates to nitrites rather slowly. It is a short, slight, very motile bacillus, which does not form spores. It often occurs in pairs and chains.

B. Lividus.

Found by Zimmermann in Chemnitz water. It closely resembles the B. Janthinus; but the bacillus is non-motile and forms spores. The colonies in gelatine plates have an irregular margin and resemble a Liver acinus.

B. Violaceus Berolinensis.

Found rather commonly in Spree water by Plagge and Proskauer and C. Fränkel. The same bacillus was also found by Frankland in the Thames and deep-well water. The colonies enclosed in the gelatine appear as small air-bubbles, which have

an irregular margin, and from the commencement of the rather quick liquefaction they send out thread-like branches; later a violet colour is developed. In gelatine-stab there is liquefaction in the form of a funnel and a violet-coloured deposit. There is a growth in broth and a violet-coloured deposit. On agar there is a deep blue growth. It reduces nitrates. It is a small motile bacillus, often in pairs, and forms spores.

B. Violaceus Lutetiensis.

Isolated by Macé from water. This organism is probably identical with the above. It is a short bacillus, which liquefies gelatine very rapidly.

B. Amethystinus (Eisenberg).

Found by Jolles in well-water (B. membranaceus amethystinus). In gelatine plates the surface colonies resemble typhoid colonies; at first they are colourless; later, they acquire a dark violet colour, and the gelatine is only liquefied in ten to fourteen days. When liquefaction has taken place the colony floats as a large violet pellicle on the liquefied gelatine. In gelatine-stab it forms a yellowish white expansion on the surface, which in ten to fourteen days assumes a violet colour and the gelatine is slowly liquefied. After about four weeks a thick, dark violet pellicle covers the surface of the liquefied gelatine. On agar it forms a yellowish-white growth, which in about ten days assumes a dark violet colour with a metallic lustre. On potato it forms a dirty yellow or olive-green coloured growth. In broth it forms a violet-coloured pellicle on the surface, and the broth itself is coloured brown. It is a small non-motile bacillus, which does not grow at 37° C.

B. Amethystinus Mobilis.

Described by Germano. Its colonies resemble the above by forming violet-coloured membranes resting on slowly liquefied gelatine. It is, however, a large motile bacillus, which produces a brown growth on potato.

B. Cœruleus (Smith).

Found by Smith in river water. It is a thin bacillus, often

in masses, and does not form spores. The colonies are round with a bluish colour and slowly liquefy gelatine. In gelatine-stab the medium is slowly liquefied in a funnel form. On agar it forms a bluish growth. On potato there is a beautiful dark-blue expansion.

B. Cœruleus (Voges).

Described by Voges. It is a small motile bacillus which is decolourised by Gram. It has one polar flagellum, and does not form spores. It will grow at 37° C., and form pigment. The colonies in the depth are small; on the surface they grow out and have a typhoid-like form; later, they become greyish-blue and slowly liquefy the gelatine. In gelatine-stab it grows slowly, and there is no pigment along the stab. In broth a pellicle is formed, and a grey colour appears in the medium. On agar there is a grey-coloured growth. Milk is not coagulated; the creamy layer has a sky-blue colour. On potato there is a greyish-blue growth, which later becomes dark blue in colour.

B. Indigoferus (Voges).

Found in the Kiel water supply. It is a small motile bacillus, which does not form spores. It is not coloured by Gram. There is a slight growth at 37° C., but no pigment is produced at this temperature. The colonies in the depth are small; on the surface, they grow out, are iridescent, and have a distinct blue colour. In gelatine-stab there is a marked blue growth on the surface, but no colour is formed in the stab; the gelatine is not liquefied. In broth there is a thin pellicle of a blue colour. Milk is unchanged; it has a bluish-grey colour on the surface. On agar there is a beautiful dark-blue layer. On potato there is a greenish-blue expansion, looking like a layer of caviar.

B. Indigonaceus (Schneider).

Found by Cläessen in Spree water. It closely resembles the B. indigoferus. The growth is, however, somewhat quicker. In broth there is no pellicle. On potato the growth is deep indigo blue when the medium is acid, and dark green when it is alkaline.

The B. indigoferus described by Zimmermann resembles the above organisms, but is said to form spores.

GROUP VIII.

This group includes the B. albus of Eisenberg, B. ubiquitus of Jordan, B. aquatilis of Lustig, and B. umbilicatus of Zimmermann.

B. Albus.

Eisenberg describes this organism as a small motile bacillus which does not grow at 37° C. In gelatine plates the colonies are round and have a pin-head appearance; the gelatine is not liquefied. In gelatine-stab there is a slow growth; on the surface it forms a pin-head appearance. On agar it produces a milk-white layer. On potato there is a dirty yellowish-white growth limited to the line of inoculation.

I have found in water an organism corresponding to the above description, which also failed to coagulate milk, and did not produce gas in glucose media, indol in peptone-water, nor reduce nitrates.

The B. aquatilis of Lustig resembles the B. albus in most of its cultures, but it reduces nitrates to nitrites.

B. Ubiquitus.

This organism was isolated by Jordan from Lawrence sewage and stated to be abundant everywhere. It is a small, short, plump bacillus closely resembling a micrococcus. It is non-motile, and grows as well at 37° C. as at 22° C. In gelatine plates the colonies at first are small, round, and of a yellowish tinge; in two days they form glistening white projections resembling drops of milk. Under a low power the colonies have a smooth edge and granular contents. In gelatine-stab there is a good growth both on the surface and along the line of inoculation; the whole growth resembles a nail. At first the colour of the growth is a lustrous porcelain white; later, it is a dull brownish-grey. On agar there is a whitish-grey growth having a somewhat metallic lustre. On potato there is a white shining growth limited to the line of inoculation. Milk is quickly coagulated with a strong acid reaction. In broth there

is a diffuse growth with a marked deposit, and in old cultures the formation of a slight pellicle is seen. Nitrates are vigorously reduced.

I have frequently found in water an organism which closely resembled the above. Milk, however, was not coagulated by my cultures.

B. Umbilicatus.

This organism was frequently isolated from Chemnitz water by Zimmermann. It is a short bacillus with rounded ends, occurring singly and in pairs; at times it forms long chains. It has a rotatory movement and does not form spores. Gram's method causes decolorisation of the bacillus. The optimum temperature varies between 18° C. and 30° C. In plate cultures, under a low power, the colonies at first appear as yellow points, which enlarge and become granular. On the surface the colonies show a wart-like appearance, but later they grow out and have an irregular margin. To the naked eye the colonies appear at first as greyish-white drops, which soon acquire an umbilicated centre. In stab-gelatine on the surface there is a thin irregularly-lobed growth; along the line of inoculation there is a good growth, often accompanied by the development of gas bubbles. On agar there is a thin greyish-white growth with an irregular margin. On potato there is a thin, smooth, yellowish-grey growth. Broth becomes opalescent, and a white sediment forms at the bottom of the tube.

GROUP IX.

This group includes a number of organisms which produce a brown colour in nutrient media.

B. Brunneus.

Isolated by Adametz from water. It is a small, thin, non-motile bacillus which forms spores. The colonies in gelatine appear on the surface as small, dirty white drops. The under-part of the colonies is at first white, then grey, and finally brown in colour; the gelatine is not liquefied. In gelatine-stab it forms a slimy growth on the surface, which later becomes

brown in colour; along the line of inoculation there is also a brown growth.

B. Fuscus.

Found by Zimmermann in the Chemnitz water supply. It is a thin non-motile bacillus which varies considerably in length. Sometimes the rods are bent; no spore formation has been observed. It is ærobic and grows best at 30° C. In gelatine plates the deep colonies appear as small brown granular dots; on the surface the colonies form pin-heads, which later become surrounded by a thinner margin, which is often thrown into folds. The centre of the colony has a dark brown colour; the gelatine is not liquefied. In gelatine-stab it produces a projecting growth, which later expands and acquires a chrome-yellow colour. On agar and potato the same chrome-yellow growth is produced.

B. Fuscus Limbatus.

Isolated by Scheibenzuber from bad eggs; also found by Tataroff in water. It is a short motile bacillus, which often forms threads. The colonies in gelatine are brownish lumps; some of them are surrounded by a lighter zone, two or three times as wide as the original colony; the gelatine is not liquefied.

In gelatine-stab there is very little growth on the surface; along the line of inoculation there is a growth from which buds spread out horizontally; the gelatine in the neighbourhood is coloured brown. On agar there is a superficial expansion, and the medium becomes coloured brown. On potato it produces a brown growth.

B. Hydrophilus Fuscus.

This organism was discovered by Sanarelli in a well-water of the University of Siena. It is pathogenic for frogs and other animals. It is a thin, very motile bacillus of variable length. It does not form spores and is not stained by Gram; grows best at 37° C. In gelatine plates the colonies are round and transparent; the gelatine is rapidly liquefied. In gelatine-stab the gelatine is rapidly liquefied in the form of a funnel; in a very short time the whole of the tube is liquefied, and there is

a copious deposit. On agar it forms a thin greyish-blue growth, which later assumes a brown colour; gas bubbles appear in the depth of the agar. There is a diffused growth in broth, and a thin pellicle forms on the surface. On potato there is a light yellow growth, which later becomes dark brown in colour.

GROUP X.

This group includes the micrococci which are commonly found in water.

Micrococcus Agilis.

It is a motile micrococcus of rather large size, and is often found as diplococci, streptococci and tetrads. In gelatine plates the colonies appear as round pinkish-red films, which slowly liquefy the gelatine. In gelatine-stab the same pinkish-red growth appears on the surface and the gelatine is slowly liquefied. On agar and potato there is a pinkish-red growth. In glucose-gelatine there is no gas formation. In broth there is a diffused growth, and a pinkish-red deposit forms at the bottom of the tube. It has no reducing action on nitrates.

The Micrococcus carneus and Micrococcus cinnabareus are allied to the above, but they are not motile and do not liquefy gelatine. The M. carneus produces a flesh-coloured pigment and the M. cinnabareus a pigment the colour of sealing wax.

Micrococcus Flavus Liquefaciens.

This organism forms rather large non-motile cocci, which are sometimes arranged as diplococci and in masses. In gelatine plates, after forty-eight hours, small yellowish colonies are seen, which gradually increase and become surrounded by a circle of liquefaction; under a low power the centre is granular, yellowish-brown in colour, and sends out processes to the periphery like the spokes in a carriage wheel. In gelatine-stab there is slow liquefaction, and on the seventh day a funnel, containing a deposit of yellow pigment, appears in the upper part of the tube. On potato there is a moist growth of a yellow colour. On agar there is a yellowish-coloured growth. In glucose-gelatine there is no gas formation. Milk is unchanged.

Micrococcus Aquatilis.

This organism consists of very small cocci, often arranged in irregular heaps. In gelatine plates the surface colonies are circular, with a narrow homogeneous marginal zone and a darker centre, which strongly resembles a section of a Liver acinus; the colonies in the depth, under a low power, have a yellow colour and resemble a mulberry in appearance. In gelatine-stab and on agar there is a white growth, which is not characteristic.

Micrococcus Luteus.

This organism forms large elliptical cocci. In gelatine plates the surface colonies are raised, sulphur-yellow in colour, and have an irregular margin; the gelatine is not liquefied. In gelatine-stab and on agar and potato the same sulphur-yellow growth appears.

The Micrococcus aurantiacus closely resembles the M. luteus, but the colour produced is orange-yellow instead of sulphur-yellow.

Micrococcus Fervidosus.

Found by Adametz in water. It is a small round coccus, which occurs as diplococci and also in small groups. In gelatine plates the colonies in the depth appear after four or five days as small white dots. Under a low power they are faint yellow in colour and smooth rimmed. The surface colonies are transparent and yellow, with a serrated edge and numerous lobular projections; the gelatine is not liquefied. In gelatine-stab it forms a thin finely-serrated surface expansion; along the stab there is a granular growth. In cane-grape and milk-sugar it develops slowly. On agar it forms a milk-white slimy expansion. On potato it forms a dirty white growth.

Micrococcus Flavus Tardigradus.

Described by Flügge. It forms large round cocci, which at times exhibit characteristic dark poles; usually arranged in heaps. It grows extremely slowly; after six days the deep colonies are still very small, round or oval in shape and dark chrome-yellow in colour. The surface colonies have a smooth

appearance, and project slightly above the gelatine; under a low power the deep colonies are circular, smooth rimmed, and uniformly dark olive-green in colour; the surface colonies at the margin are lighter in colour, viz., more greyish-yellow. The gelatine is not liquefied. In gelatine-stab there is a growth after six or eight days, a row of small yellow colonies being seen along the inoculation line.

Micrococcus Radiatus.

Described by Flügge and found by Adametz in water. It occurs as medium-sized cocci, at times arranged in short chains, more often in small heaps. In gelatine plates it forms in two days large colonies, which have a yellowish-green iridescence. Under a low power the colonies appear granular, sharply outlined, with a slightly irregular margin; at times a row of rays grows out giving the colony a starfish-like appearance. The gelatine at this stage liquefies, and in two more days the starfish rays appear as a delicate and regularly arranged circlet of rays. In two or three days a second circlet of rays may appear, and eventually even a third circlet may be formed. In gelatine-stab horizontal rays pass out from the stab, and above a funnel is formed by slow liquefaction of the gelatine.

Micrococcus Flavus Desidens.

Found by Flügge in air and water. It consists of small cocci, chiefly arranged as diplococci; also found in short chains and as triangles. In two days the colonies in gelatine appear as yellowish white points, which often show on one side a finely granular yellowish-brown projection. The surface colonies have a lighter colour near the margin. After four days the colonies in the depth are little changed; the surface colonies, however, have grown out to a diameter of 5 to 10 mm., and are circular and lobular, of a pale yellow or brownish colour, forming slimy expansions which slowly sink into a flat circular depression formed by gradual liquefaction of the gelatine. In gelatine-stab it forms a confluent, porcelain white, growth in the depth, and a yellowish-brown slimy expansion on the surface, which does not reach the margin of the tube; in about eight days the

gelatine is softened into a thick fluid, in which the surface growth sinks.

Micrococcus Versicolor.

Described by Flügge and found in water by Tils. It consists of small cocci arranged in pairs or masses. In gelatine-plates the colonies in the depth appear after forty-eight hours as round centres which have a yellow colour; on the surface they form large expansions of irregular shape, often four-cornered with lobular projections; they are slimy yellowish-green in colour and the centre is often raised. The gelatine is not liquefied. In gelatine-stab small yellowish round colonies appear in the stab; on the surface there is a mother-of-pearl growth with an irregular frayed edge.

Micrococcus Viticulosus.

Found by Flügge in air and water. It forms oval cocci of variable size arranged in thick zooglœa masses. In gelatine-plates the colonies in the depth form hair-like extensions from the centre of the colony, which unite to produce a delicate network. Under a low power the network is seen to consist of zooglœa masses of cocci arranged side by side. On the surface the colonies form thin rapidly-growing layers, from which fine threads penetrate into the depth of the gelatine. In gelatine streak it forms radial extensions from the line of inoculation, producing an appearance like a feather. In gelatine-stab the same network appears in the stab, which is soon covered up by the thin rapidly growing surface expansion.

Micrococcus Candicans.

Described by Flügge and also by Frankland. It forms rather large, round cocci, which are arranged in irregular heaps. In gelatine-plates the colonies in the depth are white centres; on the surface they are smooth, of a pure milk-white colour, and measure 2 mm. in diameter. Under a low power the deep colonies appear circular, granular, and deep brown in colour; the surface colonies are slightly irregular at the margin, deep brown in the centre but lighter towards the periphery. In gelatine-stab there is a nail-like growth; Frankland states that the gelatine is

slowly liquefied as a long narrow funnel. Flügge does not mention any liquefaction. On agar it produces a thick Chinese white growth (Frankland).

GROUP XI.

This group comprises the Sarcinæ found in water supplies. The most common variety appears to be the Sarcina aurantiaca which produces small round yellow colonies in gelatine-plates; later the colonies appear granular and acquire an orange-gold colour; the gelatine is slowly liquefied. The same orange-gold growth is seen on agar and potato. The Sarcina alba closely resembles the above, but its cultures have a white colour. The Sarcina lutea is very common in air and has been found by Tils in water; the cocci of which it is composed are larger than those of Sarcina aurantiaca, and its cultures have a yellow instead of an orange-gold colour. The cocci in all the Sarcinæ are arranged in twos or fours and in packets, which occupy three dimensions in space.

GROUP XII.

This group includes the various Spirilla which have been found in water. The Vibrio aquatilis of Günther, the Vibrio Finkler Prior, Vibrio Metchnikovi, and the Spirilla isolated from water by Sanarelli, which strongly resemble the Spirillum Choleræ Asiaticæ, will be found fully described under the diagnosis of the Spirillum Choleræ from allied organisms.

The following Spirilla have also been found in water from various sources.

Vibrio Aureus.

Isolated by Weibel. The Spirillum is 1½ times as thick as Koch's Comma spirillum; it produces long and short forms, but the tendency to produce spirals is not marked; it readily undergoes degeneration, is not motile, and does not stain with Gram. The colonies on the surface of gelatine plates are circular, flat expansions of a yellow colour. Under a low power they are smooth-rimmed, circular, granular, and golden-yellow in colour; the gelatine is not liquefied. On gelatine-stab there is a bowl-shaped yellow-ochre growth on the surface, and a yellow-ochre

granular growth along the stab. On agar and potato the same yellow-ochre growth appears.

The Vibrio flavus described by Weibel, only differs from the above in that the colour produced is pale yellow instead of yellow-ochre. The Vibrio flavescens, also described by Weibel, is a variety of the Vibrio aureus; the colour of the colonies is dirty greenish-yellow.

Spirillum Rubrum.

Isolated by Esmarch, and also found in water by Adametz. It is about twice as thick as Koch's Vibrio and in most cultures forms from one to three spirals; in broth, however, it may form twenty to fifty spiral turns. Spore formation is doubtful.

In gelatine plates the colonies grow extremely slowly; in about eight days small red centres are seen, which, under a low power, appear granular with a smooth margin; gradually the colonies assume a wine-red tint; the gelatine is not liquefied. In gelatine-stab there is a growth along the stab consisting of wine-red colonies; on the surface there is no colour produced. On agar it produces a greyish-white growth, which is red in the thickest part of the growth. On potato it forms small, deep red colonies. It renders broth slightly turbid, and a red, deposit appears at the bottom of the tube.

Spirillum Rugula.

Found by Müller in the buccal cavity; also isolated from stagnant water and fæces. It is often found with the B. butyricus and is probably anærobic. It forms long rods, which are sometimes thin and sometimes very thick; it may be simply bent or form flat spiral turns; at times it forms long chains. It has a lively rotatory movement on its long axis. It forms spores at one end of the bacillus and just before the appearance of the spore a swelling is formed, giving the bacillus a comma-like form. In gelatine plates, under anærobic conditions, it forms yellowish-white, ball-shaped discs; the gelatine is liquefied. In gelatine-stab it forms a pin-head growth. On agar at 37° C. it forms a white, slightly-folded growth. On potato at 37° C. it forms a wrinkled growth, which later becomes yellow in colour.

Spirillum Concentricum.

Isolated by Kitasato from putrid blood, and found by Lustig in water. It forms small screw-shaped forms; in broth cultures there may be from five to twenty spiral turns. It is very motile, with a screw-like movement. It does not form spores. In gelatine plates it forms circular discs, composed of concentric rings; the gelatine is not liquefied. In gelatine-stab it grows chiefly on the surface, forming a cloud-like expansion. On agar it forms a diffused growth, which adheres strongly to the medium. On potato there is no growth. In broth there is a diffused growth, and a slimy deposit appears at the bottom of the tube, above which the broth is clear.

The Spirillum volutans is found in stagnant water; it forms long threads with dark granular contents, each thread has 2½ to 3½ turns and has a long cilium at each end; it is motile; at times, however, the threads appear quite motionless. The Spirillum undula is found in stagnant water; it forms long threads, which have 1½–3 turns; there is a bunch of cilia at each end of the bacillus.

The Spirillum serpens is very common in stagnant waters; it forms long threads with 3 or 4 turns; at times the bacilli are joined into long chains. It is very motile.

The Spirillum tenue is found in stagnant water, and forms long thin threads with 1½ screw turns. It is very motile.

CHAPTER VII.

QUALITATIVE ANALYSIS—*continued.*

NITRIFYING AND DENITRIFYING MICRO-ORGANISMS.

THE idea that ammonia in water and soil is converted into nitrates through the influence of micro-organisms was first suggested in 1878 by Schloesing and Muntz. These observers proved that nitrification was caused by bacteria, but they failed to isolate pure cultures of the organisms in question. Warington, Emich, and other workers repeated and confirmed Schloesing and Müntz's observations. In 1886 Heraeus published a very important paper on this subject. He obtained pure cultures of micro-organisms from water and soil and tested their power of nitrifying salts of ammonia. The first twelve cultures investigated showed no nitrifying action; two of them, however, displayed distinct power of reducing nitrates. It appeared probable that the reduction to the state of ammonia only commenced when all the nitrates had been reduced to nitrites, and that when ammonia was also present in conjunction with nitrates the nitrogen from the ammonia would be assimilated before the nitrates were attacked. By adding 50 grammes of garden earth to 500 c.c. of water containing one gramme of ammonium carbonate, powerful oxidation was set up; the fluid appeared clear, but on the surface a thin iridescent membrane was noticed. When plated in gelatine the membrane produced two species of bacteria of special importance. The first species was very common, and to the naked eye the colonies appeared in the depth as small points and on the surface as milk-white, pin-head growths, which, under a low power, resembled smooth, round, yellow scales. The colonies of the second species closely resembled the first, but were more oval in form, and of a darker brown colour. The behaviour of these organisms was tested in

a medium free from organic matter and containing 0·05 gramme potassium phosphate, 0·01 gramme magnesium sulphate, 0·05 gramme calcium chloride, and 1 gramme ammonium carbonate per litre; the same medium was also employed with the addition of 1 gramme of glucose per litre. Growth and oxidation were most marked in the medium free from sugar, in strong contrast to the reducing micro-organisms which worked most powerfully in the medium containing sugar.

Heraeus also examined certain well-known pathogenic and non-pathogenic organisms, but as these did not grow well in media containing only salts and sugar, sterilised urine, diluted 1 in 5, and broth, diluted 1 in 10, were used for the experiments. He found that the M. prodigiosus, B. ramosus, B. anthracis, B. typhosus, Staphylococcus citreus, and the spirilla of Finkler and Miller produced nitrous acid in urine.

Heraeus considered that in nature there existed nitrifying and denitrifying organisms, and whether the oxidation or the reduction process gained the ascendency depended on the supply of nutriment present. If there was plenty of food material, especially organic substances, the denitrification process was active; but if the food supply was poor the oxidising organisms gained the upper hand. When no other source of nitrogen except nitrates existed in the water it seemed possible that the oxidising bacteria might reduce the nitrates in order to obtain food necessary for their growth; the same reduction also appeared to take place when the entrance of air into the solutions was prevented.

Winogradsky considered that the amount of nitrites and nitrates obtained by Heraeus was not sufficiently great to establish beyond question the presence of a true nitrification process. Nitrites and nitrates are constantly present in the air of rooms and are readily absorbed by alkaline fluids; consequently, unless this source of error is avoided and a sufficiently large quantity of an ammonium salt is completely oxidised by the pure cultures under examination, it is impossible to ascribe to them true oxidising powers. These conditions were not fulfilled by the organisms investigated by Heraeus, so it is doubtful whether he really succeeded in isolating true nitrifying bacteria.

P. F. and G. C. Frankland tested Heraeus' statements that M. prodigiosus and B. ramosus possessed nitrifying powers, but failed to obtain any evidence of nitrifying action; on the contrary both organisms were found to be capable of reducing nitric to nitrous acid. The Franklands believed that the indications of nitrous acid which Heraeus obtained on growing these organisms in diluted urine were due to reduction of the small quantity of nitrates almost invariably found in normal urine, and not to any oxidation of the ammonia at all. They also experimented with no less than thirty-three different cultures obtained from air and water, but in every case the results were negative. They found, however, that nitrification was readily induced when a small quantity of ordinary garden soil was introduced into a solution containing potassium phosphate, crystallised magnesium sulphate, fused calcium chloride, ammonium chloride, and carbonate of lime. By passing a few drops of the nitrified fluid through a series of dilutions in distilled water, a fluid was obtained which contained numerous micro-organisms of a very short bacillary form which could not be cultivated on gelatine-peptone. A portion of this fluid induced strong nitrification in a sterile ammoniacal solution. It was noticed that the solutions which had undergone nitrification by the organisms contained in the fluid remained perfectly clear, whilst solutions which underwent nitrification from a mixture of the nitrifying and non-nitrifying bacteria generally exhibited a thin surface film. The pure culture of the nitrifying organism, obtained by the method of fractional dilution, showed only a very short bacillus, about 0·8 μ. long, hardly longer than broad. and exhibiting only vibratory motion. This micro-organism transformed ammoniacal into nitrous nitrogen in amounts which could be quantitatively estimated by the usual chemical means. The formation of nitric nitrogen was not observed in solutions inoculated with a pure culture of the organism in question.

Winogradsky, in 1890, made some very important contributions to our knowledge of nitrifying organisms. In his earliest experiments he employed a medium containing 1 gramme of ammonium sulphate, 1 gramme of potassium phosphate, to the litre of water from Lake Zurich; to 100 c.c. of this fluid, placed in a flask, 0·5 to 1 gramme of basic carbonate of magnesia,

suspended in a little distilled water and sterilised by boiling, was added. A small drop of a recently nitrified liquid placed in a flask produced marked nitrification, and at the expiration of fifteen days all traces of ammonia had disappeared. The oxidation was found to be produced by zooglœæ of bacteria which formed on the surface of the magnesium carbonate at the bottom of the flask. The bacteria which appeared on the surface of the fluid in the flask had no nitrifying action.

In later experiments Winogradsky prepared an absolutely pure sulphate of ammonium and employed calcium carbonate instead of magnesium carbonate. Portions of the zooglœa mass distributed over gelatine plates produced no growth, but the deposit of crystals of calcium carbonate introduced into flasks containing the salt solution produced marked nitrification after the lapse of three weeks, the delay in the oxidation process being caused by the small quantity of the nitrifying organisms placed in the flask. In order to obtain plate cultures of nitrifying organisms Winogradsky employed a silica which he prepared in the following manner: Commercial sodium silicate was diluted with three times its volume of water and 100 c.c. of the dilution added to 50 c.c. of dilute hydrochloric acid, the mixture was then dialysed. The dialyser was kept in running water for twenty-four hours and then in distilled water, frequently changed, for two days. The solution so obtained was placed in a flask plugged with cotton wool and sterilised. The following solution of salts was required to produce gelatinisation: Sulphate of ammonia 0·4 gramme, sulphate of magnesium 0·05 gramme, phosphate of potassium 0·1 gramme, chloride of calcium a trace, carbonate of soda 0·6 to 0·9 gramme, distilled water 100 c.c. The two sulphates with the chloride and the phosphate with the carbonate were dissolved and sterilised separately, and then mixed when cool. Small glass dishes, having a diameter of 5 centimetres, were employed for the preparation of plates. The solution of silica was evaporated to about half its volume, or until two or three drops mixed with one drop of the saline solution produced gelatinisation in about five minutes. The silica solution was then placed in the glass dishes by means of a pipette and one-third to one-half its volume of the salt solution added. Sometimes magnesium carbonate was used instead of

sodium carbonate in the saline solution. This salt rather destroyed the transparency of the medium but drew attention to the colonies which attacked the magnesium carbonate and then seemed to be surrounded by a limpid zone. The colonies in the depth of the jelly appeared to the naked eye as small white points.

Duclaux was the first to suggest that the process of nitrification occurred in two stages, the first being the oxidation of ammonia to nitrites, and the second the conversion of nitrite into nitrate. Warington's experiments led him to entertain the same idea, but he failed to isolate separate nitrous and nitric organisms. Winogradsky, however, succeeded in cultivating the organisms which produced these chemical changes. He divided the organisms which act in the first stage into two classes—*i.e.*, the Nitrosococcus and Nitrosomonas. The Nitrosococcus was found in earth obtained from Europe, Africa, and Japan; it appeared as a non-motile spherical cell, about 3 μ in diameter. The Nitrosomonas was isolated from earth found in Java; it was motile and had a short ellipsoidal form. The organism of the second stage, or Nitrobacter, was found to be a small non-motile rod, about 0·5 m. long and 0·25 μ broad. It grew readily in the saline fluid containing potassium nitrate instead of an ammonium salt, and also on silica jelly plates. Burri and Stutzer have also isolated a nitrate producing organism from earth by means of silica jelly plates. It is larger than Winogradsky's Nitrobacter, and is motile. It can be cultivated in broth and gelatine, but then loses its nitrifying action. Winogradsky found that, as a rule, the oxidation of nitrates did not commence until all the ammonia had disappeared. Occasionally oxidation of ammonia and nitrites were found at the same time, but the oxidation of the nitrites always commenced later than the oxidation of the ammonia. The nitric ferment had no action on ammonia, and in the absence of the nitrous ferment nitrification did not take place. Nitrification in earth was then compared with the same process in fluid media. It was found that normally only nitrates were produced in soil, nitrites being rarely detected. It was also noticed that the oxidation of nitrites took place immediately after their formation, even in the presence of large quantities of ammonia.

The differences observed appear to be due to the great porosity of earth; as compared with a fluid the surfaces exposed in soil are enormous, and the nitrate ferment is never repressed by a want of oxygen unless the ground is saturated with moisture or dosed with too much ammonia.

The power of reducing nitrates to nitrites and ammonia, the converse of the oxidation processes just considered, appears to be common to many bacteria. The B. fluorescens liquefaciens, B. mycoides, and the varieties of B. arborescens and B. nubilus, which I have isolated from water, reduce nitrates very vigorously. Special denitrifying bacteria, however, have been isolated by Stutzer, Burri, and Jensen.

Bacillus Denitrificans I.

This organism was isolated by Stutzer and Burri from horses' fæces. It is a small motile bacillus, which does not form spores. In gelatine plates there is a limited growth on the surface, and small round colonies appear in the depth; the gelatine is not liquefied. Stroked over a gelatine-slope it produces a dull bluish grey layer. On agar plates the colonies are large, round, and very thin. On an agar slope there is a thin grey layer. On potato, especially at blood temperature, there is a limited reddish-brown growth. In symbiosis with B. coli or B. typhosus it reduces nitrates very vigorously.

Bacillus Denitrificans II.

This bacillus was isolated by Stutzer and Burri from straw. It is a small motile bacillus, which does not form spores, and grows best at blood temperature. The surface colonies in gelatine plates are very characteristic; they appear as large, coarse growths, marked with radial ridges, which branch as they pass outwards and then bend in and interlace at the margin; the gelatine is not liquefied. On agar a similar characteristic growth is seen. On potato there is a slimy, reddish-coloured growth. In broth a pellicle forms on the surface. This organism acts in symbiosis with B. coli, in the same manner as Bacillus I. According to Weissenberg, the B. coli reduces the nitrates to nitrites, which are then acted upon by the denitrifying bacilli.

Bacillus Denitrificans (Jensen).

A culture of this organism obtained from Kral's laboratory gave the following cultural characteristics: The surface colonies in gelatine plates were very thin and translucent, with a foliated margin and a dark granular centre. In broth there was a diffused growth and a slight film on the surface. On potato a yellowish-brown rather slimy looking growth appeared. In glucose and lactose media there was no gas formation. Milk remained unchanged in appearance. On an agar-slope there was a thick growth along the line of inoculation surrounded by a thin transparent film with a crenated margin. In stab-gelatine there was a thick white growth on the surface and along the stab. In peptone solution no indol reaction was obtained on adding a nitrite and a little pure sulphuric acid. In nitrate-broth there was a good growth and the nitrates were found to be reduced; the reduction process was found to be very marked when the bacillus was grown in conjunction with B. coli. Microscopically the bacillus resembled B. subtilis in its movement, but was smaller in size. It did not stain Gram; no growth was obtained under anærobic conditions.

CHAPTER VIII.

QUALITATIVE ANALYSIS—*continued*.

CLASS II.

This class contains the micro-organisms which are common in sewage, but are rarely found in pure water supplies. Many of them are derived from the intestinal canal of human beings and animals; others are associated with putrefactive processes. The recognition and proper interpretation of these organisms is a matter of the first importance. It often happens during the examination of water supplies suspected to have caused zymotic disease, that chemical analysis, enumeration of micro-organisms, and search for pathogenic organisms, all fail to give sufficient data upon which a reliable opinion may be based as to the purity of the supply. Under these conditions the detection of the organisms in this class will either enable a proper decision to be made, or suggest a rigid examination of the supply when possibly unsuspected sources of contamination may be detected.

Bacillus Coli Communis.

The most important organism in the sewage class is the B. coli communis, often called Escherich's bacillus, after the name of its discoverer. Studies of sewage and normal fæces, however, have shown that the B. coli usually described in the text-books is the type of a large group containing many varieties; the cultural characteristics, morphology, and serum reactions of the typical bacillus will be first considered, after which the more important varieties will be compared with the type. The typical B. coli communis is usually described as follows:

Gelatine Plates.—In the *depth* the colonies are circular or

oval in shape and dark-brown in colour. On the *surface* after twenty-four to forty-eight hours they appear as thin bluish-grey transparent films with an irregular margin. Under a low power they sometimes resemble flakes of glass with faint surface markings, but more commonly they appear somewhat opaque with a faint yellowish-brown colour, and show tracings of ridges and depressions running from the centre to the periphery; at times wavy lines parallel to the margin are seen. The gelatine is not liquefied.

Gelatine-stab.—On the surface an expansion appears like the surface colonies just described, only whiter and thicker; along the stab there is a white growth.

Gelatine-streak.—A broad white growth with a crenate margin.

Agar.—A thick white growth, not characteristic.

Potato.—As a rule a thick brownish-yellow layer is produced; occasionally the growth is an almost transparent film like that of B. typhosus.

Milk.—Generally coagulated after twenty-four to forty-eight hours incubation at 37° C.

Litmus-whey.—Rendered acid; after seven days incubation at 37° C., the amount of acid produced varies extremely, it always requires more than 6 per cent. $\frac{N}{10}$ alkali and usually from 20 to 40 per cent. of $\frac{N}{10}$ alkali is required, to neutralise it.

Glucose-gelatine "shake" (22° C.) } Abundant gas formation
Glucose-agar-stab (37° C.) } in 24 hours.

Witte's Peptone and Salt solution.—After 7 days incubation at 37° C. indol reaction is given on the addition of 1 c.c. of potassium nitrite solution (0·02 per cent.) and a few drops of pure sulphuric acid; very often the reaction is obtained after 48 hours incubation.

Broth.—Rendered turbid in 24 hours at 37° C.; later, a slight film usually forms on the surface.

Neutral-red Glucose-agar.—Red colour changed to yellow and greenish fluorescence produced.

Nitrate-broth (0·1 % KNO_3, 5 % broth).—Nitrates reduced to nitrites.

Gelatine (25 % at 37° C.).—A distinct thick white film appears on the surface.

Proskauer and Capaldi, No. I.—After twenty-four hours incubation at 37° C. there is a growth, and the medium is rendered strongly acid.

Proskauer and Capaldi, No. II.—After twenty-four hours incubation at 37° C. there is a growth, and the reaction of the medium is either unchanged or rendered slightly alkaline.

Glucose- and Lactose-broth.—After twenty-four hours incubation at 37° C. there is marked growth, and bubbles of gas form on the surface.

Microscopical Characters.—It usually appears as a very short bacillus, often strongly resembling a coccus; but longer forms are frequently seen especially in old cultures. As regards motility it varies considerably; sometimes it is almost motionless, at other times it appears to be as motile as the B. typhosus. As a rule it possesses from one to three flagella, which are very brittle and difficult to stain; occasionally eight to twelve long wavy flagella are present. It does not form spores.

Staining Reactions.—Stains readily with basic dyes; it is decolourised by Gram's method.

Reactions to Specific Sera.—It is not agglutinated by highly dilute anti-typhoid serum.

The above description gives the chief characteristics of the typical B. coli; but when studies are made of sewage and stools derived from human beings many varieties are found which do not conform to the type. The most common varieties are the following :

(1) The colonies exactly resemble the type; gas is produced in sugar media and indol formed in peptone; but milk is not coagulated, though the amount of acid produced in litmus-whey may be considerable.

(2) The colonies are typical; gas is produced in sugar media; milk is coagulated ; but indol is not formed in peptone solution.

(3) The colonies are typical; gas is produced in sugar media; but indol is not formed and milk is not coagulated.

(4) The colonies are typical; but there is no coagulation of milk, production of gas, nor formation of indol.

(5) The colonies are typical; indol is produced in peptone solution; but there is no formation of gas in glucose media nor coagulation of milk.

Gilbert and Lion investigated the varieties of B. coli found in the stools of sixty healthy men; they divided the bacilli into the following groups:

A. Motile bacilli:

(a) Very motile—cultural reactions; milk coagulated, milk sugar fermented, indol reaction sometimes present.

(b) Slightly motile—cultural reactions: (α) milk coagulated, milk sugar fermented, indol reaction sometimes present; (β) milk sugar not fermented, milk not coagulated, indol reaction present.

B. Non-motile bacilli:

(a) Milk sugar fermented, milk coagulated.

Sub-variety: (α) colonies thick and opaque, indol sometimes present.

Sub-variety: (β) colonies thin and transparent, indol sometimes present.

(b) Milk sugar not fermented, milk not coagulated, no indol produced.

Gordon has made a careful study of the varieties of B. coli; he investigated over one hundred organisms, and classified them according to (1) their reactions, and (2) their flagella averages, and in this way differentiated sixteen varieties. Two other groups of organisms were also investigated, which resembled B. coli in morphology; but the members of the first group produced alkali in litmus-tinted media, and the members of the second group slowly liquefied gelatine. The following table taken from Gordon's paper in the Journal of Pathology and Bacteriology gives the reactions of the different varieties of B. coli:

Name.	Source.	Ordinary gelatine shake.	Glucose gelatine shake.	Milk 20° C.	Milk 37° C.	Broth.	Potato.	Reaction.	Flagella average.	Lique-faction.	No. of speci-mens.
B. coli com.	Normal stool	Gas 1 day	Gas 1 day	Clot 13 days	Clot 1 day	Indol	Brownish-yellow	Acid	3		24
Sub-variety *a*	,,	,,	,,	Clot 23 days	,,	,,	,,	,,	1		
,, *b*	Sewage	,,	,,	Clot 11 days	Clot 20 days	,,	,,	,,	8		
Variety 1	Jaundice stool	,,	,,	No clot	Clot 2 days	,,	,,	,,	3		21
,, 2	Mussel	,,	,,	Clot 9 days	No clot	,,	,,	,,	1		1
,, 3	Water	,,	,,	Clot 15 days	Clot 2 days	No indol	Colourless growth	,,	10		12
,, 4	,,	,,	,,	No clot	Clot 9 days	,,	Brownish-yellow	,,	3		9
,, 5	,,	,,	,,	Clot 18 days	No clot	,,	Yellow	,,	4		2
,, 6	,,	,,	,,	No clot	,,	,,	Brownish-yellow	,,	6		3
,, 7	,,	,,	,,	,,	,,	,,	,,	,,	3		8
,, 8	Sewage	Gas 2 days	No gas	Clot 27 days	,,	Indol	,,	,,	3		1
,, 9	,,	,,	Gas 2 days	No clot	,,	No indol	,,	,,	4		1
,, 10	Milk	No gas	Gas 1 day	Clot 14 days	Clot 2 days	Indol	Colourless growth	,,	4		1
,, 11	Normal stool	,,	No gas	Clot 21 days	Clot 4 days	,,	Brownish-yellow	,,	3		5
,, 12	Sewage	,,	,,	No clot	Clot 7 days	,,	,,	,,	4		5
,, 13	,,	,,	,,	Clot 14 days	Clot 2 days	No indol	,,	,,	1		2
,, 14	Normal stool	,,	,,	No clot	Clot 5 days	,,	,,	,,	1		1
,, 15	Urine	,,	,,	,,	No clot	Indol	,,	,,	3		2
,, 16	Sewage	,,	,,	,,	,,	No indol	,,	,,	3		1

Name.	Source.	Ordinary gelatine shake.	Glucose gelatine shake.	Milk 20° C.	Milk 37° C.	Broth.	Potato.	Reaction.	Flagella.	Lique-faction.
Alkali producers										
1	Sewage	Gas 1 day	Gas 1 day	No clot	No clot	Indol	Colourless growth	Alkali	8–10	
2	Milk	No gas	No gas	,,	,,	,,	Brownish growth	,,	1	
3	Sewage	,,	,,	,,	,,	No indol	,,	,,	1	
Slowly liquefying										
I. 1	Milk	Gas 1 day	Gas 1 day	Clot 10 days	Clot 2 days	Indol	Brown growth	Acid	1	36th day
2	Oyster	No gas	No gas	Clot 19 days	Clot 5 days	No indol	,,	,,	10	24th day
3	,,	,,	,,	Clot 21 days	Clot 3 days	Indol	,,	,,	1	17th day
4	,,	,,	,,	No clot	Clot 2 days	No indol	,,	,,	8	6th day
II. 1	Sewage	,,	,,	,,	Clot 7 days	Indol	Colourless growth	Alkali	8	22nd day
2	Polluted water	,,	,,	,,	Clot 2 days	No indol	,,	,,	3	20th day
III. 1	Water, well	Gas 1 day	Gas 1 day	Clot 7 days	Clot 6 days	,,	Brown growth	Acid	4	12th day

With regard to the organisms which slowly liquefied gelatine, Gordon found that sub-cultures varied in the date of commencement of the liquefaction; some gaining, others losing, power of producing liquefaction.

During the present year I have carefully studied the cultural reactions of 150 varieties of B. coli isolated partly from normal and partly from typhoid stools. Between 50 and 60 per cent of these cultures produced gas in sugar media, soured milk, formed indol after seven days incubation at 37° C. in peptone, and gave a coloured growth on potato. About 30 per cent produced gas in sugar media, but failed to coagulate milk and form indol. The remaining cultures failed in only one of the typical reactions; the coagulation of milk was the reaction most frequently absent. Some of the varieties of B. coli isolated from typhoid stools were very peculiar. The colonies up to forty-eight hours incubation were typical; but later on wedge-shaped processes grew out from the margin of the colony so that it slowly acquired a rosette-like appearance, and the gelatine slowly liquefied. When stabbed into gelatine tuft-like processes grew out from the growth along the line of inoculation. All these colonies produced gas in sugar media, soured milk, and gave very marked indol reaction; in many respects they resembled the Proteus cloacinus, described by Laws and Andrewes as one of the commonest organisms in sewage.

A consideration of Gordon's table suggests that possibly the varieties may be derived from the typical B. coli as a result of unfavourable surroundings, want of food, &c. Some support to this view is derived from observing the changes produced in B. coli after prolonged immersion in sterile sewage. I found that a typical B. coli, freshly isolated from a normal stool, after forty-two days immersion in sewage, gradually lost its power of producing indol, and became much less resistant to the action of carbolic acid. But agglutination experiments made with the serum of animals immunised against B. coli, point strongly to each race being quite distinct. I have found that cultures of B. coli, derived from the same source and quite indistinguishable by cultural tests, produced sera which were quite distinct, each serum reacting to its own bacillus, but failing to influence in any way the other cultures. Jatta's

experiments on the action of coli-serum on different races of B. coli showed that the serum always acted in the highest dilution on the variety used to inoculate the animal. Very often the serum had no action on other varieties ; but when it did act the dilution was much lower than that which agglutinated the bacillus used for the preparation of the serum. He also showed that in the stools of the same person several varieties of B. coli might appear.

Lee Smith showed that all the B. coli cultures isolated from the stools of a child "at the breast" were agglutinated by a serum made with any one of the cultures. The same serum, however, failed to agglutinate cultures of B. coli, isolated from the stools of other children fed under the same conditions. The action of anti-typhoid serum on B. coli will be fully considered when the value of the agglutination test for the diagnosis of the B. typhosus is discussed. Here it will be sufficient to state that though many varieties of B. coli are quite uninfluenced by anti-typhoid serum, still races do occur which are agglutinated by anti-typhoid serum in low dilutions. The virulence of the B. coli does not appear to have any relation to the action of the anti-typhoid serum upon it. Jatta passed cultures of the B. coli through guinea-pigs, so as to increase their virulence ; the test serum, however, acted on the organisms in just the same manner after the treatment as before it. It has also been found that a coli-serum from an immunised animal sometimes causes agglutination of the B. typhosus, but the dilution which produces the agglutination is always much lower than that which acts on the culture of B. coli used to produce the serum. It also appears that those varieties of B. coli, which are influenced by anti-typhoid serum, produce, when injected into animals, sera which act on the B. typhosus.

The Bacterium lactis aërogenes, the Bacillus cavicida, the Bacterium tholœidum, and the Bacillus Neapolitanus, isolated from fæces, appear to be closely allied to the B. coli.

The Bacterium Lactis Aërogenes was isolated by Escherich from the intestinal tract of animals and people fed on milk, and especially from suckling children. It was found once in raw milk. In gelatine plates the surface colonies are convex,

isodiametric, and porcelain white in colour; the colonies in the depth are yellowish, round, granular centres. In gelatine-stab it grows well along the line of inoculation, and on the surface forms a nail-head shaped expansion; the gelatine is not liquefied. On potato it produces white raised colonies, permeated with gas bubbles, which often run together and form a cream-like layer. It produces gas in sugar media, and coagulates milk.

Escherich does not mention the formation of indol; but a pure culture from Kral's laboratory, which I examined, gave a marked indol reaction in Witte's peptone. Microscopically it appears as a non-motile short rod with rounded ends. The most characteristic growth of this organism is the creamy layer containing gas bubbles which appears on potato. The cultures which I examined did not always show the convex isodiametric colonies; very often the surface colonies grew out with a thin irregular margin like the typical B. coli.

Bacillus Cavicida.—This organism was isolated by Brieger from fæces. Its colonies on gelatine plates are said to be very characteristic, showing a white growth, with concentric rings, like the arrangement of the scales on a tortoise-shell. On potato it forms a yellow slimy layer, and in sugar media produces propionic acid. It does not liquefy gelatine. Microscopically it appears as short rods, which do not form spores. A culture of this bacillus from Kral's laboratory produced gas in glucose-gelatine shake, and traces of indol in peptone solution. It did not, however, coagulate milk. It appeared to correspond to one of the varieties of B. coli.

Bacterium Tholœideum was isolated by Gessner from the intestinal canal of healthy men, and strongly resembles the B. lactis aërogenes. In gelatine plates the surface colonies appear as nail-headed, slimy, opaque growths; later, they form large expansions, lose their slimy character, and show a grey centre surrounded by concentric rings. Under a lower power they are circular, with a colourless, bright shining margin; towards the centre fine radiating brownish lines are seen. The deep colonies are whetstone-shaped, of a yellowish-white colour. Under a low power they appear at first olive-green in colour; later, they become dark grey-green, and resemble date-stones in

shape. Gelatine-stab culture shows on the surface a moist shining convex growth, and along the line of inoculation a yellowish-white thick band, with closely packed, small, round extensions having thick button-shaped ends. On potato it forms a thick, yellowish expansion, and on one occasion bubbles of gas were seen in a culture four days old. It does not liquefy gelatine, and microscopically it appears as a short rod with rounded ends.

B. Neapolitanus.—This organism was discovered by Emmerich in the blood and organs of cholera patients in Naples. Weisser found it in the normal stools of human beings and in air. On the surface it forms thin round colonies, yellow in the centre but whiter at the margin; in the depth the colonies are whetstone in shape, and have a yellowish-brown colour. It does not liquefy gelatine. In gelatine-stab culture it grows like B. typhosus. On potato it forms a slimy, yellowish-brown layer. Gas production has not been observed. Microscopically it is about the same size as B. typhosus, but it is not motile. It does not form spores, and is decolorised by Gram.

Deeleman has described four coliform organisms, isolated from urine and fæces, which are important from a diagnostic point of view. The chief reactions of these organisms are as follows:

B. Coloides Virescens.—In gelatine plates the colonies, under a low power, show a leaf-like picture, with irregular, well-defined edges. On gelatine-streak there is a greyish-white growth, which has a greenish iridescence; the gelatine is not liquefied. On agar plates there is a diffuse veil-like growth without any markings, which later shows a feeble greenish iridescence. On potato there is a yellow, slightly raised growth, limited to the line of inoculation. In broth there is a diffused growth with formation of a greenish iridescent pellicle and a moderate deposit. It ferments grape-sugar, cane-sugar, and milk-sugar. Milk is coagulated in twenty-four hours. In 10 c.c. of litmus-whey, after ten days at 37° C., it produces from 13·6 to 14·3 c.c. of N acid. It gives no indol reaction. It is pathogenic for mice and guinea-pigs. It is not stained by Gram's method, and microscopically appears as a small motile bacillus.

B. Coloides Rubescens.—In gelatine plates under a low power the colonies show a leaf-like pattern. On gelatine-stroke culture there is a greyish-white growth with a reddish iridescence; the gelatine is not liquefied. On agar it forms a diffused veil-like growth, which shows a reddish iridescence. On potato there is a restricted yellow growth. In broth it produces a diffused growth with a reddish pellicle. It ferments grape-sugar and milk-sugar, but not cane-sugar; milk is completely coagulated in twenty-four hours. In 10 c.c. litmus-whey, after ten days at 37° C., it forms from 11·4 to 10 c.c. N acid. It gives a strong indol reaction. It is decolorised by Gram, and microscopically appears as a small motile bacillus.

B. Vesicæ.—In gelatine plates the colonies have a net-like appearance, with a very delicate system of ridges and furrows. On gelatine-streak there is a dull-grey opaque granular growth. In broth there is a diffused growth, with a delicate pellicle on the surface. It does not ferment grape-sugar, cane-sugar, nor milk-sugar. It coagulates milk and produces indol. In 10 c.c. litmus-whey, after ten days at 37° C., it produces 7·5 to 8·75 c. c. of N acid. It is a small motile bacillus, which does not strain with Gram.

B. Fæcalis Alkaligenes, variety.—In gelatine plates it produces colonies like the B. vesicæ. On gelatine-streak culture there is a smooth, greyish-white growth, and the gelatine is not liquefied. On agar it produces a greyish-white growth. On potato it forms a circumscribed, slightly raised, yellowish-brown growth; on the fifth day there is gas production, and later the potato substratum acquires a brown colour. A diffused growth occurs in broth, and a tough pellicle is formed on the surface. Cane-sugar and grape-sugar are fermented, but milk-sugar remains unchanged. Milk is not coagulated, and acquires an alkaline reaction. In 10 c.c. of litmus-whey, 8·8 to 11·0 c.c. of N alkali are produced after ten days incubation at 37° C. Indol is not formed. Microscopically it appears as a small motile bacillus. It is decolorised by Gram.

Methods Employed for the Isolation of the B. coli from Water Supplies.

The various methods which are employed for the isolation of

B. coli are intended either to repress the growth of the ordinary water-bacteria, so as to enable the B. coli to develop without the chance of being crowded out by these microbes, or tc provide a medium which will be so favourable to the growth of B. coli that it will develop in advance of the competing organisms.

The methods which are commonly used are as follows:

(1) Parietti's method. In this plan a series of broth tubes are taken and inoculated with gradually increasing quantities of the following mixture:

Carbolic acid	5 grammes
Hydrochloric acid (pure)	4 grammes
Distilled water	100 c.c.

As a result of the admixture the broth tubes contain from 0·05 to 0·3 per cent. of carbolic acid. Many bacteriologists omit the hydrochloric acid from the solution, and do not use the tubes which contain more than from 0·15 to 0·2 per cent. of carbolic acid. When the B. typhosus is supposed to be present with B. coli it is wise to employ only the tubes which contain 0·05 per cent. of carbolic acid; the reason for this will be explained under the section devoted to B. typhosus. The broth tubes are then seeded with varying quantities of water, and incubated at 37° C. All the tubes which show growth after one to three days incubation at 37° C. are then plated out in ordinary or carbolic acid (0·05 per cent. gelatine, and the colonies which develop are "fished" and submitted to the various tests described under B. coli. In my own experiments I found that B. coli, freshly isolated from stools, would grow in 0·2 per cent. carbol-broth but if the bacillus were allowed to remain in sterile sewage for six weeks it would not grow in more than 0·1 per cent. carbol-broth.

(2) Vincent suggested cultivating the seeded broth tubes at 42° C., at which temperature the B. coli will grow, but water organisms are either unable to develop or do so very feebly.

(3) Pakes recommends that the water should be cultivated under anærobic conditions (Buchner's tube or hydrogen gas) in glucose-formate broth (2 per cent. glucose, 0·4 per cent. sodium formate) at 42° C.

(4) In the investigations of the State Board of Health, Massachusetts, the method employed in testing waters for B. coli varied slightly with the water under examination. For waters in which B. coli was very rarely found, a qualitative test was used; while, for waters generally containing that organism in some numbers, a quantitative method was used.

The qualitative test consists in inoculating a Smith's fermentation tube (a bent tube with one end closed), containing glucose-bouillon, with one cubic centimetre of the water. This tube is incubated at 38° C., and if after twelve hours growth gas has collected in the closed arm the tube is taken out and species tests made. The absence of gas is considered final, there being no B. coli in the water. In order to make the species tests, some of the bouillon from the fermented tube is taken out and diluted with sterile water; a portion is then plated on lactose-litmus-agar. This plate is grown for twelve hours at 38° C.; if at the end of this time any red colonies are found on the plate a few of these are fished, planted out on agar-slopes, and grown for twelve hours as before. If the culture resembles B. coli it is then sub-cultured in milk, nitrate solution, Dunham's solution (1 per cent. salt and 1 per cent. Witte's peptone), and Smith's solution (meat bouillon containing 2 per cent. glucose and 1 per cent. peptone); a gelatine-stab is also made. The Smith tubes, nitrate solution and milk, are grown for twelve hours at 38° C. The Dunham's solution is grown at the same temperature for three days, when it is tested for indol in the usual manner. The gelatine must be grown at 20° C. for at least seven days, as some species of bacteria resembling B. coli liquefy gelatine very slowly.

In quantitative work, when dealing with waters in which B. coli is known to be present, the preliminary test in Smith's solution is omitted. One cubic centimetre of the water is plated direct on lactose-litmus-agar, and the plates grown for twelve hours at 38° C. At the end of this time the plates are taken out and the number of red colonies counted. The red colonies are fished and sub-cultured as in the qualitative method. The qualitative method is considered the more delicate, as duplicate tests often showed B. coli present by the gas test but absent by the plate method. This is probably due

to the influence of the slightly alkaline media, and to the fact that better development is always obtained in fluid than in solid media. The State Board of Health points out that "the accuracy of these tests depends largely on attention to details, time of growth, temperature, reaction, and composition of media. B. coli will develop well at a temperature of 38° to 40° C. in from eight to twelve hours. The thermal death point of most species of water-bacteria lies below that temperature, and some species, which would grow, require more time, usually thirty-six to forty-eight hours, for development." According to the Board the cultural characteristics of the Colon bacillus are as follows:

Agar.—On the surface of glycerine-agar it produces a luxuriant, smooth, moist, white growth, which is never stringy to the needle.

Gelatine.—It grows well in nutrient gelatine, without liquefying that medium.

Lactose-litmus-agar.—In neutral agar, containing milk-sugar and blue litmus, it grows rapidly, producing acid and turning the litmus red around the colonies.

Milk.—Grown in milk, acid is produced and the milk is quickly coagulated.

Smith's Solution.—In bouillon containing glucose fermentation at once takes place; when the medium is placed in a bent tube with one arm closed the gas is collected in the closed arm of the tube.

Nitrate Solution.—In nutrient solution, containing small amounts of potassium nitrate, the nitrate is quickly and completely reduced to nitrite.

Indol.—In solutions containing small amounts of peptone and salt, or in bouillon free from carbo-hydrates and containing peptone and salt, indol is produced. "A culture which gives characteristic reactions with all of the above media belongs to the Colon group undoubtedly."

(5) Elsner and Holz recommended a medium consisting of faintly acid potato gelatine for the differentiation of B. coli and B. typhosus, and stated that water-bacteria did not grow well on it. Further experience of this medium soon showed that other bacteria were able to develop almost as luxuriantly as B. coli. Rémy's modification of the potato medium has a constant

chemical composition, and B. coli forms very characteristic colonies in it.

The methods above mentioned will be found discussed in greater detail in the section devoted to the B. typhosus. My own experience has shown that if a pure water supply, *i.e.*, one containing few water-bacteria, has been *recently* polluted with *fresh* sewage, it is comparatively easy to isolate the B. coli by any of the methods described. If, however, the water contains a large number of water-bacteria and the pollution is not recent or has been caused by old sewage, it is sometimes by no means easy to isolate the B. coli from the supply. The B. coli does not live indefinitely in water; the numbers, as a rule, diminish rapidly unless considerable quantities of nitrates are present. Consequently, if some time has elapsed since the pollution, it may be necessary to examine considerable quantities of the water in order to isolate the bacillus; in other words, the water has to be concentrated by pumping one or two litres through a Berkefeld bougie, and the deposit on the candle, after diffusion in 10 c.c. of sterile water, must then be examined. But under these conditions the water-bacteria are present in enormous numbers, and I have found that carbolised media are quite unable to sufficiently restrain the growth of these organisms unless about 0.15 to 0.2 per cent. of carbolic acid is used: but if this amount is employed there is always a chance that the B. coli, enfeebled by existence in sewage, may not be able to develop. Under these conditions, I have obtained the best results with Pakes' method of cultivation, which combines anærobic conditions with incubation at 42° C. But even with this procedure many organisms may be found besides B. coli, so that it is necessary to make several dilutions, *i.e.*, at least three or four gelatine plates, from the glucose-formate broth tube after twenty-four hours incubation at 42° C. It is also very useful to plate the broth in litmus-lactose-agar and then subculture the colonies which turn the blue litmus red.

In order to deal with larger quantities of water, Abba suggested the following solution:

Lactose	200 grammes.
Peptone	100 „
Sodium chloride	50 „
Water	1,000 c.c.

This is heated in the steam steriliser for half an hour, then filtered and again sterilised. A litre of the suspected water is taken and 100 c.c. of this solution added to it, together with 0·5 c.c. of a 1 per cent. alcoholic solution of phenolphthalein, and sufficient (about 2 to 3 c.c.) of a cold saturated solution of sodium carbonate to render the mixture a permanent pink colour. The mixture is divided into five or six Erlenmeyer flasks and incubated at 37° C. At the same time sterile agar plates are prepared and placed in the incubator with the flasks. After twelve, sixteen, or twenty-four hours the fluid in one or more of the flasks will be decolorised if the Colon bacillus be present, and from the upper layer of the fluid a small loopful is removed and rubbed over one of the sterile agar plates; the process is repeated for the other colourless flasks, the plates incubated, and sub-cultures made from likely colonies.

The Value of the B. coli communis as a sign of Sewage Contamination of a Water Supply.

The interpretation which is to be placed on the discovery of B. coli in a water supply has been much debated. Kruse, in 1894, stated that the "Bacterium coli is in no way characteristic of the fæces of men and animals. Such bacteria are found everywhere—in the air, in earth, and in water from the most varied sources." Freudenreich and Miquel believe that B. coli can be found in any water if a sufficient quantity is cultivated. Levy and Bruns, however, considered that the B. coli found in pure waters differed from the B. coli derived from fæces in the absence of pathogenicity. The chief arguments, therefore, which have been advanced against the acceptance of B. coli as an indication of sewage contamination are as follows: (1) It is abundant everywhere; (2) it multiplies readily outside the animal body; (3) it occurs in the excreta of mammals and birds as well as in the intestine of man. If B. coli be abundant everywhere, it is strange that Houston only found it in four out of twenty-one samples of soil derived from different sources, viz., from orchards, gardens, moorland, pasture, sand-pits, sea-shore, fields, &c. It is true that nine of the samples yielded in phenol-gelatine colonies which resembled those of B. coli; but six of these failed to respond to the gelatine shake-culture test, and

the remaining three failed in one or more of the reactions considered characteristic of the typical B. coli. The statement that B. coli exists abundantly in all waters and soils appears to be based on a very elastic interpretation of the characteristics of B. coli. That this is a probable explanation is rendered manifest by the experiments just published by Weissenfeld. This observer examined water from thirty good wells, and found B. coli in 1 c.c. of seven of the specimens but only in one litre of the remaining samples. He, however, found B. coli in 1 c.c. of the water from twenty-four out of twenty-six bad wells. Weissenfeld gives the cultural characteristics of B. coli as follows:—Superficial colonies on gelatine like a vine-leaf; produces gas in sugar-agar-stab; more or less motile; often not motile; not stained by Gram's method. He considers the souring of milk and the production of indol to be of no value as distinguishing characteristics. These cultural reactions, in my opinion, are not sufficient to differentiate the B. coli from a large class of coliform organisms which are found in very many waters from perfectly unpolluted sources. Whether these coliform organisms are derived from a typical B. coli, which by prolonged immersion in pure water has lost the power of souring milk and producing indol, it is impossible to say; but the important point is that these organisms are found alone and without any admixture of the typical forms of B. coli in pure waters. Now when pollution of a water is produced by sewage, probably at least half the cultures of B. coli present will show all the typical characteristics of this organism, the remaining cultures presenting the varying reactions of the atypical members of this group. In my experience pollution by sewage is never characterised by the presence solely of the variety, which only produces gas in sugar media. The other typical members of the group must also be present to warrant the suspicion of sewage contamination.

The statement that B. coli multiplies outside the animal body is only true under certain very favourable conditions. There is every reason to believe that when the physical conditions are unfavourable its growth is inhibited, and it soon loses its vitality and dies out. Also it is not justifiable to assume that the excreta of animals are harmless to man, and in any case a water

so polluted could not be considered desirable for drinking purposes.

Lastly, many English bacteriologists will not accept the presence of the typical B. coli as an indication of sewage contamination without reference to the amount of water in which it is found. Houston states that "it is not the mere presence of B. coli that should tend to condemn a water, but its relative abundance therein." This opens a difficult question, viz., What quantity of water containing typical B. coli is to be considered indicative of contamination with sewage? Most bacteriologists would agree that if the typical bacillus were found in 1 c.c. of the water the supply ought to be condemned; but if the bacillus could not be detected, except in such large quantities as one or two litres, the water might be passed as fit for drinking purposes. Now, on examining a supply supposed to be polluted; it often happens that typical B. coli cannot be found except in 50, 100, or 200 c.c. of the water. Is such a water to be considered contaminated? Experimental investigations have shown that when a water supply has been *recently* polluted by sewage, even in a dilution of 1 in 100,000, it is quite easy to isolate the B. coli from 1 c.c. of the water; but if several weeks have elapsed since the pollution occurred, the bacillus cannot be isolated unless the water is concentrated. The purer the water the more quickly the B. coli dies out; but, even at the end of two months, I have always been able to find the bacillus in 200 c.c. of the water. Consequently I would say that a water which contained B. coli so sparsely that 200 c.c. required to be tested in order to find it, had probably been polluted with sewage, but the contamination was not of recent date. Pakes' work, however, led him to the following conclusions:

"Drinking-water from a deep well should contain no B. coli in any quantity. Water from other sources which contains the B. coli in 20 c.c. or less should be condemned; that which contains the organism in any quantity between 20 and 50 should be looked upon as suspicious, between 50 and 100 as slightly suspicious, and only in greater quantities than 100 c.c. as probably safe. If no B. coli can be obtained from the whole two litres I should consider it absolutely safe." In the case of a recent contamination of a drinking-water, there is no doubt that

B. coli affords a much more delicate test of pollution than any chemical examination which can be made. Experiments have shown me that when pure water is polluted with a highly concentrated sewage the limit of the dilution in which the contamination can be detected chemically is 1 in 1000. Dilutions of 1 in 10,000 and 1 in 100,000 showed no chemical signs of pollution; but when examinations were made for B. coli, this organism was detected with the greatest ease in 1 c.c. of the 1 in 100,000 dilution.

Klein and Houston examined eight samples of sewage obtained from different sources in various dilutions. They found that, as a rule, a dilution of 1 in 1000 could be detected chemically, but the dilutions of 1 in 10,000 and 1 in 100,000 gave no chemical indication of pollution; but in *all* the dilutions the presence of B. coli was usually demonstrated *directly* without resorting to the "filter brushing method."

The statement of Levy and Bruns that B. coli from stools are pathogenic, whereas those found in pure waters are not, has been re-investigated by Weissenfeld. Blachstein, in 1893, pointed out that if a tube of broth be inoculated with 1 c.c. of good drinking water and incubated at 30° C. for forty-eight hours, 2 c.c. of the mixture can be injected intra-venously into a rabbit or intra-peritoneally into a guinea-pig without inconvenience to either animal. If, however, the water be from a contaminated source, such as the Seine, both animals, as a rule, rapidly succumb. The lethal effects of the impure water were attributed to the B. coli and its varieties. Sims Woodhead and Cartwright Wood injected guinea-pigs subcutaneously instead of intra-peritoneally, and took the presence or absence of local reaction as the test of contamination or purity because they found it was quite the exception for water supplies in England to be so contaminated as to exert a lethal action. Even when the organisms present in tap-water were allowed to grow for weeks in broth and so heap up their products, they produced practically no effect.

Weissenfeld's experiments were made either with pure broth cultures of the B. coli, which he isolated from the wells before mentioned, or else with a mixture of the waters and broth cultures as in Blachstein's experiments; 1 c.c. of a

two-days-old culture was injected intra-peritoneally into guinea-pigs of medium weight. He found that varieties of B. coli isolated from pure waters sometimes killed the guinea-pigs, while some of the varieties isolated from the impure waters had no effect on the animals, and therefore concluded that the isolation of a virulent B. coli from a water supply did not necessarily indicate that the water had been contaminated with fæces.

CHAPTER IX.

QUALITATIVE ANALYSIS—*continued.*[1]

The Bacillus Enteritidis Sporogenes.

In October 1895, on the occasion of an epidemic outbreak of diarrhœa amongst patients of St. Bartholomew's Hospital, Klein isolated an anærobic spore-bearing bacillus—B. enteritidis sporogenes—which when injected subcutaneously into guinea-pigs, caused acute fatal disease with sanguineous exudation. Andrewes discovered the same bacillus in the milk supplied to the patients and in the bowel discharges of severe cases of diarrhœa; he did not find it, however, in the "evacuations of casual commonplace diarrhœa." Klein also stated in his first report that the spores of the microbe could not be found in the intestinal evacuations of healthy individuals. Later investigations showed that the B. enteritidis sporogenes has a wide distribution in nature, being found in the evacuations of cases of diarrhœa, in sewage, water, soil, dust polluted with sewage, and in horses' dung. Klein now states that most specimens of sewage contain on an average about 500 to 600 spores of this organism per cubic centimetre. The B. enteritidis sporogenes is an anærobic organism, and can be best isolated by Klein's method, which is as follows:

"A small portion of any material suspected to contain the organism is placed in a test-tube containing 15 c.c. of recently sterilised milk; this is heated to 80° C. for ten to fifteen minutes, and then cooled. The milk-tube is next placed in a large cylindrical test-tube (Buchner's cylinder) containing about 120 grains of pyrogallic acid, about 10 c.c. of strong liquor potassæ are added, and the Buchner test-tube closed as quickly as possible with a well-fitting india-rubber stopper, which is then sealed with melted paraffin. The whole

apparatus is then incubated at 37° C.; the next day, or at latest after thirty to thirty-six hours, the milk will be found changed in a characteristic manner. The cream is torn or altogether dissociated by the development of gas, so that the surface of the medium is covered with stringy, pinkish-white masses of coagulated casein, enclosing a number of gas bubbles. The main portion of the tube formerly occupied by the milk now contains a colourless, thin, watery whey, with a few casein lumps adhering here and there to the sides of the tube. When the tube is opened the whey has a smell of butyric acid, and is acid in reaction. Under the microscope the whey is found to contain numerous rods, some motile, others motionless."

The microbe, when obtained from a typical milk culture, consists of rods, measuring from 1·6 to 4·8 μ in length and 0·8 μ in breadth; it may form short chains. In respect of length and thickness the bacilli closely resembles the B. anthracis; they are distinctly thicker than the bacilli of malignant œdema. The bacilli form spores, but they are not seen in the milk cultures; in liquefying gelatine cultures, in serum cultures, and in the subcutaneous exudation from inoculated animals, spores under certain conditions are readily formed. When fully developed they are oval bodies, 1·6 μ in length, and as much as 1·0 μ or even 1·2 μ in thickness. The B. enteritidis sporogenes is pathogenic; if 1 c.c. of the whey produced in the milk culture be injected into the groin of a guinea-pig, weighing from 200 to 300 grammes, swelling appears in the groin after six hours, which gradually extends on to the abdomen and thigh, usually the animal is found dead after eighteen to twenty-four hours. The skin then will be found separated from the muscles over a considerable area, and the subcutaneous tissue gangrenous, containing an offensive sanguineous exudation. If the examination is made immediately after death the exudation will be found to contain numerous cylindrical bacilli; but if the examination is deferred for twenty-four hours, large, oval or egg-shaped spores will be found, both free in the fluid and at the end of the bacilli. The microbe grows well in 2 per cent. glucose-agar under anærobic conditions. In this medium there is very marked gas formation; the colonies in the depth are round, white in reflected light,

but brown and granular in transmitted light. The agar is not liquefied, and the bacilli do not form spores. On the surface of agar anærobic plates, after twenty-four to forty-eight hours incubation the colonies are circular, flat, moist, grey and translucent. "Examined under a low power, each colony is a little thicker and less transparent in the middle part, thinned out and more transparent in the marginal portion. At the same time the colony is distinctly granular, the granules being placed closer and denser in the middle part, and less close in the marginal part." Spores are not formed in these colonies. In glucose-gelatine the bacillus produces gas and causes liquefaction of the medium. When spores are planted in grape-sugar gelatine, which is then heated to 80° C. for ten to fifteen minutes, and then cooled, sealed, and incubated at 22° C., fine, dot-like colonies appear in the lower part of the tube, which after forty-eight hours form spherical liquefied masses of gelatine. If bacilli, free from spores, are planted in sugar-gelatine, the colonies appear non-liquefying, or liquefy very late and slowly. When tested as to its staining reactions, the bacillus is found to be stained by the ordinary basic aniline dyes, and also by Gram's method.

The above description gives the chief characteristics of the typical B. enteritidis sporogenes; but Klein found sometimes that the spores from subcutaneous exudation, serum and gelatine cultures, when placed in milk and heated to 80° C. for ten to fifteen minutes, produced no change at all in the milk for the first two days. About the third or fourth day, however, the milk began to clear under the unchanged layer of cream. By the end of a week or ten days the milk culture showed on the top a layer of unaltered cream, below which the medium consisted in the main of a clear or slightly turbid pale yellow watery fluid; at the bottom of the tube there was a white mass of caseine. The whey from this milk culture was found to be offensive, alkaline in reaction, and to contain bacilli and spores which had no pathogenic action. Such an atypical milk culture was at first considered to be produced by B. enteriditis sporogenes which had lost its virulence. But further experiments showed that the appearances produced in the atypical milk culture were caused by a new bacillus, called by Klein the

B. cadaveris, which resembles the B. enteritidis sporogenes, in that it is an obligatory anærobe. It produces gas in glucose-agar without causing liquefaction, but liquefies glucose-gelatine and solidified blood serum ; it forms drum-sticks and terminal spores, and is conspicuously motile. Striking differences between the two organisms are seen when anærobic cultures are made on the slanting surface of ordinary agar or on a surface anærobic plate culture of agar. It is then found that the B. cadaveris forms angular dark granular colonies, from which extend numerous filamentous processes, which later on, as incubation proceeds, form a more or less densely reticulated mass of considerable extent ; at the same time this dark granular material is embedded on a lobulated, finely granular, translucent, filmy basis. In some cultures the filmy basis is absent altogether. On the other hand the colonies of B. enteritidis are circular discs, and there is never the reticulated expansion from the centre, which is seen in the colonies of the B. cadaveris. The B. cadaveris also forms spores on agar and in milk, which is never the case with B. enteritidis. The milk culture of B. cadaveris also has a putrid odour, and its reaction is amphoteric, or perhaps a little alkaline. "The milk cultures, liquefied gelatine cultures, agar cultures and liquefied serum cultures of the B. cadaveris are without pathogenic effect on guinea-pigs ; as much as 2 and 3 c.c. of a liquefied sugar-gelatine, or of a milk culture, can be injected without physiological effect." According to Klein, the B. enteritidis can be distinguished from the bacillus of malignant œdema, &c., by the following points :

B. Enteritidis Sporogenes.

(a) It is thicker ; (b) spores at the end of the bacillus ; (c) flagella chiefly in a bundle at the end of the bacillus ; (d) gangrenous condition of the tissue at site of injection in a guinea-pig ; (e) butyric acid smell and acid reaction of milk culture, which does not contain spores ; (f) stains with Gram ; (g) gelatine-stab has not lateral off-shoots.

Bacillus of Malignant Œdema.

(a) Thinner ; (b) spores in the middle of the bacillus ;

(c) flagella disposed at the sides, not specially at the end of the bacillus; (d) subcutaneous tissue hæmorrhagic; (e) gas not formed so abundantly in milk culture; slow separation of the fluid and coagulated part, which commences near the cream; the whey is not acid, has no butryic acid smell, and contains spores; (f) it does not stain with Gram; (g) gelatine-stab possessed of many short lateral off-shoots.

Bacillus of Symptomatic Anthrax.

(a) It does not stain with Gram; (b) gelatine-stab has many short lateral off-shoots; (c) it does not produce subcutaneous gangrene, but a hæmorrhagic swelling containing gas bubbles.

Bacillus Butyricus of Botkin.

This bacillus resembles the B. enteritidis sporogenes morphologically and culturally in sugar-gelatine, in agar, and particularly in milk; but it is without pathogenic action when injected in large doses subcutaneously into guinea-pigs or rabbits.

The characteristics and means of diagnosis of the B. enteritidis sporogenes have been considered in detail, as many bacteriologists claim that the discovery of this bacillus has given a fresh impetus to the bacteriological examination of water. Houston states: "It is safe to anticipate that this discovery is destined to largely enhance the value of the bacteriological test of potable waters. For the spores of this anærobe cannot be demonstrated in pure water (by this is meant that the bacillus is absent, not only in 1 c.c. of a pure water, but that it may be absent in 500 c.c. or more of such water), whereas in impure water it can readily be shown to be present, and in raw sewage it is specially abundant. My own records, which extend over a year, and deal with a very large number of samples of sewage, show that the spores of this microbe are present in numbers varying usually from 100 to 1000 or more per cubic centimetre. It cannot fairly be said of this anærobe that it is likely to multiply outside the animal body, and the only possible objection to accepting its presence as an index of dangerous contamination of water is the fact that its being a sporing anærobe weakens somewhat its usefulness as evidence of recent and, therefore, presumably specially dangerous,

pollution. Nevertheless, and as far as can be seen at present, it not only equals, but far exceeds any known test, chemical or bacteriological, for inferring the wholesomeness or otherwise of a drinking water." Klein and Houston examined eight specimens of sewage as to the total number of microbes, the number of B. coli, and the number of spores of virulent B. enteritidis contained in 1 c.c. of the fluid. Some of their results are given in the following table :

Sample of sewage.	No. of microbes per 1 c.c. of crude sewage.	No. of B. coli per 1 c.c. of crude sewage.	No. of spores of virulent B. enteritidis per 1 c.c. of crude sewage.
Chiefly domestic .	14,240,000	260,000	2000
Mixed	7,800,000	180,000	200
Chiefly domestic .	4,800,000	500,000	2000
Mixed with a large amount of trade liquid	36,000,000	1,100,000	400
Hospital . . .	2,800,000	200,000	30
Domestic, with a large amount of trade liquid .	4,100,000	500,000	56
Wholly domestic	28,000,000	2,000,000	50
Mixed	21,000,000	1,000,000	35

From this table it appears that there is no definite relation between the total number of bacteria and the number either of B. coli or of spores of B. enteritidis ; also there is no parallelism between the number of B. coli and the number of spores of B. enteritidis. It is also evident that to detect this organism in a polluted water it may be necessary to resort to "the filter brushing method," and to concentrate the water to small bulk. Unless the pollution is very great, *i.e.*, a dilution of sewage less than 1–1000, it may be impossible to detect B. sporogenes in 1 c.c. of a water polluted with sewage containing even 2000 spores per c.c. But as some specimens of sewage contain only from 30–35 spores per c.c., a considerable quantity of the water must be examined in order to detect the B. enteritidis. As a matter of routine, it would seem advisable to concentrate always at least 2000 c.c. of a polluted water, by pumping through a Pasteur's filter, and diffusing the deposit in 5 c.c. of sterile water. One c.c. of this should be added to each of five milk tubes, which are then heated to 80° C. for

ten to fifteen minutes, cooled, and cultivated anærobically in Buchner's tubes. If the typical changes are seen in any one of the milk tubes, the presence of B. enteritidis is probable; but to obtain a decisive result the pathogenic action of the organism must be tested by injecting 1 c.c. of the whey into a guinea-pig. The fact that Botkin's bacillus butyricus exactly resembles the B. enteritidis, culturally and morphologically, renders the animal experiment absolutely imperative; unfortunately, the necessity of resorting to an animal experiment militates against the extensive use of this test in ordinary bacteriological examinations for hygienic purposes. Still, if the typical appearances in milk are found in conjunction with the presence of typical B. coli in a small quantity of the water, the fact of sewage contamination may be considered sufficiently proved for all practical purposes.

CHAPTER X.

QUALITATIVE ANALYSIS—*continued.*

Species of Streptococci and Staphylococci.

The value of these organisms as indicative of sewage contamination was prominently brought forward by Houston in the supplement to the report of the Local Government Board for 1898–99. Laws and Andrewes, however, in 1894, pointed out that a small streptococcus was the commonest organism present in fresh sewage from St. Bartholomew's Hospital. The colonies of this organism were minute, grew slowly, and possessed the power of coagulating milk in twenty-four to thirty-six hours, when incubated at 37° C. In a specimen of sewage from Snow Hill the same streptococcus was very abundant. An old specimen of sewage from Barking showed numerous colonies of the streptococcus and a yellow staphylococcus, but did not appear to contain any B. coli. This last observation is of the greatest importance in relation to the contamination of potable waters by old sewage. In my own work I have repeatedly examined waters which, from their local surroundings, must have been polluted with sewage, and yet I have been unable to find the slightest traces of B. coli in them; streptococci were, however, nearly always present. On considering the question, I thought that possibly B. coli died out in sewage kept under the conditions of an old and little-used cesspool; and on testing the point experimentally I found that B. coli gradually disappeared from many specimens of sewage kept in the dark at the temperature of an outside verandah. But the commonest organisms which persisted in these old specimens of sewage were varieties of streptococci and staphylococci, and I proceeded to examine the cultural reactions of the commonest types of these microbes, believing that their isolation might be

of great service in solving the difficult problem of sewage contamination. The following are the chief types observed up to the present time:

Streptococcus A.

Gelatine Plates.—The surface colonies were extremely small, with an irregular margin, from which here and there "streamers" ran out over the surface of the gelatine. An "impression" preparation showed that the colony consisted of small cocci tightly packed together at the centre, but forming short and long chains at the periphery. The gelatine was not liquefied.

Agar-slope.—The growth consisted of numerous small isolated colonies with a circular outline and granular centre.

Gelatine-stab.—On the surface there was a very small circumscribed film, and a fine thin growth along the stab consisting of small colonies massed together. The gelatine was not liquefied.

Milk.—Clotted after six days incubation at 37° C.

Litmus-whey.—Faintly acid after twenty-four hours incubation at 37° C. The acidity required 1·1 c.c. per cent. of $\frac{N}{10}$ alkali to neutralise it. After seven days incubation the acidity equalled 2·4 per cent. $\frac{N}{10}$ alkali.

Broth.—After twenty-four hours incubation at 37° C. a diffuse growth appeared with a slight deposit. After ten days the broth was not clear.

Peptone and Salt Solution.—After seven days incubation at 37° C. no indol was produced.

Glucose-gelatine.—No gas formation.

Gelatine (*ordinary stab*) *at* 37° *C.*—A diffused growth and slight deposit.

Potato.—Moist glistening surface; growth hardly apparent. A portion examined under the microscope showed small cocci in chains and masses.

Microscopical Characters.—A twenty-four-hour broth culture showed medium-sized cocci arranged in short chains and small masses.

Staining Reactions.—Stains with basic aniline dyes and by Gram's method.

Streptococcus B.

Gelatine Plates.—Small circular colonies, with clear sharp edges, thicker in the centre than at the periphery; under a low power granular, but no streamers observed. Gelatine was not liquefied.

Agar-slope.—The growth consisted of numerous small isolated circular colonies.

Gelatine-stab.—On the surface a very small circumscribed film, and along the stab a fine growth consisting of small colonies massed together.

Gelatine-stab at 37° C.—After twenty-four hours incubation at 37° C, the tube was nearly clear with a thick deposit at the bottom.

Glucose-gelatine.—No gas formation.

Milk.—Coagulated after forty-eight hours incubation at 37° C.

Litmus-whey.—After twenty-four hours incubation at 37° C. the acidity equalled 3·3 per cent. $\frac{N}{10}$ alkali; after seven days incubation the acidity equalled 13 per cent. $\frac{N}{10}$ alkali.

Peptone and Salt Solution.—No formation of indol.

Broth.—A diffused growth with a marked deposit at the bottom.

Potato.—Moist, glistening surface, growth hardly apparent.

Staining Reactions.—Stains with Gram's method.

Microscopical Characters.—Twenty-four hours broth culture showed chains consisting of seven to eleven cocci.

Streptococcus C.

Gelatine Plates.—Small circular colonies with clear, sharp edges, thicker at the centre than the periphery; no streamers observed; gelatine not liquefied.

Agar-slope.—Very small circular colonies, uniting below to form a thin greyish-white growth.

Gelatine-stab.—On the surface a very small circumscribed film, and along the stab a very fine growth, consisting of small colonies massed together.

Gelatine-stab at 37° C.—Diffused growth through the tube.

Glucose-gelatine.—No gas formation.

Milk.—Coagulated in forty-eight hours.

Litmus-whey.—After twenty-four hours incubation at 37° C. the acidity equalled 5 per cent. $\frac{N}{10}$ alkali. After seven days incubation at 37° C. the acidity equalled 14 per cent. $\frac{N}{10}$ alkali.

Peptone and Salt Solution.—No formation of indol.

Broth.—A diffused growth with deposit at the bottom of the tube; after ten days growth the tube was still turbid.

Potato.—Glistening transparent growth.

Staining Reactions.—Stains with Gram's method.

Microscopical Characters.—Twenty-four hours broth culture showed very long chains, consisting of at least thirty cocci.

Note.—Streptococcus C. was only distinguished from B. by the small size of its colonies on agar and the extreme length of the chains in the broth culture.

Streptococcus D.

This culture very closely resembled streptococcus C. in the size of its colonies on agar, its re-actions in milk, litmus-whey, and glucose-gelatine, and on potato. It, however, only produced very short chains in a twenty-four hours broth culture, and gave an indol reaction in peptone.

Streptococcus E.

Gelatine Plates.—Colonies circular, larger and more opaque the above varieties, consisting of very small cocci.

Agar-slope.—A thick, white, opaque growth; at the margin, large, white, and opaque, discrete colonies like those on the gelatine plates.

Gelatine-stab.—Thick, white, opaque growth on the surface and along the stab. After fourteen days at 22° C. gelatine slowly liquefied.

Gelatine-stab at 37° C.—Tube clear, with a deposit at the bottom after twenty-four hours incubation.

Broth.—At the end of twenty-four hours at 37° C. faintly

turbid ; after forty-eight hours the broth cleared and a deposit formed at the bottom ; on shaking the tube, long thin spiral threads rose up from the bottom.

Milk.—Unchanged after ten days incubation.

Litmus-whey.—No acid produced. After ten days incubation at 37° C. the reaction was slightly alkaline.

Glucose-gelatine.—No gas formation.

Peptone and Salt Solution.—After seven days incubation at 37° C., a marked indol reaction.

Potato.—At first only a thin glistening film, but after ten days incubation at 37° C., a distinct yellow growth was apparent.

Staining Reactions.—Stained by Gram's method.

Microscopical Appearances.—A twenty-four hours broth culture showed short chains and masses of very small cocci.

NOTE.—This organism seemed to be intermediate between the streptococci and the staphylococci. In its growth on potato and liquefaction of gelatine it resembled staphylo-cocci. But the growth in broth, milk, and litmus-whey, and the microscopical appearances showed that the culture was also closely related to the Streptococcus pyogenes.

None of the cultures when injected into guinea-pigs produced any effects, either constitutional or local.

Staphylococcus F.

Gelatine Plates.—Large, white, opaque, circular colonies, which later acquired a lemon-yellow colour, and slowly liquefied the gelatine.

Agar-slope.—A thick white growth, which later acquired a lemon-yellow colour.

Broth.—Diffuse growth, with a slight deposit at the bottom.

Milk.—Unchanged.

Potato.—A rather dry, light yellow growth.

Litmus-whey.—No acid produced.

Peptone and Salt Solution.—No indol produced.

Glucose-gelatine.—No gas formation.

Gelatine-stab at 37° C.—A diffused growth with a small deposit.

Gelatine-stab at 22° C.—White thick growth on the surface and along the stab; later the growth acquired a light yellow colour, and the gelatine slowly liquefied.

Staining Reactions.—Stained by Gram's method.

Microscopical Characters.—Large cocci in masses and in pairs; no chains observed.

This organism appeared to be closely allied to the Staphylococcus citreus.

As a result of my experiments, I arrived at the conclusion that streptococci presenting the above characteristics might be extremely useful to the water-bacteriologist; for if they were found in the absence of B. coli, they would probably indicate pollution of the water-supply by old sewage.

Houston, however, is inclined to regard streptococci as indicating recent and objectionable pollution. He states that streptococci "as a class may be thought of as germs especially liable to discouragement by unfavourable physical conditions; and, indeed, as surviving only when the conditions are almost ideally propitious. In the present state of our knowledge, therefore, the presence of streptococci in a substance, be it soil or sewage or water, suggests recent association of certain ingredients of that substance with an animal host." The following descriptions of streptococci, which were isolated from water, are given to show the characteristics of the organisms described by Houston.

Streptococcus I_2. *Surface Colonies on Gelatine Plates.*—Yellowish-grey, transparent, showing wavy granulation, edge sinuous, and made up of loops of streptococci. No liquefaction. *Surface-agar Plates.*—Yellowish-brown, semi-transparent colonies of irregular shape and small size. The chains of cocci give the colonies a wavy, granulated appearance. The loops of cocci are readily seen at the periphery. *Broth.*—Clear; at foot of tube there are woolly masses, which tend to cohere, and are somewhat viscous. *Litmus-milk.*—Practically no change even after six days. *Gelatine-slope.*—Transparent coarsely granular colonies, which tend to remain separate and not form a continuous growth. No liquefaction. *Morphology.*—Stains with Gram. Long chains of cocci; also masses made up of chains.

Streptococcus, J_{10}. *Surface-gelatine Plates.* — Colonies minute, circular, transparent, yellowish-grey in colour, showing very faint granulation. The edge is clean, there are no loops visible, no liquefaction. *Surface-agar Plates.*—The colonies are darker, less transparent, and the granulation, which is wavy in character, is much more marked. The colonies are usually more or less circular in shape; the edge is usually clean. *Broth.* —Diffuse but not very abundant cloudiness in twenty-four hours. At foot of tube a white and somewhat viscous deposit. *Litmus-milk.*—In three days distinct acidity but no clot; no clot sixth day. *Gelatine-slope.*—Minute, circular, transparent colonies. *Morphology.*—Stains with Gram. Cocci chiefly in adherent masses, but also separate chains.

Streptococcus K_{11}. *Surface-gelatine Plates.* — Minute, transparent, yellowish-grey, nucleated, faintly granular colonies with clean edge; no liquefaction. *Surface-agar Plates.* The colonies are darker and less transparent and show a wavy and coarse granulation; some of the colonies are quite circular with clean or only very slightly frayed edge. Other colonies are irregular in shape with a broken-up edge. *Broth.*—Remains quite clear, on the sides of the tube and at the foot fluffy white masses are to be seen, which are of a somewhat viscous nature. *Litmus-milk.*—Practically no change visible after eight days at 37° C. *Gelatine-slope.*—Minute circular transparent colonies. *Morphology.*—Stains with Gram. Cocci chiefly in masses, many of them of large size and of irregular shape; ? also chains.

Streptococcus L_{12}.—This appeared to be practically the same as K_{11}, but it grew more slowly.

Streptococcus C_3. *Surface-gelatine Plates.*—Circular, transparent, minute, faintly granular, yellowish-white colonies. No loops of cocci visible at the edge of the colonies; no liquefaction. *Surface-agar Plates.*—More or less circular minute colonies of yellowish-white colour, faintly granular, with unbroken edge. *Broth.*—Only slight diffuse cloudiness, not much deposit at the foot of the tube. *Litmus-milk.*—Very feeble acidity, no clot after fifteen days at 37° C. *Gelatine-slope.*— Minute greyish-white circular colonies, nearly transparent. *Morphology.*—Stains with Gram. Chains of cocci of medium length, not matted in masses.

Houston also isolated seven other varieties of streptococci from "soil-washings" which were characterised by colonies with clean edges. The broth cultures were clear, with a deposit at the bottom of the tube. Four of the organisms produced strong acidity but no clotting in milk. None of the cultures showed pyogenic or pathogenic properties.

In the third Report to the London County Council on the Bacterial Treatment of Crude Sewage, Houston stated that the type of streptococcus most abundant in sewage presented the following characteristics :—

Sewage Streptococcus (Houston).

Source.—Barking and Crossness crude sewage and effluents from bacterial coke-beds.

Abundance.—Usually more than 1000 per c.c. of crude sewage and effluents.

Temperature.—Grows well at blood heat.

Morphology.—Stains well by Gram's method. The chains of cocci are usually short.

Agar and Gelatine-plate Cultures.—The colonies are small and transparent. They are nearly circular in shape with a *clean* edge. The granulation is faint. The colonies in agar are usually more granular and darker-looking than in gelatine cultures. The gelatine is not liquefied.

Streak Cultures. (Agar at 37° C. and gelatine at 20° C.)—The growth usually shows itself as a delicate white film, which on close observation is seen to be made up of numerous separate minute transparent-looking colonies. The gelatine is not liquefied.

Broth Cultures (37° C.).—Abundant diffuse cloudiness. On gently shaking the tube a viscous white deposit rises from the foot of the tube in a spiral form.

Litmus-milk Cultures (37° C.).—Acidity, but usually no clot.

Roscoe and Lunt isolated from sewage a streptococcus, which appears closely allied to the varieties I have already described. The cultural characteristics of this organism are as follows :

Streptococcus Mirabilis (Roscoe and Lunt).

Gelatine Plates.—It grows badly. The colonies in the depth of the gelatine after four days incubation are mere dots or

gnarled and convoluted thread-like masses. Surface colonies exhibit a faint and transparent expansion. Under a low power the colony is seen to consist of a mass of long threads, sometimes throwing out processes into the surrounding gelatine, which is not liquefied.

Gelatine Tubes.—Produces a faint transparent film almost invisible to the naked eye.

Agar.—Growth resembles that on gelatine.

Potatoes.—Growth inappreciable.

Broth.—In forty-eight hours a fine mass resembling delicate cotton-wool collects at the bottom of the tube and the broth above remains perfectly clear.

Microscopical Characters.—Streptococci forming very long chains. The individual cocci are 0·4 μ thick. It is not motile.

Three other varieties of streptococci have been described, which differ from the above in that they liquefy gelatine. They were not very fully investigated by their discoverers, so that it is difficult to follow the descriptions. They are as follows:

The Streptococcus Albus.—This organism was isolated by Maschek from water. On gelatine plates it forms flat expansions with a white periphery; under a low power a small dark yellow cloud is visible in the centre. Liquefaction proceeds rapidly. In gelatine tubes it forms a flat expansion, which rapidly liquefies the gelatine and produces a white deposit. On potatoes it grows rapidly, producing a slimy expansion.

The Streptococcus Vermiformis.—This organism was also isolated from water by Maschek. On gelatine plates it forms yellowish-white centres, which sink into the gelatine. The centre is light, whilst the periphery is composed of a dark ring. Under a low power the contents of the colony are granular, whilst the rim exhibits a radiated structure. It liquefies the gelatine very rapidly. On potatoes it forms a dirty yellow expansion, which grows very rapidly. The individual cocci are almost always arranged in filaments which show a slow vermiform movement.

The Streptococcus Coli Gracilis.—This streptococcus was found by Escherich in the intestinal canal and fæces of flesh-eaters. It consists of cocci with an average diameter of 0·2–0·4 μ; in recent gelatine cultures it shows S-shaped forms

consisting of six to twenty cocci. On gelatine plates it forms, at first, round, sharply-outlined colonies; later they are dark and surrounded by a broad clear funnel of liquefaction. In gelatine stab there is a funnel-shaped liquefaction. After six to eight days, at the bottom of the completely liquefied gelatine there appears a white, finely granular deposit. On agar there is a scanty surface growth. On potato a scanty growth appears in the form of small white raised points. It grows best at blood-heat, and gas production has not been observed.

The varieties of streptococci which I have found most commonly in polluted water-supplies correspond to the streptococcus B., streptococcus E., and the "sewage streptococcus" of Houston. In many of the waters which contained these organisms the B. coli was not detected, and an opinion was formed that the waters in question had been polluted with old sewage; the opinion was usually found to be justified by a local examination of the supply. My experience does not support the contention that streptococci probably indicate a dangerous contamination. As I have already mentioned, the sewage streptococci appear to maintain their vitality in sewage for a much longer time than B. coli. Specimens of barrack sewage preserved in a laboratory cupboard for three months and then diluted 1-100 or 1-1000 with tap-water, show, when examined by the usual methods, large numbers of streptococci but few or no B. coli. Old sewage from which B. coli has disappeared will be unlikely to present conditions favourable to the prolonged vitality of the B. typhosus or Sp. choleræ. Consequently it appears that streptococci alone cannot be considered as necessarily indicating a dangerous contamination. It is true that when the dilutions of old sewage are kept for a few days the streptococci rapidly disappear; so their presence in a water-supply undoubtedly indicates a *recent* contamination, but the contamination is not necessarily dangerous unless the streptococci are accompanied by B. coli.

CHAPTER XI.

QUALITATIVE ANALYSIS—*continued.*

The Proteus Group.

Members of the proteus group are often found in sewage, but are very rare in pure waters. They do not necessarily indicate sewage contamination, but if they are found in conjunction with B. coli there is very little doubt that such pollution has occurred. The three chief members of the group were first isolated from putrid meat by Hauser.

Proteus Vulgaris.

Gelatine Plates.—The young colonies are very variable in outline; they are generally yellowish-brown in colour and rapidly liquefy the gelatine. Under a low power the edge of the colony may be seen to be set with fine bristles, which appear to be continued into the centre of the colony; from the margin wandering processes grow out over the gelatine, and in these the bacilli are seen to be packed side by side. Sometimes instead of the colony with a bristly edge, there appears an oval or spindle-shaped form from which processes or "swarmers," often bent on themselves, run out all over the gelatine. In the depth of the gelatine oval colonies are seen in which the bacilli appear packed in concentric circles. Zooglœa forms are also common. After twenty-four hours incubation the plates to the naked eye often show nothing but circular or irregular pits of liquefaction with slimy contents.

Gelatine-stab.—The gelatine is rapidly liquefied in a more or less funnel-shaped manner, and at the bottom of the liquefaction an opaque flocculent mass is seen.

Agar.—A moist greyish-white growth.

Potato.—A dirty yellowish-white moist growth.

Glucose-gelatine.—No gas formation.

Broth.—Uniformly turbid.

Peptone and Salt Solution.—No indol reaction produced.

Milk.—Unchanged.

Nitrate-broth.—Nitrites and ammonia produced.

Litmus-whey.—Reaction becomes slightly alkaline.

Microscopical Characters.—Thin motile rod varying greatly in length. It does not form spores and grows best at from 20° to 24° C. It is decolorised by Gram's method of staining. Involution forms are often seen.

Proteus Zenkeri.

Gelatine Plates.—The surface colonies are yellowish-white, and from the margin processes extend in every direction, so that the whole colony somewhat resembles a mould. Under a low power many of the processes may be seen to be beaded ; others are spiral, and join with processes from neighbouring colonies. The gelatine is not liquefied for a long time, and then it is only slightly marked. Zooglœa forms are said not to be seen in the depth.

Gelatine-streak.—Along the streak a white growth appears, from which fine processes run out on both sides to the margin of the gelatine ; often the branches are curved with the concavity upwards.

Gelatine-stab.— A white surface growth, with processes running out to the margin ; along the stab a white growth appears, from which numerous hair-like branches penetrate horizontally into the gelatine.

Agar.—A thin white growth.

Potato.—Yellowish-white growth.

Milk.—Unchanged.

Peptone and Salt Solution.—No indol reaction produced.

Glucose-gelatine.—No gas formation.

Microscopical Characters.—A thin motile bacillus, varying greatly in length ; some forms are almost like cocci. It does not form spores. It grows best at 20° to 24° C.

Proteus Mirabilis.

Gelatine Plates.—The surface colonies, after twenty-four

hours incubation, appear as thin white expansions, more or less circular in outline, with granular contents; later, the margin becomes irregular, and wandering processes pass out in all directions, slowly spreading over the gelatine. Zooglœa forms are commonly seen. Gelatine is slowly liquefied.

Gelatine-stab.—On the surface there is a thin white growth, from which processes run out to the margin; along the stab there is a white growth from which processes penetrate almost horizontally into the surrounding gelatine. The gelatine is very slowly liquefied.

Gelatine-streak.—There is a white growth along the line of inoculation, from which processes run out to the margin, producing a growth somewhat like a feather. The growth is very like that of Proteus Zenkeri, but the processes are shorter and thicker. The gelatine is slowly liquefied.

Agar.—A thin white growth.

Potato.—Greenish-yellow, moist, slightly raised growth.

Glucose-gelatine.—No gas formation.

Milk.—Unchanged.

Nitrate-broth.—Nitrites and ammonia produced.

Peptone and Salt Solution.—No indol reaction produced.

Microscopical Characters.—A motile bacillus; often resembles a coccus, and at times grows out to a length of 3 to 4 μ. Involution forms are common.

Proteus Zopfi.

A culture of this organism, which I obtained from Kral's laboratory, gave reactions in the various media, closely resembling the Proteus Zenkeri. The colonies in gelatine plates showed the same beaded wandering processes. The swarmers were, however, not quite so marked, and on gelatine streaks the lateral processes were shorter and thicker, more resembling Proteus mirabilis.

All the varieties of Proteus when "plated" in 5 per cent. gelatine show, after a few hours incubation and before liquefaction has commenced, a peculiar motility of the bacilli.

Micro-organisms Suggestive of Sewage Contamination.

The following organisms appear to be frequently present in waters contaminated with sewage, but are rarely, if ever, found

in pure supplies. Waters containing these bacteria should be regarded with suspicion, and the source of the supply carefully investigated.

Bacillus Megaterium.

This organism was first isolated by De Bary from boiled cabbage leaves. It was found by Tils in the Freiburg water-supply. I have also found it in a water derived from a polluted upland surface.

Gelatine Plates.—The surface colonies appear as circular masses with a "woolly" margin; later they appear depressed in the gelatine owing to liquefaction. Under a low power they show a dark granular centre surrounded by circular lines, from which fine branching processes pass off at the margin.

Gelatine-stab.—A delicate growth at first appears along the needle track, from which fine hair-like processes spread out into the gelatine. Liquefaction rapidly takes place, and a funnel-like excavation is formed, containing an opaque yellowish-white layer at the bottom.

Agar.—A thick white growth appears with a feathery margin.

Potato.—A thick yellowish-white growth.

Glucose-gelatine.—No gas formation.

Milk.—Coagulated, and then partially digested.

Peptone and Salt Solution.—No indol reaction obtained.

Nitrate-broth.—Powerful reducing action, large quantities of ammonia being produced.

Microscopical Characters.—A fat bacillus, often very long, but by the use of dehydrating agents, separate divisions can be seen, so that the bacillus then appears to be made up of a series of short rods, each containing a spore. It possesses a slow waggling movement, and is stained by Gram's method.

Bacillus Lactis Cyanogenus.

This bacillus was first isolated from milk by Hueppe. Jordan found it frequently in Lawrence sewage.

Gelatine Plates.—The surface colonies are circular and dirty white in colour; later the colour changes to an iron-grey, and the surrounding gelatine acquires a deep blue colour. Under a

low power the colonies have a yellowish colour and a dark, almost opaque, granular centre.

Gelatine-stab.—There is a white growth on the surface and along the line of inoculation. The gelatine is not liquefied, and acquires a steel-blue colour, which later becomes almost black.

Agar.—A greyish-white growth appears, and the medium becomes almost brown in colour.

Glucose-gelatine.—No gas formation. The gelatine acquires a deep Prussian-blue colour.

Milk.—Not coagulated after several days incubation, and becomes slightly alkaline, with a faint bluish tinge. In unsterilised milk it produces a sky-blue colour.

Broth.—A diffused growth. Later, a pellicle forms on the surface, and the broth acquires a blue colour.

Potato.—It forms a yellow restricted growth, and the potato assumes a greyish-blue colour.

Blood-serum.—It grows, but does not produce any pigment.

Microscopical Characters.—A thin motile bacillus, which forms spores at the ends of the rods. According to Flügge, the spores seen by Hueppe are only involution forms. It grows at room temperature and blood-heat. It does not stain with Gram.

NOTE.—Jordan stated that the specimens which he isolated from sewage did not grow as well at 37° C. as at 21° C., and were not "very motile." He also failed to observe any true spores.

Bacillus Acidi Lactici.

This bacillus was isolated by Hueppe from sour milk, and was found in the Freiburg water by Tils.

Gelatine Plates.—The surface colonies appear as circular white masses with an irregular outline and resemble those of B. coli; later, leaf-like expansions grow out from the margin. The gelatine is not liquefied.

Gelatine-stab.—There is a white growth on the surface, which later shows leaf-like expansions. Along the stab there is a white growth consisting of small white centres.

Agar-slope.—A thick, white, adhesive growth.

Potato.—Thick yellowish-brown growth.

Milk.—Coagulated in twenty-four hours at 37° C.

Peptone and Salt Solution.—A marked indol reaction obtained.

Broth.—A diffuse growth. A slight surface pellicle is formed.

Nitrate-broth.—Nitrates are largely reduced to nitrites.

Glucose-gelatine.—Marked gas formation.

Microscopical Characters.—Small non-motile bacillus, usually in pairs. Spore formation present.

It will not grow below 10° C.; requires from 10° to 12° C.; at 15° C. acid is produced, which ceases at 45·5° C.

Bacillus Ureæ.

This micro-organism was isolated from ammoniacal urine by Jaksch. It was found by Tils in Freiburg water, and by Lustig in river water.

Gelatine Plates.—The surface colonies form small, transparent, finely granular films, with an irregular margin, somewhat resembling the colonies of B. typhosus.

Gelatine-stab.—A surface growth like the colonies; also a fine white growth along the stab.

Glucose-gelatine.—No gas formation.

Milk.—Unchanged.

Potato.—A thin white growth.

Agar.—A white expansion, not characteristic.

Broth.—A diffused growth and a deposit forms at the bottom of the tube.

Peptone and Salt Solution.—No indol reaction obtained.

Microscopical Characters.—A small motile bacillus. It converts urea into ammonium carbonate.

Bacillus Fluorescens Putridus.

This microbe was isolated from water by Flügge. It is considered by Macé to be characteristic of foul waters. A culture from Kral's laboratory gave the following cultural reactions:

Gelatine Plates.—The colonies both in the depth and on the surface are small; but, later on the surface they grow out as greyish-white expansions, with a circular or slightly irregular margin. They are thicker at the centre than at the

margin, and often there is a slight knob in the centre of the colony. Under a low power they appear round, finely granular, with a dark centre; the colour is yellow in the centre, and clear grey at the margin. The gelatine around the colonies acquires a green colour.

Gelatine-stab.—Surface growth is white and opaque; along the stab there is also a delicate white growth. The gelatine assumes a bright green colour.

Agar.—A thick white growth; the agar acquires a green colour.

Glucose-gelatine (shake).—No gas formation.

Peptone and Salt Solution.—No indol reaction obtained.

Broth.—Diffuse growth; after several days a pellicle appears on the surface. The broth acquires a green colour.

Nitrate-broth.—No reduction of nitrates.

Potato.—At first there is a greenish-yellow growth, which later forms a brownish, slimy expansion.

Milk.—Unchanged.

Microscopical Appearance.—A small motile bacillus with rounded ends, often in pairs. It grows best at room temperature, and does not form spores.

In my experience this organism is often found in polluted waters. Some of the varieties isolated, when sub-cultured in broth, produced pathogenic effects when 1 c.c. was injected sub-cutaneously into guinea-pigs. Lepierre isolated a similar organism from a cistern at Coimbra, but the bacillus was not motile.

Bacillus Erythrosporus.

This micro-organism was isolated from putrefying liquids by Eidam; it has also been found in water by Bolton and Migula.

Gelatine Plates.—The surface colonies form white centres, with a slightly irregular margin. Under a low power the centre is dark brown in colour; the margin shows radial streation, and has a yellowish-green colour. The gelatine is not liquefied, and around each colony acquires a green fluorescence.

Gelatine-stab.—There is a white growth along the stab and on the surface; the gelatine acquires a green colour.

Glucose-gelatine (shake).—No gas formation.

Milk.—Unchanged.

Peptone and Salt Solution.—No indol reaction obtained.

Nitrate-broth.—The nitrates are not reduced.

Broth.—A diffuse growth, the broth acquires a green colour.

Potato.—A yellowish-brown restricted growth, later, nut-brown in colour.

Microscopical Appearance.—Thin motile bacillus, with rounded ends. From two to eight spores, which have a red colour, appear in each rod. The red spores are visible when the bacillus has been stained with methylene blue.

Bacillus Pyocyaneus.

The colour of green pus is due to the products of this bacillus; it was described by Gessard and Charrin. The same bacillus was found by Tils in the Freiburg water-supply.

Gelatine Plates.—The colonies develop rapidly. They are not sharply circumscribed, and usually present a fringe of delicate filaments, surrounding a dark granular centre; as growth advances the gelatine is liquefied, and acquires a bright green colour. Under a low power the centre of the colony appears filled with dark rounded masses, and surrounded by a wide, thin, filmy growth, which has an irregular margin.

Gelatine-stab.—There is rapid liquefaction of the gelatine, which in the earliest stage takes the form of a long narrow funnel. The gelatine assumes a bright green colour.

Agar.—There is a dry greenish-white growth; the agar is bright green.

Potato.—There is a dry brownish growth which, when touched with a platinum needle, sometimes becomes green; the colour lasts about ten minutes. This is known as the chameleon phenomenon of Ernst.

Broth.—There is a floccular growth; a pellicle forms on the surface, and the broth assumes a green colour.

Milk.—An acid reaction is produced and the milk coagulated.

Peptone and Salt Solution.—Indol is produced.

Microscopical Appearance.—It is a delicate rod with rounded ends; sometimes it is very short and resembles a coccus; in old

cultures longer forms are seen. It is very motile and possesses a flagellum at one end. According to Flügge it forms spores, though this is denied by other observers.

Micrococcus Ureæ.

This name was given by Pasteur to the organism which converted urea into ammonium carbonate. Leube described the following biological characteristics:—It is a medium-sized coccus, which usually occurs as diplococci, sometimes as tetrads and long chains. In gelatine plates the colonies appear as smooth, mother-of-pearl like growths, which in ten days grow out to the size of a sixpence and look like drops of wax; under a low power the margin is finely granular and the centre opaque; the gelatine is not liquefied. In gelatine stab it forms a thin, tough, thread-like growth along the inoculation line.

Micrococcus Ureæ Liquefaciens.

This micro-organism was isolated in Flügge's Institute; it also rapidly converts urea into ammonium carbonate. It forms large cocci, which are arranged in chains or masses. After two days the deep colonies appear as small white points, which, under a low power, are seen to be granular; on reaching the surface the colonies form yellowish-brown discs, often with a cavity in the centre; the margin is irregular, and liquefaction slowly takes place. In gelatine stab there is a white growth along the stab, the gelatine is soon liquefied, and the tube becomes filled with fluid containing a yellowish-white deposit.

Cladothrix Dichotoma.

This organism was first described by Cohn. It is found in fresh and stagnant water, and especially in water rich in organic matter; it is also common in sewage.

Gelatine Plates.—Small yellow dots appear in four or five days; each growth is surrounded by a brown colour, which gradually extends over the gelatine. Under a low power, each colony is seen to consist of a mass of branching threads closely felted together and strongly resembling a mould. Later a white mouldy growth forms on the surface of the colonies, and the gelatine is slowly liquefied.

Gelatine-stab.—Growth occurs at the surface and along the line of inoculation; the gelatine acquires a brown colour around the growth, and is slowly liquefied at the surface in the form of a cup. A white incrustation appears in the liquefied mass after a few days.

Agar.—There is a thick adherent growth, which later becomes covered with a white incrustation. The medium acquires a brown colour.

Broth.—A white growth appears on the surface and a deposit forms at the bottom; the intermediate portions of the medium are quite clear.

Glucose-gelatine (shake).—There is no gas formation.

Milk.—A white growth appears on the surface, but the milk is not coagulated.

Potato.—There is a localised greenish-yellow growth, which becomes covered with a white incrustation.

Microscopical Appearance.—In a hanging-drop long branching filaments, with hyaline contents, are seen. The long filaments break up into smaller segments, which appear to be motile and have a tendency to form zooglœa masses.

CHAPTER XII.

QUALITATIVE ANALYSIS—*continued.*

"Sewage-bacteria" described by E. O. Jordan.

In 1890 Jordan made a special study of bacteria occurring in sewage. He believed that sewage was probably inhabited by species peculiarly adapted to the conditions of life, and thought there was good reason for speaking of "sewage bacteria," *i.e.*, those bacteria normally found in sewage, and composing its special flora. The following micro-organisms were found in the Lawrence sewage, and appeared to be peculiar to this medium. The descriptions are taken from Jordan's paper in the Report of the Massachusetts State Board of Health for 1890.

Bacillus Cloacæ.

This bacillus appeared to be one of the commonest bacteria in sewage.

Plate-cultures.—In twenty-four to forty-eight hours a round, yellowish colony becomes visible in the gelatine. On coming to the surface this forms a slight bluish expansion, with irregularly notched edges, and almost immediately begins to liquefy the gelatine. Under a low power of the microscope, the colony is seen to have a dark centre, an outer translucent zone and a darker edge; the interior is finely granular. The whole plate is soon liquefied (in from three to four days).

Gelatine-tubes.—A rapid growth, liquefying the gelatine. A good growth along the inoculation line. An iridescent scum forms on the surface, and there is a heavy, whitish, flocculent precipitate. Grows as well in slightly acid gelatine as in the slightly alkaline medium.

Agar-tubes.—A moist, slimy, porcelain-white surface growth. Excellent growth along the inoculation line.

Potato-cultures.—In two days a good yellowish-white growth, which projects from the surface.

Milk.—In about four days the milk is coagulated, and gives a strong acid reaction.

Broth.—The broth becomes very cloudy in two days. In ten to fourteen days considerable whitish precipitate is produced. A slight skin forms on the surface, but falls to the bottom on shaking the tube. After some time—two to three weeks—the broth is still cloudy.

Nitrate-broth.—Reduces nitrates in broth vigorously.

Microscopical Characters.—Short, plump, oval bacilli, with well-rounded ends. No spore formation observed. Quite variable in size; slightly longer and thicker on potato than on agar. They are frequently grouped in pairs. About 0·8 μ to 1·9 μ long, 0·7 μ to 1 μ broad. Very motile. Grow better at 37° C. than at 21° to 23° C. Require oxygen; grow very scantily under a mica plate.

Bacillus Superficialis.

Found frequently in Lawrence sewage.

Plate-cultures.—The colonies become plainly visible to the naked eye in about two days. Under a low power the colony itself is seen to be approximately round, but is divided by irregular lines into angular lumps, giving a somewhat cracked appearance to the whole colony. On coming to the surface a regular, round, homogeneous, finely granular expansion is formed. To the naked eye the whole colony then appears as a projecting translucent drop. The colony grows slowly, and gradually liquefies the gelatine. When the liquefaction has proceeded for several days the colony has a yellowish-brown opaque centre and a translucent edge.

Gelatine-tubes.—A very slow growth, taking about ten days to liquefy the gelatine to the walls of the tube. The growth is almost wholly on the surface, and there is only the scantiest growth along the inoculation line. Even after standing for several weeks only the gelatine in the upper part of the test tube is affected by the growth.

Agar-tubes.—On agar there is a moist, lustrous, grey, translucent growth. After several weeks the growth is still smooth and shiny and has assumed a light brown tint.

Potato-culture.—Potato does not seem to be a favourable medium for the development of this species. Repeated trials at different temperatures have failed to induce a growth.

Milk.—There is no visible change in twenty days. The reaction, however, is slightly acid.

Broth.—Becomes turbid very slowly. A scanty white precipitate is formed after some time. No skin.

Nitrate-broth.—There is no reduction to be observed in forty days.

Microscopical Characters.—Fair-sized, plump bacilli, with rounded ends. Generally occur singly or in pairs. No spore formation observed. About 2·2 μ long, 1 μ broad; motile. Grow better at 37° C. than at 21° C., and develop feebly under a mica plate.

Bacillus Rubescens.

Isolated from Lawrence sewage.

Plate-cultures.—A slow growth. The young colonies beneath the gelatine are usually round, sometimes oval. On coming to the surface they rise into a projecting porcelain-white drop. The colonies increase in size slowly, and eventually take on a slight brownish tint.

Gelatine-tubes.—The growth is slow and mainly on the surface, where a porcelain-white, nail-head projection is found. There is only a slight growth along the line of inoculation. There is good growth in slightly acid gelatine.

Agar-tubes.—There is a rapid surface growth, white and lustrous. At first the growth is smooth, but later becomes crinkly, and the whole skin is much wrinkled. In cultures about three weeks old a slight pinkish tinge can be seen.

Potato-cultures.—There is a rapid luxuriant growth on potato. At first the colour of the growth is light brown, but this slowly changes to pink. In three weeks there is a luxuriant, projecting growth, tinted a delicate flesh-pink. The potato itself is not coloured.

Milk.—The milk is not coagulated, and gives a good alkaline reaction. In long-standing cultures a slight pinkish tinge is observed at the surface of the milk.

Broth.—The broth becomes slightly turbid and a heavy white precipitate is formed. In several weeks a thick tenacious skin forms on the surface; the main body of the broth is then clear.

Nitrate-broth.—No change in the nitrates after fifty days.

Microscopical Characters.—Large, long bacilli with well-rounded ends. Often occur in pairs and short strings. Many of the individual bacilli are slightly curved. No spore formation observed. About 4 μ long and 0·9 μ broad. A slow, sluggish movement. Grow better at 21° C. than at 37° C., and develop very poorly under the mica plate.

Bacillus Hyalinus.

Isolated from the sand of Tank 13 at a time when the tank was nitrifying well. Found in large numbers in the sand.

Plate-cultures.—Fast-growing. In twenty-four hours the colonies can be plainly seen with the naked eye. In all, even in the smaller colonies, there is a dark centre surrounded by a broad translucent zone, which gives a hazy appearance to the colonies. With a low power the interior is seen to be coarsely fibrillar, with short fibrils radiating from the edge. In two days the colonies are large—about one and a half centimetres in diameter; the contour is evenly round, the interior slightly translucent; the rim is distinct, opaque, yellowish; the edge still shows radiating fibrils.

Gelatine-tubes.—In two days a long, narrow, funnel-shaped growth. The gelatine is rapidly liquefied, and is at first cloudy, with a precipitate at the bottom of the funnel. In about eight days the tube has assumed a highly characteristic appearance: a lustrous, tenacious, white scum, a slight, flocculent precipitate, and perfectly transparent liquefied gelatine between. Grows very well in acid gelatine.

Agar-tubes.—A rapid, dry, spreading, greyish growth. Dull, tough, and rather thin. When about four days old warty projections appear.

Potato-cultures.—In two days the growth is just visible; in four days good, spreading, whitish-grey growth. Later, small protuberances appear on the surface.

Milk.—In seven days strongly coagulated, reaction acid.

Broth.—Soon becomes cloudy, a stringy precipitate, and a thick skin on the surface.

Nitrate-broth.—Reduces nitrates vigorously and rapidly.

Microscopical Characters.—Large, long, stout bacilli, with rounded ends. Usually gathered in short strings. No spore formation observed. About 3·6 to 4 μ long and 1·5 μ broad. Highly motile; grow better at 37° C. than at 21° to 23°: develop well under a mica plate.

Bacillus Delicatulus.

Isolated from the effluents of nearly all the tanks.

Plate-cultures.—When young, whitish, homogeneous, with a regular, radiating edge. In two days the gelatine becomes liquefied; later, the centre becomes darker than the surrounding zone.

Gelatine-tubes.—In two days the gelatine is liquefied well down into the inoculation line. The gelatine is completely liquefied in about seven days. There is a thick whitish skin on the surface, and a heavy, flocculent, brownish precipitate at the bottom.

Agar-tubes.—At first a crinkly greyish growth which, when older, becomes porcelain-white and glistening. Grows well both on the surface and below.

Potato-cultures.—A grey spreading growth; not projecting.

Milk.—The milk is coagulated, and gives a strong acid reaction.

Broth.—Soon becomes cloudy. A white precipitate and a white scum.

Nitrate-broth.—Reduces nitrates to nitrites very rapidly and completely.

Microscopical Characters.—Medium-sized, plump bacilli; often joined in pairs and in short strings, 2 μ long and 1 μ broad. Spore formation was not observed. Very motile. Grows better at 37° C. than 21° C. This species is very sensitive to low temperatures; it refuses to grow at about 15° C. The cultures in tubes appear to live only a short time.

Sewage-bacteria described by Laws and Andrewes.

In 1894 Laws and Andrewes presented to the London County

Council a report on the micro-organisms of sewage. They investigated specimens of sewage from various sources, and described the following new species. The descriptions are taken from the above-mentioned report:—

Proteus Cloacinus.

Source.—Found in every sample of sewage.

Form and Arrangement.—A short bacillus of variable length; 0·4 to 0·5 μ in breadth; stained specimens from a two-days old agar culture vary from 0·5 to 1 μ in length; the shorter forms almost resembling cocci. On gelatine some longer forms are seen up to 1·6 μ in length, and these may be as much as 0·6 μ in breadth. In a one-day-old broth culture the average length is 0·8 μ. It is actively motile. It does not form spores, and grows well at 37° C. and 20° C.

Gelatine Plates.—The colonies are visible to the naked eye in nineteen hours, though still minute. Under the microscope the surface colonies are thin, transparent, and irregular in outline; the deeper colonies globular and brownish by transmitted light. In two days the surface colonies are more circular and regular in outline. The largest are about 1 mm. in diameter; a few show a thin spreading edge, but no "swarming islands" are given off. No further change occurs, except that the colonies increase in size up to 2 mm. in diameter, and become raised at the edge; they are of a greyish-white colour. No liquefaction occurs even at the end of a fortnight. The plates have a sour smell.

Gelatine-streak.—Rapid growth with irregular edges, but shows no "swarms" as a rule. The growth strongly resembles that of B. coli communis. Liquefaction occurs in about three weeks. Occasionally the gelatine is liquefied in three to five days, and in these cases swarming islands are thrown out like the common Proteus.

Gelatine-stab.—Growth is rapid and well-marked along the needle track. Scanty gas bubbles are formed. Liquefaction first commences in from two to three weeks. The growth is white and nodular in the depth.

Glucose-gelatine (shake).—There is profuse formation of gas bubbles in twenty-four hours.

Agar-plates.—Incubated at 37° C. show exceedingly rapid and characteristic growth. A single colony spreads in thirty-six hours as a thin irregular dendritic film, covering almost the whole surface of the plate. The film does not thicken on further incubation.

Agar-streak culture.—Growth is very rapid both at 37° C. and 20° C. Even at the latter temperature the whole surface of the tube is covered in twenty-four hours by a thin homogeneous semi-transparent greyish film, advancing at the edge by irregular processes. On the second day the growth is somewhat thicker and whiter, and has reached its full development. There is a scanty development of gas bubbles in the depth of the agar-agar, but no odour of putrefaction is ever perceptible.

Potato.—Grows rapidly, forming a slimy layer of a yellowish-grey colour.

Broth.—In twenty-four hours the broth is turbid, and in a few days a slight film is present on the surface and a yellowish-white deposit at the bottom of the tube. In a week there is a marked putrefactive odour. Tested in eleven days no trace of indol reaction was obtained.

Milk.—Grows without producing any change in this medium.

Micrococcus Aurora.

Source.—Found in fresh sewage from St. Bartholomew's Hospital.

Form and Arrangement.—Slightly oval cocci, 0·7 to 0·8 μ in diameter, often as diplococci separated by a cleft, rarely in short chains of three or four elements. In broth cultures the cocci are often arranged in irregular packets.

Motility.—Not motile.

Gelatine Plates.—Colonies first appear on the third day, minute and circular, not yet pink in colour. Under the microscope they appear granular and slightly irregular in outline. On the fifth day the surface colonies are less than 0·5 mm. in diameter, circular, prominent, and pinkish. After a week the largest colonies are 0·7 mm. in diameter, those in the depth 0·25 mm., and of a pale pink tint. Later the colonies attain a diameter of 1 to 1·5 mm., and liquefaction slowly occurs after two to three weeks.

Gelatine-streak.—After two days a narrow, prominent line of growth of a pale pink tint, which eventually attains a width of 1·5 to 2 mm., and a bright pink colour. Liquefaction occurs after fourteen to sixteen days.

Gelatine-stab.—Definite growth in three days, almost entirely confined to the surface; in the depth of the gelatine little or no development occurs. A flat layer, 4 mm. in diameter and of a light pink colour, is present on the surface at the end of a week. Liquefaction is extremely tardy, but some depression of the surface occurs after a fortnight, and distinct liquefaction begins in sixteen days.

Agar-agar.—Streak-cultures at 20° C. show distinct growth in two days, semi-transparent and barely pink. At the end of a week the growth is opaque and of a bright pink colour. At 37° C. no growth occurs.

Potato.—A thick layer of a brilliant deep pink colour, which eventually covers the whole surface.

Broth.—The fluid gradually becomes turbid, and after a week there is a light pink sediment at the bottom of the tube.

Temperature.—Grows well at 20° to 22° C. No growth on any medium at 37° C.

Spore-formation.—Not observed.

Gas-production.—Not observed.

Need of oxygen.—Growth very scanty away from the surface.

Pigment-production.—Produces a bright pink pigment, readily soluble in alcohol, only slightly so in water.

Relations.—Appears most closely related to Micrococcus agilis and Micrococcus roseus, but differs from the former in the absence of motility and the lighter pink colour of the cultures, and from the latter in the absence of any growth at 37° C.

Bacillus Cloacæ Fluorescens.

Source.—Found in Homerton sewage and sewage from St. Bartholomew's Hospital.

Form and Arrangement.—Motile rods, on an average twice as long as broad. Breadth, 0·6 to 0·75 μ; length, 1 to 2 μ. The rods are sometimes attached in chains. It is actively motile. It is unable to grow at 37° C. No spore formation.

Gelatine Plates.—Minute colonies become visible in twenty-four hours. In two days the surface colonies begin to liquefy, and are circular, 2 to 2½ mm. in diameter, and under the microscope are seen to have a very delicate, transparent, irregular, spreading margin not yet liquefied. The circular liquefied portion is turbid, with a granular deposit in the centre, and shows slight but distinct greenish fluorescence. On the third day the colonies are 5 to 6 mm. in diameter, and partly fused with one another; the whole plate being largely liquefied with bright yellowish-green fluorescence, and having a sour, unpleasant smell.

Gelatine-stab.—Growth chiefly on the surface; hardly any to be seen along the depth of the track. Liquefaction begins at the surface on the second day in a cone, extending some way along the needle tract. It gradually spreads, chiefly at the surface, till it reaches the wall of the tube in seven days. In fourteen days the gelatine is liquefied from 10 to 12 mm. from the surface, and spreads downwards in a horizontal plane. The liquefied portion has a yellowish-green fluorescence.

Agar.—No growth at 37° C. At 20° C. whitish, semi-opaque growth, with an irregular edge. The agar has a brilliant yellowish-green fluorescence.

Potato.—A dirty brown layer, spreading from the line of inoculation.

Broth.—Rapid growth causing dense turbidity. A thin film forms on the surface, and the top layer of the broth acquires a marked greenish fluorescence.

Milk.—No coagulation, but after four days the upper layer becomes slightly yellowish and less opaque, owing to gradual digestion.

Gas-production.—Not observed. Growth scanty away from the surface. Oxygen seems necessary for the production of the fluorescent pigment.

Bacillus Fluorescens Stercoralis.

Source.—Homerton and Barking sewage.

Form and Arrangement.—Motile rods, almost twice as long as broad, but of variable length. The breadth varies from 0·5 μ to 0·7 μ, and the length from 0·8 to 1·6 μ; longer forms rarely occur. Actively motile.

Gelatine Plates.—Tiny circular colonies are visible under the microscope in nineteen hours. In twenty-four hours they are visible to the naked eye. In two days the surface colonies are liquefied and appear as uniformly turbid circles, with a whitish flocculent deposit at bottom. The deeper colonies are white, spherical, and still very minute. In three days the colonies have increased up to 10 mm., and are fused in places, the plate being mostly liquefied with distinct greenish-yellow fluorescence, and having a sour, fæcal smell. On the fourth day the plate is completely liquefied, and the fluorescence brighter.

Gelatine-stab cultures.—In nineteen hours there is already liquefaction at the surface, as a small flat hemisphere, 2 mm. in diameter, which in two days attains a diameter of 6 or 7 mm., and in three days reaches the walls of the tube, though not to a greater depth than 3 mm. Little growth occurs along the needle track. The liquefaction always advances in a horizontal plane. Greenish fluorescence is marked in the liquefied portion by the fifth day; a thin pellicle forms on the surface, and there is a thick whitish deposit at the bottom of the liquefied portion.

Agar-agar.—No growth occurs at 37° C. At 20° C. growth is rapid. In streak cultures there is a greyish-white growth, with a thick, slightly irregular edge. The agar acquires a brilliant greenish-yellow fluorescence.

Broth.—The growth is similar to B. fluorescens liquefaciens; marked green fluorescence appears in the surface layers after the third day.

Milk.—The milk does not coagulate, but is gradually digested, the upper layer becoming yellowish and transparent after two days; this change gradually spreads downwards, and the upper layers show a yellowish-green tint.

Gas production has not been observed.

This organism is closely allied to the B. fluorescens liquefaciens, but differs from it in the mode and rate of liquefaction of gelatine, and in the greater brilliancy of the fluorescence it produces on agar cultures, in which it resembles B. pyocyaneus. From the last mentioned species it differs in its refusal to grow at 37° C.

Sewage Bacteria, isolated by Houston, from London Crude Sewage and the Effluents from Coke-beds.

The latest contribution to the study of sewage flora has been made by Houston, who, during his studies of the bacterial treatment of sewage, isolated a number of micro-organisms which appeared to be new species and peculiar to sewage. The following descriptions are taken from the second report on the bacterial treatment of sewage, presented to the London County Council.

Sewage Proteus.

Source.—Very abundant in London crude sewage; frequently as many as 100,000 per c.c. of crude sewage.

Gelatine-plate Cultures.—In less than twenty-four hours at 20° C. the surface colonies appear as delicate granular films of an irregular shape. In two days the colonies look like "punched out" circles, containing liquefied gelatine and greyish-white bacterial deposit. The masses of bacteria lying in the liquefied gelatine give the colonies a mottled appearance. Under a low power the individual bacilli can be made out, and their active movement watched. The colonies are usually exactly circular in shape, with well-defined borders, and no "swarming islands" appear to be given off as in the Proteus vulgaris. By the third or fourth day the plate is completely liquefied.

Gelatine-stab Culture.—The growth is very characteristic, in twenty-four hours at 20° C. liquefaction has occurred all the way down the path of the needle, and minute bubbles of gas may be seen rising to the surface through the turbid liquefied gelatine. In forty-eight hours the liquefaction is more pronounced, and numerous bubbles may be seen at the surface, and also bubbles in the solid gelatine. The bacteria collect at the foot of the liquefied portion, as a greyish-white deposit. In four or five days the whole of the gelatine is converted into a greyish-white liquid.

Gelatine-shake Cultures.—In twenty-four hours at 20° C. numerous gas bubbles are formed, and the gelatine is liquefied near the surface.

Agar-plate Cultures.—The colonies are more or less circular in shape, greyish-white in colour, and have a moist glistening appearance.

Agar-stab Culture.—There is growth all down the stab; gas bubbles may form in the medium; on the surface the growth is like a surface colony.

Agar-streak Culture.—A greyish-white moist glistening growth.

Potato Culture.—A slimy, thin, yellowish-white growth.

Broth.—Abundant, diffuse, cloudy growth.

Litmus-milk.—The opaque bluish-purple coloured fluid is changed into semi-transparent reddish-coloured fluid. If clotting occurs, it is imperfect in character as possibly casein is dissolved as soon as it is formed.

Indol Reaction.—Usually no indol is formed in broth cultures, but in one such culture kept for twelve days a feeble reaction was obtained.

Reduction of Nitrates.—Rapid reduction of nitrates to nitrites in twenty-four hours at 20° C.

Microscopical Appearances.—Small bacillus with rounded ends, solitary, in pairs, or sometimes in short chains; involution forms may be seen. It is actively motile, possesses a single flagellum, and does not form spores. The original culture grew better at 20° C. than at 37° C.

Remarks.—This organism differs from Proteus vulgaris; it has been called sewage Proteus, owing to its prevalence in sewage and its superficial resemblance to the members of the Proteus group. Some of the cultures isolated from sewage were pathogenic to guinea-pigs; others grew well at 37° C.

Bacillus Frondosus.

Isolated from London crude sewage.

Gelatine-plate Cultures.—The surface colonies appear as white coarsely-granular films of irregular shape, which send out curious processes of varied form, resembling bits of sea-weed flattened out. Liquefaction is slow and is shown by slight pitting near the centre of each colony. The deep colonies are not characteristic. Under a low power the superficial colonies have a laminated appearance, and show at the spreading edge processes of most varied shapes, which form patterns of great delicacy and beauty.

Gelatine-stab Culture.—The growth at the surface is like a superficial colony; the growth down the stab is not characteristic. Liquefaction sets in slowly.

Gelatine-streak Culture.—A coarsely-granular film appears, which peripherally shoots out processes of very irregular shape.

Gelatine-shake Culture.—No bubbles of gas are formed.

Agar-plate Cultures.—The colonies are white in colour and do not grow out in the same characteristic manner as on gelatine.

Agar-streak Culture.—A greyish-white film, which at the margin resembles the corresponding growth on gelatine.

Potato Cultures.—A transparent colourless film.

Broth Cultures.—Grows slowly; a white deposit forms at the bottom of the tube, leaving the liquid above fairly clear.

Litmus-milk Cultures.—In milk at 20° C. no change is visible in three days; later, the milk turns slightly acid; no clotting is visible even after one month.

Indol Reaction.—No indol is formed in broth after six days incubation at 20° C.

Reduction of Nitrates.—No reduction takes place in four days at 20° C.

Microscopical Appearances.—Large motile bacillus with rounded ends; solitary, in pairs, and in chains of varying length. It forms spores. There is no growth at 37° C.

Bacillus Fusiformis.

Isolated from London crude sewage.

Gelatine-plate Cultures.—The growth is very slow. The surface colonies are circular, of an opaque porcelain-white glistening appearance. The periphery is slightly transparent. By transmitted light the colonies are yellowish in colour. Under a low power, no delicate details of growth can be made out. No liquefaction of the gelatine takes place.

Gelatine-stab Cultures.—A white growth appears along the line of the stab, and on the surface a scanty yellowish-white layer slowly develops.

Gelatine-shake Cultures.—No gas bubbles are formed.

Agar-streak Cultures.—A thin whitish layer is slowly formed, which is not characteristic.

Agar-plate Cultures.—The colonies are white and more or less circular in shape; the growth is not characteristic.

Potato Cultures.—A porcelain-white growth slowly develops, afterwards the colour becomes dirty yellowish-white, and the bacterial layer becomes unequally thickened.

Broth Cultures.—The growth is scanty and not characteristic.

Litmus-milk Cultures.—No clotting occurs; after sixteen days very feeble acidity is produced.

Indol Reaction.—No indol reaction is obtained in broth cultures.

Reduction of Nitrates.—No reduction after twelve days at 20°C.

Microscopical Characters.—Large motile bacillus, with rounded ends; solitary, in pairs, and in chains. It forms large spindle-shaped spores.

Remarks.—This micro-organism has a somewhat negative character in all the media ordinarily in use. It has been called B. fusiformis, owing to the spores being spindle shaped.

Bacillus Subtilissimus.

Isolated from crude sewage.

Gelatine-plate Cultures.—The surface colonies are very characteristic, and grow so quickly that a single colony may cover nearly a whole plate in two days. The growth is film-like, exceedingly thin and transparent, dull grey in colour, and very faintly granular. Under a low power the colony resembles B. coli, but the growth is so delicate that it is difficult to see the delicate veining of the bacterial film. The surface colonies are more or less circular in shape, but the spreading margin is nearly always irregular.

Gelatine-streak.—The growth is like an elongated surface colony. In less than twenty-four hours a delicate film has spread nearly to the walls of the tube. The spreading edge is very irregular, and in older cultures may present a terraced appearance.

Gelatine-shake Culture.—No gas bubbles are formed in the gelatine.

Agar-streak.—A white film is formed on the surface, having a markedly irregular edge. The lateral expansion is less than in the case of gelatine cultures.

Broth Cultures.—Uniform turbidity occurs in twenty-four hours at 20° C., and a very faint scum forms on the surface.

Litmus-milk Cultures.—No clotting or production of acidity.

Indol.—No indol is formed even after twenty days at 20° C. !

Microscopical Appearances.—In most cultures it appears as a large micrococcus. In the spreading edge of the colony the elements are distinctly longer than broad; nearer the centre they are oval, and frequently united in pairs; at or about the centre they are perfectly spherical.

Remarks.—This microbe has been called B. subtilissimus on account of the thin, almost gauze-like, character of the surface colonies in gelatine-plate cultures.

Bacillus Membraneus Patulus.

Isolated from crude sewage and effluent from coke-beds.

Gelatine Plates.—The surface colonies appear as coarsely granular, greyish-white films of somewhat irregular shape. From the spreading edge of the colony processes extend in a tortuous fashion over the surface of the medium, often forming patterns of great delicacy and beauty. Beneath the surface film-like growth slow liquefaction of the gelatine occurs. Under a low power the colonies present a characteristic, granular, and striated appearance.

Gelatine-streak.—In less than two days at 20° C. a coarsely granular film forms on the surface of the gelatine, which spreads rather rapidly; from the margin processes are given off which wind over the gelatine in a characteristic way. Soon a longitudinal pitting of the bacterial film along the line of inoculation is observed, and the growth sinks down to the foot of the tube.

Agar-streak.—In one night at 37° C. the growth appears as a coarse, granular, semi-transparent, greyish-white film. In old cultures the surface assumes a tuberculated appearance.

Gelatine-stab.—The growth varies; sometimes there is liquefaction down the line of the stab with tuft-like processes extending into the solid medium, and at other times there is no liquefaction, and the processes extend to the wall of the tube. The growth on the surface is like a surface colony in a gelatine plate.

Gelatine-shake Culture.—There is no gas formation.

Broth.—It grows rapidly at 37° C., producing a flocculent growth. On the surface a skin is formed, which sinks on shaking the tube.

Indol.—There is no formation of indol in broth cultures.

Litmus-milk.—A gelatinous clot is formed and the medium becomes faintly acid.

Potato.—A dirty, faint, yellowish-grey coloured growth.

Nitrate-broth.—Great reduction of nitrates in one night at 37° C.

Microscopical Characters, &c.—A non-motile, very large bacillus, which forms long chains. The cultures resist heating to 80° C., but a satisfactory double-stained spore preparation has not been obtained. It grows well at 37° C. and at the room temperature.

Bacillus Capillareus.

Isolated from crude sewage and effluents from coke-beds.

Gelatine-plate Cultures.—The colonies in the depth have a characteristic fluffy appearance, and when they reach the surface quickly liquefy the gelatine. The growth is filamentous in the depth, and on the surface from the spreading edge of the colonies delicate film-like processes are given off, which extend over the surface of the medium to form irregular patterns. Later, the final details are lost owing to the rapid liquefaction of the gelatine, the colonies appearing as large, more or less circular, areas of liquefaction with greyish-white contents.

Agar-streak.—In twenty-four hours an opaque-white growth of limited extent appears, which along the spreading edge is slightly transparent and granular-looking.

Gelatine-streak.—A longitudinal furrow appears due to the liquefaction of the gelatine; from the edge of the furrow delicate processes may be seen, extending in irregular fashion over the solid surface of the gelatine.

Gelatine-stab.—Liquefaction takes place in funnel form, and extends down the stab to an extent varying in different cultures. Along the line of inoculation feathery processes are given off, which extend into the solid gelatine for a short distance. The

masses of bacteria gradually sink to the foot of the liquefied medium.

Gelatine-shake.—No gas is formed. Sometimes the colonies have a star-shaped appearance, but at other times they are globular.

Broth.—At 37° C. there is a diffuse cloudiness; later a skin forms on the surface, and an abundant white deposit forms at the foot of the tube, leaving the liquid above fairly clear.

Indol.—No indol is formed in broth cultures.

Litmus-milk.—No decided change after seventy-two hours at 37° C. In five days weak gelatinous clot, but no acidity.

Potato.—By the second day at 37° C. a fairly abundant creamy coating has formed on the surface, and later becomes of a dirty-brown colour.

Nitrate-broth.—Reduces nitrates to nitrites.

Microscopical Appearances, &c.—A large bacillus forming long chains. It is motile. Old cultures resist heating to 80° C.; but a satisfactory preparation of spores has not been obtained. It grows luxuriantly at 37° C. and at room temperature.

Bacillus Mesentericus (Sewage variety E.).

Isolated from London crude sewage. Gelatine plates heated to 80° C. for ten minutes.

Gelatine-plate Cultures.—The growth is not very rapid. The deep colonies have a somewhat star-like appearance; the superficial colonies appear as bluish-white, delicate granular films, which are almost colon-like in character. They are somewhat irregular in shape, and from the wavy transparent edge irregular processes are given off. Under a low power the deep colonies show a central darkish yellow spot from which root-like processes are given off. The surface colonies are transparent, granular, and striated; and from the edge delicate processes are given off, which spread over the gelatine, forming curious and intricate patterns. The colonies do not attain a large size, and liquefaction only slowly sets in.

Gelatine-stab.—The growth at the surface is slow and resembles a superficial colony. Growth takes place all the way down the stab accompanied by slow liquefaction; delicate tuft-like filaments are given off all down the line of inoculation.

Gelatine-streak.—A delicate granular film of limited extent forms on the surface of the gelatine. After some days the film changes its appearance, and shows numberless fine processes radiating outwards and upwards from the central line. Tuft-like processes also extend into the solid gelatine. Liquefaction slowly sets in and shows itself as a longitudinal furrow.

Gelatine-shake Cultures.—No gas formation. The gelatine is slowly liquefied.

Agar-streak.—In twenty-four hours at 37° C. a dirty yellowish-white film has covered nearly the whole oblique surface. The spreading edge may extend as irregular processes, forming leaf-like patterns. Later, the film darkens in colour and becomes wrinkled.

Potato Cultures.—The growth is extremely characteristic. At 37° C. in twenty-four hours the whole surface of the potato is covered with a thin, yellowish-white film, which has a characteristic, folded, wrinkled appearance. The substance of the potato takes on a bright pink colour. Later, the film becomes thicker, more deeply pitted and wrinkled, and the colour becomes brown.

Broth.—In twenty-four hours at 37° C. a greyish-white wrinkled film is formed at the surface, and the liquid below is nearly quite clear.

Litmus-milk.—In seventy-two hours a weak clot has formed and the liquid near the surface is semi-transparent. On shaking the tube a pinkish tinge develops.

Indol Reaction.—No indol is formed.

Nitrate-broth.—Great reduction of nitrates to nitrites in twenty-four hours.

Microscopical Appearances.—Long bacillus with rounded ends, solitary, and in pairs and long chains. It is actively motile, possesses numerous flagella, and forms spores. It grows very rapidly at 37° C.

Remarks.—This bacillus appears to be identical with B. mesentericus ruber.

Bacillus Mesentericus (Sewage variety I.).

Isolated from London crude sewage. Gelatine-plate cultures heated to 80° C. for ten minutes. It is constantly present in sewage in the form of spores.

Gelatine-plate Cultures.—The growth is characteristically rapid. The deep colonies quickly reach the surface, to form saucer-shaped areas of liquefaction; from the edge of these processes may be given off which are almost of the nature of "swarming islands." In the areas of liquefied gelatine the bacteria may be gathered in clumps so as to give rise to a mottled appearance. Under a low power the surface colonies may be almost completely translucent and of an irregular star-shaped form; but soon circular areas of liquefied gelatine are produced, in which the extraordinarily rapid movement of the individual bacilli can be clearly watched.

Gelatine-stab.—Rapid liquefaction takes place in the form of a funnel. The liquefied gelatine has a greyish-white flocculent appearance, due to aggregation of little masses of bacteria.

Gelatine-streak.—The growth is not characteristic owing to the rapid liquefaction.

Gelatine-shake Cultures.—No gas bubbles are formed.

Agar-streak.—In twenty-four hours at 37° C. a dirty, yellowish-white film has covered nearly the whole surface of the agar. The spreading edge may be lobed or fissured. The film darkens in colour and becomes wrinkled.

Potato Cultures.—The growth is extremely characteristic. In twenty-four hours at 37° C. the whole surface of the potato is covered with a thick, greyish-white moist skin, which is thrown into multiple folds, creasings and wrinkles. The colour rapidly changes from greyish-white to yellow and then to brown.

Broth Cultures.—The growth at 37° C. is characteristic even in twenty-four hours. A greyish-white wrinkled film is formed at the surface, and the liquid below is nearly quite clear. Later the film thickens and acquires a reddish-brown colour; the liquid below, which remains nearly quite transparent, also takes on a reddish-brown colour.

Litmus-milk.—In forty-eight hours there is no clot, but the milk is rapidly becoming transparent; in seventy-two hours the whole of the contents of the tube are semi-transparent and of a dirty yellowish colour. On gently shaking the tube, the liquid assumes a crushed-strawberry colour.

Indol.—No indol is formed.

Nitrate-broth.—No reduction of nitrates to nitrites in twenty-four hours at 37° C.

Microscopical Characters.—Medium-sized bacillus with rounded ends, solitary, in pairs, and in chains. Very motile, possesses numerous flagella, and forms spores. Grows with great rapidity at 37° C.

Remarks.—This micro-organism is probably identical with Bacillus mesentericus vulgatus.

Bacillus Subtilis (Sewage variety A.).

Isolated from crude sewage and effluents from coke-beds.

Gelatine-plate Cultures.—In two days at 20° C. it forms circular greyish-white areas of liquefaction; masses of bacteria in the liquefied gelatine may give the colony a mottled appearance. Under a low power the bacilli at the margin of the colony are seen to bore into the gelatine, side by side, in a highly characteristic manner.

Gelatine-stab.—In two days at 20° C. the gelatine is liquefied down to the foot of the stab. A scum forms on the surface, but no distinct skin is formed.

Agar-streak.—In two days at 37° C. there is an abundant creamy-white layer covering nearly the whole surface, which shows numerous minute circular areas where the growth, instead of being opaque, is semi-transparent.

Gelatine-shake.—There is no gas formation.

Broth Cultures.—Diffuse cloudiness in twenty-four hours at 37° C. A scum forms on the surface, which readily falls to the foot of the tube on shaking.

Indol.—There is no formation of indol.

Litmus-milk.—In two days at 37° C. there is complete discoloration and a clot has formed; no redness is visible. In five days a white clot occupies about one-third of the bulk of the medium, which appears to be slowly peptonised. The liquid surrounding the clot is pale yellow in colour, semi-transparent, and without pink coloration.

Potato Cultures.—In two days at 37° C. a dirty-white layer is formed with a yellowish tint.

Microscopical Appearances.—Large, long bacillus with rounded ends, frequently in long chains. It forms spores and has a waddling, sluggish movement. It grows well at 37° C.

Bacillus Subtilis (Sewage variety B.).

Isolated from crude sewage and effluents from coke-beds.

Gelatine-plate Cultures.—Rapidly forms greyish-white circles of liquefied gelatine. The masses of bacteria lying in the liquefied gelatine may present a rosette or star-shaped appearance. The parallel arrangement of the bacilli at the periphery of the colony is absent, or not so well marked as in the above variety. A skin forms on the surface of the liquefied gelatine.

Agar-streak.—In twenty hours at 20° C. there is a greyish-white layer. In forty-eight hours the growth is somewhat dry and granular looking, and in four days ridges stand out from the surface of the medium about one-sixteenth of an inch. They are usually arranged in more or less transverse folds.

Gelatine-stab Cultures.—Rapid liquefaction all the way down the stab, but as the growth proceeds the liquefaction spreads in a cylindrical fashion from above downwards. A distinct skin forms on the surface, which eventually sinks in the liquefied gelatine.

Gelatine-shake.—There is no gas formation.

Broth.—Diffuse cloudiness; on the surface a skin forms, which is brittle, and sinks on shaking the tube.

Indol.—There is no formation of indol.

Litmus-milk.—The pale-blue colour gradually fades, but no clotting or redness occurs. In eight days the milk is almost transparent, and of a pale, dirty yellow colour.

Potato Cultures.—A white rather dry-looking coat is formed. Later, portions of the growth become upraised, and sometimes present a worm-like appearance.

Microscopical Characters.—Large, long bacillus with rounded ends, often in long chains. It forms spores and has a waddling, sluggish movement. It shows little or no growth at 37° C., but grows luxuriantly at 20° C.

Thermophylic Bacteria.

The micro-organisms which are capable of growing luxuriantly at a temperature of 60° to 70° C. are of considerable interest to the water bacteriologist. They are found in the alimentary canal of human beings and animals, in sewage, and in polluted

waters, but do not appear to be present in pure waters. In 1888 Globig showed that a number of bacteria were capable of growing at a temperature of 50° to 70° C. He isolated these organisms by inoculating potatoes with soil, and then incubating them at varying temperatures. In the same year Miquel described the B. thermophylus, which he found to be very prevalent in sewage-polluted water, as many as 1000 per c.c. being detected in water from the Seine collected at the Bridge of Austerlitz. According to Miquel the organism was not present in spring-water; it was occasionally found in air, but its normal habitat appeared to be sewage. When drops of drain-water were inoculated into broth, and then maintained at 69° C., the medium was found to become turbid in twenty-four hours, and contained the B. thermophylus. On agar at 42° to 45° C. the bacillus gave rise to a white raised meniscus-shaped growth, which, on microscopic examination, showed the presence of a short, plump bacillus, with a highly refracting spore at one end. In broth it grew best at a temperature of 65° to 70° C., and produced an abundant white deposit at the bottom of the tube, the liquid above becoming clear. The microscopic appearance of the bacillus appeared to vary with the temperature at which it was cultivated. At 50° C. the bacillus was short, with an oval spore at one end; at 60° C. it formed filaments, and only a few spores appeared; at 71° to 72° C. no spores were found, and the bacillus appeared swollen. It was not motile, and produced no pathogenic effects in animals. In 1894 Macfadyen and Blaxall isolated thermophylic bacteria from specimens of earth, derived partly from the surface and partly from depths up to five feet. They also found the organisms in Thames mud and in fæces from human beings and mice. In 1895 Rabinowitsch repeated and extended Macfadyen and Blaxall's work. This observer used potato as the nutrient material for isolating the bacteria. After twenty hours incubation at 62° C. four to eight white colonies appeared on the inoculated potatoes; after forty hours incubation yellowish-grey, brown, and reddish-brown colonies also developed. Pure cultures were obtained by inoculating the growths on plates containing three per cent. agar. The thermophylic bacteria were found in earth, snow, the River Spree, and in the excre-

ment of horses, cows, dogs, guinea-pigs, pigeons, mice, frogs, &c. They were detected in gradually increasing numbers in the mouth, stomach, small and large intestines. Rabinowitsch considered that the bacteria were facultative anærobes. At high temperatures they grew well as ærobes, but very slowly as anærobes; at lower temperatures, on the other hand, they grew better under anærobic than ærobic conditions, especially when broth was used as the nutrient medium. The following table gives the characteristics of the eight varieties of thermophylic bacteria which developed at 62° C.:

Number.	Growth on potato.	Colonies on plates.	Microscopical characters.	Spores.	Reaction in broth.
1	White colonies	Coarsely granular colonies with dentate margin	Non-motile rods, often growing into threads	Oval spores at one end of the bacilli	Produces acid
2	Yellowish-grey colonies with irregular margins	Greenish colonies, not so coarsely granular as 1	Non-motile, slightly curved rods	Spores in the centre of the bacillus	Produces alkali
3	Brown colonies	Small, round, greyish, sharply outlined colonies	Thick, non-motile rods	Oval spores at one end of the bacillus	Produces acid
4	Reddish colonies	Colourless colonies, with many thin out-runners	Non-motile, often producing threads	Round spores in the centre of the bacilli	Produces a trace of alkali
5	Scanty growth of a brownish-grey colour	Colourless colonies, granular in the centre	Non-motile rods	Oval spores at one end of the bacilli	Produces a little acid
6	Moist, grey colonies	Greenish-grey colonies, granular in the centre	"	"	Produces a considerable quantity of alkali
7	Whitish-grey colonies	Coarsely granular colonies, with a dentate margin	"	"	"
8	Greyish-brown moist colonies	Round, granular, sharply outlined colonies	"	Spores in the centre of the bacilli	Produces a trace of acid

None of these varieties were pathogenic to mice or pigeons, The spores were not destroyed by exposure to current steam for five or six hours. Also specimens of earth, after drying in an oven for four or five months, produced distinct growths when planted out on potato. The optimum temperature of the thermophylic bacteria appeared to be between 60° and 70° C. Macfadyen and Blaxall, in their later experiments with thermophylic bacteria, employed salt potato-agar as a means of isolating the different varieties of these organisms. They found that the colonies of the bacteria remained discrete on this medium, so it was possible to obtain readily pure cultures of the organisms. "The potato-agar is prepared as follows: Potatoes are first steamed, peeled, and pounded. To 100 grammes of potato is added one litre of tap-water, and the mass is steamed for half an hour and filtered. To the filtrate 1·5 to 2 per cent. of agar is added, and the whole autoclaved for fifteen minutes. It was found an advantage to add 1 per cent. of salt. After neutralisation with soda, and further steaming, the potato-agar is filtered into test-tubes and sterilised once more." From the primary colonies on the salt potato-agar sub-cultures were made on the ordinary culture media. The various organisms were found to stain with ordinary dyes, but, on the whole, carbol-methylene blue gave the best results. It was usually found advisable before staining to treat the cover-slip preparation with dilute acetic acid; this cleared away a zooglœa-like membrane, which often interfered with clear staining. Macfadyen and Blaxall described fourteen thermophylic bacteria.

Bacillus I.—Was isolated from animal dejecta, and produced colonies on agar closely resembling those of the B. anthracis. It liquefied gelatine on the fourteenth day. There was no growth on potato, and no action was produced in milk. In broth there was a viscous deposit, and the supernatant broth was clear. It gave no indol reaction. It was a large, non-motile bacillus, which did not stain with Gram, and contained a spore towards the centre of the slightly swollen rod.

Bacillus II.—Also isolated from animal dejecta, produced dull, white, round, smooth colonies. In broth there was a deposit and a slight surface film; indol reaction was obtained.

There was no growth on potato, and milk was rendered viscous. It was a large, non-motile bacillus, with an oval spore towards one end. It stained with Gram.

Bacillus III.—Isolated from soil; was characterised by small pin-head colonies. There was a viscous deposit and a stringy pellicle in broth, and a positive indol reaction was obtained. On potato there was a cream-coloured growth, and milk was converted into a solid curd. In was a large, non-motile bacillus, with a terminal spore, and stained with Gram.

Bacillus IV.—From ensilage; produced dull, white spherical colonies. There was an indol reaction in broth, and milk was converted into a solid curd. There was no growth on potato. It was a large bacillus, with oscillatory movements. It stained with Gram, and contained a small oval spore towards the slightly swollen end.

Bacillus V.—From Thames mud; gave rise to spherical, yellowish-white colonies, with regular margin. Under a low power the centre appeared dark-brown and finely granular; the outer zone was pale yellow, coarsely granular, with ridged markings. Its reactions in broth, milk, and on potato were the same as No. IV. The bacillus in form was more slender than No. IV., and did not stain with Gram.

Bacillus VI.—Was obtained from Plymouth sea-water, and produced dull, white, feathery colonies. It was a slender, non-motile bacillus with a drum-stick spore.

Bacillus VII.—From soil; produced round, dry, feathery colonies. There was no perceptible growth in broth. On potato there was a vigorous, moist, brick-dust-coloured growth. Milk was converted into a solid curd. The bacillus was non-motile; about the size of the anthrax bacillus, and appeared in pairs and short chains. It stained with Gram, and had a large terminal spore.

Bacillus VIII.—From soil; gave rise to yellowish-white spherical colonies. On agar there was a pale lemon-coloured growth. On potato a moist, cream-coloured growth. There was an indol reaction in broth, and milk was coagulated with separation of whey. It was a large bacillus with oscillatory movement, and contained a large oval central spore.

Bacillus IX.—From soil; produced spherical colonies, with a

dull ground-glass appearance. On agar and potato there was a white, crusty growth. Milk was coagulated, with separation of whey. An indol reaction was obtained in broth. It was a large, non-motile bacillus with a tendency to long thread formation. It possessed a very small terminal spore, and stained with Gram.

Bacillus X.—From soil; was characterised by dull, white, circular colonies. On potato there was a dry, felt-like, reddish-brown growth. Milk was converted into a solid curd. There was no indol reaction in broth. It was a very large motile bacillus with a large, terminal oval spore.

Bacillus XI.—From soil; formed round, dull, white colonies with a moist, glistening appearance. On potato there was a moist, brownish-yellow growth. Milk was coagulated with separation of whey. There was an indol reaction in broth. It was a large bacillus, which formed long chains. It stained with Gram and had a terminal oval spore.

Bacillus XII.—From soil; produced stellate, yellowish-white colonies. On potato there was a dry, pipe-clay coloured growth. Milk was converted into a solid curd. There was an indol reaction in broth. It was a large, non-motile bacillus with a drum-stick spore. It did not stain with Gram.

Bacillus XIII.—From soil; produced round, yellowish-white colonies. On potato there was a moist, yellowish-brown growth. Milk was coagulated with separation of whey. In broth an indol reaction was obtained without the addition of a nitrite! It was a large, non-motile bacillus with square ends. It stained with Gram, and possessed a spore towards the swollen end of the bacillus.

Bacillus XIV.—From soil; gave rise to a thin surface film over agar, with discrete and circular colonies at the margin. On potato there was a moist, slate-coloured growth. Milk was coagulated, with separation of whey. There was an indol reaction in broth. The organism was motile, formed short slender rods, surrounded by a zooglœa. It stained with Gram and contained a spore in the centre of the bacillus. All the above organisms grew well at 55° to 65° C. on ordinary media.

Macfadyen and Blaxall's experiments did not support Rabinowitsch's statement that at low temperature thermophylic bacteria

grow more quickly under anærobic than ærobic conditions. The exact conditions under which these organisms find, in nature, temperatures favourable to their growth, are not yet known; they may possibly be of a chemical nature. Houston found that thermophylic bacteria were present in London crude sewage in abundance. These organisms are, however, found in soils which have not been polluted recently, so their presence in potable waters cannot be said to point to recent contamination. But as all the workers on this subject have failed to detect thermophylic bacteria in water from wells and springs, the presence of these organisms in supplies derived from such sources may safely be assumed to indicate gross contamination. Their absence, however, cannot testify to purity or safety.

CHAPTER XIII.

QUALITATIVE ANALYSIS—*continued.*

CLASS III.

The most important of all the micro-organisms which appear in water are those which produce specific disease in human beings. It is not absolutely proved that the causes of most infectious diseases may not appear in water ; but at the present time it is exceedingly difficult to show the presence of any of them except the spirillum of cholera, and perhaps the B. typhosus.

Bacillus Typhosus.

This micro - organism was first observed by Eberth, and more fully investigated by Gaffky. Its characteristics are as follows :

Gelatine Plates.—The surface colonies appear as thin bluish films with an irregular margin. Under a low power markings are seen, which look like ridges and valleys, running irregularly from the centre to the periphery ; sometimes the markings are very faint, and the colony resembles a very thin film of glass. The colonies in the depth of the gelatine are round or oval in shape, more or less translucent, and appear filled with highly refracting granules. As a rule, the colonies in gelatine, when incubated at 20° to 22° C., grow very slowly, requiring seventy-two to ninety-six hours incubation before they show their characteristic appearances. The gelatine is not liquefied.

Agar Plates. — The surface colonies are white, semi-opaque, with a circular or slightly irregular margin. Under a low power surface markings of ridges and valleys are seen, but they are not usually so distinct as in the gelatine plates. Sometimes the centre appears thicker than the periphery, giving the colony a somewhat ringed appearance. When

examined with the naked eye, the growth on agar at 37° C. is almost as rapid as that of B. coli, and it is often impossible to distinguish between colonies of B. coli and B. typhosus on agar plates. Still, as a rule, the colonies of the latter organism are much smaller and more transparent than those of the former.

Gelatine-stab.—On the surface there is a thin circumscribed growth like a surface colony, and along the line of inoculation a thin greyish growth appears, ending below in discrete white masses.

Gelatine-slope.—A thin, narrow growth developes along the line of inoculation; the margin is slightly irregular, but not so markedly crenate as the corresponding growth of B. coli.

Agar-slope.—A thin, greyish-white growth, usually more transparent than that of B. coli.

Potato.—A smooth glistening film develops, which is often difficult to recognise with the naked eye. If the potato has an alkaline reaction, a faintly brownish coloured growth may appear.

Sugar Gelatine (Shake Culture).—There is no formation of gas in lactose media, but glucose-gelatine is said to be sometimes fermented.

Witte's Peptone and Salt Solution.—After seven days incubation at 37° C. there is, as a rule, no nitroso-indol reaction when the medium is tested with 1 c.c. of potassium nitrite (0·02%), and a few drops of pure sulphuric acid. Occasionally, after standing some few hours, a faint rose tint is shown by undoubtedly pure cultures of B. typhosus.

Milk.—After one month's incubation there is no change in the appearance of this medium.

Neutral-red Glucose-agar.—In this medium, as a stab or shake culture at 37° C., the B. typhosus produces no fluorescence or change in colour. One loop of a broth culture of the suspected organism is added to the melted agar, cooled to 40° C., when a shake is to be made. Scheffler recommended 0·3 gramme of glucose and 1 c.c. of a concentrated aqueous solution of neutral-red, to be added to 100 c.c. of melted agar. B. coli, when inoculated into this medium, produces a marked greenish fluorescence after twenty-four, or at latest forty-eight, hours incubation at 37° C.

Litmus-whey.—After seven days incubation at 37° C. there is a slight development of acid, which, however, never requires more than 6 per cent. of $\frac{N}{10}$ alkali to neutralise it.

Broth.—A diffused growth takes place; an imperfect film appears on the surface after several days incubation.

Gelatine (25 per cent.).—After incubation at 37° C. no film appears on the surface, but a diffused growth takes place throughout the medium.

Proskauer and Capaldi's Medium, No. I. (Asparagin and Salts).—After twenty-four hours incubation at 37° C. there is no growth.

Proskauer and Capaldi's Medium, No. II. (Witte's Peptone and Mannite).—After twenty-four hours incubation at 37° C. there is a distinct growth, and the medium acquires a strongly acid reaction.

Microscopical Characters.—A bacillus about 3 μ long and 1 μ broad, highly motile, with a quick, serpentine movement.

Staining Reactions.—Stains readily with basic dyes. It is decolorised by Gram's method.

Flagella.—Eight to twelve long wavy flagella, disposed all round the bacillus.

Spore-formation.—It does not form spores, and is destroyed when the cultures are heated to a temperature of 65° C. for ten minutes.

Reactions with Anti-typhoid Serum.—The B. typhosus is quickly agglutinated by a highly dilute anti-typhoid serum. A small dose of anti-typhoid serum injected into the peritoneum of a guinea-pig, along with a lethal dose of B. typhosus, protects the animal from the effects of this organism.

The most important of the above tests are the following:

(1) *The Slow Growth on Gelatine Plates.*—This test is of great value; none of the varieties of B. coli grow so slowly on gelatine plates as the B. typhosus. The B. enteritidis of Gärtner, and the organisms of the so-called Gärtner group, are the only bacilli which really grow like B. typhosus on gelatine plates, though even these appear to grow more quickly than the true typhoid organisms.

(2) *The Absence of Gas in Lactose Media.*—By means of this test the typical members of the Colon group are at once excluded from the typhoid group. Lactose media are recommended for this test, as some bacteriologists declare that the B. typhosus may ferment glucose media. Up to the present time I have never found the B. typhosus produce gas bubbles in a glucose-gelatine "shake" culture, or in a glucose-agar-stab.

(3) *The Absence of Indol in Witte's Peptone and Salt Solution.*—This test is not quite so exclusive as the tests just mentioned. Two typical cultures of B. typhosus (one from Kral's laboratory) in my possession give traces of indol after seven days growth at 37° C. in this medium. Also there are varieties of B. coli which fail to produce indol when cultivated in this manner. Witte's peptone is a much better medium than broth for this test. I have often found indol produced in the peptone medium when not a trace could be discovered in broth. Muscle sugar is sometimes found in broth, and bacteria first convert this into acid and then neutralise it by producing alkali. The indol reaction is not obtained when the bacteria are unable to neutralise the acid produced. Theobald Smith recommends the use of dextrose-free broth, which is obtained by inoculating bouillon with B. coli. After twenty-four hours the broth is sterilised and filtered, and peptone and salt added in the ordinary manner.

(4) *The Unchanged Appearance of Milk.*—I have never found a true culture of B. typhosus which coagulated milk even after incubation at 37° C. for one month. At the same time there are many varieties of B. coli which do not change milk; the same is true of varieties of Gärtner's bacillus.

(5) *The Amount of Acid Produced in Litmus-whey.*—After incubation for one week at 37° C. the amount of acid is very small, as compared with the typical members of the Colon group. At the same time there are atypical coliform organisms which produce less acid than the B. typhosus, so the test is not conclusive.

(6) *The Growth on Potato.*—The colourless growth on potato is a time-honoured test of great value, but the character of the growth depends on the reaction of the potato. If the potato has an acid reaction, the growth is colourless and trans-

parent; if the reaction is alkaline, the growth acquires a brownish colour. This coloured growth is never so marked as that obtained when B. coli is grown on the same potato. Some varieties of B. coli, however, produce a colourless growth exactly like that of B. typhosus. The best way to employ the test is to inoculate the same potato on one side with B. typhosus and on the other side with the microbe under investigation.

(7) *Proskauer and Capaldi's Media.*—These media are of the greatest value as a means of diagnosis. No. I. medium has the following composition:

Asparagin	0·20 per cent.
Mannite	0·20 ,,
Sodium chloride	0·02 ,,
Magnesium sulphate	0·01 ,,
Calcium chloride	0·02 ,,
Potassium mono-phosphate	0·20 ,,

The reagents are dissolved in distilled water and sterilised for an hour and a half in the steam steriliser. The medium, which has a slightly acid reaction, is then carefully neutralised with caustic soda. No. II. medium contains:

Witte's peptone	2·0 per cent.
Mannite	0·1 ,,

The reagents, as in No. I. medium, are dissolved in distilled water and sterilised in the steam steriliser for an hour and a half. The medium will then be found to have an alkaline reaction; it must be carefully neutralised with citric acid. A solution of litmus is then added until the media acquire a deep-red colour. They are then sterilised again for half an hour, filtered and placed in sterile test-tubes, which are plugged with cotton wool and covered with a cap of parchment paper, which is fastened to the tube by an india-rubber ring.

If these media are inoculated with the B. typhosus and B. coli respectively and incubated at 37° C. for twenty hours, the following changes will be noticed:

	No. I. medium.	No. II. medium.
B. Typhosus	No growth or change in reaction	Growth, reaction strongly acid.
B. Coli	Growth, reaction acid	Growth, reaction neutral or faintly alkaline.

After two or three days incubation the acid reaction of B. coli in No. I. medium changes to an alkaline reaction.

Most of the gas-producing varieties of B. coli conform to the reaction described by Proskauer and Capaldi.

The B. enteritidis of Gärtner also behaves like a B. coli. Varieties of B. coli which I isolated from water and which strongly resembled the B. typhosus in nearly every test failed to give the characteristic reaction in these media; they all grew in No. I. medium and rendered No. II. medium alkaline in twenty hours. The B. typhosus simulans varieties (a, b, c, and d) described by Houston were also, according to Horton-Smith, at once differentiated from the typhoid bacillus by means of these media.

Radzievsky has lately tested Proskauer and Capaldi's reactions with sixty-six varieties of the B. coli. All the microbes grew in No. I. medium, and in No. II. medium the reaction was neutral or slightly acid.

Proskauer and Capaldi's tests are thus seen to be extremely valuable, as by them the B. typhosus can at once be distinguished from a large number of bacteria which strongly resemble it. At the same time it cannot be said that a bacillus which does not grow in No. I. medium and produces an acid reaction in No. II. medium is necessarily the B. typhosus. I have quite lately isolated from polluted water three varieties of B. coli which produced gas in glucose media, and yet reacted in Proskauer and Capaldi's media exactly like the true B. typhosus.

(8) *The Reactions with Anti-typhoid Serum.*—(a) The agglutination test. When serum from an animal immunised by injections of B. typhosus is mixed with a broth culture of B. typhosus, or an emulsion in broth of a twenty-four hours growth of the typhoid bacillus on agar, it is found that the bacilli become immobilised and heaped together in motionless masses, or in other words agglutinated. The reaction so produced was at first considered specific, but further study soon showed that the sera of healthy human beings and animals also possessed the power of agglutinating the typhoid bacillus. Widal's experiments showed (1) that the serum from a horse, monkey, or rabbit, diluted from 1–30 to 1–50, might agglutinate the B. typhosus; (2) that the serum of guinea-pigs had usually

no effect, but exceptionally it showed an agglutinative action in a dilution of 1–5. Biberstein and Stern examined the sera of persons who were not suffering from typhoid fever. The following results were obtained:

Biberstein.		Stern-Sklower.	
Dilution of serum.	B. Typhosus culture. Agglutinated in	Dilution of serum.	B. Typhosus culture. Agglutinated in
1–10	24 per cent. of the cases	1–10	25 per cent. of the cases
1–20	6 " " "	1–20	8 " " "
1–30	2 " " "	1–30	2 " " "
1–40	0 " " "	1–40	1 " " "

In my own experiments with normal serum from a horse I found that the B. typhosus was always completely agglutinated by the serum diluted 1–20, and a marked reaction was obtained when the serum was diluted 1–80.

From these studies it is evident that, when an *unknown* serum is being tested for a specific reaction with a *known* bacillus (Widal's reaction), the errors introduced by the action of the agglutinins present in normal blood may be avoided by working with the unknown serum in a dilution of 1–50; but when attempts are made to diagnose an *unknown* bacillus by the action of a *known* specific serum upon it, the problem is more difficult. Much recent work on this subject has shown that at least three variable quantities enter into the reaction, viz.: (1) the nature of the bacillus; (2) the strength of the specific serum; and (3) the time during which the serum is allowed to act on the bacillus. It will be necessary to consider these three points in detail:

(1) *The Nature or Species of Bacillus operated on by the Specific Serum.*—In the early days of serum diagnosis it was thought that a specific serum only agglutinated the bacillus which had caused the serum to assume its specific characters. But a very little study soon showed that a specific serum, such as an anti-typhoid serum, would agglutinate other bacilli besides the B. typhosus; and of late years an enormous amount of work has been done in order to find out the essential points required to establish a really specific reaction. The B. coli and its varieties and B. enteritidis of Gärtner being closely allied to the B. typhosus in their cultural characteristics, have necessarily received much attention, and many experiments have been made

to contrast the action of anti-typhoid serum on these organisms and the B. typhosus.

Typhoid bacilli in collection obtained from			Dilution of the serum.					
			$\frac{1}{20}$	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{100000}$	$\frac{1}{200000}$	$\frac{1}{500000}$
Louvain			+	+	+	+	+	±
Paris			+	+	+	+	+	±
Lille			+	+	+	+	+	±
Berlin			0	0	0	0	0	0
Gand			+	+	+	+	+	±
Vienna I.			+	+	+	+	+	±
Vienna II.			+	+	+	+	±	±
Breslau I.			+	+	+	+	+	±
Breslau II.			+	+	+	+	+	±
Breslau III.			+	+	+	+	+	±
Bacilli isolated from the spleens of typhoid fever cases.	Case	No. 1	0	0	0	0	0	0
		2 to 16	+	+	+	+	+	±
	Case	1 to 25	+	+	+	+	+	±
	Case	1 to 22	+	+	+	+	+	±
Bacilli from the stools of typhoid cases.	Case I. 160 cultures	No. 34	+	+	+	+	+	±
		No. 35	+	+	+	+	+	±
		No. 39	+	+	+	+	+	±
		The remainder	0 or ±	0 or ±	0	0	0	0
Bacilli from the stools of typhoid fever cases.	Case II. 143 cultures	No. 3	+	+	+	+	+	±
		No. 16	+	+	+	+	+	±
		The remainder	0 or ±	0 or ±	0	0	0	0
	Case III. 36 cultures	None of them agglutinated	0 or ±	0 or ±	0	0	0	0
Coliform bacilli from suspected waters.	300 cultures	None of them agglutinated	0 or ±	0 or ±	0	0	0	0
Bacilli isolated from coliform colonies from normal stools, or cases not typhoid.	45 cultures		0 or ±	0 or ±	0	0	0	0

NOTE.—The sign + indicates agglutination with deposit and clearing of the fluid.
The sign ± indicates agglutination without deposit.
The sign 0 indicates the absence of all reaction.

Van de Velde's experiments on the value of agglutination in

the identification of Eberth's bacilli are often quoted. The agglutination tests were made in a series of tubes of small calibre, so that each tube held a column of fluid from ½ to 1 c.m. in height. The serum was added to the culture contained in the tubes, which were then incubated at 37° to 40° C. After thirty to forty minutes the tubes were examined with the naked eye. The anti-typhoid serum employed was extremely powerful, having been obtained by injecting a horse for two years with cultures obtained from the same specimen of B. typhosus. The results obtained are shown in the table on page 169.

The cultural characteristics of these various bacilli were carefully studied, and Van de Velde found that the bacilli which corresponded in their cultures to the B. typhosus were those which were agglutinated by very small doses of the anti-typhoid serum. The culture "Berlin" was the only exception, and this on further study was found to give an indol reaction in Witte's peptone solution, so it was excluded from the list of true typhoid organisms. On the other hand, all the bacilli which corresponded to the cultures of B. coli were not agglutinated by the anti-typhoid serum.

Beco's researches on the value of agglutination by anti-typhoid serum as a means of diagnosis between the B. typhosus and coliform races form a strong contrast to Van de Velde's experiments. Beco employed young broth cultures of B. typhosus and diluted the anti-typhoid serum with distilled water or physiological fluid. A known quantity of the culture was distributed amongst a series of glasses, and the serum was added with a sterilised pipette so as to give the dilutions mentioned in the table. The glasses were examined by the naked eye, and from time to time a loopful was taken and examined in a hanging drop; at the end of two hours the test was considered finished. When agglutination occurred the homogeneous aspect of the culture disappeared and small granular masses were seen in the fluid; the appearances in the hanging-drop agreed with the changes observed by the naked eye. The specimens were also submitted to the action of formalin according to Malvoz's technique. Beco's results are shown in the following table:

Micro-organisms.	Origin.	Action of formalin.	Action of anti-typhoid serum and dilution employed.
B. typhosus I. . .	Typhoid stools	+	+, 1–100,000
,, II. . .	,,	+	+, ,,
,, III. . .	,,	0	+, ,,
,, IV. . .	Collection	+	+, ,,
,, V. . .	,,	0	+, ,,
,, VI. . .	,,	+	+, ,,
,, VII. . .	Typhoid stool	+	+, ,,
,, VIII. . .	,,	+	+, ,,
,, IX. . .	,,	0	+, ,,
,, X. . .	,,	+	+, ,,
,, XI. . .	,,	+	+, ,,
,, XII. . .	,,	+	+, ,,
,, XIII. . .	,,	+	+, 1–10,000
,, XIV. . .	,,	+	+, ,,
,, XV. . .	,,	+	+, ,,
,, XVI. . .	,,	+	+, ,,
,, XVII. . .	,,	+	+, ,,
,, XVIII. . .	,,	+	+, ,,
B. coli I. . .	,,	+	+, 1–10
Para coli II. . .	,,	+	+, 1–100
B. coli III. . .	,,	+	+, 1–10
,, IV. . .	,,	+	+, ,,
,, V. . .	,,	+	+, ,,
,, VI. . .	,,	+	+, ,,
,, VII. . .	,,	0	+, ,,
,, VIII. . .	,,	+	+, ,,
,, IX. . .	,,	0	0
,, XIII. . .	,,	0	+, 1–100
,, XIV. . .	,,	0	+, 1–1,000
,, XV. . .	,,	0	+, 1–10,000
,, XVI. . .	,,	+	+, 1–1,000
,, XVII. . .	,,	+	+, ,,
,, XVIII. . .	,,	0	+, 1–100
,, XIX. . .	Normal stools	0	0
,, XX. . .	,,	0	0
,, XXI. . .	,,	0	0
,, XXII. . .	Urine, cystitis	0	+, 1–1,000
,, XXIX. . .	Normal stools	0	+, 1–10
,, XXX. . .	,,	0	+, 1–1,000
,, XXXI. . .	,,	0	+, 1–10,000
,, XXXII. . .	,,	0	+, ,,
,, XXXIII. . .	,,	+	0
,, XXXIV. . .	Meuse water	0	+, 1–100
,, XXV. . .	,,	0	+, ,,
,, XXVI. . .	,,	0	+, ,,
B. fluorescens liquefaciens	Typhoid stool	+	+, 1–10,000
,, non-liquefaciens	,,	+	+, 1–1,000
Proteus vulgaris . .	Collection	+	+, 1–100
,, mirabilis . .	,,	+	+, ,,
,, figurans . .	,,	+	+, ,,
Proteus	Normal stool	0	+, 1–1,000

As a result of his work, Beco concluded that all the specimens of B. typhosus, whatever their origin, were agglutinated

by the anti-typhoid serum in the high dilution of 1–100,000 (the table, however, shows several specimens of B. typhosus which were not agglutinated by dilutions beyond 1–10,000). The typical B. coli of Escherich varied extremely in its reaction to the serum; some were only agglutinated by a dilution of 1–10, others reacted to a dilution of 1–10,000. Taking Beco's results as published by him in the above table, the agglutination test would not appear to be an infallible means of diagnosis, as varieties of B. coli and B. typhosus were agglutinated by the same dilution of the anti-typhoid serum.

Jatta has quite recently re-investigated this subject and arrived at some important results. He found that the serum of animals immunised by injections of B. typhosus agglutinated this organism more strongly than twenty-eight different specimens of B. coli which he investigated. The serum behaved very differently to the various specimens of B. coli; the results Jatta obtained are shown in the following table:

Serum of an immunised dog:
- When diluted 1–10,000, agglutinated the B. typhosus.
- When undiluted, had no action on fifteen out of the twenty-four cultures of B. coli.
- When diluted 1–10, agglutinated one specimen of B. coli.
- When diluted 1–100, agglutinated five specimens of B. coli.
- When diluted 1–300, agglutinated four specimens of B. coli.

Serum of an immunised sheep:
- When diluted 1–1,000, agglutinated the B. typhosus.
- When undiluted, had no action on ten specimens of B. coli.
- When diluted 1–10, agglutinated five specimens of B. coli.
- When diluted 1–30, agglutinated six specimens of B. coli.
- When diluted 1–100, agglutinated seven specimens of B. coli.

The serum of a typhoid patient when tested with the specimens of B. coli. gave the following results:

Serum of patient:
- Diluted more than 1–300, agglutinated the B. typhosus.
- Diluted 1–300, agglutinated seven specimens of B. coli.
- Diluted 1–100, agglutinated four specimens of B. coli.
- Diluted 1–10, agglutinated four specimens of B. coli.

Jatta's observations appeared to show that as the agglutinating action of the serum on the B. typhosus increased or diminished, the action on B. coli also increased or diminished. The serum of the immunised dog, mentioned above, tested

on July 25, and again on October 2, gave the following results :

July 25.			October 2.
Serum.	Culture.	Dilution of serum, which completely agglutinated the culture.	Dilution of serum, which completely agglutinated the culture.
Dog	B. typhosus	1–10,000	1–1,000
"	B. coli	1–300	1–100
"	"	1–300	1–100
"	"	1–300	1–100
"	"	1–300	1–100
"	"	1–100	1–30
"	"	1–100	1–30
"	"	1–100	1–30

These experiments are of great interest, as the dog was inoculated with B. typhosus, and the results could not be influenced by a secondary Coli infection. When the serum of a typhoid patient is tested with B. typhosus and B. coli, it is sometimes found that the serum reacts in a higher dilution with the B. coli than with the B. typhosus. Biberstein found that in five cases of enteric fever which he investigated, the serum reacted in higher dilutions with the B. coli than the B. typhosus. He considered that the patients were suffering from a secondary Coli infection. Stern and Widal have recorded similar results. Consequently when the agglutination test is used to distinguish B. coli from B. typhosus, it appears absolutely necessary that the serum of an immunised animal should be used. If the serum of a patient is employed, there is always the possibility that he may be suffering from a mixed infection, and consequently the serum may react to both B. coli and B. typhosus.

Working with sera derived from immunised horses, I have lately tested the reaction to the specific agglutinins of 150 cultures of B. coli derived partly from healthy stools, and partly from the stools of typhoid patients. The results obtained with the cultures derived from healthy stools are shown in the following table :

ACTION OF ANTI-TYPHOID SERUM ON CULTURES OF B. COLI FROM HEALTHY DEJECTA.

No. of the culture.	Dilutions of the serum.				
	1–50	1–100	1–200	1–500	1–1000
Culture 1	0	0	0	0	0
,, 2	0	0	0	0	0
,, 3	0	0	0	0	0
,, 4	0	0	0	0	0
,, 5	0	0	0	0	0
,, 6	0	0	0	0	0
,, 7	0	0	0	0	0
,, 8	+	+	—	0	0
,, 9	0	0	0	0	0
,, 10	0	0	0	0	0
Cultures 11 to 22	0	0	0	0	0
Culture 23	+	+	—	0	0
Cultures 24 to 29	0	0	0	0	0
Culture 30	+	+	—	0	0
,, 31 to 41	0	0	0	0	0
Culture 42	+	±	0	0	0
,, 43	+	±	±	—	0
Cultures 44 and 45	0	0	0	0	0
Culture 46	+	±	±	—	0
,, 47	+	±	±	—	0
,, 48	+	±	±	—	0
Cultures 49 to 51	0	0	0	0	0
Culture 52	±	—	0	0	0
Cultures 53, 54, 55	0	0	0	0	0
Culture 56	±	±	±	—	0
,, 57	0	0	0	0	0
Cultures 58 and 59	±	±	±	—	0
Culture 60	0	0	0	0	0
Cultures 61 to 63	±	±	0	0	0
Culture 64	±	0	0	0	0
,, 65	±	±	±	—	0
,, 66	—	0	0	0	0
,, 67	0	0	0	0	0
,, 68	±	—	0	0	0
Cultures 69 and 70	0	0	0	0	0

The sign + indicates complete agglutination.
,, ± ,, nearly complete agglutination.
,, — ,, partial agglutination (a few clumps but many motile bacilli).
,, 0 ,, no traces of agglutination.

The results given in the above table closely agree with Jatta's experiments, and show that the limit of the dilution of anti-typhoid serum which causes agglutination of the B. coli from healthy stools is about 1–200. The results, however, with B. coli from typhoid stools were somewhat different, and suggest

that some of these bacilli may become temporarily susceptible to agglutination by a highly dilute anti-typhoid serum.

Experiments with B. fluorescens liquefaciens and B. fluorescens putridus have shown that the limit of the dilution of anti-typhoid serum which agglutinates these bacilli is about 1–100.

Coliform organisms which reacted strongly to dilute anti-typhoid serum, have been recently isolated from water-supplies by C. Sternberg. One organism was completely agglutinated by serum diluted 1–1400, and another by serum diluted 1–600. A stronger specimen of anti-typhoid serum which completely agglutinated the B. typhosus, when diluted 1–10,000, did not agglutinate the coliform organisms in a dilution above 1–5000.

It thus becomes evident that to bring out the specific reaction it is necessary to dilute the serum from the immunised animal. This opens up the difficult question as to what dilution is to be considered necessary.

Many bacteriologists believe that the B. typhosus, no matter from what source it is obtained, will always react in the same way to the same serum. Biberstein working with ten different cultures, Bensaude and Achard with twenty different cultures, and Widal with twenty-six cultures, found no difference in the agglutination of the different cultures with anti-typhoid serum.

Jatta also failed to detect any difference in his cultures which had been grown on favourable media for periods varying from some months to many years.

Kolle, however, stated that the agglutination produced by a serum was inversely proportional to the virulence of the bacilli. Gruber advised that a culture of little virulence should be taken for the diagnosis of a serum, because such a bacillus was sensitive to its action. He also thought that by means of a serum, the agglutinating action of which had been exactly estimated, it was possible to decide as to the virulence of a culture. Mills also found that the susceptibility of a bacillus to agglutination was inversely proportional to its virulence.

My own experiments showed distinct differences in cultures from different sources. As all the races were tested in the same manner with the same serum the results are perfectly comparable. At the same time the virulence of these cultures, when

tested in Wright's laboratary at Netley, did not bear out Kolle's and Gruber's contention.

Culture G. of B. typhosus, which was not agglutinated by the anti-typhoid serum, when diluted above 1–500, possessed little or no virulence as compared with culture 13 p, which was markedly agglutinated by the serum diluted 1–10,000. Culture G^x which was isolated from the spleen of a typhoid patient, at first proved highly resistant to the action of the anti-typhoid serum, but after being preserved in milk for six months it reacted to the serum diluted 1–1000, and yet with this diminished resistance to the anti-typhoid serum there appeared to be no diminution in its virulence. Consequently it appears that there is no constant relation between the virulence of the B. typhosus and its reaction to anti-typhoid serum.

All my cultures, with the exception of G^x, were completely agglutinated by anti-typhoid serum diluted 1–500, and this I should propose as a working limit, as it would exclude all forms of Coli isolated from normal stools. At the same time the existence of such cultures as G^x shows that it is impossible to lay down a hard and fast rule. This culture was carefully worked out and compared with other undoubted cultures of B. typhosus before it was finally accepted as a true B. typhosus. The results of the various tests are shown in the table on page 232, from which it appears that G^x responds to all the cultural reactions considered typical of B. typhosus. Cultures of this nature are probably rare, as I have only met with this one during several years' work at the subject.

Sacquépée has described "Eberthiform" bacilli, which, when isolated from water, typhoid stools, and also from the spleens of fatal cases of typhoid fever, did not appear susceptible to agglutination by anti-typhoid sera. The Eberthiform bacilli also differed from the true B. typhosus by producing a brownish growth on potato. When these bacilli were preserved in closed tubes, without access of light or air, they were gradually transformed into typical typhoid bacilli, producing a colourless growth on potato and reacting readily to anti-typhoid sera. The time required for this transformation varied between six and ten months, and the susceptibility to agglutination appeared to be gradually acquired during this period. The

Eberthiform bacilli when injected into animals, before the transformation had taken place, were found capable of producing a serum which in high dilutions readily agglutinated typical typhoid bacilli. Sacquépée believes that Eberthiform bacilli found in water have been derived from typhoid stools, the transformation from the true typhoid bacillus to the Eberthiform variety having taken place within the human body. He could not convert B. coli into the Eberthiform variety, but found that true typhoid bacilli introduced into collodion bags and then placed in the peritoneal cavity of a white rat, which had been strongly immunised by injections of B. typhosus, were converted into the Eberthiform variety. The typhoid bacilli so treated lost their susceptibility to specific agglutinins, and produced a coloured growth on potato. Sacquépée's results are very interesting, but require confirmation by other bacteriologists. In the present state of our knowledge I think a dilution of 1–500 of anti-typhoid serum may be accepted as a fairly safe working guide for the diagnosis of the true B. typhosus.

(2) *The Strength of the Specific Serum.*—Having considered the first variable quantity of the agglutination test, viz., the bacillus operated upon, it is necessary to mention a few points in relation to the strength of the specific serum, because its potency is intimately related to the dilution of the anti-typhoid serum, which is to be employed in the routine test. It is well known that sera prepared by immunising animals with cultures of B. typhosus vary greatly in their power of causing agglutination of this bacillus. At one time it was supposed that the results were largely dependent on the virulence of the culture used for the process of immunisation. Later experiments have thrown some doubt on this theory, so the question must still be considered *sub judice*. Whatever the explanation may be, the fact remains that sera as prepared vary extremely in their agglutinating action. The action of an anti-typhoid serum appears to be more marked on the variety of the bacillus, by means of which it has been prepared, than upon other varieties of the organism; that is to say, a serum has a "special" action upon its own organism, and a non-special action varying in degree on other varieties of the organism. Recognising these facts, attempts have been made to prepare polyvalent

sera, and to study how far the value of an anti-typhoid serum is affected by the employment of several varieties (races) of B. typhosus, either together or in succession. Ainley Walker found that there was no cumulative action or addition of agglutinating powers of the constituent monovalent sera in the production of a polyvalent serum. That is to say, the valency of polyvalent sera against any one of the varieties of typhoid used in its production is neither greater nor less than that of the monovalent serum obtained by the use of that variety alone. Further, it appears that "the polyvalent serum has no more powerful action on an independent variety of the bacillus than the most powerful towards that bacillus of its monovalent constituents." The "special" agglutinative power already obtained towards one variety of typhoid is found to diminish when the injections are discontinued, in spite of the immunisation with another variety of the bacillus being continued. For purposes of diagnosis either monovalent or polyvalent sera may be used, though perhaps the latter would be more generally useful. Ainley Walker found that polyvalent rabbits' sera could be prepared which, when diluted in 1–3500, completely agglutinated the most pathogenic variety of the typhoid bacillus against which it was tested. Horses' sera, prepared by immunisation extending over one year, when diluted 1–500,000 completely agglutinated the same variety of the typhoid bacillus. Anti-typhoid monovalent sera, prepared by immunising rabbits and guinea-pigs for short periods, do not usually bear dilution beyond 1–1000. It is quite easy to prepare sera, which, when diluted 1–1000 will completely agglutinate the B. typhosus; but to obtain sera of greater strength requires considerable time for the process of immunisation. Also, I have found that "special" sera, which act in a high dilution, slowly lose their agglutinative action when kept at room temperature. One serum in particular, which, when diluted 1–2,000,000 completely agglutinated different races of the B. typhosus, slowly lost its agglutinative action, and at the expiration of three months it ceased to influence the typhoid bacillus when employed in a dilution above 1–10,000.

Recognising these facts, it is important that every sample of anti-typhoid serum shall be standardised with the stock cultures

of different races of the B. typhosus, before it is used to diagnose an unknown bacillus. A serum which fails to agglutinate the stock cultures when used in a dilution of 1–1000 should be rejected as being too weak to secure satisfactory results.

(3) *The Time during which the Serum is allowed to Act on the Bacillus.*—The third factor of the agglutination reaction becomes of great importance when weak anti-typhoid sera are employed. If a quickly acting powerful serum is used for the test the time limit of two hours is quite sufficient for all practical purposes. But sometimes a powerful serum cannot be obtained, and

POWERFUL ANTI-TYPHOID SERUM.

Dilution of the serum.	B. Typhosus.		B. Coli.	
	Hanging drop (2 hours).	Capillary tube (24 hours).	Hanging drop (2 hours).	Capillary tube (24 hours).
1–10	+	+	+	+
1–20	+	+	+	+
1–40	+	+	+	+
1–80	+	+	±	+
1–160	+	+	±	±
1–320	+	+	0	±
1–640	+	+	0	0
1–1280	+	+	0	0
1–2500	+	+	0	0
1–5000	+	+	0	0
1–10,000	+	+	0	0
1–100,000	+	+	0	0
1–2,000,000	+	+	0	0

WEAK ANTI-TYPHOID SERUM.

Dilution of the serum.	B. Typhosus.		B. Coli.	
	Hanging drop (2 hours).	Capillary tube (24 hours).	Hanging drop (2 hours).	Capillary tube (24 hours).
1–10	+	+	+	+
1–20	+	+	+	+
1–40	±	+	±	+
1–80	0	±	0	±
1–160	0	±	0	±
1–320	0	±	0	±
1–640	0	±	0	0
1–1280	0	±	0	0

The sign + indicates complete agglutination.
" ± " incomplete agglutination (large clumps, but a few motile bacilli).
" 0 " no trace of agglutination.

under these conditions a longer exposure is necessary to bring out the specific reaction. This is well shown by the table on page 179.

General Conclusions on the Reaction of Agglutination.

Summarising our present knowledge on the subject of agglutination the following practical rules should be observed when testing the reaction of an unknown organism to anti-typhoid serum.

(1) The serum from a patient suffering from enteric fever must not be used, as it is possible that he may be suffering from a mixed coli and typhoid infection.

(2) The serum of an animal immunised by injections of B. typhosus should be employed. It should be standardised against a stock culture of B. typhosus, and if, when diluted 1–1000, it fails to agglutinate this bacillus, it should be rejected.

(3) The agglutination reaction cannot be considered specific unless it is produced by the anti-typhoid serum, diluted 1–500 and upwards.

(4) The specific reaction must be considered as only one link in the chain of evidence, and the result of the test must be taken in conjunction with the cultural reactions of the micro-organism under investigation.

Several methods have been suggested for the performance of the agglutination test. Widal recommended the following procedures for testing an unknown serum with the B. typhosus :

(*a*) Mix the serum with sterile broth in the required proportions; then inoculate the mixture with the B. typhosus and incubate at 37° C. After four to seven hours granular masses appear, and at the end of twenty-four hours the bacilli are usually found in little white heaps at the bottom of the tube and the fluid appears nearly clear.

(*b*) Add the serum in the required proportion to a twenty-four hours broth culture of B. typhosus and incubate at 37° C. After several hours the culture loses its uniform opacity, granular masses appear, and finally the broth clears by precipitation of the masses to the bottom of the tube.

(*c*) Add the serum in the required proportion to a twenty-four hours broth culture, or an agar culture made into an

emulsion with broth, and examine in a hanging-drop. This is called the rapid method as compared with the two former, which are slow methods.

(*d*) Wright has introduced a method of making the dilutions by means of glass capillary pipettes. Normal salt solution is used to dilute the serum, and a twenty-four hours agar growth of B. typhosus is made into an emulsion with normal salt solution. The dilutions of serum, thoroughly mixed with an equal volume of emulsion (which, of course, doubles the dilution of serum already prepared), are placed in capillary pipettes, which are then sealed at one end and kept at the room temperature for twenty-four hours. When agglutination is "complete" the bacilli are found as a firm sharply-outlined mass at the bottom of the pipette; if, however, the agglutination is "incomplete" granular masses are seen throughout the tube. The appearances must always be compared with a control tube containing only the emulsion diluted with an equal volume of salt solution, which has been left at the room temperature for the same time.

Wright's method of making the dilutions is admirable, but I have often found that when normal salt solution is used to make the emulsion, and also to dilute the serum, the sharpness or sensibility of the reaction is much diminished. The following method of procedure is therefore recommended:

Place a little of the anti-typhoid serum in a watch-glass, insert the point of a capillary tube in the fluid and allow this to run up the tube for about an inch and a half, mark on the glass with a wax pencil the upper limit of the fluid, and then blow out the serum into a watch-glass. Next dilute the serum 1–5. This is easily done by inserting the point of the pipette in a little sterile broth (contained in a watch-glass), which is allowed to run up to the mark on the tube. The broth is then blown out into the watch-glass containing the serum, three other equal quantities of broth, obtained in the same manner, are next added to the serum, and the broth and serum are then thoroughly mixed. Some of the dilution of 1–5 is now converted into 1–25, by taking a portion of the 1–5 dilution and adding to it four equal portions of broth. The 1–25 dilution is made into 1–50 by taking a portion and mixing with it an equal portion of broth. The 1–50 dilution is converted into a dilution of

1–250 by taking a known volume of it in the pipette as before and adding four equal volumes of broth. The 1–250 dilution is converted into 1–500 by taking a portion of the dilution and mixing with it an equal portion of broth. In this manner the serum is diluted with broth in the following proportions: 1–5, 1–25, 1–50, 1–250, 1–500.

A twenty-four hours agar culture of the suspected organism is now rubbed up with broth in a watch-glass until a perfect emulsion is made. The end of a pipette is now placed in the emulsion, which is allowed to run up the tube until a column of any desired length, usually one-third of the tube, is obtained. The upper limit of the column is marked as before, and the culture is then blown out into a clean watch-glass; a column of fluid of equal length obtained by the same pipette from the serum diluted 1–5 is now added, and the whole thoroughly mixed. In this way a mixture of serum and culture is obtained, the dilution of the serum being 1–10. The mixture of serum and culture is finally drawn up into the pipette until it is about half filled; the point is then rapidly sealed in the Bunsen flame. The same procedure is followed with the other dilutions of the serum, and in this way tubes containing serum, mixed with an equal volume of culture, are obtained in the following dilutions, viz., 1–10, 1–50, 1–100, 1–500, 1–1000. For ordinary diagnostic work these dilutions are most useful, but any other dilutions desired can be readily obtained by applying the same principles. The tubes thus obtained are preserved at the temperature of the laboratory and examined at the end of two hours, and again at the end of twenty-four hours. The results obtained at the end of two hours by the naked eye can be confirmed in the hanging-drop by snipping off the end of the capillary tube and blowing out a drop of the mixed culture and serum on to a cover-glass and examining it in the ordinary way. The capillary tube should be sealed up again and preserved for twenty-four hours. If it is preferred, a drop of the mixed serum and culture from the watch-glass can be at once put up in a hanging-drop and kept under observation for two hours. In my own work I have found that in the hanging-drop so prepared the clumps of bacteria often settle to the bottom of the drop by the end of two hours, and it is

then difficult, if not impossible, to focus the clumps accurately, consequently I prefer to open the capillary tubes and examine the contents in the manner described ; the clumps are then seen perfectly, as there is not time for them to settle down to the bottom of the drop. Sometimes when the capillary tubes are examined with the naked eye a deposit is seen at the bottom of the tube, and without practice mere sedimentation of the bacilli might be confused with agglutination. To avoid this source of error, the tubes may be opened and the contents examined by the hanging-drop method; or more simply, the question of agglutination may be decided by placing the tubes horizontally on a table for five or ten minutes. If the clumps are the result of true agglutination, the masses at the bottom of the tubes will not have changed their shape, and the outline will be sharp and convex ; if, however, the clumps have been formed merely by sedimentation, the masses will have changed their shape and the upper margin will be oblique, gradually tapering off to the lower side of the tube.

The results obtained by the twenty-four hours observation are more delicate than those obtained at the end of two hours; but as many bacteriologists perform the test in the hanging-drop and limit the observation to two hours, it is always well to examine the tubes at the end of this time, so that the results may be compared with those obtained by other observers. Temperature has an effect on the agglutinative action of anti-typhoid serum. Biberstein found that a serum diluted 1–14,000 clumped the B. typhosus when the preparation was kept at 37° C., but at the room temperature the agglutinative action was limited to a dilution of 1–12,000. I obtained very similar results with my own experiments. The serum always acted in a slightly higher dilution at 37° C. than at the room temperature. Consequently when results are stated it is always important to give the temperature at which the experiment was made.

Again, the results obtained with an emulsion made from a twenty-four hours agar growth of B. typhosus are not comparable with those obtained with a twenty-four hours broth culture of B. typhosus. I have found very marked differences in the results obtained by the two methods when applied to the same

bacillus. Broth cultures also are not suitable for use in capillary tubes; at the end of twenty-four hours the growth is rarely sufficient to give a marked reaction in the tubes, and if the culture be incubated for a longer time false clumping is likely to occur in the culture. For these reasons it is always best to use a twenty-four hours growth on agar, and make an emulsion from it by rubbing up one or two loopfuls of growth in sterile broth.

Pfeiffer's Test.—This consists in injecting into the peritoneum of a guinea-pig varying quantities of a twenty-four hours agar growth of the microbe under investigation so as to determine the fatal dose. Ten times the fatal dose, mixed with a little serum (0·01 c.c. to 0·001 c.c. according to the strength of the serum) from a highly immunised animal, is then injected into the peritoneal cavity of a guinea-pig. If the bacillus under examination be the B. typhosus the animal will remain well whilst a control animal injected only with the bacillus will die. In the peritoneal cavity of the animal which recovers the bacilli are first converted into granular masses, and then disappear under the lysogenic action of the specific serum. If the bacillus be a variety of the B. coli, the animal will infallibly die just like the control animal. This test is of the highest importance; but, unfortunately, owing to the difficulties of the experiment it is not applicable to general hygienic work. The test also has this limitation, the typhoid bacillus may have lost its virulence, so that a fatal result may not be obtained when the control animal is injected with the agar culture.

Results obtained by Immunising an Animal with the Micro-organism under Examination.—True races of the B. typhosus when injected into an animal gradually produce a serum which will agglutinate other races of B. typhosus. The extent to which the serum so produced can be diluted, and still agglutinate other races of B. typhosus, depends on the race of B. typhosus used for, and the length of time occupied by, the process of immunisation. Experiments at Netley showed that the least pathogenic race of B. typhosus produced a serum which, when diluted 1–100, completely agglutinated all the other races of the typhoid bacillus in the laboratory collection. I have never been able to isolate from water a micro-organism belonging to the pseudo-typhoid group which would produce a serum capable

of agglutinating the B. typhosus. In doubtful cases I regard this test of immunisation as a very important means of diagnosis between the B. typhosus and varieties of B. coli. Fodor and Rigler have shown that 0·1 c.c. and 0·5 c.c. of a typhoid broth culture injected into a guinea-pig weighing 300 grammes produce a serum with less agglutinating power than that obtained when 1 c.c. is injected. They also believed from the results of their experiments that increasing the amount of broth culture to 5 c.c. per 300 gramme guinea-pig did not increase the agglutinating power of the serum obtained, and that the best results were derived from a serum taken from the animal ten days after the subcutaneous injection of the broth culture. These statements, however, did not hold good for the sera prepared for me in the pathological laboratory at Netley. It was always found that 3 c.c. of typhoid broth culture per 300 gramme guinea-pig produced a more powerful serum than 1 c.c. of broth culture per 300 gramme guinea-pig. The whole question of immunisation of experimental animals is very intricate; much depends on the health of the individual animal, it is very easy to give too big an initial dose, and time must be given for the animal to recover from the effects of the injection before proceeding to give another. By gradually increasing the dose, and extending the injections over a period of three weeks, a more powerful serum is usually obtained than when a single big dose is given. Fodor and Rigler also believe that it is quite possible for typhoid bacilli to become so altered that they will neither become agglutinated by typhoid serum nor produce a serum with agglutinating action when injected into animals. In my own experiments I have found that true typhoid bacilli may be left in water and sewage for several weeks and still retain their susceptibility to typhoid serum and the power of producing a serum when injected into animals.

The Relation of Typhoid Bacilli to other Pathogenic and Non-pathogenic Bacteria.

Freudenreich has made some interesting experiments on this subject. He inoculated flasks, containing 200 to 300 grammes of broth, with different bacteria. After these had developed the contents of the flasks were filtered through a Pasteur-

Chamberland bougie, and the filtrate divided amongst a number of small flasks. As a rule, the flasks were kept for from four to six weeks before being filtered. The flasks containing the filtrate were then incubated to ascertain whether the broth had been completely freed from bacilli. If there was no turbidity after incubation of the filtrate, the flasks were inoculated with different bacilli. It was found that when the B. typhosus was inoculated into flasks containing the toxins of the following bacilli, the results recorded below were obtained:

In the toxins of:

- Staphylococcus pyogenes aureus, the B. typhosus grew very feebly.
- Staphylococcus pyogenes albus, the B. typhosus did not grow.
- Staphylococcus pyogenes fœtidus, the B. typhosus did not grow.
- B. pyocyaneus, the B. typhosus did not grow.
- B. typhosus, the B. typhosus grew very feebly.
- B. of symptomatic anthrax, the growth of the B. typhosus was delayed.
- Chicken cholera, the growth of the B. typhosus was very feeble.
- Pneumonococcus of Friedlander, the growth of the B. typhosus was very feeble.
- B. phosphorescens, the B. typhosus did not grow.
- Spirillum of Asiatic cholera, the B. typhosus grew feebly.
- Spirillum of Finkler-Prior, the B. typhosus grew well.
- Spirillum of Miller, the B. typhosus grew very feebly.
- Spirillum of Deneke, the B. typhosus grew very feebly.

In flasks containing the toxins of the B. typhosus it was found that the following bacilli grew well, viz., Staphylococcus pyogenes aureus, albus, citreus, and fœtidus; Micrococcus tetragenus; Micrococcus prodigiosus; Micrococcus roseus; Bacillus of blue milk; Bacillus pyocyaneus; Bacillus of symptomatic anthrax; Bacillus megaterium; Bacterium phosphorescens; Pneumonococcus of Friedlander; Spirillum of Asiatic cholera; Spirillum of Finkler-Prior; Spirillum of Miller; Spirillum of Deneke. The micro-organism of chicken cholera grew feebly, and the B. typhosus very feebly in typhoid toxins. The influence of the products of B. fluorescens putridus and B. fluorescens liquefaciens on the B. typhosus have already been described. The influence of the toxins of B. coli on B. typhosus will be fully considered under the section devoted to the experimental researches on the duration of life of the B. typhosus in water and sewage.

The Relation of the Typhoid Bacillus to Acids and Alkalies in Nutrient Media.

The effects of the presence of acids and alkalies in nutrient media on the development of the B. typhosus have been carefully investigated by Kitasato. Broth and gelatine media were carefully neutralised, and varying quantities of the substances under examination added. The medium was then inoculated with a culture of the typhoid bacillus. When gelatine was employed "roll cultures" were made, and the development of the colonies of B. typhosus examined in the usual manner.

The sign + indicates growth, ± growth restrained, — no growth.

Acids.	+	±	—
	per cent.	per cent.	per cent.
Sulphuric acid	0·049	0·065	0·08
Hydrochloric acid	0·1	0·158	0·2
Nitric acid	0·1	0·157	0·2
Sulphurous acid	0·09	0·2	0·28
Phosphoric acid	0·15	0·224	0·3
Acetic acid	0·2	0·255	0·3
Carbolic acid	0·2	0·258	0·34
Formic acid	0·22	0·278	0·356
Oxalic acid	0·23	0·285	0·366
Lactic acid	0.27	0·36	0·4
Tartaric acid, Citric acid, Malic acid	0·338	0·384	0·476
Tannic acid	1·3	—	1·66
Boric acid	1·5	2·0	2·7
Alkalies.	**+**	**±**	**—**
	per cent.	per cent.	per cent.
Caustic lime	0·0725	0·0805	0·0966
Caustic potash, Caustic soda	0·1	0·14	0·18
Ammonia	0·148	0·2	0·3
Lithium carbonate	0·514	0·6	0·666
Potassium carbonate	0·566	0·74	0·81
Barium hydrate	0·65	0·83	1·00
Ammonium carbonate	0.72	0·845	1·0
Sodium carbonate	2·00	2·2	2·47
Salts.	**+**	**±**	**—**
	per cent.	per cent.	per cent.
Potassium iodide	6·66	8·0	9·23
Potassium bromide	8·0	9·23	10·37
Potassium chloride	9·1	10·6	12·0

When broth was used, the medium was inoculated with the typhoid bacillus and kept at 36° C. until a good growth was obtained. The chemical substance was then added, and the culture kept at the room temperature for four or five hours. At the end of this time, and ten to fifteen hours later, half a cubic centimetre was plated out in a "roll culture." The results obtained by Kitasato are given in the table on page 187.

Kitasato's results with carbolic acid do not agree with my observations. The percentage of caustic lime required to kill the typhoid bacillus is also much greater than that given by other observers. Liborius found that 0·0074 per cent. of caustic lime destroyed the typhoid bacillus when growing in broth. Kitasato explains the discrepancy by the fact that he used concentrated broth, whereas Liborius employed broth diluted fifteen times with distilled water. The phosphates present in the undiluted broth required to be neutralised by the lime before this substance could act on the bacilli. By using a diluted broth, Kitasato found that 0·0074 per cent. of lime destroyed the typhoid bacilli.

Micro-organisms which Resemble the B. Typhosus.

There are many organisms which may be mistaken for the B. typhosus unless great care is taken to apply the tests already mentioned. In polluted waters B. coli and its varieties occur, and, before the modern tests were elaborated, there is no doubt that this great group of micro-organisms was often confused with the B. typhosus. There is, however, at the present day not much difficulty in separating the great bulk of these organisms from the typhoid bacillus. The following table gives the reactions of the typical B. typhosus and B. coli :

B. Coli Communis.	B. Typhosus.
(1) *Surface Colonies, Gelatine Plates.* —Thicker, and grow more rapidly than those of B. typhosus. After forty-eight hours incubation at 22° C. they are usually large and characteristic.	(1) Much thinner than those of B. coli, and grow more slowly. After forty-eight hours incubation at 22° C. they are hardly visible to the naked eye.
(2) *Gelatine-stab.* — Quick growth on the surface and along the line of inoculation.	(2) Slow growth on the surface like the colonies ; along the line of inoculation the growth is much thinner, and often ends below in a few white points consisting of discrete colonies.

B. Coli Communis.	B. Typhosus.
(3) *Gelatine-slope.* — Thick, broad greyish-white growth with a crenated margin.	(3) Thin narrow greyish-white growth, crenated margin not marked as in B coli.
(4) *Witte's Peptone and Salt Solution.*—Indol produced.	(4) No formation of indol.
(5) *Milk.*—Coagulated.	(5) Unchanged after a month.
(6) *Litmus-whey*, one week at 37° C.—Acid produced, usually requiring from 20 to 40 per cent. of $\frac{N}{10}$ alkali to neutralise it.	(6) Very small amount of acid produced, requiring not more than 6 per cent. of $\frac{N}{10}$ alkali to neutralise it.
(7) *Neutral-red Glucose-agar.* — Marked green fluorescence.	(7) No change.
(8) *Glucose-gelatine and Lactose-gelatine Shake Cultures, and Glucose-agar-stab.*—Marked gas formation.	(8) No gas formation.
(9) *Gelatine*, 25 per cent. incubated at 37° C.—Thick film appears on the surface.	(9) No film appears on the surface, but a general growth takes place throughout the tube.
(10) *Potato.*—As a rule, a thick yellowish-brown growth.	(10) Thin transparent growth hardly visible to the naked eye.
(11) *Proskauer and Capaldi's Media*, No. I., after twenty hours.—Growth, medium acid.	(11) No. I., no growth or change in the reaction of the medium.
No. II., after twenty hours.—Growth, medium neutral or faintly alkaline.	No. II., Growth, medium acid.
(12) *Nitrate-broth.* — Nitrate reduced to nitrite.	(12) Reduction of nitrate not so marked.
(13) *Microscopical Appearances.*—A small bacillus often like a coccus, not motile as a rule.	(13) Usually longer than B. coli: highly motile, with a quick serpentine movement.
(14) *Flagella.*—Usually 1 to 3, short and brittle; sometimes 8 to 12, long and wavy.	(14) Usually eight to twelve, long and wavy.
(15) *Agglutination.*—As a rule, no agglutination with a dilute anti-typhoid serum.	(15) Marked agglutination with dilute anti-typhoid serum.

The "atypical" members of the Coli group which fail to coagulate milk and do not produce indol sometimes give rise to difficulties in diagnosis, but most of them, as a rule, can be distinguished by the production of gas in glucose media. In polluted waters, however, coliform organisms occur which are not so readily distinguished. The four varieties of B. typhosus simulans, described by Houston, are instances of this class of micro-organisms. When investigated they showed the following characteristics:

B. Typhosus Simulans (Houston).

	A.	B	C.	D.
Morphology	Long thin, highly motile bacilli	Long thin, highly motile bacilli	Long thin, highly motile bacilli	Long thin, highly motile bacilli
Surface colonies	Greyish-white thin films with an irregular margin, slow growth	Greyish-white thin films with an irregular margin, slow growth	Greyish-white thin films with an irregular margin, slow growth	Greyish-white thin films with an irregular margin, slow growth
Shake-gelatine	No gas	No gas	No gas	No gas
Litmus-milk	No acid, no clot	No acid, no clot	No acid, no clot	No acid, no clot
Formation of indol after five days	Trace of indol	No indol	Trace of indol	No indol
Growth on potato	Transparent colourless growth	Transparent colourless growth	Transparent colourless growth	Faint yellowish-coloured growth
Flagella	3 to 6	3 to 6	3 to 9	3 to 9
Agglutination with anti-typhoid serum	Nil	Nil	Nil	Nil

The following organisms which I have recently isolated from a polluted water are even more difficult, as some of these react to anti-typhoid serum:

B. Typhosus Simulans (Culture A.).

Gelatine Plates.—The surface colonies appeared as thin bluish films with an irregular margin. Under a low power the centre of the colonies had a yellowish tinge, and surface markings of ridges and valleys were seen running out from the centre to the periphery.

Gelatine stab.—Surface growth resembled the colonies; a thin white growth along the stab.

Milk.—Unchanged after a month.

Litmus-whey.—Faint acidity, requiring less than 6 per cent. of $\frac{N}{10}$ alkali to neutralise it.

Glucose-gelatine Shake.—No gas formation.

Witte's Peptone and Salt Solution.—No indol produced after seven days incubation at 37° C.

Potato.—Growth at first transparent, later acquired a faint yellowish tinge.

Gelatine (25 per cent.) at 37° C.—No film on the surface; a diffused growth through the tube.

Proskauer and Capaldi, No. I.—Growth, but no change in the reaction of the medium.

Proskauer and Capaldi, No. II.—Growth, medium rendered slightly alkaline.

Microscopical Characters.—A small highly motile bacillus, about the same size as B. typhosus.

Staining Reactions.—Stained by basic aniline dyes; decolorised by Gram's method.

Reaction to Anti-typhoid Serum.—Anti-typhoid serum, even in a dilution of 1–10, produced no agglutination.

This micro-organism only differed from the B. typhosus in that it failed to grow typically in Proskauer and Capaldi's media, and did not react to anti-typhoid serum.

B. Typhosus Simulans (Cultures B_3, B_4, B_5, and B_6).

These cultures all gave the following characteristics, and appeared to be the same micro-organism:

Gelatine Plates.—Identical with those of culture A.

Gelatine-stab.—Surface growth the same as the colonies, which appeared to grow more slowly than B. coli; a thin growth along the stab.

Gelatine-streak.—A thin growth, which did not show the crenated margin of B. coli, but was distinctly more copious than that of B. typhosus planted out at the same time.

Witte's Peptone and Salt Solution.—No indol produced after seven days incubation at 37° C.

Glucose-gelatine Shake.—No gas formation.

Milk.—Unchanged after one month.

Potato.—Growth at first transparent, later acquired a yellowish tinge.

Gelatine (25 per cent.) at 37° C.—No film appeared on the surface, but a diffused growth appeared throughout the tube.

Proskauer and Capaldi, No. I.—Growth, but no change in the reaction.

Proskauer and Capaldi, No. II.—Growth, but medium rendered slightly alkaline.

Microscopical Characters.—A small, highly motile bacillus, closely resembling the B. typhosus.

Staining Reactions.—Stains with basic aniline dyes; decolorised by Gram's method.

Reaction with Anti-typhoid Serum.—Practically complete agglutination with anti-typhoid serum, diluted 1–80.

This micro-organism closely resembled the B. typhosus in many of its reactions, but it did not give the typical reactions in Proskauer and Capaldi's media and the anti-typhoid serum which, when diluted 1–2,000,000, caused complete agglutination of the true B. typhosus, had no complete agglutinating action on the B. typhosus simulans when diluted above 1–80.

The B. Fæcalis Alkaligenes, isolated by Petruschky, is said to resemble the B. typhosus in the following points: (1) Active motility; (2) number of flagella; (3) decolorisation by Gram; (4) appearance of the colonies on gelatine plates; (5) growth in milk without fermentation; (6) growth in sugar media without the production of gas; (7) agglutination with anti-typhoid serum; (8) failure to produce indol. It is, however, supposed to be distinguished by producing a brown growth on potato, and rendering litmus-whey alkaline after forty-eight hours incubation.

A pure culture of this organism was obtained from Kral's laboratory and carefully studied. It was a small motile bacillus, closely resembling the B. typhosus. It did not stain with Gram. In broth a surface pellicle was produced, which fell to the foot on shaking the tube. In glucose-agar at 37° C., and in lactose- and glucose-gelatine shake cultures at 22° C., there was no formation of gas. Indol was not formed in Witte's peptone and salt-solution after seven days incubation at 37° C. Litmus-whey showed no appreciable change in reaction after seven days incubation at 37° C. On potato a yellowish-brown growth was produced. Milk was unchanged after seven days incubation. In gelatine-stab the growth was slow, and strongly resembled

that of the B. typhosus; the medium, however, acquired a greenish tinge at the surface. In an agar-stab the same greenish tint was seen in the medium; on an agar-slope the growth was thin and greyish in tint, the discrete colonies were small. In gelatine plates the colonies grew slowly: those in the depth were circular in shape, of yellowish-brown colour, and coarsely granular in the centre; on the surface, the colonies showed a wavy irregular margin and a dark centre, from which ridges and valleys ran out to the periphery. The colonies in the depth resembled B. coli; those on the surface, however, were more like the B. typhosus, both in slowness of growth and the small size and appearance of the colonies. A growth took place after twenty-four hours in both of Proskauer and Capaldi's media; in No. I. the reaction was alkaline, and in No. II. neutral. A twenty-four hours agar growth was tested as to agglutination with anti-typhoid serum; the reaction was incomplete when the serum was diluted 1–100. The same serum diluted 1–1000 completely agglutinated a stock culture of B. typhosus.

It will be noticed that this culture failed to give the characteristic alkaline reaction in litmus-whey. Germano and Maurea also found that the formation of alkali by Petruschky's bacillus was inconstant. The B. fæcalis alkaligenes appears to be fairly common in fæces, so it is important to distinguish the organism from the B. typhosus. The diagnosis can be readily made, in the absence of the formation of alkali, by the brown growth on potato and the absence of reaction to a highly dilute anti-typhoid serum.

The B. Enteritidis, isolated by Gärtner from cases of meat poisoning, seems to be intermediate between the B. coli communis and the B. typhosus. In gelatine plates the surface colonies sometimes exactly resemble those of B. typhosus, at other times the wavy margin and ridge and valley appearance are not marked; the colonies then appear coarsely granular with a nearly circular outline. The colonies grow more quickly than those of the B. typhosus, but more slowly than those of B. coli. In gelatine-stab and streak the growths resemble those of B. typhosus, but are more copious. In glucose-agar-stab at 37° C. gas is always produced, but in glucose-gelatine-shake at 22° C. it is sometimes absent. Gas does not appear to be

formed in lactose-gelatine-shake cultures. Milk is not coagulated. There is no formation of indol in Witte's peptone and salt solution after seven days incubation at 37° C. The growth on potato varies; sometimes it is colourless and transparent, like that of B. typhosus, at other times there is a yellowish slimy growth, resembling that produced by B. coli. A growth takes place after twenty hours incubation in both of Proskauer and Capaldi's media. In No. I. the reaction is sometimes acid and sometimes alkaline; in No. II. the reaction is either neutral or alkaline. The behaviour of the bacillus in litmus-whey is peculiar and very characteristic. It rapidly produces a large quantity of acid; this is followed later by the formation of alkali, which neutralises the acid, so that the litmus-whey is acid for the first two or three days and then becomes alkaline. In microscopical characters the bacillus is almost identical with B. typhosus. It is not stained by Gram's method. The flagella, as a rule, are fewer than those of B. typhosus, but more numerous than those of B. coli. In its reaction to anti-typhoid serum the B. enteritidis shows less susceptibility to the specific agglutinins than the B. typhosus. Durham states that B. enteritidis is not agglutinated by anti-typhoid serum diluted 1–50,000. As a rule, this is true, but on one occasion I obtained a culture which was completely agglutinated by anti-typhoid serum in this dilution.

The B. enteritidis was found by Karlinski four times in normal fæces, and Germano and Maurea have found bacilli closely resembling it in water.

The **Bacillus Breslaviensis** belongs to the Gärtner group, and was found by Van Ermenghem and Känsche in outbreaks of meat poisoning in Morseele and Breslau. It is very motile, possesses from four to twelve flagella, and does not stain with Gram. The colonies are coliform, broth becomes turbid, indol is not produced, and milk is not coagulated. On potato there is a thick yellowish growth. In agar and broth containing grape-sugar, milk-sugar, or cane-sugar, gas is produced, but the quantity is small, except in the media containing grape-sugar.

The **Bacillus Friedebergensis** described by Gaffky and Paak, is also closely allied to Gärtner's bacillus. It is about one-third

the size of the B. typhosus and only slightly motile. Indol is not produced and milk is not coagulated.

Schottmüller has described a number of cases of "paratyphus." The symptoms very closely resembled those seen in true cases of enteric fever. The temperature charts were so characteristic that the diagnosis of enteric fever seemed almost beyond cavil. Rose-spots, enlargement of the spleen, and diarrhœa were also observed in some of the cases, but in others the abdominal symptoms were not so marked. From twenty to twenty-five cubic centimetres of blood were removed from each patient and made into plates on agar. The colonies which resulted were carefully studied; in every case motile bacilli which did not stain with Gram were isolated. On gelatine plates and Piorkowski's alkaline urine medium the colonies resembled those of B. coli. On potato a greyish-brown, thick growth, was observed. In peptone and salt solution there was good growth but no indol reaction was observed. Gas formation was seen in glucose-broth and glucose-agar. Neutral-red agar was changed to a yellow colour, and a greenish fluorescence was observed. Litmus-whey was rendered acid for the first two or three days, but a markedly alkaline action appeared later. When tested with the serum from an undoubted case of enteric fever the bacilli showed no traces of agglutination, but when tested with serum collected from each of the cases all the bacilli became agglutinated. The reaction was observed not only between the bacilli and serum from the same case, but also between the bacilli and sera from all the cases. The effective dilutions of the sera varied from 1–100 to 1–10,000. All the cases recovered, so an examination of the spleen and internal organs could not be made. At the same time the bacteriological results obtained seem to prove conclusively that the cases of "paratyphus" were caused by a variety of the bacillus of Gärtner.

The **B. aquatilis sulcatus** varieties described by Weichselbaum produce surface colonies which at first resemble those of B. typhosus but on later development acquire a yellow colour. These bacilli do not develop indol, coagulate milk, nor produce gas in glucose media. Some of the varieties produce a yellow growth on potato. They are readily distinguished by failing to agglutinate

with anti-typhoid serum and also by growing at 0° C., at which temperature the B. typhosus does not develop.

The **B. lactis ærogenes** resembles the B. typhosus in the appearance of its colonies, but it is not agglutinated by anti-typhoid serum. It also develops indol, coagulates milk, and produces gas in glucose media.

The **B. cavicida** described by Brieger also resembles the B. typhosus in the appearance of its colonies, but it develops indol and produces gas in glucose media.

The **B. fluorescens non-liquefaciens** group of organisms occasionally gives rise to difficulties, as the surface colonies have a superficial resemblance to those of the B. typhosus. As a rule, the marked venation on the surface colonies and the green colour of the gelatine around them enables a diagnosis to be made without difficulty. Sometimes, however, the venation is not marked and the green colour is not developed for many days. Under these conditions a mistake may be made, as these bacilli do not produce gas, coagulate milk, nor give rise to gas in glucose media. They are, however, not agglutinated by anti-typhoid serum in a high dilution, though some members of the group are agglutinated by the serum when diluted 1–80.

The B. Acidi Lactici produces surface colonies somewhat like those of B. typhosus, but it is a spore-bearing organism, coagulates milk, produces indol, and forms gas in glucose media.

The B. Ureæ also gives rise to surface colonies which closely resemble those of the B. typhosus. It is distinguished by not reacting to the agglutination test and by its power of converting urea into ammonium carbonate.

The B. Psittacosis, which has been isolated from Parrots, belongs to the typhoid group of organisms. It is an actively motile bacillus, which does not stain by Gram's method. The colonies on gelatine plates closely resemble those of the B. typhosus. It does not ferment lactose, nor curdle milk, nor produce indol in Witte's peptone and salt solution. It, however, produces a coloured growth on potato like the B. coli, which serves to distinguish it from the B. typhosus.

Coliform Organisms Isolated by C. Sternberg from Water.

No. I. *Gelatine Plates.*—Typical growth.

Broth.—Diffused growth without any surface pellicle.

Witte's Peptone and Salt Solution.—No indol produced.

Milk.—Unchanged after seventeen days.

Litmus-whey.—5·5 per cent. acid.

Sugar-media.—Gas formation.

Potato.—Thick greyish-white growth.

Microscopical Characters.—Short and long motile rods, not stained by Gram.

Anti-typhoid serum.—Completely agglutinated by the serum diluted 1–1400.

No. II. Resembled No. I. in most of the tests, but the acidity was 9 per cent., and the limit of the dilution which caused agglutination was 1–600.

Methods which have been Proposed for the Isolation of the B. Typhosus from Water.

In order to study gelatine plates and make sub-cultures of suspicious organisms, numerous efforts have been made to repress the growth of the saprophytic microbes without hindering the development of the B. typhosus. Chantemesse and Widal proposed to add 0·25 per cent. of carbolic acid to the nutrient gelatine. Holz, however, showed that 0·20 per cent. of carbolic acid prevented the development of the B. typhosus in Esmarch roll tubes and on Koch's plates; the same effect was not obtained when 0·117 per cent. of carbolic acid was employed. the B. typhosus always grew well, and the liquefying bacteria were destroyed. Tiemann and Gärtner found 0·25 per cent. of carbolic acid very useful, and showed that the growth of the typhoid bacillus was only slightly hindered when this amount of carbolic acid was added to the water for three hours. Plates made at the end of this time were found to be almost completely free from foreign organisms. Holz's experiments showed that it was not safe to use more than 0·15 per cent. carbolic acid even for the three hours exposure. Parietti suggested a solution containing 5 grammes of phenol and 4 grammes of pure hydrochloric acid in 100 c.c. of distilled water: varying

quantities of this solution, usually 0·1, 0·2, and 0·3 c.c. were added to tubes containing 10 c.c. of nutrient broth. The tubes were then incubated for twenty-four hours at 37° C., and if they remained free from growth, gradually increasing quantities of the water—from two drops to 1 c.c.—were added to the tubes which were again incubated at 37° C. for from twenty-four to seventy-two hours. All the tubes which showed any growth were then plated out in gelatine; the colonies which developed were examined under a low power, and those resembling B. typhosus were fished and sub-cultured in various media. Tiemann and Gärtner very often found that the typhoid bacillus did not grow in broth containing 0·10 and 0·15 per cent. of carbolic acid, and Lösener obtained similar results. Vincent recommended the addition of five drops of a 5 per cent. solution of carbolic acid to the broth tubes, which were then incubated at 42° C. Ravitsch-Stcherba added 0·1 per 1000 of α-naphthol to the nutrient medium. Lösener did not obtain satisfactory results with this method; the surface colonies of B. typhosus were green in colour, granular, raised in the centre, and not easily recognised. Holz prepared a potato-gelatine medium, which had an acid reaction, and ten grammes of the medium required 1·6 c.c. of deci-normal alkali to effect neutralisation. Elsner added 1 per cent. of potassium iodide to the potato-gelatine; the advantage of this addition has been denied by many workers. On the iodised potato-gelatine medium the B. typhosus grows slowly, and at the end of forty-eight hours the colonies appear as small, clear dots, like minute drops of water; the B. coli, on the other hand, shows much larger colonies of a dark-brown colour. Unfortunately these appearances cannot be relied upon; the colonies of B. coli sometimes appear small and transparent like those of B. typhosus. Also many liquefying organisms develop on the medium, so that the plates cannot be kept very long under observation when the water is seriously polluted. The great advantage of the potato-gelatine is that it diminishes the number of colonies which must be sub-cultured for further study. Only the small transparent colonies need be "fished"; the large brown colonies can be at once excluded from the investigation. Uffelmann suggested that ordinary gelatine should be exactly neutralised, and then 0·01 per cent. of pure carbolic

acid and 0·002 per cent. of methyl-violet added. The colonies of B. typhosus grow in a characteristic manner and remain distinctly bluer than the surrounding medium. Stoddart recommended a medium containing 0·5 per cent. agar and 5 per cent. gelatine. A sufficient quantity of this medium is poured into a dish or flat flask, so as to fill it to a depth of 5 mm.; the dish or flask is then sterilised and allowed to cool gradually (preferably in the steriliser over night). Drops of moisture which may have condensed on the cover are drained off, and the centre of the medium touched with a platinum needle charged with the material to be examined. In order to prevent condensation the dish is enclosed in a larger one and incubated at 35° C. for twenty-four hours. If B. typhosus is present, about two-thirds of the dish will be found occupied by a circular opalescence, which later extends throughout the whole dish. Under similar conditions the B. coli produces the usual colony of limited extent. If the inoculation is made from a mixture of both organisms, there results a central disc of coli bacilli, surrounded by an opalescent halo of typhoid growth, which yields perfectly pure sub-cultures. Unfortunately many of the varieties of B. coli, which are motile, produce exactly the same halo appearance as the B. typhosus; so the test cannot be relied upon to distinguish between these two microbes.

Hankin has published a method by which he states he has been able to isolate the B. typhosus from water supplies in India. The method is as follows:

"Five tubes are taken, each containing 10 c.c. of neutral bouillon. To the first tube no addition of Parietti's solution is made. It merely serves as a control of the capacity of the bouillon used to permit the growth of microbes. To the remaining tubes are added one, two, three and four drops of Parietti's solution respectively. Each tube is then infected with a few drops of the water or other liquid to be tested. The tubes are covered with india-rubber caps and placed in the incubator at 37° C. for twenty-four hours. On the following day a variable number of the above tubes will be found to be turbid. One of the series has now to be chosen for further use. The tube containing the highest number of drops of Parietti's solution that is yet turbid should be discarded. Usually the

tube next below this in the series should be chosen. But the character of the growth in the different tubes must be taken into account. As a rule, a tube that has a thick scum on the surface, or in which growth is only visible in the deeper layers of the bouillon, or in which bubbles are seen in the liquid, should be discarded. A tube should be preferred in which there is a uniform turbidity. Usually the tube containing two or three drops of Parietti's solution is the one chosen for further use, although with the bouillon I employ, if infected with dirty water, growths would occur in a tube containing ten or twelve drops of the Parietti's fluid. Tubes containing the larger quantity of Parietti should be employed if it is required to isolate B. coli communis. The tube of bouillon chosen as above is then used to inoculate a second series of bouillon tubes to which successively increasing quantities of Parietti's solution are added. The first tube of the second series has the same number of drops of Parietti added to it as were present in the tube taken for further inoculation from the first series. For instance, supposing the tube taken from the first series was the one containing three drops of Parietti, then the first tube of the new series will also contain three drops, the next will contain four drops, the next five drops, and so on. Two or three drops of the bouillon in the tube from the first series are used to inoculate each tube of the second series. These tubes are covered with indiarubber caps and placed in the incubator as before. On the following day choice of a tube has to be made from the second series . . . the tube containing the highest number of drops of Parietti's solution that is turbid is discarded, and one of the lower tubes is taken for further use. The tube of bouillon chosen must now be inoculated on to agar, having a fairly dry surface in such a way as to produce isolated surface colonies. A small quantity of the bouillon is taken up on the end of an inoculating needle from the surface of the liquid. The needle is introduced into the agar tube and rubbed on the bottom of the slanting surface of the agar: then it is moved in a zig-zag track over the remaining portion of the surface of the agar. At least three agar tubes should be inoculated in this way. After inoculation the agar is kept in the incubator. On the following day the colonies that have developed must be

carefully examined. Each colony whose appearance is suspicious must be inoculated on to a tube of litmus-agar . . . It is not enough to inoculate two or three tubes from suspicious colonies . . . five to ten tubes may be sufficient if the water is comparatively pure. But if the water has been exposed to very obvious contamination, the resulting colonies on the agar will be very varied in aspect, and it will be necessary to inoculate from them ten to twenty or an even larger number of litmus-agar tubes." The litmus-agar contains twenty-five grammes of litmus and thirty grammes of milk sugar to the litre of nutrient agar. "On the day after their inoculation a number of the litmus-agar tubes will be found to have turned red, and may be at once discarded. After a further twenty-four hours others may turn red, and may also be discarded. . . . The remaining tubes that are still blue, and that have the naked eye appearance of the growth of enteric, must now be subjected to microscopical examination." The cultures resembling B. typhosus are finally sub-cultured in milk, potato, &c., and agglutination tested with anti-typhoid serum.

Using this method, Hankin states that he has frequently isolated the B. typhosus from suspected waters in India; and, as most modern bacteriologists working with other methods have failed to detect the B. typhosus in suspected waters, the procedure appears deserving of critical study. The tube which Hankin selects from his first series is usually that containing two to three drops of Parietti's fluid, which corresponds to 0·05 per cent. carbolic acid (1 c.c. = 30 drops), the amount generally used by bacteriologists at the present day. The second series, commencing with three drops, would run up to six drops of Parietti, or in other words up to 0·1 per cent. of carbolic acid, in which B. typhosus will usually grow, if it has not been debilitated by prolonged immersion in sewage. So that if the B. typhosus were not accompanied by B. coli it should be detected by this method, providing of course it was originally present in considerable quantity, so that typhoid bacilli might be present in the small quantity of water used for the experiment. If, however, B. coli is present with the B. typhosus (as it usually is in most polluted waters) the result will be different. B. coli will grow in 0·2 per cent. carbolic (= 12 drops of

Parietti's fluid), and following Hankin's technique if the next tube or next but one to this be taken it will contain ten to eleven drops of Parietti, or 0·15 per cent. of carbolic, in which B. typhosus often does not grow in twenty-four hours. Consequently by Hankin's method B. typhosus may not be detected when it is accompanied by B. coli. Hilbert carefully investigated Hankin's method by adding a twenty-four hours broth culture of B. typhosus to water from different sources. He first estimated the number of bacilli which would be found in varying dilutions of the twenty-four hours broth culture, and came to the conclusion that with a dilution of 1-100,000,000, three drops of the fluid would contain a typhoid bacillus. The following table gives the results of his experiments with three different samples of water:

No. and date of the experiment.	Origin of the Water.	Dilution.	Quantity used.	Result + = B. typhosus detected.	No. of drops of Parietti's solution in the tube used.	Remarks.
1. 1-12-1899	Pipe-water (filtered)	1–10,000	3 drops	+	3	—
2. 1-12-1899	"	1–1,000,000	"	+	3	—
3. 4-12-1899	"	1–1,000,000	"	+	5	—
4. 3-1-1900	"	1–10,000,000	"	+	2	—
5. 7-12-1899	"	1–110,000,000	"	—	—	—
6. 3-1-1900	"	1–100,000,000	"	+	1	—
7. 7-12-1899	"	1,1,000,000,000	"	—	—	—
8. 13-12-1899	Pump in Drummstrasse.	1–100,000	"	+	3 and 4	—
9. 13-12-1899	"	1–1,000,000	"	+	2 and 3	—
10. 4-1-1900	"	1–10,000,000	"	+	5 and 6	—
11. 4-1-1900	"	1–100,000,000	"	—	—	—
12. 19-12-1899	Pregel water	1–100,000	"	—		B. coli found
13. 19-12-1899	"	1–1,000,000	"	—		
14. 19-12-1899	"	1–10,000,000	"	—		

In pure filtered water the B. typhosus was detected when only three drops of a dilution of 1-100,000,000 was used for the experiment. In water from a pump in the Drummstrasse the same result was obtained with a dilution of 1-10,000,000. In Pregel water, which contained the B. coli, the B. typhosus could not be detected. Hilbert then endeavoured to find out

if the failure to find the B. typhosus in the Pregel water was due to the presence of the B. coli. With this end in view he mixed equal quantities of B. coli and B. typhosus in "pipe-water," and also added typhoid stools, which were known to contain B. typhosus, to the "pipe-water." The waters thus polluted were investigated by Hankin's method, with the results shown in the following table :

No. and date of experiment.	Pipe-water, mixed with	Dilution.	Quantity used.	Result.	Remarks.
1. 11-1-1900	Equal quantities of twenty hours cultures of B. typhosus and B. coli	1–10,000	3 drops	—	B. coli found
2. 11-1-1900	"	1–100,000	"	—	
3. 11-1-1900	"	1–1,000,000	"	—	
4. 30-1-1900	Typhoid stool	1–100	"	—	B. coli found
5. 21-1-1900	"	1–1,000	"	—	"
6. 21-1-1900	"	1–10,000	"	—	

It is evident from these experiments that Hankin's method will fail to detect the B. typhosus in water supplies polluted with typhoid excreta. If, however, a water supply were polluted only with urine, in which case the B. coli is usually absent, the method would probably succeed in demonstrating the B. typhosus, but under these conditions most of the other methods would also be successful, and the case of a water-supply being only polluted with urine so seldom occurs in actual practice that Hankin's method must be considered of limited value.

Piorkowski has lately recommended a method for isolating the B. typhosus, which has been successfully used to determine the presence of this organism in typhoid stools. A special medium is employed, which is made by allowing ordinary urine (specific gravity 1020) to turn faintly alkaline; 3·3 per cent. gelatine and 0·5 per cent. peptone are then added, and the medium sterilised in the "steamer" on three successive days. After fifteen hours at 22° C. in this medium B. typhosus grows into characteristic flagellated or Ramosus-like colonies, while the B. coli appears only as round colonies, from which at times short, plump processes may run out into the gelatine. Working

with Piorkowski's medium I have found that the appearances presented by B. coli and B. typhosus are very variable. It is very difficult to get the medium of a uniform composition, as the time taken by different urines to become alkaline is not constant. Also the characteristic forms of B. typhosus often appeared in ordinary faintly alkaline gelatine, containing 3·3 per cent. gelatine. In 1896 Klie investigated the growth of B. typhosus in media containing different percentages of gelatine, and at different temperatures. He figured a very characteristic colony of B. typhosus, growing at 19°–21° C. in 3·3 per cent. gelatine, consisting of long threads growing out from a central point. The appearances, however, changed after twenty-four hours incubation, round centres appearing as in the colonies of B. coli. Klie came to the conclusion that the appearances of the colonies in dilute gelatine media did not afford a sure means of diagnosis. The colony figured by Klie, however, seems to me characteristic of B. typhosus. None of the specimens of B. coli in my possession have ever shown in 3·3 per cent. gelatine, after twenty-four hours incubation at 19° to 21° C., a colony which at the centre consisted of a point or fine line, from which long threads ran out into the surrounding gelatine. The most motile forms of B. coli under these conditions always showed a more or less thick circular dark-brown centre from which threads passed out into the gelatine.

Mayer has lately investigated the growth of B. typhosus in Piorkowski's medium, and also in 3·3 per cent. neutral gelatine. In order to overcome the difficulty introduced by the difference in the time required to render urine alkaline, Mayer recommends the addition of a twenty-four hours culture of Proteus vulgaris to the morning urine. If the urine be kept at 22° C. it will become faintly alkaline within fifteen to twenty hours; gelatine (3·3 per cent.) and peptone are then added, and the medium sterilised in current steam at 100° C. In this medium (which has a neutral reaction) five forms of colonies are obtained, according to Mayer; the fifth form or group is characteristic of B. typhosus, and consists of colonies which have no appreciable centre, or simply a thick point or streak from which flagella pass off either on one side only or from all sides. This

fifth form exactly corresponds to the colonies described by Klie, and Mayer also found that the same colonies were obtained in a medium simply consisting of 3·3 per cent. gelatine and 0·5 per cent. peptone, carefully neutralised with crystallised soda or bi-potassium phosphate. It therefore appears that the characteristic growth of B. typhosus in Piorkowski's medium is chiefly dependent on the small percentage of gelatine in the medium, the temperature and length of the incubation, and the motility of the bacillus. Mayer believes that if the characteristic form appears, and the colony when planted out in glucose broth does not give rise to the production of gas, the diagnosis of B. typhosus may be made.

Nearly all the methods described aim at giving the B. typhosus time to develop, but they all fail to prevent the growth of the B. coli and its varieties. I have lately employed a modification of Parietti's method combined with the use of glucose-litmus-agar plates. Two litres of the water to be examined are concentrated by pumping through a Berkefeld or Pasteur-Chamberland candle, and the deposit on the candle is then diffused by means of a sterile brush in 10 c.c. of sterile water. One c.c. of the concentrated water is then added to each of ten broth tubes containing 0·05 per cent. carbolic acid. It has been shown that the B. typhosus which has existed for several weeks in sewage, may not develop in broth tubes containing more than 0·05 per cent. carbolic acid; consequently it is important not to exceed this amount of acid. It is true that many liquefying organisms can develop in such a broth, but this disadvantage is more than counterbalanced by the fact that the B. typhosus, no matter how debilitated it may be, will always be able to grow in the medium. The broth tubes after inoculation are incubated at 37° C., and kept under observation for seventy-two hours. All the broth tubes which show a diffused turbidity are then plated out on glucose-litmus-agar. This medium contains two per cent. glucose added to ordinary agar containing sufficient aqueous extract of litmus to give the medium a light blue colour. For use the medium is melted, cooled to 42° C., and then $\frac{N}{10}$ NaHO added, so that each 10 c.c. of the medium has an alkalinity equal to 1·8 c.c. $\frac{N}{10}$ alkali. The alkaline agar

mixture is then poured into Petri dishes and allowed to solidify. Three plates are prepared for each turbid broth tube, one loopful from which is stroked in a ring form, without recharging, over the surfaces of all three plates. The colonies of B. typhosus appear on the plates as small round *transparent drops of a bluish tint*, the colonies of B. coli are larger, more opaque, and red in colour, also the medium around them shows the same red colour. The bacilli which belong to the fluorescent group (B. fluorescens liquefaciens and non-liquefaciens), also some of the Proteus species, which are able to develop at 37° C., give rise to colonies which have a deep blue colour, and are readily distinguished from the colonies of B. typhosus and B. coli. The sewage streptococcus produced colonies which by the naked eye cannot be distinguished from the colonies of the typhoid bacillus. The transparent blue colonies are then fished and examined in a hanging-drop; if motile bacilli like the B. typhosus are seen, I am in the habit of preparing 1–50 and 1–500 dilutions of anti-typhoid serum, and then adding a loopful of the 1–50 dilution to the hanging-drop already prepared, and a loopful of the 1–500 dilution to another hanging-drop made from the same colony. If agglutination of the bacilli is observed a portion of the colony is then planted out on an agar-slope, and the growth which results is then tested on all the various media described under the cultural reactions of B. typhosus.

The special alkalinity of the glucose-litmus-agar recommended above was arrived at by testing the amount of acid or alkali produced by B. typhosus, B. coli and its varieties, and the various water bacteria. I thought at first that about 6 per cent. of $\frac{N}{10}$ alkali (0·6 c.c. per 10 c.c. tube) would suffice to neutralise the acid produced by B. typhosus, and still leave B. coli markedly acid. In litmus-whey tubes 6 per cent. $\frac{N}{10}$ alkali was sufficient to neutralise the acid, but on glucose-litmus-agar poured into Petri dishes the typhoid colonies showed a marked red colour in the presence of this amount of alkali. By gradually increasing the amount of alkali it was found that 1·8 c.c. $\frac{N}{10}$ NaHO. per 10 c.c. tube of litmus-agar sharply differentiated B. typhosus from most of the varieties of B. coli. There are a few varieties of B. coli which are also neutralised by this amount of

alkali, but they are comparatively rare and, when sub-cultured, are easily distinguished from the typhoid bacillus. By adopting the alkaline glucose-litmus-agar the labour of investigating plates is much diminished, with a little practice a large number of plates can be examined in a very short time; as a rule, only about half a dozen colonies in each plate require to be fished.

Rémy has lately suggested a modification of the potato-gelatine medium. The new gelatine has a chemical composition almost identical with the chemical analysis of potato; it consists of:

Distilled water	1,000	grammes
Asparagine	6·00	,,
Oxalic acid	0·50	,,
Lactic acid	0·15	,,
Citric acid	0·15	,,
Di-sodic phosphate	5·00	,,
Magnesium sulphate	2·50	,,
Potassium sulphate	1·25	,,
Sodium chloride	2·00	,,

After sterilisation the gelatine is acidified with semi-normal H_2SO_4, so that 10 c.c. of the medium shall have an acidity which is exactly neutralised with 0·2 c.c. of a semi-normal solution of soda. Just before use, 1 c.c. of a 35 per cent. solution of lactose and 0·1 of a 2·5 per cent. solution of carbolic acid are added to each tube. In this gelatine the deep colonies of B. coli are round, oval, or sometimes fusiform, and of a brownish-yellow colour, fine bubbles of gas arising from the decomposition of the lactose sometimes accompany the colonies. The superficial colonies of B. coli are of two kinds: the first are globular, of a brownish-yellow colour, and sometimes show vertical prolongations which are raised above the surface of the gelatine; the second are opaque films (spread out) with an irregular margin. At first they are sometimes transparent and of a bluish colour, but later they rapidly become opaque. The colonies of B. typhosus which appear at the end of two days are deep and superficial. The deep colonies are bluish-white in colour and much smaller than those of B. coli; they are, however, very distinct to the naked eye. The deep typhoid colonies are never surrounded by bubbles of gas. The superficial colonies are not generally visible until the third day. At first they

have the appearance of moulds; later they spread out, become more blue in colour, and may attain the size of a 50-centime piece. When the typhoid bacillus has great vitality, as for example, if it comes from the spleen, its superficial colonies more resemble those of B. coli. The deep colonies, however, preserve their typhoid aspect. When the B. coli is feeble, its deep colonies are less distinct; they may lose their brownish colour and become blue; in this case they have a more marked blue colour than the colonies of B. typhosus. When plates are made in the medium from a fresh mixture of B. coli and B. typhosus, there is a very marked difference between the colonies of these organisms both in the depth and on the surface; the development of gas bubbles, however, is too irregular to be of much value. The great points are the brownish colour and the opacity of the coli colonies as compared with the transparent bluish colonies of B. typhosus. At times the varieties of B. coli, especially when they have been isolated from water, show colonies in the depth which are quite indistinguishable from those of B. typhosus.

Rémy's medium is not elective; other bacteria grow on it, but not so well as B. coli and B. typhosus. It is, however, fairly easy to prepare, and, being of constant composition, might replace the Elsner potato-gelatine medium with advantage.

Rémy points out that it is better to directly plate out a suspected water on his gelatine, containing 0·25 and 0·5 per 1000 of carbolic acid, than to pass the specimen through carbolic acid broth before making the plates. After ten days association with B. coli, the B. typhosus is said to become enfeebled, and if the weakened organism is passed through carbolic broth, containing only 1 per 1000 of carbolic acid, it is apt to die out. Rémy has found that B. typhosus, after being associated for thirty-eight days with B. coli, can be easily isolated by directly plating out the water in his special medium. But when the mixture of the two organisms is passed through carbolic acid broth, the typhoid bacillus cannot be found in the gelatine plates made from the carbolised broth if more than ten days have elapsed since the admixture of the typhoid with the colon bacilli. He also suggested that typhoid

bacilli in water have become adapted to a comparatively low temperature, and incubation at 37° C. may be inimical to them. Rémy stated that typhoid bacilli isolated from a stool and kept at the room temperature of 25° to 30° C. did not develop well in broth at 37° C., but grew vigorously in broth kept at 25° to 30° C. The pernicious influence of carbolic acid on weakened forms of the typhoid bacillus is undoubted, and many bacteriologists at the present day have abandoned the use of carbolised media and prefer to make a large number of plates with ordinary gelatine. By employing a small quantity of water, diluted if necessary, for each plate, they hope to obtain comparatively few colonies, and so give the typhoid bacillus time to develop without the risk of being crowded out by liquefying organisms.

Experimental Researches on the Duration of Life of the B. Typhosus in Water, Sewage, etc.

Kraus introduced a large number of typhoid bacilli into three samples of unsterilised water, one being a pure pipe supply and the other two well-waters; the bacilli disappeared between the fifth and seventh day in all three samples. Hueppe repeated Kraus's experiments and obtained the same results. Karlinski introduced 30,000 typhoid bacilli into the Innsbruck water, and found that after seven days they had completely disappeared. Karlinski also placed typhoid dejecta, containing typhoid bacilli, in a cistern filled with river and rain water. Three experiments were made, and in all of them the B. typhosus could not be found after the third day. Bobrow studied the duration of life of the B. typhosus in a well-water containing 15,000 water-organisms per c.c.; the typhoid bacillus could not be isolated after eight days. Hueppe introduced typhoid bacilli into unsterilised well-water kept at 16° to 20° C.; out of ten experiments he found the typhoid bacilli had disappeared after five days on four occasions, after ten days on five occasions, and in one case they appeared to live up to the thirtieth day. Frankland made simultaneous experiments on water derived from the River Thames, Loch Katrine, and a deep well in the chalk. The same amount of culture was introduced into the three specimens. In the Thames water the B. typhosus could not

be found after nine days, but in Loch Katrine water it lived for nineteen days, and in the deep well-water for thirty-three days. In the deep well-water the ordinary water bacteria increased far more rapidly than in the Thames and Loch Katrine waters, so Frankland believed that the disappearance of the B. typhosus could not be due to the action of the water-organisms. Klein introduced a recent culture of B. typhosus into unsterilised tap-water derived from the West Middlesex, Kent, and New River companies, and kept the samples at room temperature. Eighteen days after the inoculation living typhoid bacilli were found in 0·1 c.c. from each of the waters. After thirty-six days typhoid bacilli were not detected in 1 c.c. of the New River and Kent waters, but they were found in the West Middlesex water. After forty-two days the typhoid bacilli appeared to have disappeared from all three waters. In *sterilised* waters the B. typhosus has usually been found to have a more prolonged existence. Straus and Dubarry found it lived for sixty-nine days in distilled water, for eighty-one days in sterile L'Ourcq water, and for forty-three days in sterile Vanne water. Hochstetter, however, stated that the B. typhosus only lived for five days in distilled water and for seven days in sterile Berlin water. Wolffhugel and Riedel, working with sterilised Panke water, found that the B. typhosus multiplied when the temperature was favourable, viz., above 16° C., but when the temperature remained below 8° C. the bacillus remained alive but did not multiply. Klein found the B. typhosus lived for eighty-five days in sterilised water derived from the New River, West Middlesex and Kent companies.

Numerous experiments have been made on the vitality of the B. typhosus in sewage. Klein found that it could be detected in ordinary *sterile* sewage three weeks after inoculation. Laws and Andrewes experimented with sewage from Barking and Crossness. At first the sewage was sterilised by boiling, but in the later experiments the sewage was twice filtered through a Berkefeld bougie and then heated to 60° C. in a sterile flask for twenty minutes on two successive days. Planted out in this sewage and incubated at 37° C. the B. typhosus appeared to die within a few days: it could not be recovered after seventy-four hours. Further experiments were made on the same lines,

but the sewage was now kept at 22° C. instead of 37° C. The results obtained were as follows:

Immediately after inoculation		225 colonies developed from one loopful of sewage.
After 24 hours	.	250 " " " " "
" 68 "	.	140 " " " " "
" 5 days	.	48 " " " " "
" 7 "	.	13 " " " " "
" 13 "	.	0 " " " " "

Experiments made with B. coli showed that this organism was able to grow and multiply abundantly in sewage sterilised by heat when incubated at 37° C., and that it could be cultivated for several generations. After growing for four days in sewage the B. coli still retained its powers of coagulating milk, of forming gas-bubbles in gelatine and producing indol in broth. As a result of these experiments, Laws and Andrewes concluded that sewage, even in the absence of the normal micro-organisms which it contains, is an unfavourable medium for the growth of the typhoid germ, whereas the colon bacillus can grow and multiply freely in it. Further experiments were then made in order to gain some idea of the influence which various non-pathogenic bacteria normally present in sewage would exert on the life and growth of the typhoid bacillus when growing side by side with it. Organisms were selected which did not grow well at 37° C., viz., B. fluorescens liquefaciens, B. albus putidus, B. fluorescens stercoralis, and B. cloacæ fluorescens. The following conclusions were arrived at:

The presence of certain non-pathogenic organisms commonly present in sewage appeared to influence the extinction of the B. typhosus. Of the four organisms tested B. fluorescens stercoralis alone seemed to have any marked effect upon the vitality of B. typhosus, and this effect was practically absent when other organisms were present at the same time. The mixture of the four non-pathogenic bacteria had no effect in hastening the extinction of B. typhosus; indeed, the reverse appeared to be the case. My own experiments at Netley gave somewhat different results from those obtained by Laws and Andrewes. The experiments were performed as follows:

A specimen of sewage obtained from Yeovil was (*a*) placed

in sterile test tubes and sterilised by heating to 65° C. for ten minutes on four successive days; (*b*) placed in test tubes and sterilised in the autoclave at 120° C. for fifteen minutes; (*c*) filtered through a Berkefeld bougie into sterile test tubes. All the test tubes were inoculated with one loopful of a forty-eight hours growth of the B. typhosus, and then kept in the laboratory cupboard at a temperature which varied between 16° and 22° C. The day following one loopful was removed from each of the tubes and plated out in gelatine; after seventy-two hours incubation at 22° C. all the plates were crowded with typhoid colonies. Four days later a loopful was again removed and rubbed over an agar slope; after twenty-four hours at 37° C. a growth appeared which, when made into an emulsion, was found to be completely agglutinated by anti-typhoid serum. At the end of three weeks similar results were obtained. After two months a loopful was removed and plated out in gelatine; very few colonies appeared, and those which did grow were black in colour with granular contents, and the margin was only slightly wavy. In fact they appeared so unlike the original colonies that it was feared some pollution had occurred. However, one of the colonies was fished and inoculated on an agar slope; after twenty-four hours a growth appeared which, when tested with anti-typhoid serum, was completely agglutinated by the serum diluted 1–1000. Besides the change in the appearance of the colonies the B. typhosus appeared to have less resistance to carbolic acid, for while the original culture gave a copious growth in broth containing 0·05 per cent. carbolic acid after twenty-four hours incubation at 37° C., the agar growth prepared from the discoloured colonies showed no growth after twenty-four hours incubation at 37° C. in 0·05 per cent. carbolic acid broth, but after forty-eight hours there was a slight loss of transparency, and after seventy-two hours there was a marked growth. Experiments on the same lines were made with the same sewage diluted 1 in 10; identical results were obtained. These experiments appeared to show that the B. typhosus will be found alive after sixty days immersion in both strong and diluted sewage containing its usual toxines and salts, but freed from other living organisms. The power of reacting to anti-typhoid serum will still be present, but the colonies may present a dark

crumpled appearance, and the bacillus will show diminished resistance to carbolic acid. This last fact is of importance in relation to Parietti's test for the isolation of the B. typhosus from water. The behaviour of the B. coli in sterile sewage was then studied. After immersion for forty-two days in sewage filtered through a Berkefeld bougie it was found that the production of indol was delayed and the bacillus seemed less resistant to carbolic acid, for whereas the original B. coli grew well in 0·2 per cent. carbolic acid broth after twenty-four hours incubation at 37° C., the bacillus from the sewage showed no growth in 0·15 per cent. carbolic acid broth after twenty-four hours at 37° C., but grew well in 0·1 per cent. carbolic acid broth. The action of B. coli on B. typhosus when growing side by side in sterile sewage was next tested. A fresh twenty-four hours culture of B. coli was added to test tubes containing sterile sewage in which B. typhosus had been planted out twenty-four hours earlier. After fourteen days at the laboratory temperature, loopfuls were removed and plated in Holz's potato-gelatine, and rubbed over the surface of ordinary gelatine solidified in Petri dishes. No signs of the B. typhosus could be detected in these plates. The experiment was repeated again, but in a slightly different manner. A large loopful of a twenty-four hours agar growth of B. typhosus was added to 10 c.c. of sterile sewage and incubated at 37° C. for forty-eight hours; the sewage was then found quite opaque, and a loopful plated out in gelatine produced innumerable colonies of the typhoid bacillus. A very small quantity of an agar growth of B. coli was now introduced into the mixture of sewage and typhoid bacilli, and the tube preserved at the laboratory temperature. The B. typhosus was isolated from the tube up to the end of five days, but after this it could not be detected. Working on the same lines with B. fluorescens liquefaciens I was not able to isolate the typhoid bacillus after the seventh day. These experiments appeared to show that the presence of B. coli and B. fluorescens liquefaciens had a marked influence on the existence of the typhoid bacillus in sewage. At the same time I was conscious that the results were by no means conclusive, as some typhoid colonies might have been present on the gelatine plates and yet escaped attention owing to the large number of colonies of B. coli which

developed. In later experiments by employing surface alkaline glucose-litmus-agar plates instead of surface gelatine plates, I have been able to sharply differentiate colonies of B. coli from those of B. typhosus, and so obviate the defects of the earlier experiments. By this means I found that when one loopful of an agar growth of B. typhosus was mixed with one loopful of an agar growth of B. coli in ten cubic-centimetres of sterile tap water, the B. typhosus could be easily isolated after twenty-eight days. The bacillus so obtained had lost none of its cultural characteristics, and as regards its reaction to the specific agglutinins contained in Berne serum it appeared, if anything, more susceptible to agglutination after the four weeks existence with B. coli than when it was first introduced into the tube. The original culture was completely agglutinated by Berne serum diluted 1–1000, and only partially agglutinated by the same serum diluted 1–10,000; but the culture of B. typhosus after association with B. coli was found to be *completely* agglutinated by the Berne serum when diluted 1–10,000. Attempts were then made to study the duration of life of the B. typhosus in unsterilised tap-water to which B. coli was also added. A loopful of B. typhosus was added to 10 c.c. of unsterilised tap-water and twenty-four hours later a loopful of B. coli was introduced. The tube was maintained at the room temperature, and loopfuls withdrawn from time to time were plated out on the alkaline glucose-litmus-agar medium. The typhoid bacillus was easily isolated after twenty-one days. In all these experiments the B. typhosus was present in large quantity, and it seemed probable that the B. coli did not multiply to any extent. When B. typhosus and B. coli were added to peptone water, it was found that the B. coli underwent rapid multiplication and no signs of the B. typhosus could be discovered after seven days. Also when a loopful of a typical typhoid stool was diluted in 10 c.c. of sterile tap-water and examined at once, B. typhosus was found to be present in considerable numbers; but after seven days had elapsed the typhoid bacillus could not be found.

Sidney Martin has investigated the growth of the typhoid bacillus in the presence of particular soil organisms. Most of the experiments were made with bacteria isolated from

Chichester soil. The organisms were obtained in pure culture, but no attempt at naming them was made; they were simply indicated as Chich. 1, Chich. 2, &c. "The culture media of experiment were a flask containing 200 c.c. of sterile distilled water and about 10 c.c. of ordinary peptone broth; and sterilised soil, the soil used in each instance being that from which the particular soil bacillus of experiment had been isolated. The temperature at which the experiments were performed was either that of the 37° C. incubator, the temperature of an outside shed (3°–14° C.) or, in a few cases, the temperature of the laboratory (13°–19° C.). The general method of investigation consisted in inoculating the different media with a definite quantity of a pure broth culture of one of the soil organisms, and at the same time with an equal quantity of pure broth culture of the typhoid bacillus, both cultures being of the same age." The relative proportion of the two sorts of bacteria present was determined at stated intervals by brushing two or more agar plates with a sterile brush dipped in the nutrient fluid. Surface colonies, generally discrete, were obtained, and from these plates the relative proportion of each organism present could be ascertained. Chichester 1 was a fluorescent organism which did not liquefy gelatine. When grown in a liquid medium at 37 C. with B. typhosus, the colonies of both organisms could be easily detected twenty-four hours after the inoculation; but, after six days, no colonies of B. typhosus could be detected. The same result was obtained when the medium was kept at 4° to 12° C. In sterile soil at 37° C. Chichester I. caused the disappearance of the typhoid bacillus in six days, but at 8° to 12° C. the typhoid bacillus could be detected after six days, though it was not found at the end of fourteen days. Experiments on the same lines were made with Chichester 2, which was a large, stout bacillus, and formed spores freely. It was easily distinguished from the typhoid bacillus. In all the experiments conducted at 37° C. the typhoid bacillus at first grew more rapidly than Chichester 2. As growth proceeded, however, Chichester 2 gradually increased more rapidly than the typhoid bacillus, and eventually gained the upper hand. At shed temperature, Chichester 2 maintained the ascendency at the

first, and in two weeks was the only organism that could be found; but later the typhoid bacillus, present apparently in too few numbers to be seen on the plate when growing at the shed temperature, gradually increased, and in the final experiment at 37° C. assumed the upper hand. The experiments made with Chichester 3 were of special interest. Chichester 3 was a stout bacillus with rounded ends, forming long chains. On potato it formed an abundant yellow growth, but it did not produce indol nor form gas in sugar media. It liquefied gelatine slowly, and formed dense opaque colonies on agar plates. No confusion could arise between this organism and the typhoid bacillus. The experiments showed that Chichester 3 had difficulty in growing in the presence of the typhoid bacillus. It grew readily enough in a mixture of water and broth when it was the only organism present; but when the typhoid bacillus was also present Chichester 3 could not establish itself, it did not grow or was killed out altogether. This took place at low temperature and at 37° C. When, however, Chichester 3 was grown in the sterilised soil from which it was obtained, its chances of growth and multiplication were greatly increased, and in one case it so far gained the mastery that no typhoid bacilli could be found after fifteen days.

The latest contribution to the study of the B. typhosus in relation to other micro-organisms is furnished by the researches of Rémy on the antagonism of the B. coli and the B. typhosus. This observer believes that the failure to isolate the B. typhosus when growing side by side with other bacteria is not due to the disappearance of the typhoid organism but to the imperfection of the methods employed to isolate it. He added to a flask containing a litre of peptonised water (peptone 3 per cent., sodium chloride 0·5 per cent.), exactly neutralised, 5 c.c. of a twenty-four hours culture of B. typhosus and 5 c.c. of a twenty-four hours culture of B. coli. The same procedure was followed with a flask containing peptonised water to which, after neutralisation, 0·5 per 1000 of H_2SO_4 was added. The flasks were kept at the room temperature, and examined at intervals by plating out the contents in Rémy's special medium. After five days association the B. typhosus was found not to be agglutinated by anti-typhoid serum, diluted 1–10, though

originally it was agglutinated by the serum, diluted 1-70,000; the B. coli maintained its characters unchanged. After fifty-one days association the B. typhosus colonies were found to develop more slowly than before, and the B. coli colonies were smaller and resembled those of B. typhosus. After eighty-two days association the B. typhosus remained as before, but the B. coli isolated from the neutral peptone water, though it still fermented sugar media, had lost the power of producing indol. In the acidified flask, however, the B. coli maintained its characters unchanged. After 100 days it was difficult to distinguish the colonies of B. coli and B. typhosus from the neutral flask; all the colonies failed to produce gas and indol and to agglutinate with anti-typhoid serum. In the flask containing the acidified water, however, the B. coli still gave the indol reaction, and fermented lactose, and the B. typhosus did not react with anti-typhoid serum. A week later the colonies from the neutral water were exceedingly feeble in their growth, and, as before, failed to produce indol or gas or to agglutinate with anti-typhoid serum. In the acidified water the B. typhosus had now disappeared, and only B. coli could be isolated, which, however, still produced indol and gas. In these experiments there was a very marked difference between the results obtained in the neutral and acid waters; in the former medium the B. coli and B. typhosus speedily lost their special characteristics; but in the latter the B. coli maintained its power of forming gas and indol right up to the end of the experiment, and the B. typhosus only lost its power of reacting to anti-typhoid serum after 100 days association with B. coli. Control experiments were then made to see if the B. coli and B. typhosus lost any of their special characteristics when growing *separately* in the neutral peptone water. After four months the two organisms were still found alive, and they both maintained their specific reactions. Other races of B. coli and and B. typhosus when grown together in neutral peptone water were not found to be affected in the same way as the B. coli and B. typhosus which were used for the first experiments. Rémy then endeavoured to decide whether the colonies which had perfectly negative reactions when isolated after 100 days from the neutral peptone water were B. coli or

B. typhosus. To decide this question he immunised guinea-pigs with the cultures; of the sera so prepared, some agglutinated the B. typhosus, while others agglutinated the B. coli, used originally to infect the flasks; consequently these atypical bacilli appeared to be true descendants of the original cultures. As a result of these experiments Rémy arrived at the following general conclusions :

(1) The idea of the effacement of the B. typhosus by the B. coli is not confirmed by researches on the antagonism of these two organisms.

(2) Association can profoundly modify the properties of the two organisms, the B. typhosus losing its sensibility towards agglutinins and the B. coli being deprived of its specific characteristics.

(3) The colonies of B. coli in the depth of the gelatine, after the third or fourth week, insensibly approach in dimensions and appearance the colonies of B. typhosus.

(4) The diminution of the vital energy of the organisms shows itself not only in a diminution in the size of the colonies, but also by a delay in their appearance.

(5) If the agglutination of a bacillus, presenting the characters of the B. typhosus, by an experimental anti-typhoid serum in a high dilution, suffices to authorise us to consider the organism as typhoid in nature, the absence of sensibility towards the specific agglutinins does not permit us to reject the organism from the group of typhoid bacilli.

(6) Bacilli which possess the attributes of the B. typhosus, but which are no longer agglutinated by experimental anti-typhoid serum, ought to be considered as typhoid bacilli if a guinea-pig, which has received every second day 2 c.c. of a forty-eight hours broth culture of the organism, produces after fifteen days a serum which agglutinates a typical B. typhosus in a minimum dilution of 1–40.

(7) There may exist true typhoid bacilli which cannot be agglutinated by anti-typhoid serum, and the typhoid nature of which it is impossible to determine, either by the procedure given above or by the means of diagnosis actually at our disposal.

Rémy's results are very interesting, but it is strange that the effects of the association of B. coli and B. typhosus were only

obtained with two particular cultures of these organisms, and in neutral peptone water. My own experiments showed that the B. typhosus, when growing with B. coli maintained its power of reacting to anti-typhoid serum so long as it could be isolated from the mixture. Also experiments made at Netley on the inter-action of B. coli and B. typhosus when planted out in large quantities in tap-water, showed that even when employing Rémy's medium B. typhosus could not be isolated after the thirteenth day. The colonies of B. coli acquired much the same appearances as those of B. typhosus, so that it was necessary to sub-culture all the colonies before a diagnosis could be made between the two organisms.

The whole question of the duration of life of the B. typhosus in water and sewage is very complex; temperature, the amount of nutriment, the presence of other organisms and their toxines, the number and vitality of the typhoid bacilli introduced, are powerful factors which influence the result in various ways. In most experiments the typhoid bacilli have been introduced in considerable numbers, but examination of the stools of typhoid patients has shown that typhoid bacilli may not be present in any quantity when water is fouled by typhoid dejecta. Wathelet only found ten colonies of B. typhosus among six hundred colonies having characters common to B. coli and B. typhosus, which he isolated from typhoid stools. Consequently it seems extremely probable that under natural conditions comparatively few typhoid bacilli will gain access to a water supply, and to detect them it will be necessary to examine a considerable quantity of the water. The bulk of the experimental evidence seems to show that, unless the specific pollution is continuous, it is extremely unlikely that typhoid bacilli will be detected in a water if more than a week has elapsed since the actual pollution occurred. The outbreak of typhoid fever at Maidstone affords considerable support to this view. Washbourn made a bacteriological examination of the infected water about ten days after the specific infection had taken place. He failed to find the B. typhosus, though the presence of B. coli showed that sewage contamination of the water had occurred. When, however, sewage containing the specific excreta passes into a cesspool, and percolates at once into the surrounding ground, the

conditions of life of the B. typhosus are different. Robertson's experiments appeared to show that typhoid bacilli may exist in earth under natural conditions for several months. Martin's latest studies on the behaviour of B. typhosus in soil, however, throw some doubt on these results ; everything seems to depend on the nature of the soil and the other organisms present in it. Still, the endemic prevalence of enteric fever year after year in certain localities, characterised by polluted soils, renders it extremely probable that the life of the B. typhosus in soil is longer than in water-supplies. If this be so the bacilli may be carried from the soil into drinking water by rising ground water or rain, and in this way outbreaks of enteric fever may occur long after the initial infection.

Cases in which the B. Typhosus has been Isolated from Water-supplies.

The discovery of the B. typhosus in water-supplies has often been announced. Moers, in 1886, was the first to isolate the bacillus from a contaminated well supplying water to a number of people amongst whom cases of enteric fever had occurred. Michael, in the same year, claimed to have found the bacillus in a well-water suspected to have caused enteric fever in Dresden. Chantemesse and Widal, Thoinot, Loir, and Vincent have all found the typhoid bacillus in the water of the river Seine. Fodor stated that he found the bacillus five times in the water-supply of Budapest. Most of the early announcements of the discovery of the B. typhosus must be accepted with great reserve, as at that time the appearance of the colonies on gelatine plates was considered sufficient to prove the presence of the bacillus, and most of the tests now required were not applied. Cassedebat studied with great care the water-supply of Marseilles in 1890, employing special methods, but he failed to discover the typical B. typhosus of Eberth and Gaffky. He, however, isolated three other bacilli which he called pseudo-typhoid ; these organisms bore a close resemblance to the true typhoid, and could only be distinguished from it with great difficulty. Cassedebat made about two hundred and fifty cultures, employing ten drops of water for each culture, and was careful to state that his failure to discover the typhoid bacillus

did not prove anything for or against the existence of this bacillus in water, owing to the small amount of water with which he worked. At the same time it is evident that his so-called pseudo-typhoid bacilli would have been described by earlier observers as true typhoid organisms. Klein examined the water-supply of Worthing, where an outbreak of enteric fever had occurred. A sample was collected drop by drop during a period of twelve hours from the rising main in the yard of the Worthing waterworks; 1500 c.c. of this water were passed through a Berkefeld filter, and the residue on the bougie was distributed among phenolated broth tubes and phenolated gelatine plates. "In the tubes and plates B. coli developed abundantly, and in addition a few other colonies were detected —two in one plate and one in another plate—which on sub-culture presented, morphologically as well as culturally, all the characters of enteric fever bacilli." The following features were given as characteristic of B. coli and B. typhosus:

B. Coli.	B. Typhosus.
Shorter and less motile than the enteric fever bacilli.	Longer and more motile than B. coli.
Forms gas bubbles in gelatine-shake culture.	Does not form gas bubbles in gelatine-shake culture.
Curdles milk in one to two days at 37° C.	Does not curdle milk.
Forms indol in broth after several days.	Gives no indol reaction.

The tests given under B. typhosus would be considered at the present day as quite insufficient to establish the diagnosis of the typhoid bacillus. Consequently the Worthing epidemic must be relegated to the large class of cases in which there is still a doubt as to the presence of B. typhosus in the water-supply.

In 1897 Remlinger and Schneider examined thirty-seven specimens of water obtained from wells and rivers both during outbreaks of enteric fever and in the absence of all manifestations of this disease. Nine of the specimens contained a bacillus presenting all the characters which they considered typical of B. typhosus, viz., (1) aspect of cultures on gelatine; (2) active motility of the bacillus; (3) large number of flagella; (4) failure to stain by Gram's method; (5) absence of gas forma-

tion in sugar-media; (6) milk unchanged; (7) indol not produced in peptone water; (8) acid produced in litmus-whey not exceeding 3 per cent. of $\frac{N}{10}$ alkaline solution; (9) typical growth on potato; (10) pathogenic action; (11) agglutination with anti-typhoid serum. Two of the specimens came from towns (Meaux, Saint-Omer) where typhoid fever existed at the time. The B. typhosus was only transitory in these two cases, for new specimens of the water taken a month later did not contain the bacillus. Six other specimens were obtained from towns where enteric fever had existed, but at the time was not in epidemic form. The examinations of the water from Châteaudun and Dijon were of special interest. An epidemic of typhoid fever appeared in Châteaudun during the winter of 1895-6 amongst the civil and military population. The water-supply was first examined on January 21 by means of phenolated media; the B. typhosus was not found. A second examination was made on March 15 by Elsner's method; on this occasion the typhoid bacillus was isolated. On May 10 and again on June 15 the B. typhosus was detected, though from the beginning of March the outbreak lost its epidemic form and only appeared as rare and isolated cases. Typhoid fever also appeared in an epidemic form in Dijon during the winter of 1895-6, but examinations of the water-supply, both in 1895 and early in 1896, by phenolated media failed to demonstrate the presence of the B. typhosus. In April 1896, however, when enteric fever no longer existed in the town, Elsner's method showed that a bacillus, identical with the B. typhosus, was present in the water-supply. In May and June the same bacillus was isolated, though no enteric fever existed in either the civil or military population. In this outbreak water apparently containing the B. typhosus was consumed for three months without any epidemic of enteric fever appearing.

In 1899 Kubler and Neufeld examined specimens of water from a farm where enteric fever existed. By means of Elsner's method B. typhosus was isolated from the water in a well supplying drinking-water to the farm. The tests on which the authors relied for the differentiation of the typhoid bacillus were as follows: Characteristic growth on potato, gelatine, and in litmus-whey, absence of indol in broth, absence of gas formation

in sugar media, large number of flagella, and agglutination with a very dilute anti-typhoid serum. Also a three hundred gramme guinea-pig was killed by one loopful of a twenty-four hours growth on agar; but with the same amount of growth and 0·01 c.c. of anti-typhoid serum, a guinea-pig of the same weight remained quite well. About four weeks later two cultures were obtained from the same well, which in appearance, motility, chemical reactions, and agglutination resembled the typhoid bacillus; but these cultures were not pathogenic, one loopful of a twenty-four hours agar culture had no effect when injected into a guinea-pig. The authors regard Pfeiffer's reaction as the most important diagnostic sign of the B. typhosus, because it indicates that the bacillus is in a virulent condition. In this outbreak B. coli was not found in the well-water, and the typhoid bacilli were present in large numbers. Kubler and Neufeld, therefore, believed that the pollution of the water was caused by urine from a typhoid patient, for when infection of the water-supply was produced by fæcal contamination B. coli was usually present in large numbers. The absence of B. coli from the water in this case rendered the isolation of the typhoid bacillus a comparatively easy matter, and probably accounted for the persistence of the bacillus in the water supply.

In 1899 Genersich also investigated an outbreak of typhoid fever in Pécs, supposed to have been caused by infected water. In the epidemic there were 209 cases and 28 deaths, which occurred in a portion of the town supplied with water from the Alsó-Rókus wells. Suspicion fell on cisterns in the Kis Rókus-Gasse and in the "Kreuzer-kaserne." Twenty-eight gelatine plates were made with water taken from the cisterns and the wells. Suspicious colonies which developed in the plates were fished and planted out in gelatine-stabs. The growths which resulted were compared with a true typhoid culture; and the motility and staining reactions were also examined. The cultures which resembled B. typhosus were then planted out on potato, control preparations of B. coli and B. typhosus being made at the same time. Characteristic cultures were then inoculated into sugar-agar broth and milk. The growths in broth were tested for the indol reaction. Agglutination experiments were made with a twenty-four hours broth culture of the

suspected organisms and the serum from typhoid patients, and also with the serum from a guinea-pig (immunised by injecting 1 c.c. of a forty-eight hours broth culture per 0·3 kilogramme of body-weight) diluted 1–50. Lastly, according to Fodor and Rigler's recommendation, forty-eight hours broth cultures of the suspected organisms were injected into guinea-pigs and dogs. Specimens of blood from these animals were taken after eight to ten days, centrifugalised, and then tested with true cultures of B. typhosus, B. coli, and the organisms which had been used for the immunisation. From the 28 plates 157 suspicious cultures were obtained, but of these only 61 corresponded to B. typhosus in motility, staining reactions, and growth in gelatine-stab. Out of 61 cultures, however, only 31 grew typically in sugar-agar, milk, and broth, and only 11 of these were agglutinated by the serum from typhoid patients diluted 1–50. Out of 9 cultures tested 7 were agglutinated by the serum of the guinea-pig immunised with B. typhosus diluted 1–50. Eight guinea-pigs and three dogs were then inoculated with the 11 cultures which had shown a reaction with the serum of the typhoid patients. The serum diluted 1–50 from each of the guinea-pigs and dogs agglutinated the special organisms which had been used for their immunisation; and also, for the most part, the serum from all the animals agglutinated all the organisms which had been used for the injections. But, with one exception, the sera failed to agglutinate a broth culture of a true typhoid bacillus. Genersich could not explain the cause of this failure, but suggested that the supposed typhoid bacilli, isolated from water, had much less virulence than the typhoid bacilli isolated from the spleens of typhoid patients, and so were unable to produce a serum which would agglutinate the latter bacilli.

B. Fischer and G. Flatau in January 1901 reported the discovery of the typhoid bacillus in water from a well in the town of Rellingen. Plates were made with varying quantities of the water added to ordinary and carbolic acid (0·05 per cent.) gelatine; surface plates with the same media were also prepared. On the surface plates made with carbolic acid five suspicious colonies appeared, but only one of these failed to produce gas in glucose-agar. This organism was then carefully studied. It

was a motile bacillus, about the same size as the typhoid bacillus, and did not stain with Gram. On potato it produced a moist transparent growth. Milk was not coagulated after eight days incubation at 37° C. Indol was not produced. In Petruschky's litmus-whey the amount of acid formed was very small. The agglutination test was then tried with serum from a goat immunised by repeated injections of the B. typhosus. This serum, diluted 1–200, caused agglutination of the bacillus which had been used to prepare it, the clumps being visible, macroscopically, in a quarter of an hour. It also caused agglutination of the bacillus isolated from water when employed in dilutions of 1–50, 1–100, and 1–200, but the clumps were not visible, macroscopically nor microscopically, for from one to two hours. The same delayed agglutination was also seen when two true typhoid cultures from different sources, *i.e.*, a typhoid spleen and a typhoid abscess, were tested with the serum. The bacillus isolated from water was also tested with a serum which, in a dilution of 1–500, caused agglutination of its own bacillus in one to two hours. The "water bacillus" was agglutinated by this serum diluted 1–500, but the reaction was not apparent microscopically for four hours. Pfeiffer's test was also applied, but with some difficulty, as the typhoid serum had not a powerful bacteriolytic action, and the minimal fatal dose of the water bacillus varied from 0·046 mgm. to 0·092 mgm. per 100 grammes of body-weight of the experimental animal. Experiments showed, however, that 0·05 c.c. of the typhoid serum protected against ten times the lethal dose of the bacillus, whereas the same amount of normal serum from men and goats had no protecting action against the same dose of the bacillus. The chain of evidence seems fairly complete, and the bacillus isolated by Fischer and Flatau must be regarded as the true B. typhosus. Four weeks later the authors again examined the water from Rellingen, but failed to find any traces of the B. typhosus.

Apart from the Isolation of the B. Typhosus, are there any means of Diagnosing Pollution of a Water Supply with the Specific Dejecta of Cases of Enteric Fever?

The cases just recorded show that, with one or two exceptions, it has been impossible to isolate the B. typhosus from suspected

water supplies during epidemics of enteric fever. The cause of this failure has been variously interpreted. Some bacteriologists believe it is due to the feeble vitality of the B. typhosus when associated with other micro-oganisms, especially the B. coli, so that the specific organism dies out of the water supply before the outbreak of disease has directed attention to the necessity of a bacteriological examination of the water. Other bacteriologists would, however, attribute the failure to the imperfection of modern bacteriological methods of investigation. A French school, headed by Rodet and Roux, believes in the existence of transitional forms between the typical B. typhosus and the typical B. coli. Rodet has quite lately described such transitional forms which are of great interest to students of epidemiology. In May 1897, Rodet isolated from the spleen of a fatal case of enteric fever a bacillus which resembled the typhoid bacillus in its morphology, mode of growth on gelatine and agar, absence of gas production in sugar media, and failure to produce indol in peptone solution. Immediately after isolation it was not agglutinated by anti-typhoid serum, even in a dilution of 1–10, and produced a brownish-yellow growth on potato; consequently the organism appeared to be an atypical coli. The microbe was preserved for a year and its power of reacting to anti-typhoid serum tested from time to time. It was found that the reaction to specific agglutinins gradually increased until, finally, the bacillus was agglutinated by a specific serum diluted 1–100,000. In October 1897, a similar bacillus, which showed a steady increase in its reaction to anti-typhoid serum, was also isolated from the spleen of a fatal case of typhoid fever. A third bacillus, which more strongly resembled B. coli and produced gas in glucose-agar, was recovered from the spleen of a fatal case of enteric fever in December 1897. Six months after its isolation this microbe, which at first was not affected by anti-typhoid serum, was found to be agglutinated by two different sera in exactly the same manner as a typical B. typhosus.

On the other hand, Remlinger and Schneider state that they have isolated the B. typhosus from the stools of patients who were not suffering from enteric fever. Four of the bacilli isolated were pathogenic to guinea-pigs, and preventive injections of anti-typhoid serum preserved animals from infection. Independently of the above bacteria the same observers stated that

they often found in water, soil, and human fæces bacilli which strongly resembled the B. typhosus in all points except reaction to specific agglutinins and pathogenicity to animals. They believe that there are varieties of the true B. typhosus, and consider that this organism exists naturally in air, water, and earth, but how it obtains its pathogenic action is not yet known; possibly privation and fatigue are important factors.

Whether it be true that B. coli can be converted into B. typhosus, as Rodet believes, or that the B. typhosus naturally exists in water, &c., in an abortive form, as Remlinger and Schneider consider probable, the fact remains that up to the present time most bacteriologists have failed to isolate the typhoid bacillus from suspected water supplies. Consequently it would be a very important matter for hygienists if they could, in the absence of the discovery of the B. typhosus, say that a water supply had been infected by the specific dejecta of enteric fever. With this end in view I have lately made a special study of the varieties of B. coli which are associated with the typhoid bacillus in the dejecta of patients suffering from enteric fever. I hoped that the varieties in question might show cultural characteristics or reactions to specific sera, which would enable them to be distinguished from the varieties of B. coli present in the stools of healthy people. One hundred and fifty different cultures were examined, eighty being derived from the stools of patients suffering from typhoid fever and seventy from the stools of healthy men. The stools of the enteric fever patients were obtained during the third and fourth weeks of the disease and also from the intestines after death had occurred. A loopful of the stool was stroked over the surface of a series of plates containing solidified gelatine. The first two plates generally liquefied, but the remaining plates showed discrete colonies which developed satisfactorily. Typical colonies were fished and planted out on agar slopes, which were then incubated at 37° C. for twenty-four hours. The growths which resulted were used for agglutination experiments and planted out in the various media.

Agglutination Experiments.—The twenty-four hours growth was made into an emulsion with broth and then mixed with an equal amount of horse's anti-typhoid serum, so as to make final dilutions of the mixed emulsion and serum in the

proportion of 1–50, 1–100, 1–200, 1–500, 1–1000. The experimental investigation of the 150 cultures of B. coli extended over a period of four months, consequently it was necessary to employ different batches of anti-typhoid serum; and to make the results strictly comparable, the various specimens of serum were standardised week by week with two stock cultures of B. typhosus. All through the experiments the sera used gave a complete agglutination in a dilution of 1–1000 and a marked reaction in a dilution of 1–10,000 with the stock cultures of B. typhosus. The results obtained with the 80 cultures of B. coli isolated from enteric stools are shown in the following table:

Table A.

VARIETIES OF B. COLI FROM ENTERIC STOOLS TESTED WITH ANTI-TYPHOID SERUM.

No. of culture.	Dilution of the serum.				
	1–50	1–100	1–200	1–500	1–1000
1	+	+	±	0	0
2	+	—	0	0	0
3	+	—	0	0	0
4	+	+	±	0	0
5	0	0	0	0	0
6	+	+	±	0	0
7	0	0	0	0	0
8	0	0	0	0	0
9	0	0	0	0	0
10	0	0	0	0	0
11	0	0	0	0	0
12	+	+	±	0	0
13	0	0	0	0	0
14	0	0	0	0	0
15	+	+	±	0	0
16	+	+	±	0	0
17	0	0	0	0	0
18	0	0	0	0	0
19	0	0	0	0	0
20	0	0	0	0	0
21	0	0	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0
25	0	0	0	0	0
26	0	0	0	0	0
27	+	+	±	—	0
28	+	+	±	—	0
29	+	+	±	—	0
30	+	+	±	—	0

TABLE A.—*continued.*

No. of culture.	Dilution of the serum.				
	1-50	1-100	1-200	1-500	1-1000
31	+	+	±	—	0
32	+	+	±	—	0
33	—	0	0	0	0
34	—	0	0	0	0
35	—	0	0	0	0
36	+	+	±	0	0
37	+	+	±	0	0
38	+	+	±	0	0
39	+	+	—	0	0
40	+	+	±	0	0
41	+	+	±	0	0
42	+	+	±	0	0
43	+	+	±	0	0
44	+	+	±	0	0
45	0	0	0	0	0
46	0	0	0	0	0
47	0	0	0	0	0
48	0	0	0	0	0
49	0	0	0	0	0
50	+	—	0	0	0
51	+	±	0	0	0
52	+	±	±	0	0
53	±	±	±	0	0
54	+	±	±	0	0
55	+	±	±	0	0
56	+	±	—	0	0
57	+	+	±	0	0
58	+	+	+	—	0
59	+	±	0	0	0
60	+	+	+	0	0
61	+	+	+	0	0
62	+	+	+	+	+
63	+	+	+	+	—
64	+	+	+	+	—
65	+	+	+	+	—
66	+	+	+	+	±
67	+	+	+	+	±
68	+	+	+	—	0
69	+	+	+	+	—
70	0	0	0	0	0
71	0	0	0	0	0
72	0	0	0	0	0
73	0	0	0	0	0
74	0	0	0	0	0
75	0	0	0	0	0
76	0	0	0	0	0
77	0	0	0	0	0
78	0	0	0	0	0
79	0	0	0	0	0
80	0	0	0	0	0

NOTE.—The sign + indicates complete agglutination.
" ± " nearly complete agglutination.
" — " partial agglutination (many free bacilli).
" 0 " no traces of agglutination.

This table shows that seven cultures of B. coli were completely agglutinated by the anti-typhoid serum, diluted 1–500; two cultures were markedly agglutinated by the serum, diluted 1–1000, and one culture was completely agglutinated by the serum in this dilution. This last culture was further tested at once with the serum in still higher dilutions, and was found to be completely agglutinated by the serum when diluted 1–2500. The serum was then tested with four cultures of B. typhosus isolated from the spleens of the fatal cases which furnished the stools under investigation. The results, together with the effects of the serum on the two stock cultures, are shown in the following table:

Table B.

Cultures of B. typhosus.	Dilution of anti-typhoid serum.					
	1–50	1–100	1–200	1–500	1–1000	1–10,000
G	+	+	+	+	—	0
G^x	—	0	0	0	0	0
G^x	+	+	+	+	+	—
M	+	+	+	+	±	0
A	+	+	+	+	+	0
Stock { K	+	+	+	+	+	±
Stock { 13 p.	+	+	+	+	+	±

It will be observed that all the specimens with the exception of G^x were completely agglutinated by the anti-typhoid serum diluted 1–500; with the higher dilutions, however, the results varied, culture B. typhosus G and culture B. coli 62 were obtained from the same case, and it will be noticed that the B. coli was completely agglutinated by the anti-typhoid serum in a higher dilution than was effective with the B. typhosus. If the serum employed had been obtained from the patient it would have been easy to explain the result by supposing that the patient suffered from a mixed "typhoid" and "coli" infection. In the present case, however, there could be no question of a secondary coli infection, as the anti-typhoid serum was obtained by vaccinating a horse with pure cultures of B. typhosus. It therefore appears that coliform organisms in typhoid stools, as a result of their surroundings, may become agglutinated by a highly dilute anti-typhoid serum. That the sensibility to agglutination is only

acquired by environment, and is not truly specific as in the case of the B. typhosus, is shown by the fact that when the cultures were removed from their associations and preserved for several months on agar slopes, the sensibility to the agglutinins in the anti-typhoid serum gradually declined, and at the end of six months the results shown in the following table were obtained :

TABLE C.

CULTURES OF B. COLI, FROM TYPHOID STOOLS, RE-TESTED WITH ANTI-TYPHOID SERUM AFTER BEING PRESERVED FOR SIX MONTHS ON AGAR.

No. of coli culture.	Dilution of anti-typhoid serum.				
	1–50	1–100	1–200	1–500	1–1000
62	±	±	0	0	0
63	±	0	0	0	0
64	+	+	0	0	0
65	+	+	0	0	0
66	+	±	0	0	0
67	+	±	0	0	0
68	—	0	0	0	0
69	+	+	±	—	0

The maximum dilution which caused complete agglutination was now only 1–100. The races of B. typhosus, however, showed no change, the results previously given were again obtained at the end of six months. The culture B. typhosus G^x was peculiar; when first isolated from the spleen it only showed traces of agglutination with the anti-typhoid serum diluted 1–50; but, after being preserved in milk for six months, a subculture was completely agglutinated by the anti-typhoid serum diluted 1–1000.

The typhoid culture G^x was carefully studied, and it was found to possess cultural characteristics absolutely identical with undoubted races of B. typhosus. The cultural characteristics of G^x and the races of B. typhosus shown in Table B. are given in Table D on page 232.

The cultural characteristics of the coliform cultures Nos. 62 to 69 were then examined; it was thought that as they came within the "typhoid range" as regards agglutination they might show an approximation to the cultural reactions of

Table D.

RACES OF B. TYPHOSUS.

	G	G^{x}	A	M	K	13 p
Agar	Thin greyish-white growth	Thin greyish-white growth	Thin greyish-white growth	Thin greyish-white growth	Thin greyish-white growth	Thin greyish-white growth
Broth	Turbid, no film on the surface	Turbid, no film on the surface	Turbid, no film on the surface	Turbid, no film on the surface	Turbid, no film on the surface	Turbid, no film on the surface
Glucose or lactose-gelatine (shake)	No gas formation	No gas	No gas	No gas	No gas	No gas
Witte's peptone and salt solution, after seven days at 37° C.	No indol	Traces of indol	No indol	No indol	Traces of indol	No indol
Potato	Moist, colourless growth	Colourless growth	Colourless growth	Colourless growth	Colourless growth	Colourless growth
Milk, three weeks at 37° C.	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged
Litmus - whey, seven days at 37° C., acidity equals	5·7 % $\frac{N}{10}$ alkali	5·1 % $\frac{N}{10}$ alkali	5·6 % $\frac{N}{10}$ alkali	5·4 % $\frac{N}{10}$ alkali	5·6 % $\frac{N}{10}$ alkali	5·5 % $\frac{N}{10}$ alkali
Proskauer & Capaldi's medium, No. I., twenty-four hrs. at 37° C.	No growth or change in reaction	Growth, but no change in reaction	No growth or change in reaction	No growth or change in reaction	No growth or change in reaction	Growth, but no change in reaction
Ditto, No. II., twenty-four hours at 37° C.	Strongly acid	Strongly acid	Strongly acid	Strongly acid	Strongly acid	Strongly acid
Gelatine plates	Colonies small and growth slow	Colonies small and growth slow	Colonies small and growth slow	Colonies small and growth slow	Colonies small and growth slow	Colonies small and growth slow
Motility (twenty-four hours in bro h)	Highly motile	Highly motile	Highly motile	Highly motile	Highly motile	Highly motile
Stained by Gram's method	No	No	No	No	No	No

B. typhosus. All the cultures, however, were found to present the chief typical reactions of B. coli; cultures 62 and 66 only varied from the type by failing to produce indol. Culture No. 70, however, which did not react at all with anti-typhoid serum, showed considerable variations from the type; it was highly motile, did not produce acid in litmus-whey, and failed to give the characteristic reactions of B. coli in Proskauer and Capaldi's media.

Forty-five of the other cultures shown in Table A were also examined as to the production of indol in peptone solution, acid in litmus-whey, souring of milk and gas formation in sugar media. Twelve of the cultures showed characteristic colonies and gave gas in glucose media, but failed to produce indol, or change milk, and the acid formed in litmus-whey was comparatively small, requiring only from 8 to 16 per cent. of $\frac{N}{10}$ alkali to neutralise it. As regards the three tests usually considered typical of B. coli, viz., the production of indol, the formation of gas in sugar media, and the souring of milk, 64 per cent. of the colonies examined gave all three reactions, 4·5 per cent. failed to produce indol, 4·5 per cent. also failed to sour milk, and 27 per cent. gave only one reaction, the production of gas in sugar media. The results obtained by the study of the eighty varieties of B. coli derived from typhoid stools were then compared with those obtained by an investigation of seventy cultures of B. coli obtained from the stools of healthy men. The same procedure was followed as before. As regards agglutination with anti-typhoid serum not one of the cultures from healthy men was agglutinated by the serum diluted 1–500; a complete reaction was only obtained twice with the serum diluted 1–100, and a marked reaction ten times with a dilution of 1–200. As regards cultural characteristics 48 per cent. gave the three typical coli reactions, 52 per cent. gave only two reactions, usually the souring of milk was absent. The acid produced in litmus-whey required from 27 to 47 per cent. of $\frac{N}{10}$ alkali to neutralise it. There were no constant cultural characteristics by which the cultures of B. coli which reacted to anti-typhoid serum could be distinguished from those which were completely uninfluenced by the serum.

Conclusions arrived at from this research may be summarised as follows:

(1) As regards the appearances of the cultures on the various media: the varieties of B. coli in typhoid stools cannot be distinguished by cultural characteristics from the varieties of B. coli found in healthy stools.

(2) As regards reaction to anti-typhoid serum: the varieties of B. coli isolated from typhoid dejecta show much greater sensibility to agglutination than the varieties of B. coli found in normal stools. Consequently if cultures of B. coli isolated from suspected water supplies are found to come within the typhoid range of agglutination, there appear to be fair grounds for assuming that the water supply in question has been polluted with typhoid dejecta.

Having ascertained that the B. coli present in the intestines of patients suffering from enteric fever may acquire an increased sensibility to the agglutinins existing in anti-typhoid serum, it appeared desirable to test experimentally the possibility of producing the same sensibility *in vitro*. With this object in view a pure culture of the colon bacillus, isolated from a typhoid stool, was grown in Berne anti-typhoid serum for six months at blood temperature. It was then planted out on agar, and the resulting growth tested as to its sensibility to the agglutinins contained in Berne serum. No change in the sensibility was observed; the bacillus reacted to the serum in exactly the same manner as before the experiment was commenced. The specific serum having failed to influence the B. coli, an experiment was then devised by which the culture was kept under the influence of the toxins produced by the typhoid bacillus. A sterile Berkefeld candle, placed in a glass mantle, was connected by means of a short piece of india-rubber tubing, previously fitted with a pinch-cock, to a sterile glass tube passing through an india-rubber bung fitted into a Kitasato flask. The arm of the flask and the mouth of the glass mantle were plugged with sterile wool. Sterile broth was then introduced into the mantle and the flask, the former being inoculated with a culture of B. typhosus and the latter with a culture of B. coli, which, when isolated from a typhoid stool, showed no sensibility to agglutination. By releasing the

pinch-cock every third day, about 10 c.c. of the toxins, excreted by the typhoid bacillus, were allowed to filter through into the Kitasato flask. The amount of fluid filtered from the mantle was replaced by sterile broth. The procedure was continued for two months until the flask was filled nearly to the level of the arm. A loopful was then withdrawn and planted out on agar. The growth which resulted was tested for agglutination with Berne anti-typhoid serum, and sub-cultured in various media. No change was observed in its sensibility to agglutination or in its cultural reactions. The flask containing the mixture of colon bacilli and typhoid toxins was kept at a temperature of 25° C. for a further two months. A loopful was then withdrawn as before, and the growth tested with Berne serum. No increased sensibility to agglutination was noticed.

It is impossible to imitate by experiment the complex condition of things which exists in the human body suffering from a typhoid infection, so it is not surprising that the agglutinins and toxins *in vitro* failed to influence the sensibility of the colon bacillus to agglutination.

CHAPTER XIV.

QUALITATIVE ANALYSIS—*continued.*

Spirillum Choleræ Asiaticæ.

This micro-organism was first isolated by Koch, in 1883, from the stools of cases of cholera which he investigated in Egypt. Later, more extended researches were made in India, and Koch came to the conclusion that the association of this microbe with Asiatic cholera was constant.

On cultivation the following appearances are seen on the various media:

Gelatine Plates.—In twenty-four to forty-eight hours the colonies appear as minute white points, which under a low power show an irregular or furrowed margin. Later, liquefaction of the gelatine is produced, and the colonies sink into small cups formed by the liquefaction; the plate then shows small sharply-marked rings around the colonies. After about seventy-two hours, owing to the increasing liquefaction, the colonies appear as granular masses, with torn or broken up margins, lying in the bottom of circular cups of liquefied gelatine. The granular masses gradually became fainter, and the whole surface of the plate is completely liquefied.

Agar Plates.—Under a low power the surface colonies appear as circular discs of brownish-yellow colour, and are more transparent than the colonies of most other organisms.

Gelatine-stab.—During the first twenty-four hours the growth is hardly perceptible, but in forty-eight hours slight liquefaction with excavation of the gelatine is seen at the surface of the stab, and below this a white growth appears along the line of inoculation. About the fourth day a bubble-shaped depression is seen at the surface, and below this a funnel of liquefaction, covered at the surface with a white growth.

Agar-streak.—A greyish-white growth, which is not characteristic.

Broth.—A diffuse growth takes place and a thin pellicle forms on the surface.

Milk.—The microbe grows well at 37° C., and produces coagulation of the casein accompanied by an acid reaction of the medium.

Potato.—It does not grow on slightly acid potato at 22° C.; but at 37° C. it produces either a yellowish-brown or a non-glistening white growth, which is hardly perceptible except when the potato is held up to the light in a particular position. On potato which has been rendered alkaline by soaking in a 2 per cent. solution of sodium carbonate it grows both at 22° C. and at 37° C., producing a yellowish-brown growth.

Peptone (Witte's) and Salt Solution.—After twelve to twenty-four hours incubation at 37° C., both indol and a nitrite are formed, so that on the addition of a few drops of pure sulphuric acid a red colour is produced. This is usually known as the "cholera-red reaction."

Microscopical Characters.—The cholera spirillum is a small curved organism resembling a comma; usually only one curve is seen, but sometimes two spirilla are attached together and an S-shape is produced. It is actively motile and possesses a single flagellum placed at one end. It does not form spores, and involution forms are common, especially when the organism has been kept in water; it may then appear short and thick and resemble a coccus. It is not stained by Gram's method. The optimum temperature for its growth is between 35° and 38° C.; it grows, but more slowly, at a temperature as low as 17° C., but under 16° C. no growth is visible. It is strongly ærobic.

Reaction with Anti-cholera Serum.—Pfeiffer suggested the following test as a means of diagnosis: The blood serum of an animal, which has been immunised to the highest degree possible by several months treatment with cholera vibrios, is diluted 1–100 with ordinary nutrient bouillon. A loopful (2 mgm.) of a twenty hours agar culture of the vibrio to be tested is then added to 1 c.c. of the mixed serum and bouillon, and the mixture injected into the peritoneal cavity of a guinea-pig weighing 200 grammes; every five minutes up to the end of twenty minutes, when the experiment is ended, a portion of the

peritoneal contents is withdrawn with a glass capillary tube and examined in hanging-drop and "cover-glass" preparation. If the vibrios injected have been true cholera spirilla, they will become swollen up into round motionless masses which eventually become broken down into granular masses and disappear. If at the end of twenty minutes the peritoneal contents show unaltered and motile vibrios, they cannot be true cholera spirilla, but belong to some other species. If, however, the vibrios are dead but otherwise unchanged, two conditions of things are possible, viz., either the bacilli are true cholera vibrios which have been acted on by the specific bactericidal substances, or they are saprophytic vibrios which, without any question of a specific action, have been acted on by the bactericidal substances present in the normal guinea-pig. In order to diagnose between these two possibilities, one loopful of the suspected culture must be added to 1 c.c. of bouillon containing 0·01 c.c. of normal serum, and the mixture then injected into the peritoneal cavity of a guinea-pig. If the vibrios are found alive and motile at the end of the time when, in the cholera serum experiment, they were completely dissolved, then it is probable that they belong to the cholera bacilli species. Unfortunately it is possible that vibrios which appear to be killed by normal serum may still be true cholera spirilla, for it has been shown that Koch's vibrios which are enfeebled and have lost their virulence, may be destroyed by normal peritoneal fluid. If, therefore, the suspected vibrios disappear in the control experiment, Pfeiffer recommends that the suspected culture be used to immunise a guinea-pig. The serum from this animal must then be tested as to its bactericidal action on true cholera vibrios. Dömtz, acting on Pfeiffer's suggestion, showed that an old culture of cholera from Calcutta produced a serum which had a specific bactericidal effect on cholera vibrios.

The agglutination test (or Durham-Gruber reaction) may be performed in the same manner as described under B. typhosus. The serum is generally used in dilutions varying from 1–10 to 1–120. This test is more suitable for general hygienic work than Pfeiffer's reaction. It requires to be performed with care, and, as in the case of the B. typhosus, the dilution of the serum employed is a matter of great importance. Durham prepared

cholera serum with different races of cholera vibrios which had been tested as to their virulence by peritoneal injection into guinea-pigs. He found that a serum prepared from the vibrio with the greatest virulence reacted powerfully with all the races, but a serum prepared from the vibrio with least virulence had a very slight action on the most virulent vibrios. For purposes of diagnosis it is therefore important to work with a sufficiently potent sample of cholera serum. Durham also found that vibrios allied to the cholera vibrio reacted with cholera serum. The Vibrio Ivanoff reacted to the different varieties of cholera serum exactly like a cholera vibrio of medium virulence. Also, the influence of the various kinds of cholera serum upon the race of Vibrio Berolinensis agreed with the results obtained upon the different races of cholera vibrios themselves. The Vibrio Sanarelli also reacted markedly with potent cholera serum. Sera prepared by other vibrios, such as the Massowah, Danubicus, and Elwers, had a slight action on cholera vibrios, but weak dilutions of the serum had very little effect. On the other hand, cholera serum had absolutely no effect on the Vibrio Finkler-Prior. With regard to the relation of the agglutination test to Pfeiffer's reaction, Durham concludes that whatever "specific," or preferably "specialised," action a serum has upon a given vibrio, such action has already taken place before the injection is made. The reaction of Pfeiffer's is to be regarded as consisting of two parts: the one being a weakening of the microbes before injection by direct action of the active serum; the other being the normal bactericidal influence of the guinea-pig's peritoneal fluid upon these weakened microbes.

It is thus evident that the serum reactions cannot be considered absolutely diagnostic. Positive reactions, however, especially when obtained with a highly dilute serum, are undoubtedly very strong evidence in favour of the microbe being the true cholera spirillum.

The cultural reactions are also open to serious criticism. The appearance of the colonies in gelatine-plate cultures has been considered highly characteristic of the cholera spirillum, and special stress has been laid upon the irregular margin of the young colonies, the margin of the young colonies of most foreign vibrios being quite regular and forming an unbroken circle. Metchnikoff has, however, isolated vibrios

from cheese which form young colonies in gelatine with an irregular margin, resembling in every way those of the true cholera microbe. He regards this characteristic as of no importance as a means of diagnosis. The rate of liquefaction of the gelatine, once considered an important sign, has been abandoned since Koch himself described a case of cholera in which the vibrios liquefied the gelatine so slightly that the colonies appeared in the form of shields. Also, according to Metchnikoff, true cholera spirilla are met with, which liquefy gelatine more rapidly than the typical forms and produce funnels filled with masses of uniformly turbid culture. The form of the spirilla is also very variable; side by side with thick curved forms others are found which are thin and hardly curved at all. Friedrich described a Shanghai variety which closely resembled a true bacillus, and a Malta variety which appeared to be a cocco-bacillus. Koch, now, attaches only a secondary diagnostic value to these characteristics, and insists above all on the indol reaction and virulence for animals. With regard to the cholera red reaction, he states that none of the spirilla, which can possibly be confounded with the cholera vibrios, produce indol and nitrous acid at the same time in their cultures. Consequently he attaches great value to this test, but points out that the test must be performed according to certain well-defined rules. Above all, a proper variety of peptone must be employed, as every peptone does not give the reaction. Bleisch has shown that the peptones which do not give a proper reaction contain either too much or too little nitrates. The peptone to be employed must first be tested with a known cholera vibrio to see if it gives the characteristic reaction; if the peptone contains too little or no nitrates, these may be added in proper amount. Bleisch recommends the following solution: Witte's peptone 2·00 gramme, pure sodium chloride 0·5 gramme, distilled water 100 c.c., pure potassium nitrate solution (0·08 per cent.) 30 to 50 drops. He also insists on the necessity for using pure sulphuric acid, quite free from nitrous acid. Broth, even though it contains peptone, does not give the reaction so well as peptone and salt solution.

With regard to the virulence test, Koch recommends that one

loopful (1·5 mgm.) of a young agar culture be added to 1 c.c. of broth, and the mixture then injected into the peritoneal cavity of a guinea-pig, weighing 300–350 grammes. The weight of the animal must always be taken into consideration; if a guinea-pig of greater weight is employed for the experiment a larger dose of the agar culture must be given. One or two hours after the injection the animal is languid, loses its appetite, and there is a rapid fall of the body temperature, sometimes preceded by pyrexia, and death ensues from collapse. Koch states that none of the spirilla which can possibly come into question during the investigation of cholera, when employed in the above dose, give rise to the same symptoms as the true cholera spirilla.

According to Metchnikoff both the indol reaction and the virulence test are open to question. Sanarelli and Kohlbrugge have both isolated from water supplies vibrios which give the cholera-red reaction; indeed Kohlbrugge's investigations appeared to show that temperature and season of the year had an influence on this reaction.

It is thus evident that the diagnosis of the cholera spirillum, ordinarily fairly easy when the microbe is isolated from a case of cholera, may be exceedingly difficult, owing to the variability of its reactions, when it has gained access to water. Klein has shown how modifications of the cholera vibrio can be artificially induced. He inoculated non-sterile sea-water with an agar culture of a St. Petersburg cholera vibrio, and then isolated it again eleven days later. The vibrio was found to have undergone the following modifications:

(*a*) It had lost altogether its power of growing at 37° C. either on agar or in peptone.

(*b*) It grew at 20° C. in peptone and salt solution, but when tested with pure sulphuric acid, after incubation for four or more days, it gave no trace of the cholera-red reaction.

(*c*) In stained specimens it appeared thicker than the St. Petersburg vibrio.

(*d*) When injected in considerable doses into the peritoneal cavity of a guinea-pig it produced no diseased condition; the animal remained well.

(*e*) When tested with cholera serum, which quickly agglutinated the original St. Petersburg vibrio, it gave a negative

result. The vibrio was then transmitted from culture to culture for more than a year, but it retained unimpaired the cultural characteristics just given. It failed to grow at 37° C., gave no cholera-red reaction, and did not react with cholera serum *in vitro*. These results are directly opposed to the statement of Pfeiffer that a vibrio which does not react to cholera serum is not a descendant of a true cholera vibrio.

The Diagnosis of the Cholera Vibrio from Allied Forms.

Many spirilla have been isolated from various sources which in some of their cultural reactions closely resemble the true cholera spirillum. The most important of these are the following:

Spirillum of Finkler-Prior.

This micro-organism was isolated at Bonn by Finkler and Prior during an epidemic of cholera nostras. Koch asserted that these observers had only found the microbe on one occasion; but, in a second paper on the same subject, Finkler and Prior stated that they had isolated the vibrio from other cases of cholera nostras in perfectly pure culture. The cultural characteristics of the vibrio are as follows:

Gelatine Plates.—During the first stages of growth the colonies are larger than those of the cholera spirillum, and show a smooth circular margin free from crenations. In twenty-four hours the gelatine commences to liquefy, and in forty-eight hours the liquefaction is very marked, the colony which is now ragged and torn lying in a circle of liquefaction. The double contour effect seen in the case of the cholera colonies is little marked, as a large clear area results from the rapid liquefaction of the gelatine.

Gelatine-stab.—In twenty-four hours there is distinct growth along the line of inoculation, and at the upper part liquefaction is commencing with the formation of an air bubble. In forty-eight hours liquefaction has advanced considerably and produced a funnel-shaped excavation with a deposit of bacteria at the apex of the funnel.

Agar-slope.—The growth forms a moist, greyish-white layer, indistinguishable from that of the cholera vibrio.

Potato.—There is a growth at ordinary temperature con-

sisting of a slimy, greyish-yellow layer, which rapidly spreads over the potato.

Peptone and Salt Solution.—As a rule, no indol reaction can be obtained on the addition of sulphuric acid, as, though it produces indol, nitrites are not manufactured in sufficient quantity to give the cholera-red reaction. In some cases more nitrites are produced, and the reaction is then developed slowly with a faint reddish tint.

Microscopical Appearances.—The organism is a comma-shaped bacillus, slightly thicker in the middle than at the ends; it is larger than Koch's organism, highly motile, and possesses a single flagellum.

When injected into the peritoneum of a guinea-pig it causes peritonitis; and in man, after neutralisation of the acid in the stomach, it causes intestinal disturbance (Metchnikoff).

The vibrio of Finkler-Prior is easily distinguished from the typical cholera spirillum by its form, rapid liquefaction of gelatine, and failure to give the cholera-red reaction.

Vibrio Metchnikovi.

This microbe was discovered by Gamaleïa at Odessa, where it produced epidemic disease amongst fowls. It is of great interest owing to its very close resemblance to Koch's spirillum. An organism closely resembling the vibrio Metchnikovi was isolated by Pfuhl from water.

Gelatine Plates.—The young colonies may have a perfectly smooth outline, though sometimes the margin is distinctly crenated like that of the cholera colonies. The colonies generally grow more quickly, and liquefy more rapidly, than those of Koch's vibrio; in forty-eight hours the margin is often very irregular and torn, and resembles Finkler-Prior's colonies.

Gelatine-stab.—The appearances closely resemble those produced by Koch's vibrio; liquefaction, however, occurs more rapidly; the rate seems to be intermediate between Finkler-Prior's and Koch's organisms.

Agar-slope.—A greyish-white, moist growth.

Potato.—A raised, dirty-brownish growth.

Broth.—Diffuse growth in twenty-four hours; a surface pellicle is eventually formed.

Peptone and Salt Solution.—Indol and nitrites are produced, so that a red colour is obtained on adding a few drops of pure sulphuric acid.

Milk.—Becomes acid, and the casein is coagulated about the eighth day.

Microscopical Appearances.—As a rule, it appears nearly identical with Koch's spirillum; sometimes, however, larger and thicker forms are seen. It is very motile, and has one flagellum.

Pigeons are readily killed by sub-cutaneous injection of very small quantities of this organism; inoculation of the same quantity of Koch's organism produces practically no effect. This test at once enables a diagnosis to be made between the two organisms.

Spirillum of Deneke.

This spirillum was isolated by Deneke from old cheese; it is also known as the Spirillum tyrogenum. In morphology it is closely allied to Koch's spirillum.

Gelatine Plates.—The colonies, to the naked eye, have a yellowish colour, and they liquefy gelatine more rapidly than Koch's microbe. Under a low power the margins of the young colonies are seen to be smooth and regular. Later, there is often a very close resemblance to the colonies of the cholera spirillum; but, as a rule, the yellow colour and the more marked liquefaction enable a diagnosis to be made.

Gelatine-stab.—Closely resembles the appearances produced by Koch's vibrio. Liquefaction however is more rapid.

Potato.—It produces a thin yellowish layer.

Peptone and Salt.—It produces indol, but only traces of nitrites, so that the "cholera-red" reaction is produced very feebly and irregularly on the addition of pure sulphuric acid.

Vibrio Aquatilis.

This vibrio was first isolated by Günther from the water of the river Spree at Stralaw. It was also found by Kiessling in the washings of the sand used in the water-works of Altona.

Gelatine Plates.—The colonies at first are circular, with a perfectly smooth rim. They are brown in colour, and have granular contents. Liquefaction takes place very slowly, and the colony eventually sinks in a basin of liquefied gelatine.

Gelatine-stab.—There is growth on the surface, and liquefaction slowly takes place, forming a basin-shaped depression. There is very little growth along the stab.

Agar.—Forms a greyish-white growth, not characteristic.

Broth.—It is said not to grow in broth at 37° C.; and at 21° to 22° C. the growth is extremely slow.

Peptone and Salt Solution.—The cholera-red reaction is not obtained on adding a few drops of pure sulphuric acid.

Potato.—No growth takes place at 22° C. or at 37° C.

Microscopical Appearances.—It is a highly motile vibrio exactly resembling the cholera spirillum. It possesses one flagellum.

Vibrio Berolinensis.

This microbe was isolated by Neisser and Günther from Berlin pipe-water. The specimen of water had been designedly infected with true cholera bacilli some time before, so there was a possibility that the Vibrio berolinensis was a variety of the cholera spirillum, modified by existence in water.

Gelatine Plates.—It grows extremely slowly, and in twenty-four hours the colonies are only visible with the aid of the microscope, and are round and finely granular. Even in forty-eight hours the colonies are not visible to the naked eye.

Gelatine-stab.—Growth extremely slow, resembles that of the cholera vibrio.

Agar.—White growth, not characteristic.

Potato.—Growth like the cholera vibrio.

Milk.—Unchanged.

Broth.—Diffuse growth.

Peptone and Salt Solution.—Cholera-red reaction obtained.

Microscopical Characters.—A motile vibrio about the same size as Koch's spirillum; it posseses one flagellum. It is decolorised by Gram's method, and is pathogenic to guinea-pigs. It does not give Pfeiffer's reaction.

Vibrio Massowah.

This vibrio was isolated from the stools of a sick person during a small epidemic of cholera. It was at first accepted as a true cholera spirillum, but later researches showed that there were differences between the two organisms. The Vibrio

Massowah possessed four flagella, liquefied gelatine very slowly, and its colonies were quite round, sharply outlined, and had a yellowish colour. It gave the nitroso-indol reaction after twenty-four hours. Its pathogenic action was very like that of the Vibrio Metchnikovi.

Vibrio Gindha.

This vibrio was isolated by Pasquale from the water of a well in Gindha. There had been a small epidemic of cholera in the neighbourhood of the well some months before Pasquale's investigations took place. In cultural reactions the vibrio closely resembled Koch's vibrio, but it failed to give the nitroso-indol reaction after twenty-four hours incubation.

Vibrio Danubicus.

This vibrio was isolated by Heider from canal water. In many respects it resembles Koch's vibrio. But in the appearance of the colonies, its pathogenic action, and failure to give Pfeiffer's reaction, it differs from the true cholera microbe.

Vibrio Ivanoff.

This microbe was discovered by Ivanoff in the stools of a typhoid patient. Pfeiffer believes that the vibrio was the result of an accidental contamination, as Ivanoff at the time was working on the action of disinfectants on stools containing pathogenic organisms. The Vibrio Ivanoff gave Pfeiffer's reaction, and corresponded in every way with Koch's organism, excepting that it showed a tendency to grow out into fine, long, slightly curved, comma forms.

Vibrio Phosphorescens.

Dunbar and Rumpel found phosphorescent vibrios frequently in water from the Elbe, Spree, and Rhine. The same vibrios, according to Rumpel, were also found in the filtered pipe-water of Hamburg. These spirilla are very closely allied to the true cholera spirilla, both morphologically and culturally, and also in pathogenic action. Kutscher observed that the vibrios isolated by Dunbar produced in the dark a bluish-green light, which was seen on fresh agar and gelatine cultures, and also in

the peritoneal cavity of guinea-pigs which had been infected intra-peritoneally by the microbes. The phosphorescence is not an absolutely constant phenomenon; it may disappear when the microbes are cultivated for a long time on nutritive media. Dunbar found that the phosphorescent vibrios failed to give Pfeiffer's reaction. He tested a large number of these organisms and found that a pathogenic action was exerted both on the control guinea-pigs and on the guinea-pigs which had received the vibrios plus cholera serum; the vibrios when withdrawn from the peritoneal cavity were found to be still highly motile. Rumpel's statement that the true cholera vibrio can be changed into the phosphorescent variety requires confirmation.

The characteristics of Dunbar's vibrios will be seen from the following short description of some of these organisms:

Vibrio No. 17a was a rather plump, curved bacillus, with rounded ends. It was highly motile and stained unevenly. On agar there was a greyish-white, moist layer. In peptone-water at 37° C. a marked growth appeared after fourteen hours; a thin membrane formed on the surface, which later became very thick and villous; a typical "cholera-red" reaction was obtained. In gelatine-stab after four days there appeared a clear air bubble, below which the gelatine was liquefied in a capillary form along the stab. In gelatine plates there was no liquefaction apparent to the naked eye, the colonies appeared as white points. Under a low power a thin, colourless, finely granular surface growth was seen; the deep colonies were also colourless and finely granular, and rather sharply outlined. Phosphorescence was always observed.

Vibrio No. 29 in appearance exactly resembled No. 17a. On agar a greyish-white slimy growth was seen. In peptone-water there was a marked growth, with formation of a thick pellicle on the surface; a typical "cholera-red" reaction was obtained. In gelatine-stab after six days the gelatine was completely liquefied to the depth of 1 c.m.; below this the gelatine was liquefied in a capillary form. The appearance of the colonies in gelatine plates exactly resembled No. 17a. Phosphorescence was always seen.

Vibrio No. 30 was plump, highly motile, and possessed a long flagellum at one end. On agar there was a greyish slimy

growth. In peptone and gelatine-stab the appearances were the same as No. 29. The colonies were also very similar to those of 17A ; those in the depth, however, had an irregular margin and were coarsely granular.

Vibrio No. 34 was rather larger than the above, and possessed one long flagellum. On agar, in gelatine-stab and peptone, the growths were the same as No. 29. In gelatine plates small dry hollows appeared, containing at the bottom dry white colonies, which, under a low power, exactly resembled No. 17A.

Vibrio No. 45 was rather small, curved, with rounded ends and highly motile. On agar, in peptone, and in gelatine the growths resembled No. 29. In gelatine plates dry hollows which contained thin grey colonies were seen. The colonies in the depth were sharply outlined greenish-yellow, and finely granular.

Vibrio No. 51 closely resembled No. 45 both in form and in the appearance of the colonies in gelatine plates. In peptone and gelatine-stab the growths were the same as 17A.

Bacillus Choleroides *a*

This vibrio was isolated by Bujwid from river water. A little peptone was added to some of the water placed in a flask ; after incubation for three days a loopful from the surface plated out in gelatine showed, after three to six days at room-temperature (10°–12° R.), very suspicious colonies resembling the cholera spirillum. At a higher temperature, however, the colonies grew out in a more superficial manner, and were broader and did not sink so much into the gelatine. A smell of methyl-merkaptan was observed. Under a low power the colonies had a sharp, regular outline, and a smooth or very finely granular appearance. In gelatine-stab it grew chiefly on the surface, and liquefied only the upper layers. At a temperature of 10°–12° R. the gelatine was more slowly liquefied, and the air-bubble, so characteristic of the cholera vibrio, was produced. In the depth of the stab there was very little growth. On agar-slope there was a marked growth, with a smell resembling methyl-merkaptan. Broth showed very little growth, and no surface pellicle was formed. There was no indol reaction. Microscopically the vibrio was not so motile as the cholera vibrio, and longer and shorter forms were seen.

B. Choleroides β.

This organism was isolated by Orlowski, Bujwid's assistant, from a well, in the neighbourhood of which there had been several cases of cholera. It resembled the cholera vibrio more closely than the above, but grew more anærobically and produced a more marked funnel-shaped liquefaction.

Vibrios Isolated from Water by Sanarelli.

Sanarelli isolated thirty-two vibrios from water. Eight were found in the Seine below Paris; of these one gave a very marked nitroso-indol reaction, and its biological characters were as follows:

Morphology.—A motile, thin, curved vibrio, often presenting a spiral form.

Culture on Gelatine.—Forms the characteristic bubble of air after twenty-four hours, and liquefies in a typical funnel form.

Culture in Solution of Peptone-gelatine.—Rapid development; a pellicle forms at the surface in twenty-four hours.

Culture on Agar.—Marked development at 37° C. on agar made with and without broth.

Culture in Broth.—Rapid growth, with formation of a superficial pellicle in twenty-four hours.

Culture on Potato.—Brownish circumscribed growth.

Nitroso-indol Reaction.—Very marked in twenty-four hours.

Indol Reaction.—Very marked in twenty-four hours.

Nine other vibrios were isolated from the upper part of the Seine where it flows through Paris. None of these gave a marked cholera-red reaction; the colour was either absent or just perceptible after eight days incubation.

In the effluent from the sewage of Paris after irrigation, vibrios were also found which gave a marked nitroso-indol reaction. The biological characters of these organisms were as follows:

No. XXV.—Gennevilliers.

Morphology.—A motile vibrio, but longer and thicker than the true cholera microbe.

Culture on Gelatine.—Development along the line of inoculation, with funnel-shaped liquefaction at the surface.

Culture in Solution of Peptone-gelatine.—Diffuse growth, with a pellicle at the surface.

Culture on Agar.—Good growth on the agar made without broth; no growth on the agar containing broth.

Culture in Broth.—Very feeble after twenty-four hours; later very abundant with a surface pellicle.

Culture on Potato.—Insignificant.

Nitroso-indol Reaction.—Negative after twenty-four hours; very marked after eight days.

No. XXVI.—Gennevilliers.

Morphology.—Very thin motile vibrio.

Culture on Gelatine.—Development along the line of inoculation, with a small cup-shaped liquefaction at the surface.

Culture in a Solution of Gelatine-peptone.—Abundant growth with formation of a pellicle.

Culture on Agar.—Abundant growth on agar free from broth; none on the agar made with broth.

Culture in Broth.—Very small after twenty-four hours; later, more abundant, with formation of a surface pellicle

Culture on Potato.—Insignificant.

Nitroso-indol Reaction.—Negative after twenty-four hours; very marked after eight days.

No. XXVII.—Gennevilliers.

Morphology.—A motile vibrio, less curved, longer, and a little thicker than the above.

Culture on Gelatine.—Abundant growth, with liquefaction along the line of inoculation and characteristic bubble of air; after four days typical liquefaction in the form of a funnel.

Culture on Agar.—Very rapid on both forms of agar.

Culture in Broth.—Abundant, with a superficial pellicle after twenty-four hours.

Culture on Potato.—A light grey layer.

Nitroso-indol Reaction.—Slight after twenty-four hours; very marked with a scarlet colour after eight days.

In the water of the Seine at Versailles another vibrio was found closely resembling No. XXVII. It, however, gave a very marked nitroso-indol reaction after twenty-four hours incubation.

The remaining cultures out of the total thirty-two isolated showed extreme variability. Side by side with vibrios which liquefied gelatine in a manner absolutely characteristic of the true cholera spirillum, others were found which liquefied gelatine very slightly, or not at all, and which multiplied with great difficulty. Many also refused to grow on ordinary gelatine, and also in broth, except at the room temperature. It could not be said that the failure to develop at 37° C. was directly related to a condition of degeneration, for many vibrios which failed to liquefy gelatine grew at 37°, and *vice versa;* many which liquefied gelatine failed to grow at 37° C. The cultures on potato failed to be of use as a means of diagnosis. With regard to the nitroso-indol reaction, all the vibrios isolated from water were able to produce indol, but very few of them at the same time were able to reduce nitrates to nitrites. As regards pathogenicity, only four out of the thirty-two microbes were found to be pathogenic when injected intra-peritoneally into guinea-pigs; but many of them, though they did not cause immediate lethal effects, produced a condition of gradual wasting from which the animals eventually died. It was also found that if 1 c.c. of a sterilised culture of B. coli was injected into the peritoneum of a guinea-pig, and a small quantity of these vibrios at the same time into the pleura of the animal, the vibrios multiplied and caused the death of the animal. Sanarelli then endeavoured to find out whether there was any reciprocal reaction between animals vaccinated with the four pathogenic water vibrios and two other vibrios derived from undoubted cases of cholera. Guinea-pigs were vaccinated with cultures grown for eight to ten days in peptone-gelatine and then sterilised at 120°. A successful vaccination was proved by the animal resisting a fatal dose of the active culture. The results of the experiments are shown in the following table. The sign + indicates that the guinea-pig, vaccinated against a given variety, survived after inoculation with a different variety; the sign − indicates the death of the vaccinated animal. Sanarelli does not state in his paper whether the cultures used for vaccination were sterilised at 120° C. or 120° F. If the former temperature was used, the cultures would not be likely to have much protective influence.

Inoculation.	Guinea-pigs vaccinated against vibrios coming from					
Guinea-pigs vaccinated with	Courbevoie (cholera case).	Angers (cholera case).	Point-du-Jour (water).	Saint-Cloud (water).	Gennevilliers (water).	Versailles (water).
Courbevoie	...	—	+	+	—	+
Angers	—	...	—	—	—	—
Point-du-Jour	+	—	...	+	+	+
Saint-Cloud	+	—	—	...	+	+
Gennevilliers	—	+	—	—	...	—
Versailles	+	+	+	+	—	...

This table shows that there is no constant vaccinal reciprocity between the vibrios derived from different sources. Even in the case of the true cholera vibrios the spirillum from Courbevoie did not confer immunity against the spirillum from Angers, and *vice versa.*

Sanarelli also made experiments to see the effect of placing the Vibrio Saint-Cloud under conditions closely resembling those found in nature. Two flasks were filled with water from the Seine at Clichy and then sterilised at 120° C. Some drops of a culture of the Vibrio Saint-Cloud were then added to each flask, one of which was kept at the room temperature (20°–24° C.) and the other placed in ice at 5°–10° C. After a month the vibrio was again isolated from the flasks, and it was found that the vibrio from the first flask showed a feeble cholera-red reaction, as compared with the original culture, and in the case of the vibrio from the second flask the cholera-red reaction was almost imperceptible. Both vibrios had also completely lost their virulence, intra-peritoneal injections into guinea-pigs of large doses of the microbes producing no effect. A specimen of the water at Saint Cloud, from which the Vibrio Saint-Cloud was isolated, was kept at the room temperature for three months. Vibrios were then isolated from the water, but the new specimens gave no indol reaction, liquefied gelatine much more slowly than before, and had no pathogenic action on animals.

Kohlbrugge quite recently investigated water from an arm of the Rhine at Utrecht (free from cholera for seven years), and found vibrios present during every month in the year; but whereas the vibrios isolated in the summer months gave a marked cholera-red colour, those isolated in the winter failed to

give any trace of this reaction. As a result of his comparisons of the water-vibrios with true cholera spirilla, he concluded that the two groups could not be distinguished by cultural reactions, and that a diagnosis of cholera could only be made when the bacteriological examination was confirmed by epidemiological facts and the appearance of disease.

Methods Proposed for the Isolation of the Cholera Spirillum from Water.

The simplest method is to convert a considerable bulk of the specimen of water into a 1 per cent. peptone and 0·5 per cent. salt solution. This is easily done by keeping a stock solution containing 10 per cent. peptone and 5 per cent. salt; 10 c.c. of the stock solution are added to 90 c.c. of the water placed in a sterile flask, which is then incubated at 37° C. After twelve to twenty-four hours a faint pellicle forms on the surface if cholera spirilla are present. Loopfuls must then be removed from the surface and plated out in gelatine and rubbed over the surface of agar solidified in Petri dishes. If pure cultures of spirilla are obtained the colonies must be fished and planted out on the following media:

(1) *Peptone and Salt Solution.*—After twenty-four hours incubation at 37° C. the tube will become turbid, and on the addition of pure sulphuric acid the cholera-red reaction will be obtained.

(2) *Gelatine Plates.*—The characteristic colonies with *irregular* margins will appear.

(3) *Gelatine-stab.*—The typical air-bubble liquefaction will be obtained.

(4) *Agar-slope.*—The growth which appears in twenty-four hours must be tested for agglutination and for Pfeiffer's reaction with anti-cholera serum.

A portion of the colony should be examined in a hanging-drop for the characteristic appearance and motility of Koch's vibrio. If a spirillum conforms to all the tests it may be classed with the true cholera spirilla.

Metchnikoff recommended the following method: A series of flasks are prepared, and into each the following solution is poured, *i.e.*, water 50 c.c., peptone 2 grammes, salt 2 grammes,

gelatine 4 grammes, and a solution of soda sufficient to give a slight alkalinity. The flasks are sterilised in the autoclave, and then 150 c.c. of the suspected water are added to each of them. The flasks and contents are incubated at 37° C.; if a film appears on the surface after eight to twelve hours, loopfuls are removed and treated as above described.

Sanarelli employed a similar method. He placed 200 c.c. of the water in a sterilised flask sufficiently large to allow a considerable surface of the water to be exposed to the air, and then added 8 c.c. of the following nutritive mixture:

Gelatine	20 grammes.
Dried peptone	10 „
Sodium chloride	10 „
Potassium nitrate	1 gramme.

This gelatine, prepared beforehand, is kept in sterilised tubes. By adding about 8 c.c. of the nutritive mixture the water is converted into a nutritive solution having the following composition:

Gelatine	2 grammes.
Peptone	1 gramme.
Sodium chloride	1 „
Potassium nitrate	0·10 „
Water	100 grammes.

In these flasks the vibrios develop so rapidly that after twelve hours incubation at 37° C. they appear at the surface in the form of a thin pellicle. In order to obtain pure cultures of the vibrios, Sanarelli either plated out loopfuls of the pellicle in gelatine, or made several passages through the gelatine and peptone water, re-inoculations being made every six hours. In this way absolutely pure cultures were obtained, the vibrios appearing before the water bacteria had time to develop.

The Vitality of the Cholera Spirillum in Water, &c.

A large number of experiments have been made to ascertain the vitality of the cholera spirillum in water. Kraus studied the behaviour of the spirillum in three specimens of water which had the following chemical composition:

	Total solids.	Chlorine.	Nitrates.	Nitrites.	Ammonia.	Oxygen absorbed.
			(All in parts per 100,000.)			
No. I. Pipe-supply .	27·6	0·41	0·24	0	0	0·036
No. II. Well-water .	59·6	2·67	6·96	0	0	0·304
No. III. Well-water .	56·0	2·56	0·26	0	trace	0·620

Koch's vibrio was introduced into these three waters, and the following results were obtained :

		Number of days after the water was inoculated.			
		1	2	4	8
Koch's vibrios in 1 c.c. of water.	No. I.	10,000	0	0	0
	No. II.	8,700	0	0	0
	No. III.	9,420	0	0	0
Water bacteria in 1 c.c. of water.	No. I.	30	400	70,000	1,400,000
	No. II.	80	900	85,000	innumerable
	No. III.	250	2,000	100,000	„

These results showed that Koch's vibrio could not be detected forty-eight hours after inoculation, although No. II. specimen contained large quantities of nitrates, and No. III. specimen contained a good deal of organic matter. In these experiments special methods were not used to isolate the vibrios, so the results must be accepted with considerable reservation.

Nicati and Rietsch found cholera vibrios in

Sterilised distilled water after	20 days.
Marseilles canal-water after	38 „
Sea-water after	64 „
Harbour-water	81 „
Bilge-water	32 „

De Giaxa found cholera vibrios in unsterilised sea-water after two to four days ; in sterilised sea-water the vibrios appeared to multiply, and he detected them after several weeks. Koch found vibrios alive after thirty days existence in well-water ; but in a canal-water at Berlin he only found them up to six or seven days; and when the water was mixed with fæces, the vibrios seemed to disappear after twenty-seven hours. Babes found that cholera vibrios lived for seven days in Berlin tap-water. Wolffhügel and Riedel stated that cholera spirilla did not multiply in sterilised water kept at 16° to 20° C. ; and in unsterilised water kept at the same temperature they found the vibrios disappeared in a few days. Hueppe, working with sterile distilled water containing common salt, found the spirilla

alive after twenty to sixty days; he considered that the presence of common salt exercised a favourable influence on the vitality of the spirilla. Hochstetter detected cholera spirilla in sterilised Berlin tap-water, kept at 30° C., after three hundred and ninety-two days; although in some cases the water was found not to be sterile, other organisms having gained access to it, owing to the length of time covered by the experiment. The temperature at which the vibrios are kept seems to influence the result. In some experiments the spirilla appeared to live longer at a temperature of 16° C. than at a temperature of 11° C., which corresponds to that usually found in well-waters. At Altona, however, Koch found that cholera vibrios rapidly disappeared from the water in a well, but in a litre of the same water, removed from the well and kept at 3° to 5° C., he detected the vibrios after eighteen days. Uffelmann, working with a very impure harbour-water from Rostock, found cholera vibrios at a room temperature only up to the third day. These experiments, which are in harmony with the outbreaks of cholera at Nietleben and Altona in winter, show that cholera spirilla live longer in cold than warm water; probably because they are not crowded out by the water organisms which cannot multiply at a low temperature. The observations at Nietleben also showed that cholera vibrios could exist in ice-cold water under snow and ice. Uffelmann allowed water containing cholera vibrios to freeze, and every day melted just as much of the ice as he required for his experiments. Although the temperature at night fell to –24·8° C., living cholera spirilla were isolated from the melted ice-water up to the fifth day. Trenkmann made some remarkable experiments on the influence of salts on the growth of cholera spirilla. Test tubes were carefully cleaned and filled with 10 c.c. of a well-water; then into each tube one, two, or three drops (25–27 drops = 1 gramme) of a 10 per cent. solution of sodium chloride, sodium nitrite, sodium nitrate, sodium carbonate, and di-sodium phosphate were added by means of a small pipette. The test-tubes were sterilised, and then each of them inoculated with one loopful of a twenty-four hours broth culture of the cholera spirillum. The inoculated glasses were kept at 21°–24° C., and after twenty-four hours a loopful was taken from each of them with

the same platinum needle and plated out in gelatine. The following results were obtained :

						Plates after twenty-four hours.	Plates after eight days.
(1)	10 c.c. sterile well-water					580	5
(2)	do.	+ 1 drop 10 per cent.		sodium chloride		6,120	12,480
(3)	do.	+ 2 drops	do.	do.		9,240	19,560
(4)	do.	+ 3 do.	do.	do.		15,000	10,440
(5)	do.	+ 1 drop	do.	sodium nitrite		1,740	10,920
(6)	do.	+ 2 drops	do.	do.		6,600	1,460
(7)	do.	+ 3 do.	do.	do.		17,160	2,260
(8)	do.	+ 1 drop	do.	sodium nitrate		8,040	4,040
(9)	do.	+ 2 drops	do.	do.		6,660	14,760
(10)	do.	+ 3 do.	do.	do.		20,940	16,080

A control plate, made immediately after the inoculation, showed 1440 colonies.

					Plate after twenty-four hours.
(1)	10 c.c. sterile well-water				520
(2)	do.	+ 1 drop 10 per cent.		di-sodium phosphate	3,360
(3)	do.	+ 2 drops	do.	do.	7,560
(4)	do.	+ 2 do.	do.	do.	6,540

					Plate after twenty-four hours.
(1)	10 c.c. sterile well-water				580
(2)	do.	+ 1 drop 10 per cent.		sodium carbonate	7,440
(3)	do.	+ 2 drops	do.	do.	28,680
(4)	do.	+ 3 do.	do.	do.	31,560
(5)	do.	+ 2 drops 10 per cent. sodium chloride, and 1 drop sodium carbonate			54,720

A control plate, made immediately after the inoculation, showed 1800 colonies. From these experiments Trenkmann concluded that in waters containing no sodium chloride or other sodium salts, the cholera bacilli diminished in numbers, whilst in others they multiplied almost in proportion to the salt added. The greatest multiplication was seen with a combination of sodium chloride and sodium carbonate. Trenkmann also found that potassium salts gave rise to a similar multiplication of the cholera spirilla.

					Plate after twenty-four hours.
(1)	10 c.c. sterile well-water				1,020
(2)	do.	+ 1 drop 10 per cent.		potassium chloride	4,200
(3)	do.	+ 2 drops	do.	do.	38,520
(4)	do.	+ 3 do.	do.	do.	43,320
(5)	do.	+ 1 drop	do.	potassium nitrate	9,000
(6)	do.	+ 2 drops	do.	do.	19,860
(7)	do.	+ 3 do.	do.	do.	22,740

The following experiments were then made with unsterilised water :

		Plate after twenty-four hours.	
		Cholera bacilli.	Water bacteria.
(1)	10 c.c. unsterilised well-water	216	19,300
(2)	do. + 1 drop 10 per cent. sodium sulphide	0	2,370
(3)	do. + 1 do. do. sodium chloride	54	30,000
(4)	do. + 2 drops do. do.	1,940	32,400
(5)	do. + 3 do. do. do.	10,000	32,400
(6)	do. + do. do. + 1 drop 10 per cent. sodium sulphide	5,500	3,240
(7)	do. + do. do. + 2 drops do.	17,100	540
(8)	do. + do. do. + 3 drops do.	28,100	2,160

The test tubes were kept in the incubator at 21°–24° C.

It therefore appears that :

(1) In unsterilised water the cholera bacilli quickly diminish, whilst the water bacteria quickly multiply.

(2) The addition of common salt at first has little or no action on the cholera vibrios, but, with an increasing amount of salt, the vibrios undergo considerable multiplication ; the water bacteria multiply rapidly on the addition of the smallest amount of salt.

(3) After the addition of sodium sulphide the vibrios rapidly disappear.

(4) After the addition of sodium sulphide and sodium chloride the cholera vibrios undergo rapid multiplication.

When sodium chloride and sodium carbonate were added to the unsterilised well-water, the water bacteria rapidly multiplied, but the cholera vibrios could not be isolated after the fourth day. When, however, sodium sulphide, in addition to sodium chloride and sodium carbonate, was added to the water, the cholera vibrios underwent considerable multiplication and could be isolated up to the seventh day. At a lower temperature of 12·5°–16° C. the addition of sodium chloride, di-sodium phosphate, and sodium sulphide enabled the spirilla to be isolated up to the ninth day. As a result of all his experiments, Trenkmann states that the addition of sodium chloride and sodium sulphide to a water causes a rapid disappearance of many species of water bacteria, and very often only a few species, sometimes only one, remain in conjunction with the cholera vibrios. He also points out that water fouled

with urine, fæces and refuse from manufacturing processes, may contain sodium chloride and sodium sulphide, and so present conditions favourable to the duration of life of cholera vibrios. Trenkmann's experiments certainly explain the prolonged vitality of the cholera vibrios in certain epidemics, such as that at Hamburg, where the water was distinctly saline owing to the high percentage of salt discharged from salt works into the Elbe.

Summarising the results of the various experiments which have been made on the vitality of cholera spirilla, Tiemann and Gärtner give the following conclusions: "Cholera spirilla behave very differently in water. Most commonly they disappear in a few days; at 0° C. they can exist for several days, at 10° C. for weeks, and at 20° C. for months." The rapid disappearance of cholera spirilla from an infected water cannot be safely assumed, as most of the earlier experiments which appeared to demonstrate this point were made without attempting to isolate the spirilla by the special methods now in use. Gruber, employing special methods, has shown that when cholera spirilla are mixed with putrefactive bacteria, although the latter may gain the ascendency for some time, yet the vitality of the former is not extinguished, for if the struggle between the two be sufficiently prolonged, the presence of the cholera bacilli may be again demonstrated by cultivation.

The vitality of cholera spirilla in water containing carbonic acid gas is an important practical question. Hochstetter has studied this subject with great care. In eight experiments with fifty-six flasks of soda-water, he found that the cholera spirilla could be isolated in a living state after three hours exposure to the influence of carbonic acid gas, but after twenty-four hours not a single spirillum could be detected. The rapid disappearance of the spirilla was found to be due to the carbonic acid gas and not to the chemical constituents of the water. In soda-water which had been heated so as to drive off the carbonic acid gas cholera spirilla were found alive up to the eighteenth day. A pressure of two atmospheres appeared to have little influence in destroying cholera spirilla; carbonic acid gas simply passed through the water killed the spirilla in the shortest time.

The vitality of the cholera bacillus in crude sewage has quite

recently been investigated by Houston. Ten c.c. of Crossness sewage were poured into a sterile test tube, and then inoculated with a loopful of a recent agar culture of the cholera vibrio. The tube was kept in the dark at the room temperature, and from time to time a loopful of the contents was transferred to a peptone and salt solution tube. The peptone tube was incubated at 37° C., and in less than twenty-four hours a loopful was taken from the surface and a cover-glass preparation made. The results obtained were as follows: Immediately after the inoculation the cholera vibrios were present in great abundance, and almost pure cultures were obtained. On the following day the vibrios were demonstrated with difficulty. On the third and fourth days, however, the vibrios were present in abundance. From this date up to the eleventh day the vibrios were demonstrated with difficulty. On the sixteenth day a few typical vibrios were found, but on the twenty-fifth day many vibrios were isolated which were longer and thicker than the normal microbes. On the forty-second day the vibrios completely disappeared.

The experiment was repeated, but now a whole agar-slope of the spirillum, instead of one loopful, was added to the sewage. Again, the same difficulty was experienced in isolating the vibrios the day after the inoculation. On the eighth day they were found without difficulty; but after the fourteenth day the result was negative. From these experiments it appears that cholera spirilla may lose their vitality in less than a fortnight, or remain viable for nearly four weeks, in crude sewage.

The Relation of Cholera Vibrios to other Pathogenic and Non-pathogenic Micro-organisms.

Many experiments have been made on this important subject. Garré grew B. fluorescens putridus on agar, and having removed the growth of this organism planted out Sp. choleræ on the medium. He found that the products of B. fluorescens putridus were only slightly antagonistic to the growth of the cholera microbe. Freudenreich filtered a broth culture of B. pyocyaneus through a Chamberland bougie, and then planted out Sp. choleræ in the filtrate; the organism, however, refused to grow. He also found that broth, in which the cholera spirillum

had been grown, was not a favourable medium for many bacteria.

Kitasato made numerous experiments with cholera vibrios and pathogenic and non-pathogenic bacteria. The methods employed were as follows:

(1) The cholera vibrio and the organism selected were planted out in parallel streaks, and in the form of a cross, on gelatine plates.

(2) The cholera vibrio and the organism were planted out at the same time on an agar-slope, and incubated at room or blood-temperature.

(3) The vibrio and the organism were planted out in slightly alkaline broth and incubated as above.

(4) The organism was inoculated into a recent broth culture of the cholera vibrio.

(5) Cholera vibrios were planted out in a recent broth culture of the organism.

(6) Ten hours after the inoculation made under (2), (3), (4), and (5), and subsequently from time to time, a loopful of the culture was removed and plated out in gelatine.

(7) Care was also taken to plant out the above mixed cultures on fresh media, and at different temperatures, in order to make sure that the culture which had disappeared was not able to develop under more favourable circumstances.

Kitasato obtained the following results:

Pathogenic Organisms.

Cholera Vibrios and Anthrax Bacilli.—The cholera vibrios were not in any way injured by admixture with anthrax bacilli; on the other hand, the anthrax bacilli were quite destroyed at the end of two weeks.

Cholera Vibrios and Typhoid Bacilli.—The typhoid bacilli were not in any way injured by the vibrios. At first the former were slightly overgrown by the latter, but after a few days both microbes grew well and were found alive at the end of three months.

Cholera Vibrios and Friedlander's Pneumo-bacilli. — The pneumo-bacilli disappeared at the end of a month, whilst the cholera vibrios were still alive at the end of three months.

Cholera Vibrios and the Bacilli of Green Pus.—The bacilli overgrew the vibrios; at the end of three months the vibrios disappeared, but the bacilli could still be found after thirteen months. Cholera vibrios planted out in a filtered broth of B. pyocyaneus were quite unable to develop and rapidly disappeared.

Cholera Vibrios and Brieger's Bacilli.—The bacilli were at first overgrown by the vibrios; later, they developed well. At the end of three months both were found alive in a mixed culture.

Cholera Vibrios and Emmerich's Naples Bacillus.—The relation was the same as in the case of Brieger's bacilli.

Cholera Vibrios and Staphylococcus Pyogenes Aureus.—The staphylococcus was at first overgrown by the vibrios, but both were found alive at the end of three months. The same result was obtained with Staphylococcus pyogenes albus.

Cholera Vibrios and Streptococcus (Erysipelas).—The streptococci had no action on the vibrios, but were rapidly destroyed.

Non-Pathogenic Organisms.

Cholera Vibrios and B. Prodigiosus.—On mixed plates the B. prodigiosus grew so rapidly that the cholera vibrios could not be observed; so the mixed cultures were planted out on potatoes and incubated at 22° C. and 37° C. At 37° C. the cholera colonies were seen, at 22° C. the red growth of prodigiosus appeared. On agar plates at 37° C. the cholera vibrios could be detected after two months. The B. prodigiosus was found alive after thirteen months.

Cholera Vibrios and B. Indicus.—The B. indicus overgrew the vibrios; both disappeared in three months.

Cholera Vibrios and B. Fluorescens.—The fluorescent bacteria had no influence on the vibrios and died out before the latter in mixed cultures.

Cholera Vibrios and B. Violaceus.—The B. violaceus was overgrown by the vibrios and disappeared in fourteen days.

Cholera Vibrios and B. Subtilis.—The B. subtilis grew with the cholera vibrios without any unfavourable influence. The same result was obtained with the B. mycoides, B. mesentericus, Proteus species, and B. butyricus (Hueppe).

Cholera Vibrios and B. Acidi Lactici.—When grown in a medium free from sugar the B. acidi lactici had no influence on the vibrios; but in milk, owing to the production of lactic acid, the vibrios quickly died out.

Cholera Vibrios and B. Cyanogenus.—There was no unfavourable influence; both bacteria grew well together.

Cholera Vibrios and Red, White, and Orange Cocci from Air.—The vibrios gained the upper hand and the cocci disappeared in a month. The yellow sarcina behaved in the same manner. Three yeasts were also destroyed in a short time.

Bacilli isolated from fæces, singly and combined, were found to have no prejudicial influence on the cholera vibrios.

Kitasato also investigated the growth of cholera vibrios in sterilised cultures of a large number of bacteria, and found that if the cultures were recent the vibrios grew well, but the older the cultures the more feebie was the development of the vibrios. In pyocyaneus cultures, however, the vibrios always died out.

The Behaviour of the Spirillum of Asiatic Cholera in Nutrient Media containing Acids and Alkalies.

This subject has been studied by Kitasato, who employed the same methods as were used for his studies of the growth of the B. typhosus in acid and alkaline media. The results obtained are shown on the following page:

Kitasato found that in acid media the growth of the cholera vibrios was dependent on the temperature. When he added to neutral agar media 0·225 per cent. lactic acid, and then inoculated the media with cholera vibrios, no growth occurred if the plates were kept at 20° to 22° C., but if the plates were incubated at 37° C. colonies developed in three days.

The same result is seen when cholera vibrios are planted out on slightly acid potatoes; no growth occurs at 22° C., but at 37° C. the characteristic development takes place.

Liborius found that 0·0246 per cent. caustic lime destroyed cholera vibrios. Kitasato's higher figure was due to the fact that he employed undiluted broth, and some of the lime was expended in neutralising the phosphates contained in the broth.

The sign + indicates a good growth, ± restrained growth, — no growth.

Acids.	+	±	—
	per cent.	per cent.	per cent.
Sulphuric acid	0·02	0·032	0·049
Hydrochloric acid	0·052	0·08	0·132
Nitric acid	0.052	0·08	0·132
Sulphurous acid	0·07	0·09	0·148
Phosphoric acid	0·075	0·135	0·183
Carbolic acid	0·088	0·12	0·2
Acetic acid	0·1	0·153	0·2
Formic acid	0·11	0·167	0 22
Oxalic acid	0·146	0·23	0 285
Lactic acid	0·18	0·225	0·27
Tartaric acid, Citric acid, Malic acid	0·2	0·24	0·3
Tannic acid	0·9	—	1·3
Boric acid	0·43	0·8	1·33

Alkalies.	+	±	—
	per cent.	per cent.	per cent.
Caustic lime	0 0922	0·0966	0·1004
Caustic potash, Caustic soda	0·14	0·18	0·237
Ammonia	0·2	0·24	0·338
Lithium carbonate	0·6	0·666	0·72
Potassium carbonate	0·74	0·81	1·0
Ammonium carbonate	0·8	1·0	1·3
Barium hydrate	0·83	1·0	1·42
Sodium carbonate	2·2	2·47	3·45

Salts.	+	±	—
	per cent.	per cent.	per cent.
Potassium iodide	6·66	8·0	9·23
Potassium bromide	8·0	9·23	10·37
Potassium chloride	9·1	10·6	12·0

CHAPTER XV.

THE MODE OF ACTION AND UTILITY OF THE PASTEUR-CHAMBERLAND AND BERKEFELD FILTERS.

These filters have a very trifling influence on the chemical composition of water which is passed through them. They are supposed, however, to prevent the passage of bacteria by exercising a molecular influence on the microbes during the transit of the fluid containing them through the materials of the filtering candles. Numerous investigations have been made on the efficacy of these filters. In 1890 Kübler tested the Pasteur-Chamberland filter and arrived at the conclusion that this filter only delivered a germ-free filtrate for a period of four days. In his first series of experiments water, containing from 6160 to 126,720 bacteria per c.c., was passed intermittently through the filter, suction being applied by the mouth. Up to the fourth day the filtrate was sterile; on the fifth day twenty-four bacteria per c.c. were counted, and on the eighth day the bacteria were innumerable. In the second series of experiments, employing continuous filtration, a sterile filtrate was only obtained up to the third day. In the third series of experiments the surface of the filter was brushed every day, but a sterile filtrate was only obtained up to the end of the first day. Kübler noticed that the first bacteria to appear in the filtrate were the fluorescent motile bacteria; two days later, other species appeared. He accordingly came to the conclusion that the bacteria grew through the filtering candle owing to their power of multiplying in water, and this idea was supported by the fact that when the filtration was maintained at 0° C., a temperature unfavourable to the multiplication of micro-organisms, a permanently sterile filtrate was obtained.

In 1892 Freudenreich re-investigated the possibility of the passage of bacteria through the Pasteur-Chamberland filter. A bougie was sterilised, and the case having been screwed on to a tap, Seine water was passed through the bougie under a pressure of one-third of an atmosphere; 830 c.c. of the filtrate were collected in a flask containing 500 c.c. of broth; the flask was then incubated at 30–35° C. The filter was maintained in use for three days, water being allowed to pass through drop by drop; 760 c.c were then collected as before. After another three days 610 c.c. were again collected. All the flasks were found to be free from bacteria after incubation for twelve days at 35–37° C. A similar experiment was made with Ourcq water, and these flasks also remained sterile. The results of the experiments appeared to show that a sterile filtrate was delivered from the filter for a period of six days. As the results were quite at variance with the experiments of Kübler, Freudenreich then devised an experiment to test the possibility of micro-organisms going through the walls of the filtering candle. A porcelain candle of known structure was taken, and a pipette, with a globular enlargement above was passed through the opening to the bottom of the bougie; the mouth of the pipette was plugged with cotton-wool and the whole sterilised in the autoclave. After sterilisation the junction of the pipette to the bougie was closed with melted paraffin; the bougie was then placed in a glass vessel filled with water and kept at a known temperature. At various intervals some of the water which had filtered through the bougie was aspirated into the expansion on the pipette and then inoculated in broth.

The same apparatus was next used to see if typhoid bacilli were able to grow through the walls of the filtering candle. Broth placed in the glass vessel was inoculated with a B. typhosus culture and the apparatus was kept at the room temperature. Next day there was a marked growth in the broth. At various intervals, up to the end of fourteen days, portions of the filtered broth were aspirated through the pipette but were found quite clear. On the fourteenth day 3 c.c. were drawn through the pipette and placed in 200 c.c. of sterile broth; this, however, remained quite sterile. In three other experiments the filter was kept at 35° C., and portions of the filtrate examined after twelve, fifteen,

and twenty-two days were found quite sterile. It appeared, therefore, that, under the conditions of the experiments, the B. typhosus was not able to grow through the walls of the filtering candle. Freudenreich believed that the failure was due to the products secreted by the B. typhosus passing through the bougie and exerting a negative chemiotaxis on the typhoid bacilli, which were thus prevented from growing through the walls. This explanation appeared probable, as it was found that when some of the filtrate was inoculated with a fresh culture of B. typhosus the bacilli were unable to grow in it.

Thirty experiments were made with water filtration, fifteen at a temperature of 35° C., when the water filtrate remained sterile after periods of filtration varying from two to fourteen days, and fifteen at a temperature of 22° C., in the latter experiments the filtrate was sterile after from seven to eighteen days filtration. Experiments were also made at 15–18° C.; at these temperatures the filtrate remained sterile after fifteen to twenty-one days. These results also showed that water bacteria were able to grow through the walls of the filtering candle, but the rate of growth depended on the temperature. Freudenreich stated that when typhoid bacilli were present in a water supply, negative chemiotaxis would not be exercised by the products present in the filtrate; but in any case the typhoid bacilli would never grow through the filtering candle more quickly than water bacteria; consequently, at ordinary room temperatures, the Pasteur-Chamberland could be depended upon to produce a germ-free filtrate for about eight days.

With regard to the Berkefeld filter, the results of the various experiments have caused bacteriologists to express diametrically opposite opinions as to the usefulness of the filter. Nordtmeyer and Bitter considered that the Berkefeld filter gave a germ-free filtrate for a longer time than the Pasteur-Chamberland filter. Prochnick found the filtrate from the Berkefeld filter to be germ free after the filter had been in continuous use for thirty-eight days. Weyl, as the result of his experiments in Berlin, stated that the Berkefeld filter produced a germ-free filtrate for three days, and when the candles were brushed the filtrate remained sterile for six days. In 1893, Kirchner made a large number of experiments with the Berkefeld filter, and arrived at

the conclusion that the filtrate was not completely germ free for a longer period than one day. He also tested the possibility of pathogenic organisms passing through the filter. Broth cultures of cholera vibrios, typhoid bacilli, and staphylococcus pyogenes aureus were filtered through the bougie into a glass vessel, in which there was a negative pressure of 500–600 mm. of mercury; one hour after the commencement of the experiment, and then every twenty-four hours, a drop of the filtrate was plated out in gelatine. Cholera vibrios and staphylococci were found in large numbers in the filtrate received after twenty-four hours, the typhoid bacilli, however, did not appear until after seventy-two hours. A further experiment was then made by adding 100 c.c. of a broth culture of cholera vibrios to one litre of water which contained 30,000 bacteria per c.c., and then filtering the mixture through a Berkefeld candle; cholera vibrios were again found in the filtrate at the end of twenty-four hours. A similar experiment was made with B. typhosus, and this organism was detected in the filtrate at the end of forty-eight hours. Kirchner then concluded that the Berkefeld filter operated in exactly the same manner on pathogenic as on non-pathogenic bacteria. Gruber criticised Kirchner's work, and pointed out that, in the filtration of water, only those bacteria could grow through a filter which found sufficient nutriment in water to enable them to multiply in the pores of the filter material. He considered that ordinary drinking water did not contain sufficient nutriment to enable pathogenic bacteria to multiply in this manner. In Kirchner's experiments pure broth cultures, or broth cultures diluted ten-fold with water, were employed, and Gruber contends that in this manner an amount of nutriment was supplied which enabled the pathogenic organisms to multiply in the filter; such an amount of nutriment he believes would never be found in an ordinary drinking water. Gruber also considers that the counting of the bacteria in the filtered and unfiltered water is not a good test of a filter, and recommends the following points for consideration when examining the power of a bougie to prevent the passage of bacteria. The direct passage of microbes must be sharply differentiated from the gradual growth of bacteria through the material composing the filtering

candle. The direct passage points to some imperfection in the filter, but the gradual growth through the material is common to all filters, and does not indicate danger of infective disease. He considers that the best way to test a filter is to pass through it some easily recognisable special organism, and if this cannot be detected in the filtrate, the filter may be considered to give security against the passage of pathogenic organisms.

Schöfer's experiments, which were made in 1893, strongly supported Gruber's contention that typhoid bacilli, when inoculated into pure and impure natural waters, will not grow through the walls of a filtering candle owing to a deficient supply of nutriment. Schöfer employed a Berkefeld bougie placed in a glass cylinder. Sterile distilled water and sterile well water were filtered through the bougie for sixteen and twelve days respectively without any sign of the B. typhosus appearing in the filtrate, although a culture of this organism was inoculated into the contents of the glass cylinder, not only on the first day but about every second day throughout the experiment. A considerable quantity of water was passed through the filter, and the temperature during the experiment varied between 19° and 26° C. A similar experiment was made with the spirillum of cholera, but no traces of this vibrio could be detected in the filtrate. Schöfer then tried the effect of filtering a polluted sterile well water inoculated with typhoid bacilli. The well water contained in one litre: 1205 mgms. of total solids, 38 mgms. of organic material, marked ammonia, traces of nitrous acid, 251 mgms. of nitric acid, 120 mgms. of chlorine, 158 mgms. of sulphuric acid, 245 mgms. of calcium oxide, and 28 mgms. of magnesium oxide. The experiment was continued for twenty-four days; during this time a culture of B. typhosus was added twelve times to the contents of the glass cylinder, and the temperature was maintained between 18° and 25·2° C. The B. typhosus, however, was never detected in the filtrate. Other experiments were made with a canal water, which were of special interest, as they appeared to show that the only cause of the non-appearance of the B. typhosus in the filtrate was a deficiency of nutrient material. The canal water contained in one litre: 215 mgms. of solids, 47 mgms. of suspended material, 8 mgms. of chlorine, and 2 mgms.

of ammonia. The water was sterilised, and filtered through a new bougie for thirteen days. B. typhosus cultures were added five times during this period, but the organism could not be detected in the filtrate. Five c.c. of broth were then added to the water in the glass cylinder, and two days later typhoid bacilli were found in the filtrate. Schöfer concluded from his experiments that only those bacteria were able to grow through the filter which found sufficient nutrient material in the water to enable them to grow out and multiply.

Sims Woodhead and Cartwright Wood in 1897 repeated and extended the experiments made by Schöfer. These observers first tested the filtration of the New River Company water supply through Pasteur-Chamberland and Berkefeld filters attached to taps and used as pressure filters. In the case of the Pasteur-Chamberland filter it was found that only one of the four micro-organisms present in the water appeared in the filtrate, and this microbe was so devitalised that its power of liquefying gelatine and producing a yellow pigment was much delayed; it required several weeks cultivation before it recovered its usual characteristics. In the case of the Berkefeld filter the same organism appeared in the filtrate on the second or third day of filtration, and was accompanied by the B. fluorescens liquefaciens. These organisms, however, did not display any evidence of the devitalising influence which was found in the experiments with the Pasteur-Chamberland filter. Experiments were then made with chromogenic organisms placed in the reservoir of the filters before these were attached to the tap. The Micrococcous prodigiosus, Staphylococcus pyogenes aureus and Bacillus violaceus were employed for the tests; but none of these organisms could be discovered in the filtrate, although the experiments were carried on for several weeks and large quantities of the filtrate were added to concentrated broth and incubated for some time. The test organisms at the end of the experiments were easily recovered from the reservoir of the filter, showing that their non-appearance in the filtrate was not due to the death of the micro-organisms.

The next tests were made with pathogenic organisms, the filters being fed day by day with emulsions of cholera and typhoid bacilli in sterilised water. After the lapse of six and eight weeks

sterile filtrates were obtained from both filters, although inoculations from the outer surface of the candles showed that living pathogenic germs still remained. The addition of a little broth, one-fiftieth of the bulk of the water, caused the pathogenic organisms to appear in the filtrate. Typhoid and cholera bacilli were then added to Thames water, taken near Waterloo bridge at low tide, and sterilised before use. Negative results were obtained with both filters, showing that these pathogenic organisms, even when present in a highly polluted water, do not pass through into the filtrate. "As a final and conclusive test as to the possibility of disease germs traversing the walls of these filters when fed with a water highly contaminated with organic matter, an experiment was carried out with undiluted sewage." A cholera culture was added to sterilised London sewage, which was then filtered through a sterilised candle; spirilla could not be found in the filtrate although the experiment was continued for eighteen days. A mixture of cholera vibrios and sterile sewage was also passed through a sterilised candle coated with slime resulting from previous filtration; the results in this experiment were negative. Lastly, a culture of cholera was added to unsterilised sewage and then filtered through an unsterilised candle coated with slime; spirilla could not be found in the filtrate. Experiments on similar lines were also made with B. typhosus, but these again gave an absolutely negative result. All these results support the authors' contention that waters naturally highly contaminated, and even sewage itself, do not appear to supply pathogenic organisms with a sufficiently favourable pabulum to enable them to traverse the walls of Pasteur-Chamberland and Berkefeld candles.

In 1897 Lunt made an investigation of the capabilities of the Berkefeld filter, in order to obtain an independent confirmation of the results obtained by Schöfer and others, as to the power of this filter to intercept pathogenic bacteria—especially typhoid—even when water bacteria are able to pass through. Lunt's experiments appeared to justify the following conclusions:

(1) When the Berkefeld filters are efficiently sterilised and used in an approved manner, they give on the first day of using an absolutely sterile filtrate. This is the case with all the forms of filtering candles examined, not only with natural water but

also with water artificially infected with enormous numbers of bacteria from cultures, and also with broth cultures teeming with bacilli.

(2) Water bacteria, however, begin to appear on the second or third day of using, so that to obtain a continuously sterile filtrate it is necessary to re-sterilise the filter daily.

(3) A long series of experiments, in which London tap-water was strongly infected with B. coli communis, showed that this organism could not be detected in the filtered water over a period of thirty-nine days. During this period the B. coli communis could be detected on the outside of the filtering candle as late as the thirty-ninth day.

(4) Exhaustive experiments showed that with London tap-water strongly infected artificially with the typhoid bacillus, not a single typhoid bacillus was detected in the filtered water over a period of twenty-six days, though it was detected in the unfiltered water up to the sixth day. As is well known, typhoid bacilli ultimately perish in water which contains the water bacteria which are always present in natural water, but during the whole time the typhoid bacilli were demonstrated to be alive in the unfiltered water, they were never once detected in the water which had passed through the filter.

(5) London tap-water strongly infected artificially with the cholera bacillus was found, after filtration through the filter, to be absolutely sterile, and therefore entirely free from the cholera organism.

(6) The method of using filters with a combined suction and force pump, causing as it does sudden and violent changes of pressure on the outside of the filter, gives very bad results with filtering candles, which produce an absolutely sterile filtrate when used by a better method. The principle of the filters in which atmospheric pressure forces the filtered water into an exhausted receiver is free from the above objection, and when used by this method absolutely sterile filtrates are obtained, even when identically the same filtering candles are used which previously passed large numbers of bacteria from the very first, when used by the suction and force-pump method.

(7) Seeing that the bacteria which first appear in the filtered water are harmless water bacteria, and that such organisms as

B. coli communis and the typhoid bacillus do not accompany them, even when they are present in large numbers in the unfiltered water, it seems that in all cases in which a drinking water is not under suspicion of being contaminated, a semi-weekly or weekly cleansing and sterilisation of the filtering candle is all that is necessary as a precautionary measure against water-borne disease. Nevertheless, in all cases of contaminated water supplies, or supplies known to be infected with disease organisms, a daily sterilisation of the filtering candle is advisable, thus ensuring an absolutely sterile filtrate.

All the experiments hitherto described appear to prove conclusively that typhoid bacilli will not be able to grow through either a Pasteur-Chamberland or Berkefeld filtering candle. Unfortunately, my own work has led me to a different conclusion in regard to the Berkefeld candles. The experiments I made were as follows:

Experiment I.—A Berkefeld candle (No. 12 in the catalogue) was placed in a mantle and firmly fixed in position by means of a binding screw working on a thread on the metal pipe delivering the filtered water. This metal pipe was connected, by means of a short piece of stout india-rubber tubing previously fitted with a pinch-cock, to a short glass tube which just passed through an india-rubber cork fitting tightly into a Kitasato's flask. The lateral arm of the flask, and the mouth of the mantle surrounding the filtering candle, were tightly plugged with sterile wool. The whole apparatus was then placed in the autoclave and exposed to a temperature of 115° C. for half an hour. The apparatus was allowed to cool, and the filtering candle and mantle connected in the upright position to an ordinary burette stand. The pinch-cock being placed in position on the india-rubber tubing so as to separate the mantle completely from the flask, all the joints of the apparatus were thickly covered with melted paraffin. The mantle was then filled with broth so as to cover the bougie completely, but not touch the cotton wool. Fifty cc. of broth were placed in the flask and, the bung being replaced, the joint was made tight with melted paraffin. These manipulations were done with every precaution, and carried out as quickly as possible,

so as to avoid contaminating the broth. By opening the pinch-cock 10 c.c. of the broth from the mantle were now allowed to filter through the bougie into the flask. The filtration occurred simply as the result of atmospheric pressure, and the bougie worked under conditions which would allow its molecular influence full play. The apparatus was then placed in an incubator, the temperature of which averaged 25° C. After twenty-four hours exposure to this temperature the broth in the mantle and flask was found perfectly clear, showing that the manipulations had been performed without introducing any contamination. The broth in the mantle was then inoculated with a loopful of a twenty-four hours agar culture of B. typhosus. Next day the broth in the mantle was found uniformly turbid, but the broth in the flask was perfectly clear; the pinch-cock was then opened and 10 c.c. allowed to filter through into the flask. On the following day the broth in the flask being still perfectly sterile, 10 c.c. were again filtered through from the mantle. The same filtration process was continued day by day, and the amount of broth filtered each day from the mantle was replaced by fresh sterile broth, the manipulation being carried out with every precaution. The broth in the flask remained perfectly clear for four days, showing that there was no imperfection in the connections of the filtering bougie with the Kitasato flask. On the fifth day of filtration, however, the broth in the flask was found slightly turbid. A loopful was then withdrawn from the contents of the flask by means of

	B. typhosus in the mantle.	Organism in the filtrate.
Agglutination with Berne serum	Complete 1–1000 Marked 1–10,000	Complete 1–1000 Marked 1–10,000
Potato	Thin colourless growth	Thin colourless growth
Milk	Unchanged	Unchanged
Proskauer and Capaldi, I.	No growth	No growth
" " " II.	Growth, medium acid	Growth, medium acid
Peptone solution	No indol reaction	No indol reaction
Litmus-whey, seven days at 37° C.	Acidity less than 6 per cent. $\frac{N}{10}$	Acidity less than 6 per cent. $\frac{N}{10}$
Glucose-gelatine Lactose-gelatine	No gas formation	No gas formation
Gelatine-stab and plates	Very slow growth	Very slow growth
Motility	Very motile	Very motile
Stained by Gram	No	No

a bent platinum wire passed through the lateral arm, and planted out on an agar-slope. The thin greyish growth which resulted was then sub-cultured and tested for agglutination. The B. typhosus used for the inoculation of the mantle was also tested at the same time. The results are given in the Table on the previous page.

The organism which appeared in the Kitasato flask was evidently the B. typhosus which had taken five days to grow through the walls of the No. 12 filtering candle. This result is in harmony with the results of Schöfer and Sims Woodhead.

Experiment II.—The same form of apparatus as in Experiment I. was employed, a new Berkefeld bougie, size No. 12, being fixed in the mantle. Fifty c.c. of sterile broth having been placed in the Kitasato flask, and all the joints made tight with melted paraffin, the mantle was filled with a sample of sterile barrack sewage. This sewage on chemical analysis gave the following results, expressed as parts per 100,000: Total solids 610 parts, chlorine 52 parts, free ammonia 1·44 parts, albuminoid ammonia 1·846 parts, oxygen absorbed (Tidy, four hours) 8 parts. By releasing the pinch-cock 10 c.c. of the sterile sewage were allowed to filter through into the broth; the apparatus was then incubated at 25° C. Next day the broth in the flask being perfectly clear, the sewage in the mantle was inoculated with a sub-culture of the B. typhosus employed for the first experiment. Ten c.c. of the sewage were then filtered into the broth every twenty-four hours, the amount filtered being replaced by sterile sewage. The broth remained perfectly clear until the fifth day, when a distinct turbidity was noticed. A loopful of the turbid broth in the flask was withdrawn through the lateral arm and planted out on agar. The growth which appeared in twenty-four hours was tested with Berne serum for agglutination and sub-cultured in the usual media. The following results were obtained:

The bacillus was completely agglutinated by the Berne serum diluted 1–1000, and partially agglutinated by the serum diluted 1–10,000. On potato, there was a moist transparent growth. In peptone solution, no indol was produced. Milk was unchanged. In glucose-gelatine there was no gas formation. In litmus-whey, after seven days at 37° C. the acidity equalled 4 per cent. $\frac{N}{10}$ alkali.

Proskauer and Capaldi's media, No. I. showed no growth, No. II. was rendered acid. In gelatine plates, growth was slow, the colonies were small, and resembled those of B. typhosus. Microscopical examination showed a small highly motile bacillus, decolorised by Gram.

The tests showed that the bacillus which appeared in the filtrate was identical with the B. typhosus planted out in the sewage in the mantle. As the fittings were absolutely tight, and the bacillus did not appear in the filtrate until the fifth day, the experiment appeared to show that the sewage, just like the broth, had supplied sufficient nutriment to enable the typhoid bacillus to grow through the walls of the filtering candle.

Experiment III.—The same form of apparatus was employed as in the two previous experiments. The mantle, however, was filled with sterile barrack sewage, largely diluted with distilled water. The dilute sewage was found to have the following composition : Total solids, 7 parts ; chlorine, 0·3 part ; oxygen, absorbed in four hours, 1·1 parts ; free ammonia, 0·1248 part ; albuminoid ammonia, 0·2016 part ; nitrites, absent ; nitrates (NO_3), 0·926 part ; all expressed per 100,000. The dilute sewage was inoculated with a sub-culture of the same typhoid bacillus and filtration carried on daily ; the amount filtered was replaced by sterile sewage, so that the filtering candle was never allowed to become dry or exposed to the air. The broth in the Kitasato flask remained perfectly clear for nine days, but on the morning of the tenth day of filtration a slight turbidity was noticed. A loopful of the turbid broth was removed through the arm and planted out on agar. The growth which resulted was tested in the same manner as before, and found to be a pure culture of B. typhosus. The results of the agglutination and cultural tests exactly corresponded to those of the B. typhosus introduced into the mantle. In this experiment the typhoid bacillus was not supplied with as much nutriment as in the two former experiments, and it appeared to require nine days to grow through the walls of the filtering candle.

Experiment IV.—The same form of apparatus was employed as before, a perfectly new No. 12 candle being fixed in the mantle. Surface water from a ditch was sterilised and placed in the mantle. This water on analysis gave the following results,

expressed as parts per 100,000: Total solids, 44 parts; volatile solids, 16 parts; chlorine, 6·2 parts; free ammonia, 0·526 part; albuminoid ammonia, 0·60 part; oxygen absorbed in four hours, 2·2 parts; nitrites, absent; nitrates (NO_3), 1·005 part. The water in the mantle was inoculated with a loopful of a twenty-four hours agar culture of B. typhosus, and filtration carried on daily as in the previous experiments. The broth in the flask remained perfectly clear for eight days, but on the ninth day it was found to be turbid. A loopful of the turbid broth, when examined as before, was found to contain a pure culture of B. typhosus. In this experiment the nutriment supplied was greater than in Experiment III., but not so great as in Experiments I and II. Corresponding apparently to the nutriment supplied, the time taken by the typhoid bacillus to grow through the candle was eight days as compared with four days in Experiments I. and II., and nine days in Experiment III.

Experiment V.—In this experiment the mantle was filled with a specimen of sterile barrack sewage, which contained a large quantity of organic matter. The results of the analysis expressed as parts per 100,000 were as follows: Total solids, 55 parts; volatile solids, 15 parts; chlorine, 9·5 parts; free ammonia, 8·28 parts; albuminoid ammonia, 5·07 parts; oxygen absorbed in four hours, 6·4 parts; nitrites, absent; nitrates, absent. The sewage was inoculated with a twenty-four hours agar culture of B. typhosus, and filtration carried on in the same manner as in the previous experiments. The broth in the flask remained quite clear for four days, but was found turbid on the fifth day. The turbidity was caused by B. typhosus. In this experiment the available organic matter was much greater than in Experiments III. and IV., and consequently the bacillus only required four days to grow through the walls of the candle.

Experiment VI.—The same form of apparatus was used, a new No. 12 Berkefeld candle being fitted into the mantle. A specimen of water collected from a reservoir at Netley was sterilised and then analysed. The following results, expressed as parts per 100,000, were obtained: Total solids, 18 parts; volatile solids, 5 parts; chlorine, 3·3 parts; free ammonia, 0·00816 part; albuminoid ammonia, 0·0158 part; oxygen absorbed in four hours, 0·5 part; nitrites, absent; nitrates (NO_3), 0·8 part.

This water was placed in the mantle, inoculated with B. typhosus, and filtration carried out as in the previous experiments. The broth in the flask remained perfectly sterile until the ninth day, when faint turbidity was noticed. A loopful withdrawn through the lateral arm was planted out on agar, and tested in the usual manner. The growth was completely agglutinated by Berne serum diluted 1–1000, and when planted out on the various media, the results obtained were identical with those given by a true culture of B. typhosus.

Experiment VII.—The same water was used for this experiment, which was conducted in exactly the same manner as Experiment VI., a fresh No. 12 candle, however, being employed. The B. typhosus again appeared in the filtrate on the ninth day.

Experiment VIII.—The reservoir water was again employed, but a large candle, No. 10, was used and fitted into a mantle holding about 500 c.c. of water. The water in the mantle was inoculated on three occasions with a twenty-four hours agar culture of B. typhosus. The broth in the flask remained perfectly sterile for ten days, but on the eleventh day turbidity was noticed, which was found to be due to a pure culture of the typhoid bacillus.

Experiment IX.—A large candle, size No. 10, was employed, and the mantle filled with sterile barrack sewage. A twenty-four hours agar culture of the typhoid bacillus was planted out in the sterile sewage, and filtration carried out in the same manner as before. On the fifth day of filtration the typhoid bacillus was found in the broth placed in the flask.

Experiment X.—The bougie used in Experiment VI. was removed fiom the mantle and thoroughly cleaned with a stiff brush, the metal delivery pipe was also thoroughly washed and flamed. The bougie was then replaced in a sterile mantle and the apparatus fitted up in the usual manner. Sterile reservoir water was then filtered through the candle, and next day the broth in the flask was found to be turbid. The typhoid bacillus was easily isolated from the turbid broth. This result appeared to show that carefully cleaning a bougie would not remove the B. typhosus which had evidently penetrated into the interior of the material and was washed through into the filtrate by the passage of the sterile water.

Experiment XI.—In this experiment the bougie used in Experiment VII. was sterilised in an autoclave and then fitted up in a sterile apparatus. Sterile reservoir water was filtered through into the flask for eleven days without the slightest trace of turbidity appearing in the broth.

Experiments on the same lines were then made with Pasteur-Chamberland candles, fitted into glass mantles by means of india-rubber bungs. The delivery pipe of the candle in each case was connected by a short piece of india-rubber tubing, fitted with a pinch-cock, to a Kitasato flask containing sterile broth. As in the previous experiments, all the joints were made tight with melted paraffin. In the first trial, the mantle was filled with sterile broth, which was then inoculated with a twenty-four hours culture of B. typhosus. Next day the broth in the mantle was extremely turbid; filtration was then commenced, 10 c.c. of broth being filtered into the flask every day and 10 c.c. of sterile broth being put into the mantle. After filtration for three weeks, the broth in the flask was found quite sterile. One loopful of the broth from the container, however, contained a large number of typhoid bacilli. Trials were then made with the other candles; sterile barrack sewage, sterile sewage effluents, polluted ditch water and reservoir water, repeatedly inoculated with large doses of B. typhosus, were filtered for three weeks, but no traces of B. typhosus could be detected in the broth placed in the Kitasato flasks. The failure to pass through the filtering candles was not due to the absence of B. typhosus from the fluid in the mantles, as in each experiment one loopful of the fluid in the mantle at the end of the trial was found to contain large numbers of living, vigorous, typhoid bacilli.

The following conclusions appear to be justified.

(1) The B. typhosus is not able to grow through the walls of a Pasteur-Chamberland candle, and, if proper care be taken to prevent the direct passage of organisms through flaws in the material and imperfections in the fittings, the Pasteur-Chamberland filter ought to give complete protection from water-borne disease.

(2) Typhoid bacilli can grow through the walls of Berkefeld candles, the time required for the passage being largely dependent on the nutriment supplied to the organisms by the filtering fluid. The large size of the lacunar spaces in the Berkefeld candle,

which cannot be avoided if a fair delivery is to be obtained, appears to militate against the immobilising and devitalising influences which operate so strongly in filters made with very narrow lacunar spaces. It is well known that the typhoid bacillus can grow out into long thread-like forms, and if this takes place in the lacunar spaces of the Berkefeld candle the bacillus may find its way into the cavity in the interior of the candle. It is also probable that, owing to deficient immobilisation, the bacilli are gradually washed through the filtering candles. This often takes place when filters are worked with a pump bearing directly on the bougie; if, however, an air cavity is introduced between the pump and the filtering candle, the pressure is equalised, and this source of danger is removed.

(3) When a highly polluted liquid containing typhoid bacilli is filtered through a Berkefeld candle the bacilli may appear in the filtrate in four days. Consequently in order to obtain protection from water-borne disease, when these filters are employed, it is necessary to sterilise the candles every third day. This cannot be done by simply brushing the candles, they must be sterilised by boiling water or by exposure to saturated steam.

Methods of Testing Water Filters.

It is clear from what has been said, that when examining filters as to their powers of removing bacteria from water, it is necessary to distinguish between the *direct* passage of micro-organisms through the filter, due to some imperfection in the filtering material itself or in its connection with the receptacle for filtered water, and the *indirect* passage caused by the growth of the micro-organisms through the walls of the filtering material. The direct passage of bacteria may be determined in the case of non-pressure filters by placing a rich emulsion of some special organism, such as the B. prodigiosus, in the water to be filtered and examining the filtrate for this organism by means of plate cultures. If the special organism be found in the filtrate, this may be caused by some flaw in the filtering material or defects in the fitting of the material to the delivery pipe. If the filtering material be in the form of a bougie it should be placed in a vessel containing an emulsion of the special organism and then be connected to a sterile partially exhausted flask. It is

important to feed the vessel containing the emulsion with water so as to prevent the bougie from becoming dry. The filtrate which collects in the exhausted flask is then examined in the usual manner. In the case of filters which require considerable pressure, the receptacle for the unfiltered water must be filled with the special emulsion of a non-pathogenic organism, and then connected to a service supply or to a tank raised sufficiently to give the required head of pressure. When the delivery of filtered water is very rapid, it is necessary to collect a considerable amount of the filtrate, especially during the first few minutes of working, as Gruber has shown that this is the critical time of the experiment. I have found that under these conditions it is best to pump several litres of the filtrate through a Pasteur-Chamberland candle and diffuse the deposit in 5 c.c. of sterile water. The whole of this mixture should then be plated out in gelatine, gradually increasing amounts being used for each plate.

In order to test the indirect passage of micro-organisms through the filter the plan already described under the experiments made with the Berkefeld candles is very convenient. If, however, there is no mantle supplied with the filtering candle this must be connected to a partially exhausted sterile Kitasato flask by means of stout rubber fitted with a pinch-cock. The candle is then placed in a narrow-necked jar, containing either an emulsion of B. typhosus or Spirillum Choleræ Asiaticæ, in sewage or polluted water. The filtering portion of the candle only must be placed in the fluid, and the neck of the jar is then plugged with sterile wool. By releasing the pinch-cock a few c.c. are allowed to filter through the candle every day, taking care to replace the amount of fluid filtered so that the surface of the candle may not become dry. The filtrate in the flask in the case of the B. typhosus should be mixed with a large quantity of broth, and then incubated at 37° C.; if a turbidity occurs, loopfuls should be plated out and tested in the usual manner. In the case of the Spirillum of cholera the filtrate should be converted into 1 per cent. peptone and ½ per cent. salt solution by adding a sufficient quantity of the stock peptone and salt solution. The flask is then incubated at 37° C., and loopfuls removed from the surface after ten, eighteen, and twenty-four hours are examined in the usual manner.

CHAPTER XVI.

SUMMARY OF THE PROCEDURE RECOMMENDED FOR THE BACTERIOLOGICAL EXAMINATION OF WATER AND PREPARATION OF NUTRIENT MEDIA.

A. Quantitative Analysis:

Shake a portion of the sample gently, but thoroughly, so as to break down clumps of bacteria. Ascertain roughly the probable number of micro-organisms present by means of a cover-glass preparation, and if necessary dilute the specimen with sterile tap-water. Make three gelatine plates with an amount of the water which will not produce more than 300 colonies in each plate. As a rule, $\frac{1}{10}$ c.c., $\frac{1}{4}$ c.c., and $\frac{1}{2}$ c.c. will be found suitable amounts for the plates. Incubate the plates at 22° C. for as long as possible. Count the colonies day by day, and record the average number which develop per c.c.

B. Qualitative Analysis:

(1) If the specimen does not contain many bacteria, pump one or two litres through a Berkefeld or Pasteur-Chamberland candle, and diffuse the deposit in 5 to 10 c.c. of sterile water. Treat this as follows:

(*a*) Add 1 c.c. of the concentrated water to 15 c.c. of sterile whole milk, heat to 80° C. for ten minutes, cool, and then incubate at 37° C. in a Buchner tube. Note if any changes characteristic of *B. enteritidis sporogenes* are produced within twenty-four to thirty-six hours.

(*b*) Plate in carbolic acid gelatine (0·05 per cent. carbolic acid) from 0·1 to 1 c.c. of the concentrated water.

(*c*) Add from 0·1 to 1 c.c. of the concentrated water to broth tubes containing 0·05 per cent. carbolic acid, and incubate the tubes at 37° C.; *or* add the same amounts of the concentrated

water to glucose-formate broth tubes, and then incubate in Buchner's tubes at 42° C. (Pakes' method). Plate out all the tubes which show any turbidity after twenty-four hours incubation in gelatine and on alkaline glucose-litmus-agar.

(*d*) Examine all the plates for B. coli and other sewage organisms.

Sub-culture the coliform colonies in :

Glucose-gelatine shake, for gas formation.

Milk, for acidity and coagulation of casein.

Peptone (Witte) water, for the indol reaction.

Agar-slope, for agglutination test.

If B. typhosus be suspected, all the tests given under the description of this organism must be applied.

(2) If the specimen of water contains a very large number of micro-organisms, it is useless to plate out the concentrated sample directly in carbol-gelatine.

The best plan is to cultivate the concentrated water in amounts varying from 0·1 to 1 c.c. in glucose-formate broth, according to Pakes' method, and in carbolic acid broth containing not more than 1·0 per cent. carbolic acid. The growths which result must be plated out on alkaline glucose-litmus-agar.

(3) If the water is suspected to contain the cholera spirillum, to 90 c.c. of the sample add 10 c.c. of the stock peptone solution (10 per cent. peptone, 20 per cent. gelatine, and 5 per cent. salt) and cultivate the mixture at 37° C. After twelve hours incubation remove loopfuls from the surface, and examine in a hanging-drop for spirilla ; if these are not in pure culture inoculate a loopful into a second flask, containing 1 per cent. peptone and ½ per cent. salt. Plate out loopfuls from the surface of the pure culture in gelatine and on agar. Then inoculate peptone tubes for the indol reaction, and stab-gelatined for the typical liquefaction ; also plant out a loopful on an agar-slope, and employ the growth, which results in twenty-four hours, for Pfeiffer's reaction and the agglutination test.

PREPARATION OF CULTURE MEDIA.

Nutrient Broth (Peptone Bouillon).—One pound of beef free from fat must be finely minced, then infused in a litre of cold distilled water and allowed to stand in a cold place for twenty-four hours. The whole mass is then strained through a cloth and distilled water added to the filtrate so as to make up the volume of fluid to one litre. Ten grammes of peptone and five grammes of common salt are now added to the litre of fluid, which is then boiled in the steam steriliser for one hour. The medium is next made slightly alkaline with a 1 per cent. solution of sodium carbonate, litmus being used as the indicator, and again boiled in the steamer for half an hour. Coagulated material is then removed by filtration through moistened "Chardin" paper. The medium is finally sterilised in the steamer on three successive days for ten to fifteen minutes each day. Sometimes it is impossible to obtain good beef; Liebig's extract may then be employed with advantage. The broth obtained is perfectly reliable for ordinary purposes, and though perhaps not so nourishing as that made with the best beef, it has the advantage of being more uniform in quality. Five grammes of Liebig's extract are added to a litre of tap-water containing ten grammes of Witte's peptone and five grammes of common salt. The mixture is then boiled in the steam steriliser or over a Bunsen flame, made faintly alkaline with sodium carbonate and filtered into a sterile flask. The flask is placed in the steam steriliser for three-quarters of an hour and coagulated material removed by filtration through moistened "Chardin" paper. The medium may then be used to prepare nutrient gelatine or agar-agar; if not required for this purpose, it is poured into a series of sterile test-tubes, 10 c.c. in each tube, which are then sterilised on three successive days.

Another method which is largely used in some laboratories is as follows: Take one pound of beef free from fat, mince finely, and diffuse in a litre of tap-water, boil for twenty minutes and allow to stand all night in an ice chest. Next day strain the fluid, add ten grammes of Witte's peptone, five grammes of salt, and make up the bulk to one litre, dissolve by heat and then carefully neutralise with a 28·6 per cent. solution of sodium

carbonate. Next sterilise the fluid in the autoclave at 115° C. for fifteen minutes, and then filter through moistened "Chardin" paper. Place in tubes or a sterile flask and again sterilise at 115° C. for half an hour.

Nutrient Gelatine.—To broth prepared as above add 15 per cent. "gold label" gelatine, and place in the steam steriliser until all the gelatine is dissolved. Then make faintly alkaline with the sodium carbonate solution, cool to 50° C., and add the white of an egg dissolved in 50 c.c. of distilled water. Boil for an hour or until the fluid is clear, and then filter through moistened "Chardin" paper placed in a hot-water filter. Divide the filtrate into sterile tubes, 10 c.c. in each, and sterilise in the steam steriliser at 100° C. on three successive days for half an hour each day.

Agar-Agar.—Broth prepared as above is placed in a flask and rendered *distinctly* alkaline. Two per cent. of agar power is then added to the alkaline fluid; if agar is added to an acid medium it is converted into galactose. The flask is heated in the steam steriliser until the agar is dissolved; the contents are then cooled to 50° C., and the white of an egg dissolved in 50 c.c of distilled water added. The flask is now heated in the autoclave at 115° C. for three-quarters of an hour, and the contents are then filtered through moistened "Chardin" paper placed in a hot-water funnel. The filtrate is divided amongst sterile test-tubes, 7 c.c. in each, which are then sterilised in the autoclave at 115° C. for half an hour.

Glucose-broth, *glucose-gelatine*, and *glucose-agar* are made by adding 2 per cent. of glucose to each of the media prepared as above, but before sterilisation has been effected. Sugar media should not be sterilised at a temperature above 100° C.

Cane-sugar-gelatine and *lactose-gelatine* are prepared in the same manner as the glucose media.

Peptone Solution.—Witte's peptone, 1 per cent., and common salt 0·5 per cent., are dissolved in water with the aid of heat (as a rule the fluid does not require the addition of an alkali, the peptone being sufficiently alkaline). The mixture is then boiled, filtered, and divided amongst sterile tubes, which are then sterilised in the autoclave at 115° C. for twenty minutes.

Potato.—A large potato is well washed and scrubbed with a brush. A cylinder is then bored from its interior and cut obliquely. The brown peel is cut off from the ends, and the wedges so obtained are allowed to soak overnight in distilled water so as to get rid of the excess of starch. Each wedge is then placed in a potato tube previously fitted with a pad of wool at the bottom and filled for about an inch in depth with distilled water. The tubes are plugged with cotton wool and then sterilised in Koch's steamer for one hour. Before use the potato tube should be incubated at 37° C. for twenty-four hours, in order to make sure that sterilisation has been affected. Sometimes it is necessary to autoclave the medium, but this should be avoided if possible. If sterilisation for one hour at 100° C. renders the potato too soft, discontinuous sterilisation for twenty minutes on three successive days should be practised.

Milk.—Fresh milk is steamed for fifteen minutes in the Koch's steriliser, and placed in a cool place over-night to facilitate separation of the cream. The milk is then siphoned off from beneath the cream and placed in sterile test-tubes. The tubes are plugged and sterilisation effected by steaming at 100° C. for twenty minutes on three successive days. For cultivation of the B. enteritidis sporogenes *whole* milk should be placed in sterile tubes and then sterilised as above.

Litmus-whey.—This medium was suggested by Petruschky for the study of the amount of acid produced by the fermentation of lactose. Fresh milk is warmed, and the casein coagulated by means of a little hydrochloric acid. The separated casein is then filtered off and the clear whey neutralised with dilute sodium hydrate solution. The fluid is then steamed for two hours; a little acid albumen usually separates and is filtered off. The filtrate should now be clear, colourless, and neutral in reaction. To the filtrate 5 per cent. of a saturated alcoholic solution of litmus is added, and the medium placed in tubes and sterilised. For accurate work it is better to add neutral litmus to the neutral whey, and then sterilise as usual.

Lactose Litmus Solution.—This medium is made as follows: Dissolve two grammes of lactose in 40 c.c. of broth and add water to make up to 100 c.c. Carefully neutralise and boil in

the steamer for twenty minutes and filter. Then add neutral litmus so as to produce a reddish-purple colour. Divide into test-tubes and sterilise in the steamer on three successive days.

Neutral Litmus.—This solution is prepared by adding 300 c.c. of rectified spirit to two ounces of commercial litmus. After standing for a fortnight the spirit is poured off and a second 300 c.c. added, and allowed to stand for a second fortnight. The alcohol is then poured off, and all traces of the spirit removed by exhausting the flask with an air-pump. The litmus is now added to 800 c.c. of water placed in a flask and allowed to stand for twenty-four hours. The watery extract so obtained is filtered into a flask, and a few drops of pure sulphuric acid added until the solution has an acid reaction. Baryta solution is then added in excess, and the fluid left until it has an alkaline reaction. The solution is then filtered, and carbon-dioxide passed through it until an acid reaction is again obtained. The Barium carbonate is filtered off, and the solution placed in a test-tube. Sterilisation is effected in steam steriliser on three successive days.

Potato-gelatine.—Crush 500 grammes of peeled potato and macerate in a litre of water for three or four hours. Sift and allow to stand for twenty-four hours, then decant, and make up the volume to 1000 c.c. Dissolve 150 to 200 grammes of gelatine by heat, and boil for a few minutes. Then add a solution of soda until the reaction is feebly but distinctly acid. Heat to 115° C. for five minutes, and filter through "Chardin" paper placed in a hot funnel. Divide into tubes and sterilise at 112°–115° C. Just before use Elsner recommends the addition of 1 per cent. of potassium iodide to the gelatine previously melted in the "plate bath."

Glucose-formate-broth.—For the study of bacteria under anærobic conditions Kitasato recommended the addition of 0·4 gramme of sodium formate and two grammes of grape sugar to ordinary broth. Pakes' medium for the isolation of B. coli and B. typhosus is made as follows: "To ordinary meat infusion are added 1 per cent. peptone, 0·5 per cent. sodium chloride, 2 per cent. glucose, and 0·4 per cent. sodium formate. When these have all been dissolved by heating the medium is neutralised, and after neutralisation 4 c.c. of normal soda solution per

litre are added. The broth is then boiled in the steam steriliser for twenty minutes, filtered, poured into the test-tubes, and sterilised for twenty minutes on the day it is made and the two succeeding days."

Nitrate-broth.—This medium is prepared by adding 0·1 per cent. of potassium nitrate to nutrient broth, diluted with sterile water, so that the medium contains only 5 per cent. broth, viz., KNO_3 0·1 gramme, broth 5 c.c., sterile water 95 c.c. If pure broth is used the medium turns almost black on adding Nessler solution, so that it is impossible accurately to determine the presence of ammonia.

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