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ELEMENTS

OF

WATER BACTERIOLOGY

WITH SPECIAL REFERENCE TO

SANITARY WATER ANALYSIS.

BY

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IN THE

MASSACHUSETTS INSTITUTE OF TECHNOLOGY.

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S. C. PRESCOTT
AND
C.-E. A. WINSLOW

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BOSTON, U.S.A.

DEDICATED

TO

William Thompson Sedgwick

BY TWO OF HIS PUPILS,

AS A TOKEN OF GRATEFUL AFFECTION.

PREFACE TO FIRST EDITION

THE general awakening of the community to the importance of the arts of sanitation — accelerated by the rapid growth of cities and the new problems of urban life — demands new and accurate methods for the study of the microbic world. Bacteriology has long since ceased to be a subject of interest and importance to the medical profession merely, but has become intimately connected with the work of the chemist, the biologist, and the engineer. To the sanitary engineer and the public hygienist a knowledge of bacteriology is indispensable.

In the swift development of this science during the last ten years perhaps no branch of bacteriology has made more notable progress than that which relates to the sanitary examination of water. After a brief period of extravagant anticipation, and an equally unreasonable era of neglect and suspicion, the methods of the practical water bacteriologist have gradually made their way, until it is recognized that, on account of their delicacy, their directness, and their certainty, these methods now furnish the final criterion of the sanitary condition of a potable water.

A knowledge of the new science early became so indispensable for the sanitary expert that a special course in the Bacteriology of Water and Sewage has for some years been given to students of biology and sanitary engineering in the Biological Department of the Massachusetts Institute of Technology. For workers in this course the present volume has been especially prepared, and it is fitting, we think, that such a manual should proceed from an institution whose faculty, graduates, and students have had a large share in shaping the science and art of which it treats. We shall be gratified, however, if its field of usefulness extends to those following similar courses in other institutions, or occupied professionally in sanitary work.

The treatment of the subject in the many treatises on General Bacteriology and Medical Bacteriology is neither special enough nor full enough for modern needs. The classic work of Grace and Percy Frankland is now ten years old; and even Horrocks' valuable "Bacteriological Examination of Water" requires to be supplemented by an account of the developments in quantitative analysis which have taken place on this side of the Atlantic.

It is for us a matter of pride that Water Bacteriology owes much of its value, both in exactness of method and in common-sense interpretation, to American sanitarians. The English have contributed researches of the greatest importance on the significance of certain intestinal bacteria; but with this exception the best work

on the bacteriology of water has, in our opinion, been done in this country. Smith, Sedgwick, Fuller, Whipple, Jordan, and their pupils and associates (not to mention others) have been pioneers in the development of this new field in sanitary science. To gather the results of their work together in such form as to give a correct idea of the best American practice is the purpose of this little book; and this we have tried to do, with such completeness as shall render the volume of value to the expert and at the same time with such freedom from undue technicality as to make it readable for the layman. It should be distinctly understood that students using it are supposed to have had beforehand a thorough course in general bacteriology, and to be equipped for advanced work in special lines.

BOSTON, *March 10, 1904.*

PREFACE TO SECOND EDITION

SINCE the appearance of the first edition of this work the investigation of various phases of the bacteriology of water has been advanced with zeal by many workers on both sides of the Atlantic. As a result a considerable mass of new evidence has accumulated which has established the bacteriological examination of water on a firmer basis than ever, and shown it to be the most direct, accurate and practical method at the disposal of the sanitarian.

The study of presumptive tests for the colon bacillus has advanced, with highly satisfactory results. The methods for the isolation of specific pathogenic organisms have also been notably improved.

An excellent volume on Water Bacteriology by Dr. W. G. Savage has appeared during the past year, which shows the English methods of investigation and interpretation to be closely in accord with those used in America.

It has been our aim in preparing this new edition to include the results of the work of the last four years which bear on the practical investigation of sanitary questions connected with water supply. Considerable additions have been made to the treatment of the prob-

lems of self purification in Chapter I, to the description of methods for the isolation of the typhoid bacillus in Chapter V, to the treatment of the interpretation of the colon test in Chapter VII, to an account of the newer presumptive tests for B. coli in Chapter VIII, and to the discussion of the significance of intestinal bacteria, other than B. coli, in Chapter IX. A new chapter has been introduced on The Bacteriology of Sewage and Sewage Effluents, in recognition of the growing importance of this branch of the subject.

THE BIOLOGICAL LABORATORIES,
MASSACHUSETTS INSTITUTE OF TECHNOLOGY,
Boston, January 1, 1908.

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ELEMENTS OF WATER BACTERIOLOGY

CHAPTER I.

THE BACTERIA IN NATURAL WATERS.

BACTERIA are the most numerous and the most widely distributed of living things. They are present not merely at the surface of the earth or in the bodies of water which partially cover it, as is the case with most other living things, but in the soil itself, and in the air above, and in the waters under the earth.

Probably no organisms are more sensitive to external conditions, and none respond more quickly to slight changes in their environment. Temperature, moisture, and oxygen are of importance in controlling their distribution; but the most significant factor is the amount of food supply. Bacteria and decomposing organic matter are always associated, and for this reason a brief consideration of the general relation of bacteria to their sources of food supply must precede the study of their distribution in any special medium.

The bacteria possess greater constructive ability than any animal organisms. They lack, however, the power of

green plants to build up their own food from compounds like carbon dioxide and nitrates which have no stored potential energy. The food requirements of various bacterial types differ, however, widely among themselves. Fischer (1900) has divided the whole group into three great subdivisions according to the nature of their metabolism. The Prototrophic forms are characterized by minimal nutrient requirements, including organisms like the nitrifying bacteria which require no organic compounds at all but derive their nourishment from carbon dioxide or carbonates, nitrites and phosphates, or from inorganic ammonium salts. A second group, the Metatrophic bacteria, includes those forms which require organic matter, nitrogenous and carbonaceous, but are not dependent on the fluids of the living plant or animal. Finally, the Paratrophic bacteria are the true parasites, which exist only within the living tissues of other organisms. These subdivisions, like all groups among the lower plants, are not sharply defined; and the metatrophic bacteria in particular exhibit every gradation, from types which grow in water with a trace of free ammonia, to organisms like the colon bacillus which normally occur on the surface of the plant or animal body, feeding upon the fluids or on the extraneous material collected upon its surface.

The vast majority of bacteria belong to the second, or metatrophic group, living as saprophytes on dead organic matter wherever it may occur in nature, and particularly

in that diffuse layer of decomposing plant and animal material which we call the humus, or surface layer of the soil. Wherever there is life, waste matter is constantly being produced, and this finds its way to the earth or to some body of water. The excretions of animals, the dead tissues and broken-down cells of both animals and plants, as well as the wastes of domestic and industrial life, all eventually find their way to the soil. In a majority of cases these substances are not of such chemical composition that they can be utilized at once by green plants as food, but it is first necessary that they go through a fermentation or transformation in which their chemical composition becomes changed; and it is as the agents of this transformation that bacteria assume their greatest importance in the world of life.

We may take the decomposition of a comparatively simple excretory product, urea, as an example of the part which the bacteria play in the preparation of plant food. Through the activity of an enzyme produced by certain bacteria this compound unites with two molecules of water and is converted into ammonium carbonate,

$$CO\begin{matrix}\diagup NH_2 \\ \diagdown NH_2\end{matrix} + 2\,H_2O = (NH_4)_2\,CO_3.$$

This, however, is only part of the process. While green plants can derive their necessary nitrogen in part, at least, from ammonium compounds, it is a well-established fact that this element may be obtained much more readily

from nitrates, and there are other bacteria which as a further step oxidize the ammoniacal nitrogen to a more available form. This process of oxidation is known as *nitrification*, and takes place in a succession of steps, the organic nitrogen being first converted to the form of ammonium salts, and these in turn to *nitrites* and *nitrates*, the oxygen used coming from the air. Several groups of organisms are instrumental in bringing about this conversion. It is generally assumed that one group attacks the ammonium compounds and changes them to nitrites, while another group completes the oxidation to nitrates. In the latter form nitrogen is readily taken up by green plants to be built up into more complex albuminoid substances (organic nitrogen) through the constructive power of chlorophyll.

This never-ending cycle is illustrated in the accompanying figure, devised by Sedgwick (Sedgwick, 1889) to illustrate the transformations of organic nitrogen in nature, the increasing size and closeness of the spiral on the left-hand side indicating the progressive complexity of organic matter as built up by the chlorophyll bodies of green plants in the sunlight, and the other half of the figure the reverse process carried out largely by the bacteria. In nature there are many short circuits, as, for instance, when dead organic matter is used as food for animals and built up into the living state again without being nitrified and acted upon by green plants; but the complete cycle of organic nitrogen is as indicated on the diagram.

We have dwelt thus at length upon the general relation between bacteria and organic decomposition because in this relation will be found the master key to the distribution of bacteria in water as well as in other natural habitats. It is true that certain peculiar forms may at times multiply in fairly pure waters; but in general large numbers of bacteria are found only in connection with the organic matter upon which they feed. Such organic

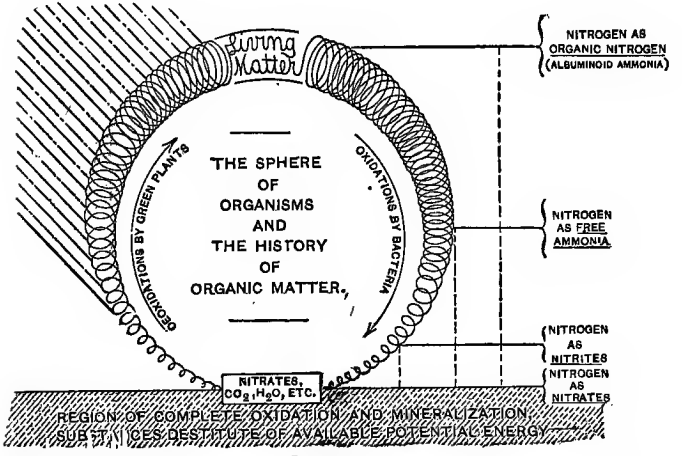

FIG. 1.

matter is particularly abundant in the surface layer of the soil. Here, therefore, the bacteria are most numerous; and in other media their numbers vary according to the extent of contact with the living earth.

Natural waters group themselves from a bacteriological standpoint in four well-marked classes, according to their

relation to the rich layers of bacterial growth upon the surface of the globe. There are first the atmospheric-waters which have never been subject to contact with the earth; second, the surface-waters immediately exposed to such contamination in streams and pools; third, the lakes and large ponds in which storage has reduced bacterial numbers to a state of comparative purity; and fourth, the ground-waters from which previous contamination has been even more completely removed by filtration through the deeper layers of the soil.

Even rain and snow, the original sources of our potable waters, are by no means free from germs, but contain them in numbers varying according to the amount of dust present in the air at the time of the precipitation. After a long-continued storm the atmosphere is washed nearly free of bacteria, so that a considerable series of sterile plates may often be obtained by plating 1-c.c. samples. These results are in harmony with the observations of Tissandier (reported by Duclaux, 1897), who found that the dust in the air amounted to 23 mg. per cubic meter in Paris and 4 mg. in the open country. After a rain-fall these figures were reduced to 6 mg. and .25 mg., respectively.

With regard to what may be considered normal values for rain, satisfactory data are not abundant. Those obtained by Miquel (Miquel, 1886), during the period 1883–1886, showed on the average 4.3 bacteria per c.c. in the country (Montsouris) and 19 per c.c. in Paris. Snow

shows rather higher numbers than rain. Janowski (Janowski, 1888) found in freshly fallen snow from 34 to 463 bacteria per c.c. of snow-water.

As soon as the raindrop touches the surface of the earth its real bacterial contamination begins. Rivulets from ploughed land or roadways may often contain several hundred thousand bacteria to the cubic centimeter; and furthermore the amounts of organic and mineral matters which serve as food materials, and thus become a factor in later multiplication of organisms, are greatly increased.

In the larger streams several conditions combine to make these enormous bacterial numbers somewhat lower. Ground-water containing little microbic life enters as a diluting factor from below. The larger particles of organic matter are removed by sedimentation; many earth bacteria, for which water is an unfavorable medium, gradually perish; and in general a new condition of equilibrium tends to be established. It is difficult, however, to find a river in inhabited regions which does not contain several hundreds or thousands of bacteria to the cubic centimeter. Furthermore, heavy rains which introduce wash from the surrounding watershed may at any time upset whatever equilibrium exists, and surface-waters are apt to show sudden fluctuations in their bacterial content. Particularly in the spring and fall high numbers manifest themselves, and seasonal variations arise, such as are shown in the appended table.

SEASONAL VARIATIONS IN BACTERIAL CONTENT OF RIVER WATERS. BACTERIA PER C.C. MONTHLY AVERAGES.

River.	Year.	Jan.	Feb.	Mar.	Apr.	May.	June.
Thames[1] . .	1905–6	2075	1679	1161	277	1064	382
Lea[1]	"	5192	3083	1308	471	1350	598
New[1]	"	1455	1304	291	149	352	198
Mississippi[2] .	1900–01	972	2871	1795	3597	2152	2007
Potomac[3] . .	1906–7	4400	1000	11,500	3700	750	2300
Merrimac[4] . .	1905	14,200	14,800	10,300	3600	1900	9600
Susquehanna[5]	1906	9510	21,228	31,326	39,905	6187	2903

River.	Year.	July.	Aug.	Sept.	Oct.	Nov.	Dec.
Thames[1] . .	1905–6	952	...	...	...	1633	740
Lea[1]	"	1190	...	...	...	3946	2050
New[1]	"	450	...	...	...	718	621
Mississippi[2] .	1900–01	1832	805	...	...	...	2021
Potomac[3] . .	1906–7	2700	3000	6200	2300	1800	6900
Merrimac[4] .	1905	3900	19,500	13,500	39,800	8700	...
Susquehanna[5].	1906	685	1637	836	7575	26,224	37,525

[1] Houston, 1906a, 1906b.
[2] New Orleans, 1903.
[3] Figures obtained through courtesy of F. F. Longley.
[4] Massachusetts, 1906.
[5] Harrisburg, 1907.

In general, bacterial numbers are highest for river-waters in the winter and spring months. The rainfall is the main factor which affects seasonal variations, but its specific effect differs with different streams. The immediate result of a smart shower is always to increase contamination by introducing fresh wash from the surface of the ground. More prolonged moderate rain, however,

exerts an opposite effect, and after the main impurities which can be washed away have been removed, may dilute the stream with water purer than itself. In the Kennebec River at Waterville, for example, Whipple (1907) found that bacterial counts were highest at the times of largest stream flow. What the net effect of rain may be depends on the character of the stream. A river of fairly good quality shows its highest numbers in rainy periods. With a highly polluted stream, on the other hand, the constant influx of sewage overbalances occasional contributions of surface contamination. Thus Gage (1906) shows in the following table that the bacterial content of the Merrimac is highest when the stream is lowest, that is when its sewage content is least subject to dilution.

RELATION BETWEEN VOLUME OF FLOW AND BACTERIAL CONTENT IN THE MERRIMAC RIVER.
(GAGE, 1906.)

Flow of Stream. Cubic feet per second per square mile of Watershed.	Bacteria per c.c.		B. coli. per c.c.	
	Canal.	Intake.	Canal.	Intake.
Less than 1	7500	10,800	66	88
1–2.	6800	6200	50	51
2–4.	3600	5600	29	39
Over 4	3400	3100	16	29

The contrast between the two classes of rivers is well brought out in a study of the Lahn and the Wieseck, by Kisskalt (1906); and the table below, compiled from his data, gives an excellent idea of the total numbers of bac-

teria and their seasonal fluctuations in a stream of fair quality (the Lahn) and a highly polluted one (the Wieseck). In the former case the bacterial numbers are highest when rain brings surface pollution; in the latter, when the sewage constantly present is least diluted.

MONTHLY VARIATIONS OF BACTERIA IN A NORMAL AND A POLLUTED STREAM.

(KISSKALT, 1906.)

Date.	Bacteria per c.c.		Date.	Bacteria per c.c.	
	Lahn.	Wieseck.		Lahn.	Wieseck.
1904			1904–5		
July . .	318	104,000	Dec.[1] . .	1220	21,200
July . . .	132	156,800	Jan.[1] . . .	3668	29,920
Aug. . . .	840	98,400	Feb.[1] . .	5380	11,900
Oct.[1] .	1235	28,400	Mar.[1] . .	1210	8250
Oct.[1] . . .	420	58,000	Apr.[1] . . .	4925	5910
Nov. . . .	2340	39,200	May . . .	570	14,800
Nov.[1] . . .	1740	52,000	June . . .	686	50,180
Dec.[1] . . .	780	28,600			

[1] Rain or high water due to previous thaw.

In standing waters all the tendencies which make for the reduction of bacteria are intensified, and when a river passes into a natural or artificial reservoir a more notable reduction in numbers occurs. The following table shows the striking effect produced upon the water of the Potomac River by its successive passage through the three reservoirs of the Washington water supply in the first nine months of 1907. We owe these figures to the courtesy of Mr. F. F. Longley, the engineer in charge of the Washington filter plant.

REDUCTION OF BACTERIA IN WASHINGTON RESERVOIRS.
BACTERIA PER C.C. MONTHLY AVERAGE, 1907.

	Potomac Reservoir.	Dalecarlia Reservoir.	Georgetown Reservoir.	Washington City Reservoir.
Jan.	4400	2400	2200	950
Feb.	1000	950	1000	750
Mar.	11,500	8300	7200	3600
Apr.	3700	2100	1400	475
May	750	350	325	130
June	2300	950	600	100
July	2700	600	350	160
Aug.	3000	275	425	80
Sept.	6200	...	1900	230

When the water which enters a pond or a reservoir has already undergone considerable storage and reached a comparatively stable condition, the diminution due to additional storage may be almost negligible. Thus Philbrick (1905) found that the influent water of the Chestnut Hill Reservoir of the Metropolitan Water Works contained on the average, during the eleven years, 1893–1903, 220 bacteria per c.c., and the effluent 179. In many individual months, and in some whole years, the effluent contained more than the influent.

The seasonal variations in the bacterial content of a large pond or lake follow a somewhat different course from those observed in a stream. Philbrick, in the paper just cited, gives the figures tabulated on following page for the Chestnut Hill Reservoir of the Metropolitan Water Works (Boston). The averages are based on weekly analyses covering the eleven years, 1893–1903.

MONTHLY VARIATIONS IN BACTERIAL CONTENT OF CHESTNUT HILL RESERVOIR, 1893–1903.

Month	J.	F.	M.	A.	M.	J.	J.	A.	S.	O.	N.	D.
Bacteria per c.c.	82	73	71	123	69	73	82	95	134	89	103	96

The marked increase in April and September is the notable feature of these analyses; and this is due to the effect of the spring and fall overturns which, in the months in question, stir up the decomposing organic matter at the bottom and distribute it through the reservoir. The slight, but steady, increase during the warm months from May to August is also probably significant.

On the whole it may be said that the bacterial content of a lake or pond should not be more than one or two hundred per c.c. and may often be under a hundred. The student will find numerous analyses of natural waters in Frankland's classic work (Frankland, 1894). He notes, for example, that the Lake of Lucerne contained 8 to 51 bacteria per c.c., Loch Katrine 74, and the Loch of Lintralthen an average of 170. The water of Lake Champlain examined by one of us (S. C. P.) in 1896 contained on an average 82 bacteria per c.c. at a point more than two miles out from the city of Burlington. Certain surface water-supplies near Boston, studied by Nibecker and one of us (Winslow and Nibecker, 1903), gave the following results:

City.	Number of Samples.	Average Number of Bacteria per c.c.
Wakefield	7	59
Lynn	6	16
Plymouth	6	35
Cambridge	5	94
Salem	5	232
Medford	5	524
Taunton	4	13
Peabody	3	141

In sea-water, too, bacterial numbers are small as noted by Russell at Naples (Russell, 1891) and Wood's Hole (Russell, 1892), and in salt as in fresh water the amount of bacterial life decreases in general as one passes downward from the surface and outward from the shore. Otto and Neumann (1904) obtained the results summarized below at various points on the high seas between Portugal and Brazil. Near the European coast, numbers were much higher.

BACTERIA IN THE ATLANTIC OCEAN.

(OTTO AND NEUMANN, 1904.)

Bacteria per c. c.

Nearest Land.	Depth in Meters.			
	5	50	100	200
Canary Islands	120	76	20	1
Cape Verde Islands	58	16	64	6
St. Paul Island	20	480	54	4
Pernambuco	48	168	83	14

The decrease in numbers which takes place when a surface water is stored in a pond or reservoir, indicates that the forces which tend to produce bacterial self-purification are important ones. It is necessary to consider in somewhat more detail just what these forces are, in order to gauge their potency in any particular instance.

Chief of them appear to be sedimentation, the activity of other micro-organisms, light, temperature, and food-supply, and perhaps more obscure conditions such as osmotic pressure.

The subsidence of bacteria either by virtue of their own specific gravity or as the result of their attachment to particles of suspended matter is unquestionably partly, if not largely, responsible for changes in the number of bacteria in the upper layers of water at rest or in very sluggish streams. The results of numerous investigations by different workers seem to indicate that sedimentation of the bacteria themselves takes place slowly, and that the difference in numbers between the top layer and the bottom layer of water in tall jars in laboratory experiments of a few days' duration is very slight or quite within the limits of experimental error (Tiemann and Gärtner, 1889). Different species may, of course, be differently affected (Scheurlen, 1891). It must be remembered, however, that in natural streams bacteria are to a great extent attached to larger solid particles upon which the action of gravity is more important. Spitta (1903) found that from one-fifth to one-half of the bacteria in

canal water may be attached to gross particles, as evidenced by their sedimentation in a few hours. Jordan (Jordan, 1900) is firmly of the opinion that in the lower part of the Illinois River, where there is a fall of but 30 feet in 225 miles, the influences summed up by the term sedimentation are sufficiently powerful to obviate the necessity for summoning another cause "to explain the diminution in numbers of bacteria," and he further adds: "It is noteworthy that all the instances recorded in the literature where a marked bacterial purification has been observed, are precisely those where the conditions have been most favorable for sedimentation."

Little is known as to the share of other organisms in hastening the decrease of bacteria in stored water. Doubtless predatory Protozoa play some part in the process. Huntemüller (1905), after infecting water containing Protozoa with typhoid bacilli, found the Protozoa crowded with bacteria; and he observed under the microscope the actual ingestion of the living and motile bacilli. Korschun (1907) and others have obtained similar results and consider the activity of Protozoa to be an important factor in self-purification.

Fehrs (1906) found that typhoid bacilli would live for 7 days in unsterilized Göttingen tap water, for 46 days in the same water sterilized, and for 13 days in water inoculated with a culture of flagellate Protozoa after sterilization. Water bacteria were of course added with the Protozoa.

Certain bacteriologists have held that the toxic waste products of the bacteria themselves may render water unfit for their own development. Horrocks (Horrocks, 1901), Garré (Garré, 1887), Zagari (Zagari, 1887), and Freudenreich (Freudenreich, 1888) have shown that an "antagonism" exists when bacteria are grown in artificial culture media such that the substratum which has supported the growth of one form may be rendered antiseptic to another. Frost (1904) has exhaustively studied the phenomenon of antagonism by exposing typhoid bacilli in collodion sacs to the action of certain soil and water bacteria growing in broth. Artificial culture media, however, offer conditions for bacterial development which are scarcely paralleled in natural waters. It is difficult to believe that under ordinary conditions poisons are produced of such power as to render a stream or lake specifically toxic for any particular type of bacteria. It appears indeed from the experiments of Jordan, Russell and Zeit (1904), and Russell and Fuller (1906), which will shortly be referred to more fully, that the life of typhoid germs is shorter in water containing large numbers of other bacteria than in that of greater purity. Horrocks (1899), too, found freshly isolated typhoid bacilli alive in sterile sewage after sixty days; while they disappeared in five days when B. coli was also present. These phenomena may be due, however, to a struggle for oxygen, or for food, rather than to the assumed presence of highly toxic bacterial products of which there is no independent evidence.

Temperature has a direct relation to bacterial life, and the number of parasitic bacteria at least may be quickly lessened by the action of cold. Sedgwick and one of us (Sedgwick and Winslow, 1902) have shown that of typhoid or colon bacilli in ice or cool water, over 40 per cent will perish in three hours and 98 per cent and upwards in two weeks. This diminution is not of course due to cold alone, but at high temperature the decrease is much less marked. At Harrisburg, Pa. (1907), an actual increase of colon bacilli was observed in a small reservoir during a warm period when its temperature approximated blood heat.

Many investigations conducted since the pioneer researches of Downes and Blunt (Downes and Blunt, 1877) have confirmed the results reported by them, which showed that direct sunlight is fatal to most bacteria in the vegetative state and even to spores if the exposure be sufficiently long, while diffused light is harmful in a less degree. Opinions vary as to the degree to which light is active in destroying the bacteria in natural waters. Buchner (Buchner, 1893) found by experiment that the bactericidal power of light extends to a depth of about three meters before it becomes imperceptible. On the other hand, Procaccini (Procaccini, 1893) found that when sunlight was passed vertically through 60 cm. of drain-water the lower layers contained nearly as many bacteria after three hours' treatment as before exposure. The middle and upper portions showed a great falling off in numbers, however.

But few studies have been made of the effect of light on bacteria in flowing water. Jordan (Jordan, 1900) has investigated several Illinois streams, and arrived at the conclusion that in moderately turbid water, at least, the sun's rays are virtually without action. On the other hand, Rapp has observed a considerable reduction of the bacteria in the Isar at Pullach after the period of diurnal insolation, as shown by the table on page 20.

It is unnecessary to dwell in detail upon the effect which the lack of nutritive elements must exert upon intestinal bacteria and soil bacteria in waters of ordinary purity. Comparative studies of culture media, to be quoted in the succeeding chapter, will show how delicately the bacteria respond to comparatively slight changes in their food supply. Wheeler (1906) found that typhoid bacilli would persist in almost undiminished numbers in sterilized water from a polluted well, containing considerable organic matter and kept in the dark at 20 degrees, while in purer water or in the light they died out in from two to six weeks.

Whipple and Mayer (1906) have called attention to another important factor in the general problem. They find that the presence of oxygen is essential to the persistence of typhoid and colon bacilli in water, although in nutrient media both forms may thrive under anærobic conditions.

EFFECT OF OXYGEN ON VIABILITY OF TYPHOID BACILLI IN STERILE TAP WATER.
(WHIPPLE AND MAYER, 1906.)

Period in Days.	Tubes kept in Air.		Tubes kept in Hydrogen.	
	Bacteria per c.c.	Per Cent.	Bacteria per c.c.	Per Cent.
0	600,000	100.0	600,000	100.0
2	455,000	76.0	2,400	0.4
4	190,000	32.0	25	0.004
8	120,000	20.0	0	0.0
12	67,000	11.0	0	0.0
18	25,000	4.2	0	0.0
26	9,250	1.5	0	0.0
33	2,150	0.6	0	0.0
40	132	0.02	0	0.0
47	6	0.001	0	0.0
54	0	0.000	0	0.0

Various inorganic constituents of the medium undoubtedly exercise an important influence upon the life of bacteria in water; and the mutual interaction of the different substances present is a highly complex one. Thus Winslow and Lochridge (1906) report that five parts of dissociated hydrogen per million parts of tap water (.005 normal HCl) is fatal to typhoid bacilli, while ten times as much acid is required for sterilization when one per cent of peptone is present to check the dissociation of the hydrogen.

Although it is hard to estimate the exact importance of each factor, the general phenomena of the self-purification of streams are easy to comprehend. A small brook immediately after the entrance of polluting material from the surface of the ground contains many bacteria from a diversity of sources. Gradually those organisms adapted

EXAMINATIONS OF THE ISAR AT PULLACH.

(RAPP, 1903.)

(A) Carried out September 26, 1898, no rain having fallen for three weeks.

Temperature		Time of the Experiment.	Bacteria per c.c.
of the Water.	of the Air.		
13.0° C.	8.8° C.	7.30 p.m.	146
12.1° C.	7.0° C.	9.30 p.m.	270
10.5° C.	6.2° C.	5.00 a.m.	370
10.2° C.	8.2° C.	8.00 a.m.	320

(B) Carried out November 28, 1898, no rain having fallen for some time.

5.5° C.	3.0° C.	6.00 p.m.	266
5.5° C.	2.5° C.	8.00 p.m.	402
5.5° C.	2.0° C.	10.00 p.m.	482
5.0° C.	2.0° C.	3.00 a.m.	532
4.5° C.	2.5° C.	7.30 a.m.	400

to life in the earth or in the bodies of plants and animals die out, and the forms for which water furnishes ideal conditions survive and multiply. It is no single agent which brings this about, but that complex of little-understood conditions which we call the environment. If any one thing is of prime importance it is probably the food-supply, for only certain bacteria are able to multiply in the presence of the small amount of organic matter present in ordinary potable waters. As Jordan (Jordan, 1900) has said: "In the causes connected with the insufficiency or unsuitability of the food-supply is to be found, I believe, the main reason for the bacterial self-purification of streams."

It is obvious that the efficiency of all the agencies which tend to decrease the number of bacteria in surface waters will increase with the prolongation of the period for which they act. Time is the great measure of self-purification. The longer the storage period, the greater the improvement.*

* The absolute time necessary to remove disease bacteria and render a polluted water safe for drinking is impossible to fix with any certainty. Food supply, light, temperature and the activity of other living forms vary widely, and in deposited material conditions are different from those which obtain in the water itself. Jordan, Russell and Zeit (1904), in an important series of experiments, added typhoid bacilli to the unsterilized waters of Lake Michigan, the Chicago River and Drainage Canal and the Illinois River, in collodion sacs suspended in the respective bodies of water. From the relatively pure Lake Michigan water the specific organisms could be isolated for at least a week, but in the polluted waters of the rivers and the Drainage Canal they were not found after three days except in a single instance. Russell and Fuller, (1906) confirmed these general results, finding that typhoid bacilli would live for ten days in the unsterilized water of Lake Mendota while they could isolate them only after five days when immersed in sewage. Other observers record much greater viability for the typhoid bacillus. Savage (1905) added a heavy dose of the organism to unsterilized tidal mud and found it living after five weeks. Hoffmann (1905), after inoculating a large aquarium with a rich typhoid culture, was able to isolate the germ from the water after four weeks and from the mud at the bottom after two months. Konrádi (1904) reports the persistence of typhoid bacilli in unsterilized tap water for over a year.

Under certain conditions it is even possible for intestinal bacteria to multiply in water. At Harrisburg, Pa., a series of B. coli examinations made in the midsummer of 1906 showed positive results in 7 per cent of the samples of water entering the storage reservoir and in 27 per cent of the samples leaving it. The storage period in this case was about two days and the temperature of the water in the reservoir nearly at blood heat (Harrisburg, 1907). It is improbable that true pathogenic germs like the typhoid bacillus could increase even under such exceptional conditions.

In general we have seen that surface-waters tend continually to decrease in bacterial content after their first period of contact with the humus layer of the soil. In that other portion of the meteoric water which penetrates below the surface of the earth to join the reservoir of ground-water, later to reappear as the flow of springs and wells, this diminution is still more marked since the filtering action of the earth removes not only most of the bacteria but much of their food material as well. The numbers of bacteria in the soil itself decrease rapidly as one passes downward. Kabrhel (1906) found several million per c.c. in surface samples of woodland soil, a few thousands or tens of thousands half a meter below and usually only hundreds in centimeter samples collected at depths greater than a meter. Many observers formerly believed that all ground-waters were nearly free from bacteria, because often no colonies appeared on plates counted after the ordinary short periods of time. If, however, a longer period of incubation be adopted considerable numbers may be obtained.

For convenience we may divide ground-waters into three groups, namely: shallow open wells, springs, and "tubular" (driven) or deep wells. This division is important because ordinary shallow wells form a group by themselves in respect to the possibility of aërial and surface contamination, their water often being fairly rich in bacterial life. Egger (Wolffhügel, 1886) examined 60 wells in Mainz and found that 17 of them contained over 200

bacteria to the cubic centimeter. Maschek (Maschek, 1887) found 36 wells out of 48 examined in Leitmeritz which had a bacterial content of over 500 per c.c. Fischer (Horrocks, 1901) reported 120 wells in Kiel which gave over 500 bacteria per c.c. and only 51 with less than that number.

In the ordinary standard 48-hour period very few bacteria develop from normal spring-waters. Thus in an examination of spring-waters made by the Massachusetts State Board of Health in 1900 (Massachusetts State Board of Health, 1901), of 37 springs which were practically unpolluted and had less than 0.10 parts per 100,000 excess of chlorine over the normal, 54 samples were examined and gave an average of 41 bacteria per c.c. Only 6 samples showed figures over 50.

It now remains to consider the other great division of ground-waters, namely, deep, "driven," or "tubular" wells, which, if carefully constructed, should ordinarily be free from all surface-water contamination, and should show low bacterial counts. The results tabulated below obtained by Houston in the examination of a series of deep wells of high quality at Tunbridge Wells are fairly typical.

BACTERIAL CONTENT OF DEEP WELL WATERS.
(HOUSTON, 1903.)

Bacteria per c.c.				
36	6	9	4	1
16	17	4	3	12
2	4	10	5	2

Fifteen driven wells in the neighborhood of Boston, examined in 1903, showed at the end of 48 hours an average of only 18 colonies per c.c.; and the results of certain examinations of other wells and springs, recently made by the authors, are given in the table below.

BACTERIA IN DEEP WELL AND SPRING WATERS.

Town.	Bacteria per c.c.	Town.	Bacteria per c.c.
Worcester, Mass. .	10	Saranac Lake, N.Y.	11
Waltham, Mass. . .	3	Ellenville, N. Y. .	0
Newport, R. I.. . .	7	Hyde Park, Mass.	12

It is plain that water absolutely free from bacteria is not ordinarily obtained from any source. In deep wells, however, their number is small; and the peculiar character of the organisms present is manifested in many cases by the slow development at room temperature (frequently no growth until the third day), the entire absence of liquefying colonies, and the abundance of chromogenic species.

CHAPTER II.

THE QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF WATER.

THE customary methods for determining the number of bacteria in water do not reveal the total bacterial content, but only a very small fraction of it, as becomes apparent when we consider the large number of organisms, nitrifying bacteria, cellulose-fermenting bacteria, strict anærobes, etc., which refuse to grow, or grow only very slowly in ordinary culture media, and which, therefore, escape detection. On the one hand, certain obligate parasites cannot thrive in the absence of the rich fluids of the animal body; on the other hand, the prototrophic bacteria adapted to the task of wrenching energy from nitrates and ammonium compounds are unable to develop in the presence of so much organic matter. Winslow (1905), in the examination of sewage and sewage effluents, found 20–70 times as many bacteria by microscopic enumeration as by the gelatin plate count. Certain special media enable us to obtain much larger counts than those yielded by the ordinary gelatin method. The Nährstoff Heyden agar, for example, has been strongly advocated by Hesse (Hesse and Niedner, 1898) and other German bacteriologists upon this ground. In this country Gage and Phelps (Gage and Phelps, 1902) showed that the numbers obtained

by the ordinary procedure were only from 5 to 50 per cent of those obtained by the use of Heyden's Nährstoff agar. For practical sanitary purposes, however, our methods are fairly satisfactory. Within limits, it is of no great importance that one method allows the growth of more bacteria than another. When we are using the quantitative analysis as a measure of sewage pollution the essential thing is that the section of the total bacterial flora which we obtain should be thoroughly representative of that portion of it in which we are most interested — the group of the quickly growing, rich-food-loving sewage forms. In this respect meat-gelatin-peptone appears to be unrivalled; and it is in this respect that such media as Nährstoff agar fail. Müller (1900) showed that the larger counts obtained by plating on the Nährstoff medium are due to the fact that it specially favors the more prototrophic forms, among the water bacteria themselves. Intestinal organisms and even the ordinary putrefactive germs, when plated in pure culture, show no higher counts on Nährstoff agar than on gelatin. Gage and Adams (1904) found by plating pure cultures of the common laboratory bacteria, saprophytes, and parasites, that Nährstoff counts were actually lower than those obtained by the use of gelatin. When sewage and highly polluted waters are examined, counts are slightly higher on Nährstoff media, while with purer waters the Nährstoff numbers are far in excess of those obtained with gelatin. Winslow (1905) found the ratio of Nährstoff agar to gelatin count to be 1.7 to 1.0 for

sewage, and 4.8 to 1.0 for sand filter effluent. With waters of still better quality the ratio goes higher, reaching a maximum when the bacteria which increase and multiply in pure water are most abundant. Müller (1900) found, for example, that water which normally showed six times as many bacteria on Nährstoff agar as on gelatin might give a Nährstoff-gelatin ratio of 20–30 after it had been standing for some time in the supply pipes. The table below, taken from the valuable paper by Gage and Phelps (1902), shows strikingly the different Nährstoff-gelatin ratios for waters of various grades of purity.

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON REGULAR AGAR AND ON NÄHRSTOFF AGAR FOR DIFFERENT CLASSES OF WATERS.

(GAGE AND PHELPS, 1902).

REGULAR AGAR.

Class of Water.	Days' Count.						
	2	3	4	5	6	7	8
Ground-water . .	0	5	6	6	6	6	6
Filtered water . .	6	7	7	7	7	7	7
Merrimac River .	6	7	7	8	8	9	9
Filtered sewage .	14	17	18	19	19	19	19
Sewage . .	34	44	46	46	46	46	46
NÄHRSTOFF AGAR.							
Ground-water . .	6	43	78	88	93	100	100
Filtered water .	37	69	80	92	98	100	100
Merrimac River .	29	78	93	97	97	99	100
Filtered sewage .	26	65	93	95	97	99	100
Sewage	39	75	95	100	100	100	100

It is obvious from all these facts that the effect of using the Nährstoff medium is to increase disproportionately the bacterial counts obtained from purer waters and thus to diminish the difference in bacterial content between normal and contaminated sources. The ordinary gelatin medium, on the other hand, is adapted to the growth of intestinal and putrefactive forms and, therefore, serves best the prime object of bacteriological water examination.

The first requisite in a procedure for water analysis is, that it should be adapted to the end in view, the differentiation of pure and contaminated waters. The second and equally important requirement is, that the procedure should be a standard one, so that results obtained at different times and by different observers may be comparable. In this respect the work of G. W. Fuller, G. C. Whipple, and other members of the Committee on Standard Methods of the American Public Health Association has placed the art of quantitative water analysis in this country in a very satisfactory state by contrast with the varying practices which prevail in England and Germany. The first report on this question was made in 1897 (Committee of Bacteriologists, 1898). A permanent Committee on Standard Methods was then formed which reported in 1901 (Fuller, 1902), and again in 1904 (Committee on Standard Methods of Water Analysis, 1905), recommending in considerable detail a standard routine procedure for the quantitative

and qualitative bacteriological examination of water for sanitary purposes. These reports have had a far-reaching effect in simplifying and unifying the methods of water analysis. Similar results may be expected from the work of the English Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water which reported in 1904, although this committee unfortunately did not consider the process of media making in great detail. The last report of the American Committee on Standard Methods (1905) will be adhered to in this and succeeding chapters unless otherwise specifically stated; and that portion of its report which deals with methods of making media will be found in full in the Appendix.

The procedure for the quantitative determination of bacteria in water consists, in brief, in mixing a definite amount of a suitably collected specimen of the water with a sterile solidifiable culture medium and allowing it to develop for a sufficiently long time to permit reproduction of the bacteria and the formation of visible colonies which may be counted. The process is divided naturally into four stages — sampling, plating, incubating, and counting.

Sampling. — All samples of water for bacteriological examination should be collected in clean, sterile bottles with wide mouths and glass stoppers, preferably of the flat mushroom type. It is desirable that these bottles should have a capacity of at least 100 c.c.

They should be cleaned thoroughly before using, by treatment with sulphuric acid and potassium bichromate or with alkaline permanganate of potash, followed by sulphuric acid, dried by draining, and sterilized by dry heat at 160° C. for at least one hour, or by steam at 115–120 degrees for fifteen minutes. If not to be used immediately the neck and stopper should be protected against dust or other contamination by wrapping with lead-foil. For transportation the bottle should be enclosed in a suitable case or box.

The greatest care must be taken that the fingers do not touch the inside of the neck of the bottle or the cone of the stopper, as the water thereby would become seriously contaminated and rendered unfit for examination. It is well known that bacteria are found abundantly upon the skin, and Winslow (Winslow, 1903) has shown that even B. coli is present upon the hands in a considerable number of cases.

In order to obtain a fair sample, great precautions must be taken, and these will vary with the different classes of waters to be examined and with local conditions. If a sample is to be taken from a tap, the water should be allowed to flow at least five minutes (if from a tap in regular use) or for a longer period in case the water has been standing in the house service system. In the small pipes, changes in bacterial content are liable to occur, certain species dying and others multiplying.

If a sample is to be taken from a pump similar pre-

cautions are necessary. The pump should be in continuous operation for five minutes at least, and preferably for half an hour before the sample is taken, in order to avoid excessively high numbers due to the growth of bacteria within the well and pump, the bacterial condition of the water as it passes through the ground being what we wish to determine. Thus Heræus (Heræus, 1886), in a well-water which had been but little used during the preceding thirty-six hours, found 5000 organisms per c.c.; when the well was emptied by continuous pumping, a second sample, after an interval of half an hour, gave only 35. Maschek (Tiemann and Gärtner, 1889) obtained similar results shown in the following table:

EFFECT OF PUMPING ON THE BACTERIAL CONTENT OF WELL-WATER.

Well-water	after continuous pumping for fifteen minutes	458
"	after continuous pumping for many hours	140
"	later	68
"	after continuous pumping for fifteen minutes	578
"	after continuous pumping for many hours	179
"	later	73

After a proper interval of pumping the sample of a well-water may be collected from the pet-cock of the pump or from a near-by tap. With a hand-pump, such as is found in domestic shallow wells, the water is, of course, pumped directly into the sample bottle. The difficulties in securing an average sample from this latter source are often great, since if the flooring about the pump is not tight, as is

usually the case, continued pumping may wash in an unusual amount of surface pollution.

In sampling surface-waters, the greatest precautions must be observed to prevent contamination from the fingers. In still waters the fairest sample is one taken from several inches down, as the surface itself is likely to have dust particles floating upon it. The method most frequently recommended is to plunge the bottle beneath the surface to a depth of a foot or so, then removing the stopper and allowing the bottle to fill.

Another method which is comparatively free from objection, and which has been employed by the writers, is to remove the stopper first and then, holding the bottle by the base, plunge it mouth downward into the water, turning it at the desired depth so as to replace the enclosed air by the water. Whenever any current exists, the mouth of the bottle should be directed against it in order to carry away any bacteria from the fingers. If there is no current, a similar effect can be produced by turning the bottle under water and giving it a quick forward motion. In rapidly flowing streams it is only necessary to hold the bottle at the surface with the mouth pointed up-stream.

For taking samples of water at greater depths, a number of devices have been employed, all of which are fairly satisfactory. The essentials are, first, a weight to carry the bottle down to the desired depth, and, second, some method of removing the stopper when that depth

is reached. The student will find one good form of apparatus described in Abbott's "Principles of Bacteriology" (Abbott, 1899); an admirable one was devised by Hill and Ellms (Hill and Ellms, 1898); and Thresh (1904) figures an ingenious device for the same purpose. Miquel and Cambier (Miquel and Cambier, 1902) and other authors recommend the use of a sealed glass bulb with a capillary tube which can be broken off at the desired moment.

As soon as a sample of water is collected, its conditions of equilibrium are upset and a change in the bacterial content begins. Even in the purest spring-waters, which contain but few bacteria when collected, and in which the amount of organic matter is infinitesimal, enormous numbers may be found after storage under laboratory conditions for a few days or even a few hours. In some cases the rise in numbers is gradual, in others very rapid. The Franklands (Frankland, 1894) record the case of a deep-well water in which the bacteria increased from 7 to 495,000 in three days. Miquel (Miquel, 1891) from his researches, arrived at the conclusion that in surface-waters the rise is less rapid than in waters from deep wells or springs, and that in the latter case the decrease, after reaching a maximum, is likewise rapid and steady. Just how far protection from light, increase in temperature, and a destruction of higher micro-organisms is responsible for the increase, and to what extent an exhaustion of food-supply or the formation of toxic waste

products causes the succeeding decrease, we are not aware; but the facts are well established.

Whipple has exhaustively studied the details of this multiplication of bacteria in stored waters, and has shown in the table given below that there is first a slight reduction in the number present, lasting perhaps for six hours, followed by the great increase noted by earlier observers. It is probable that there is a constant increase of the typical water bacilli, overbalanced at first by a reduction in other forms, for which the environment is unsuitable.

BACTERIAL CHANGES IN WATER DURING STORAGE.

(WHIPPLE, 1901.)

Sample.	Initial Temperature.	Temp. of Incubation of Sample.	Number of Bacteria per c.c.				
			Initial.	After 3 Hours.	After 6 Hours.	After 24 Hours.	After 48 Hours.
	C.	C.					
A	7.6°	17.0°	260	215	230	900	27,000
B	7.6°	17.0°	260	245	255	720	10,850
C	7.6°	12.5°	260	270	231	600	2,790
D	7.6°	12.5°	260	270	245	710	1,800
E	7.6°	2.4°	260	243	210	675	1,980
F	7.6°	2.4°	260	235	270	560	1,980
G	11.0°	12.8°	77	55	58	101	10,250
H	11.0°	12.8°	77	53	74	87	2,175
I	11.0°	23.6°	77	51	52	11,000	41,400
J	6.7°	20.0°	430	375	245	...	385,000[1]
K	6.7°	20.0°	430	345	405	...	750,000[1]
L	23.2°	23.0°	510	340	230	8,000	20,000
M	23.2°	2.5°	525	300	220	380	2,200

[1] 0.0005 per cent peptone added to the water.

EFFECT OF SIZE OF VESSEL UPON THE MULTIPLICATION OF WATER BACTERIA DURING STORAGE.

(WHIPPLE, 1901.)

Sample	Bottle.	Temp. of Incubation.	Number of Bacteria per c.c.					
			Initial.[1]	After 3 hrs.	After 6 hrs.	After 12 hrs.	After 24 hrs.	After 48 hrs.
		C.						
A	1-gallon	13°	77	63	65	47	42	175
B	2-quart	13°	77	59	63	60	45	690
C	1-quart	13°	77	63	63	47	46	325
D	1-pint	13°	77	57	61	36	38	630
E	2-ounce	13°	77	55	58	47	101	10,250
F	1-gallon	24°	77	81	97	275	290	300
G	2-quart	24°	77	92	59	62	180	250
H	1-quart	24°	77	84	77	46	340	900
I	1-pint	24°	77	51	46	100	2,950	7,020
J	2-ounce	24°	77	51	52	145	11,000	41,400

[1] Average of five plates.

Wolffhügel and Riedel (Wolffhügel and Riedel, 1886) noted the dependence of this multiplication on the air-supply, vessels closed with rubber stoppers showing lower numbers than those plugged with cotton. Similarly, Whipple found that the multiplication of bacteria was much greater when bottles were only half full than when they were filled completely; and also, as shown in the above table that the size of the bottle markedly influenced the growth.

An important series of investigations by Kohn (1906) suggests that this phenomenon of multiplication during storage may be due in part to the solution of certain constituents of glass which favor bacterial life, since the

increase is notably greater in bottles of the more soluble glasses.

Whipple's tables, quoted above, show that the multiplication during storage was greater at a higher temperature; and this is a well recognized general rule. In order to obviate the abnormal results of storage increase it is therefore obvious that samples must be examined shortly after collection, and that they must be kept cool during their necessary storage. If fairly pure waters are placed upon ice and kept between 0 degrees and 10 degrees, they will show no material increase in twelve hours. With polluted water, however, another danger is here introduced. Samples of such water when packed in ice show a marked decrease due to the large number of sensitive intestinal bacteria present. Jordan (Jordan, 1900) found that three samples of river-water packed in ice for forty-eight hours fell off from 535,000 to 54,500; from 412,000 to 50,500, and from 329,000 to 73,000, respectively. It is, therefore, important that even iced samples should not be kept too long; and it is desirable to adhere strictly to the recommendations of the Standard Methods Committee that the interval between sampling and examination should not exceed twelve hours in the case of relatively pure waters, six hours in the case of relatively impure waters, and one hour in the case of sewage.

Plating. — The bottle containing the sample of water is first shaken at least twenty-five times in order to get an

equal distribution of the bacteria. If the number of bacteria present is probably not greater than 200, 1 c.c. is then withdrawn with a sterile 1 c.c. pipette and delivered into a sterile Petri dish of 10 cm. diameter. To this is added 5 c.c. of standard 10 per cent gelatin at a temperature of about 30° C. or standard agar (7 c.c.) at 40°–42° C. Should the number of bacteria per c.c. probably exceed 200, dilution is necessary. This is best accomplished by adding 1 c.c. of the water in question to 9, 99 or 999, etc., c.c. of sterile tap water according to the amount of dilution required. The diluted sample is then shaken thoroughly and 1 c.c. taken for enumeration. In order to determine the number of bacteria originally present it is only necessary to multiply by the factor 10, 100, or 1000, etc.

When a sample of water from an unknown source is to be examined it is generally desirable to make two check plates at each of the above dilutions, selecting those dilutions which give nearest to 200 colonies on the plates after incubation as the ones on which to rely for the count. A much smaller number will not give average figures, and if more than 200 colonies are present on a plate many bacteria will be checked by the waste products of those which first develop and the count obtained will be too low. After the addition of the diluted sample and the nutrient medium, their thorough mixture in an even layer on the bottom of the plate is obtained by careful tipping and rotation.

It was formerly customary to mix the water with the gelatin in the tube before pouring into the plate, but this method is objectionable because there is always a residuum of medium remaining in the tube which will retain varying numbers of bacteria and thus interfere with the accuracy of the count. Before pouring the medium into the plate the mouth of the tube should be flamed to remove any possibility of contamination.

The exact composition of the medium is, of course, of prime importance in controlling the number of bacteria which will develop. The figures previously cited in connection with the discussion of Hesse's Nährstoff agar show how bacterial counts may vary with media of widely different composition. The table on page 39, quoted from Gage and Phelps (1902), shows the considerable differences which may be due to the presence or absence of meat infusion, peptone, etc., in media of generally similar character (compare the figures for plain gelatin, pepton gelatin, and meat gelatin). Much slighter variations than this, however, are significant. The reaction of the medium was found as early as 1891 to be important, for Reinsch (Reinsch, 1891) showed in that year that the addition of one one-hundredth of a gram of sodium carbonate to the liter increased sixfold the number of bacteria developing. Fuller (Fuller, 1895) and Sedgwick and one of us (Sedgwick and Prescott, 1895), working independently, established the fact that an optimum reaction existed for most water bacteria, and that a

deviation either way decreased the number of colonies developing.

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON MEDIA OF DIFFERENT COMPOSITIONS.

(GAGE AND PHELPS, 1902.)

Medium.	Days' Count.							
	2	3	4	5	6	7	8	9
Nährstoff agar	19	60	78	85	95	99	99	100
Nährstoff pepton agar	10	22	26	28	30	30	30	30
Pepton agar	11	16	22	23	24	24	24	24
Meat agar	8	13	16	17	17	17	17	17
Plain agar	8	10	13	14	14	14	14	14
Regular agar	7	9	11	11	11	11	11	11
Nährstoff glycerin agar	6	10	11	11	11	11	11	11
Nährstoff meat agar	7	7	8	8	10	10	10	10
Meat gelatin	12	19	24	26	26	26	26	26
Pepton gelatin	7	12	18	20	20	20	20	20
Standard gelatin	8	10	11	12	13	13	13	13
Plain gelatin	1	6	12	13	13	13	13	13
Nährstoff gelatin	5	6	9	11	13	13	13	13

Whipple (Whipple, 1902) has shown that not only the particular kind of gelatin used but its exact physical condition as affected by sterilization and other previous treatment will materially affect the results obtained. Gage and Adams (1904) found marked differences in counts as the result of the use of the two best known commercial peptones. A long series of waters plated on agar made up with Merck's and Witte's peptones, respectively, showed the following average relative results:

AVERAGE RELATIVE NUMBER OF BACTERIA ON PEPTON AGAR WITH DIFFERENT PEPTONS.

(GAGE AND ADAMS, 1904.)

Days	2	4	6	8	10	12
Merck's	9	33	51	67	89	98
Witte's	38	53	100	100	100	100

The same authors showed that the composition of the water used exercised a marked selective action upon the development of bacteria. Agar made up with sewage permitted a maximum growth of sewage bacteria and showed no colonies when inoculated with filtered city water. On the other hand agar made up with city water showed 100 per cent of the bacteria present in city water and river water, three-quarters of those present in sewage and less than half of those present in sewage effluents.

Hesse (1904) found that the number of bacteria developing on Nährstoff agar varied with the composition of the glass tubes in which the media had previously been sterilized. The more soluble glasses yielded sufficient alkali to the medium to inhibit four-fifths of the bacteria present in certain cases.

All these facts make it evident that only the strictest adherence to a standard method can ensure comparable results; the ordinary nutrient gelatin should then in all practical sanitary work be made up from distilled water, meat infusion, pepton and gelatin, in exact ac-

cordance with the directions of the Standard Methods Committee.

Even the standard procedure fails to ensure uniformity in one important respect. The meat infusion which it calls for is in itself a highly variable quantity. Gage and Adams (1904), in the examination of fifteen lots of beef infusion, found variations of nearly one per cent in organic solids (calculated on the weight of the whole infusion), after the final filtration. The organic constituents of the meat infusion varied, therefore, among themselves by nearly the total amount of peptone added. It is to be hoped that the standard methods may soon be so revised as to eliminate this necessarily uncertain constituent of nutrient media. Criticisms of detail must, however, give way to the importance of securing fairly comparable results; and the confusion which would follow the use by individual bacteriologists of media made without meat would outbalance the errors inherent in the standard procedure.

Incubation. — Incubation should take place in a dark, well-ventilated chamber where the temperature is kept substantially constant at 20 degrees and where the atmosphere is practically saturated with moisture. It has been shown by Whipple (Whipple, 1899) and others that the number of bacteria developing in plate cultures is to an appreciable extent dependent upon the presence of abundant oxygen and moisture. Thus, reckoning the number of bacteria developing in a moist chamber at 100, the

percentage counts obtained in an ordinary incubator were as follows: 75 when the relative humidity of the incubator was 60 per cent of saturation; 82 when it was 75 per cent; 98 when it was 95 per cent. This source of error may be avoided by the use of ventilated dishes and by the presence of a pan of water in the incubating chamber.

According to American and German practice, plates made for sanitary water analysis are counted at the end of forty-eight hours. The English Committee appointed to consider the standardization of methods for the Bacterioscopic Examination of Water (1904) fixed the time at 72 hours. French bacteriologists, and some Germans (Hesse and Niedner, 1906), still recommend longer periods, and the table on p. 43 from Miquel and Cambier (Miquel and Cambier, 1902) shows that many bacteria fail to appear in our ordinary procedure. It is, however, in the main, the characteristic water bacteria which develop slowly, sewage bacteria almost without exception being rapid growers. The longer period of incubation is, therefore, not only inconvenient but undesirable, since it obscures the difference between good and bad waters.

Counting. — The number of bacteria is determined by counting the colonies developed upon the plate, with the aid of a lens magnifying at least five diameters. For convenience in counting, the plate may be placed upon a glass plate ruled in centimeter squares and set over a black tile, or the tile itself may be ruled. As has already been said, it is desirable that the number of colonies should not

exceed 200, for when the number is very high the colonies grow only to a small size, making counting laborious and inaccurate, and many do not develop at all. The best results are obtained with numbers ranging from 50 to 200.

EFFECT OF THE LENGTH OF INCUBATION OF WATER BACTERIA IN GELATIN UPON THE NUMBER OF COLONIES DEVELOPING.

(MIQUEL AND CAMBIER, 1902.)

Length of Incubation.	Colonies Developed.	Length of Incubation.	Colonies Developed.
1 day	20	9 days	821
2 days	136	10 days	859
3 days	254	11 days	892
4 days	387	12 days	921
5 days	530	13 days	951
6 days	637	14 days	976
7 days	725	15 days	1000
8 days	780		

When it is possible to do so, all the colonies on the plate should be counted. When they exceed 400 or 500 it is often easier, and fully as accurate, to count a fractional part of the plate and estimate the total number therefrom. This should not be done, however, except in case of necessity.

It is customary in determining numbers to make plates in duplicate, thereby affording a check upon one's own work. Owing to the lack of precision in the method, the limit of experimental error is a wide one. It should be

possible for careful manipulators to obtain results within 10 per cent of each other, but a closer agreement than this is hardly to be expected. It has been suggested by the Committee of the American Public Health Association, that the following mode of expressing results be adopted in order to avoid the appearance of a degree of accuracy which the methods do not warrant.

NUMBERS OF BACTERIA FROM

1–50	shall be recorded to the nearest unit						
51–100	"	"	"	"	"	"	5
101–250	"	"	"	"	"	"	10
251–500	"	"	"	"	"	"	25
501–1000	"	"	"	"	"	"	50
1001–10,000	"	"	"	"	"	"	100
10,001–50,000	"	"	"	"	"	"	500
50,001–100,000	"	"	"	"	"	"	1,000
100,001–500,000	"	"	"	"	"	"	10,000
500,001–1,000,000	"	"	"	"	"	"	50,000
1,000,001–5,000,000	"	"	"	"	"	"	100,000

The determination of numbers of bacteria in water in the field has frequently been attempted. Since the laboratory method of "plating out" is difficult to use in field work, the Esmarch tube process has often been employed. This consists in introducing into a tube of melted gelatin or agar 1 c.c. of the water, and then rotating the tube until the medium has solidified in a thin layer on the inner wall. Other bacteriologists have devised ingenious field kits for adapting the plate method to this purpose. The opportunity for air infection in work done outside a proper

laboratory is, however, always great, and it is almost impossible to secure proper conditions for incubation in any temporary establishment. On the whole, the authors are of the opinion that laboratory examinations are to be preferred to those made in the field, if a laboratory can be reached within twelve hours of the time of collection of the samples.

CHAPTER III.

THE INTERPRETATION OF THE QUANTITATIVE BACTERIOLOGICAL ANALYSIS.

THE information furnished by quantitative bacteriology as to the antecedents of a water is in the nature of circumstantial evidence and requires judicial interpretation. No absolute standards of purity can be established which shall rigidly separate the good from the bad. In this respect the terms "test" and "analysis" so universally used are in a sense inappropriate. Some scientific problems are so simple that they can be definitely settled by a test. The tensile strength of a given steel bar, for example, is a property which can be absolutely determined. In sanitary water examination, however, the factors involved are so complex, and the evidence necessarily so indirect, that the process of reasoning much more resembles a doctor's diagnosis than an engineering test.

The older experimenters attempted to establish arbitrary standards, by which the sanitary quality of a water could be fixed automatically by the number of germs alone. Thus Miquel (Miquel, 1891) published a table according to which water with less than 10 bacteria per c.c. was "excessively pure," with 10 to 100 bacteria,

"very pure," with 100 to 1000 bacteria, "pure," with 1000 to 10,000 bacteria, "mediocre," with 10,000 to 100,000 bacteria, "impure," and with over 100,000 bacteria, "very impure." Few sanitarians would care to dispute the appropriateness of the titles applied to waters of the last two classes; but many bacteriologists have placed the standard of "purity" much lower. The limits set by various German observers range, for example, from 50 to 300. Dr. Sternberg (Sternberg, 1892) in a more conservative fashion, has stated that a water containing less than 100 bacteria is presumably from a deep source and uncontaminated by surface drainage; that one with 500 bacteria is open to suspicion; and that one with over 1000 bacteria is presumably contaminated by sewage or surface drainage. This is probably as satisfactory an arbitrary standard as could be devised, but any such standard must be applied with great caution. The source of the sample is of vital importance in the interpretation of analyses; a bacterial count which would condemn a spring might be quite normal for a river; only figures in excess of those common to unpolluted waters of the same character give the indication of danger. Furthermore, the bacteriological tests are far more delicate than any others at our command, very minute additions of food material causing an immense multiplication of the microscopic flora. This delicacy necessarily requires, both in the process of analysis and the interpretation of results, a high degree of caution. As pointed out in the previous chapter, the touch of a

finger or the entrance of a particle of dust may wholly destroy the accuracy of an examination. Even the slight disturbance of conditions, incident upon the storage of a sample after it has been taken, may in a few hours wholly alter the relations of the contained microbic life. It is necessary, then, in the first place, to exercise the greatest care in allowing for possible error in the collection and handling of bacteriological samples; and in the second place, only well-marked differences in numbers should be considered significant.

In the early days of the science, discussion ran high as to the interpretation of bacteriological analysis, and particularly as to the relation of bacterial numbers to the organic matter present in a water. Different observers obtained inconsistent results, and Bolton (Bolton, 1886) concluded that there was no relation whatever between the chemical composition of a water and its bacterial content. Tiemann and Gärtner (Tiemann and Gärtner, 1889) furnished the key to the difficulty in their statement that there are two classes of bacteria, the great majority of species, normally occurring in the earth or in decomposing organic matter, which require abundance of nutriment, and certain peculiar water bacteria which can multiply in the presence of such minute traces of ammonia as are present in ordinary distilled water. Even these prototrophic or semi-prototrophic forms require a definite amount of food of their own kind.

Kohn (1906) determined the minimal nutrient material requisite for certain of the latter, and found that they could develop in the presence of 198×10^{-10} to 198×10^{-13} per cent of dextrose, 66×10^{-13} to 66×10^{-17} per cent ammonium sulphate and 66×10^{-13} to 66×10^{-19} per cent ammonium phosphate. Such minute amounts of organic matter are found in the purest of natural waters, and under exceptional conditions certain species of bacteria may therefore multiply in bottled samples, or, at times, in a well or the basin of a spring. In normal surface-waters, such growths of the prototrophic forms do not apparently occur. Here it is found as a matter of practical experience that the number of bacteria present depends upon the extent to which the water has been contaminated with decomposing organic matter, either by pollution with sewage or by contact with the surface of the ground. The bacterial content varies as the extent and character of the contamination varies. It measures not merely organic matter but organic matter in a state of active decay, and like the ammonias and other features of the sanitary chemical analysis, indicates fresh organic pollution, with the added advantage that the presence of the stable nitrogenous compounds often present in peaty waters introduces no error in the bacteriological analysis.

In judging of a surface-water the student will be aided by reference to the figures given for certain normal sources in Chapter I; the Boston tap water with 50 to 200 bacteria

per c.c. (Philbrick, 1905) and the water of Lake Zurich with an average of 71 in summer and 184 in winter (Cramer, 1885) may be taken as typical of good potable waters; and numbers much higher than these are open to suspicion, since all contamination whether contributed by sewage or by washings from the surface of the ground is a possible source of danger. The excess of bacteria in surface-waters during the spring and winter months is by no means an exception to the general rule that high numbers are significant, since the peril from supplies of this character is clearly shown by the spring epidemics of typhoid fever which at the times of melting snow visit communities making use of unprotected surface-waters. Streams receiving direct contributions of sewage exhibit a similar excess of bacteria at all times, numbers rising to an extraordinary height near the point of pollution and falling off below as the stream suffers dilution and the sewage organisms perish. Miquel (Miquel, 1886) records 300 bacteria per c.c. in the water of the Seine at Choisy, above Paris; 1200 at Bercy in the vicinity of the city, and 200,000 at St. Denis after the entrance of the drainage of Paris. Prausnitz (Prausnitz, 1890) found 531 bacteria per c.c. in the Isar above Munich, 227,369 near the entrance of the principal sewer, 9111 at a place 13 kilometers below the city, and 2378 at Freising, 20 kilometers further down. Jordan (Jordan, 1900), in his study of the fate of the sewage of Chicago, found 1,245,000 bacteria per c.c. in the drainage canal at Bridgeport, 650,000 twenty-nine

miles below at Lockport, and numbers steadily decreasing below to 3660 at Averyville, 159 miles below the point of original pollution. Below Averyville the sewage of Peoria enters and the numbers rise to 758,000 at Wesley City, decreasing to 4800 in 123 miles flow to Kampsville. Brezina (1906) found 1900 bacteria per c.c. in the Donau River above, and 110,000 at the mouth of, the Donau canal. This number fell to 85,000 one kilometer below, 62,000 four kilometers below, and 40,000 seven kilometers down the stream. Vincent (1905) records from 1000 to 46,000 bacteria per c.c. in the waters of more or less polluted French rivers. Mayer (1902), on the other side of the world, found 21 and 35 bacteria per c.c. in the Shaho River, near its source, in the vicinity of the great Chinese Wall, and from 100,000 to 600,000 in the highly polluted Whangpo, near its mouth.

In ground-waters we have seen that bacteria may occasionally be present in considerable numbers, but, if so, they are generally organisms of a peculiar character, incapable of development on the ordinary nutrient media in the standard time. Thus in forty-eight hours we often obtain counts measured only in units or tens such as have been recorded in Chapter I. When higher numbers are present, the general character of the colonies must be taken into account, since besides the slowly-growing forms certain other water bacteria, which require a comparatively small amount of nutriment, may multiply at times in a deep well or the basin of a spring. In such a

case, however, the appearance of the plates at once reveal, the peculiar conditions, for the colonies are of one kind and that distinct from any of the sewage species. Thus Dunham (Dunham, 1889) reports that the mixed water from a series of driven wells gave 2 bacteria per c.c., while another well, situated just like the others, contained 5000, all belonging to a single species common in the air. Except in such peculiar cases as this, high numbers in a ground-water mean contamination.

The process of slow sand filtration for the purification of unprotected surface-waters is essentially similar to the action which takes place in nature when rain soaks through the ground to appear in wells and springs; and it is in the examination of the effluent from such municipal plants that the quantitative bacteriological analysis finds, perhaps, its most important application. The chemical changes which occur in the passage of water through sand at a rate of 1,000,000 or 2,000,000 gallons per acre per day are so slight as to be negligible. The bacteria present should, however, suffer a reduction of 98 or 99 per cent, and their numbers furnish the best standard for measuring the efficiency of such filtration plants. At Lawrence, in 1905, Clark found an average of 12,700 bacteria per c.c. in the raw water of the Merrimac River, while the number present in the filtered water was only 70 (Massachusetts State Board of Health, 1906). Where the number of bacteria in the applied water is smaller it is difficult to obtain so high a percentage efficiency. At

Washington, for example, prolonged sedimentation generally reduces the bacterial numbers to less than a thousand, and it is almost impossible to secure a 99 per cent removal. The actual numbers of bacteria in the effluent are, however, much lower than at Lawrence. The monthly average results obtained for a year at these two plants are tabulated on page 54.

Mechanical filtration gives similar results. Fuller at Cincinnati (Fuller, 1899) records 27,200 organisms per c.c. in the water of the Ohio River between September 21, 1898, and January 25, 1899, while the average content of the effluent from the Jewell filter was 400. Data with regard to the operation of mechanical filters are now abundant, since all over the world the operation of these plants is controlled by bacteriological methods. Recently Johnson (1907) has reported some interesting results from the far East. At Osaka, Japan, an average of 200 bacteria per c.c. in the raw water of the Yodo River was reduced, in 1905, to an average of 25 by slow sand filters; at Bethmangala, India, in 1906, mechanical filters treated the water of the Palar River, containing 4350 bacteria per c.c., and yielded an effluent with only 13 per c.c. (Johnson, 1907).

The average monthly results obtained with the new mechanical filter plant at Harrisburg, Pa., are included in the table on page 54 for comparison with the figures recorded at Washington and Lawrence; and these may be taken as typical since the Harrisburg plant is the latest

of its type, as the Washington plant is the newest and most perfectly equipped of slow sand filters.

REMOVAL OF BACTERIA BY NATURAL SAND FILTERS AND MECHANICAL FILTERS. BACTERIA PER C.C. IN APPLIED WATER AND EFFLUENT.

MONTHLY AVERAGES.

Month.	Washington, 1906.		Lawrence, 1905.		Harrisburg, 1906.	
	Applied Water.	Effluent.	Applied Water.	Effluent.	Applied Water.	Effluent.
Jan. .	1500	39	14,200	110	9510	104
Feb. .	550	16	14,800	55	21,228	298
Mar. .	650	19	10,300	55	31,326	75
Apr. .	400	22	3600	170	39,905	42
May	65	17	1900	12	6187	86
June .	220	17	9600	9	2903	31
July .	160	26	3900	55	685	10
Aug.	190	14	19,500	37	1637	5
Sept. .	130	14	13,500	44	836	12
Oct. .	275	16	39,800	110	7575	63
Nov.	220	12	8,700	70	26,224	236
Dec. .	700	45	...	...	37,525	163

In well-managed purification plants the bacteria in the effluent are determined daily, and any deviation from the normal value at once reveals disturbing factors which may impair the efficiency of the process. In Prussia official regulations demand such systematic examinations, and prescribe 50 as the maximum number of bacteria allowable in the filtered water. In the same way the condition of an unpurified surface supply may be determined by daily bacteriological analyses and warnings of danger issued to the public, as has been done at Chicago and other

cities. In general, any regular determination of variations from a normal standard furnishes ideal conditions for the bacteriological methods; and the detection by Shuttleworth (Shuttleworth, 1895) of a break in a conduit under Lake Ontario by a rise in the bacteria of the Toronto water-supply may be cited as a classic example of its application.

Often, however, the expert is called to pass upon the character of a water of which no series of analyses is available and whose surroundings it may be impossible for him to inspect. It has been said that single bacteriological analyses of this kind are valueless; but this we believe cannot always be maintained. Knowing the normal bacterial range for a given class of waters, even an isolated analysis may show such an excess as to have great significance, as a few practical examples will make clear (Winslow, 1901).

In the spring of 1900 the city of Hartford, Conn., was using a double supply, from the Connecticut River and from a series of impounding reservoirs among the hills. A single series of plates showed from 4000 to 7000 bacteria per c.c. in the water of the river, while the reservoir water contained 300 to 900. The abandonment of the river supply followed, and at once the excessive amount of typhoid fever in the city was curtailed.

In the fall of 1900, Newport, R. I., experienced an outbreak of typhoid fever, and when suspicion was thrown upon the surface water-supply, chemical analysis of the latter was not wholly reassuring; but there were only 334

bacteria per c.c. in the water from the taps, while a well in the infected district gave 6100. It was no surprise to find, on a further study of the epidemic, that the well was largely at fault and the public supply was not.

In the case of ground-water the evidence is usually even more distinct. At Framingham, Mass., in 1903, high chlorin content in the public supply, drawn from a filter gallery beside a lake, had led to public anxiety. Five samples from different parts of the system showed averages of 1, 2, 2, 2, and 4 bacteria per c.c.; and taking this in conjunction with the other features of the bacteriological analysis, it was possible to report that any pollution introduced upon the gathering ground had at the time of examination been entirely removed.

CHAPTER IV.

DETERMINATION OF THE NUMBER OF ORGANISMS DEVELOPING AT THE BODY TEMPERATURE.

THE count of colonies upon the gelatin plate measures, as we have pointed out, the number of the metatrophic bacteria in general; and the distribution of these forms corresponds with the decomposition of organic matter wherever it may occur. In this great class there are some species which will grow under a wide variety of conditions. These are present in most waters in small numbers, and in sources containing much decaying vegetable matter they occur in abundance. Other metatrophic forms, however, through a semi-parasitic mode of life have become specially adapted to the peculiar conditions characteristic of the animal body; and these bacteria possess the property of developing most actively at the temperature of the human organism, 37° C., which altogether checks the growth of the majority of normal earth and water forms. The determination of the number of organisms growing at the body temperature may throw light, then, on the presence of direct sewage pollution, since the bacteria from the alimentary canal flourish

under such conditions, while most of those derived from other sources do not. Savage classifies the bacteria which may be found in water under three headings: normal inhabitants, like B. fluorescens; unobjectionable aliens (from soil), like B. mycoides, and objectionable aliens (from excreta), like B. coli. The first sort and many of the second sort are generally unable to grow at 37 degrees. This criterion is not an absolute one. Savage (1906) reports an experiment in which unpolluted soil, which had not been manured or cultivated for at least three years, was added to tap water, with the result that a 20° count of 76 was increased to 1970, and a 37° count of 3 was raised to 1630. In this case most of the bacteria in the soil were capable of development at body temperature. Experience shows, however, that the numbers of such bacteria which actually reach natural waters from such sources are seldom large. The count at 37 degrees, therefore, helps to distinguish contamination by wash of the soil of a virgin woodland from pollution by excreta, since in the former case the proportion of blood-temperature organisms is much smaller than in the latter. Furthermore, this method is free from much of the error introduced by the multiplication of bacteria after the collection of a sample, as most of the forms which grow in water during storage cannot endure the higher temperature and consequently do not develop upon incubation. Recently, for example, water from a spring of good quality was shipped to the laboratory from a considerable distance. Gelatin

plates showed 4200 bacteria per c.c., but agar plates at 37 degrees were sterile.

The body-temperature count must, of course, be made upon agar plates, otherwise the procedure is the same which has already been described for the routine quantitative bacteriological analysis. The period of incubation ordinarily adopted by the writers is twenty-four hours, as little development occurs after that time. Difficulty is sometimes caused by the spreading of colonies of certain organisms over the surface of the plate in the water of condensation which gathers; this may be avoided by inverting the plates after the agar is once well set or still better by the use of plates provided with earthenware tops as suggested by Hill. The porous earthenware absorbs the water which condenses on it, the surface of the plate remains comparatively dry, and the percentage of "spread" plates is reduced from 30 per cent to 1 per cent (Hill, 1904).

Additional evidence as to the character of a water sample may be obtained with little extra trouble by adding a sugar and some sterile litmus to the agar medium and observing the fermenting powers of the organisms present, as first suggested by Wurtz (Wurtz, 1892) for the separation of B. coli from B. typhi. It happens that the most abundant intestinal organisms, belonging to the groups of the colon bacilli and the streptococci, decompose dextrose and lactose with the formation of a large excess of acid. The decomposition of the latter sugar

on the other hand is almost entirely wanting among the commoner saprophytic bacteria, and therefore lactose is most commonly used in making sugar agar, 1 per cent being added to the medium just before the final filtration (between steps 15 and 16 in the standard process of media making given on p. 209). In pouring the plate a cubic centimeter of sterile litmus solution should be added. After incubation the colonies of the acid-forming organisms will be clearly picked out by the reddening of the adjacent agar. Only those colonies which are sharply colored should be considered as significant, since certain bacteria of the hay-bacillus group produce weak acid and faint coloring of the litmus.

When polluted waters are examined in this manner the number of organisms developing on the lactose-agar plate will be very high, almost equalling in some cases the total count obtained on gelatin. Chick (Chick, 1901), using a lactose-agar medium with the addition of one-thousandth part of phenol, found, of colon bacilli alone, 6100 per c.c. in the Manchester ship canal, 55 to 190 in the polluted River Severn, and numbers up to 65,000 per gram in roadside mud. In an examination of water from the Charles River above Boston, total 37-degree counts ranging from 9800 to 16,900 have been found. The average result of 56 examinations of Boston sewage from July to December, 1903, showed 5,430,000 bacteria per c.c. at 20 degrees, and 3,760,000 per c.c. at 37 degrees, of which 1,670,000 were acid formers. The average of

25 samples examined in July and August, 1904, showed 1,690,000 bacteria per c.c. at 20 degrees and 1,400,000 at 37 degrees, while 429,000 per c.c. were acid formers (Winslow, 1905).

In unpolluted waters not only the absolute number of organisms developing at the body temperature, but the ratio to the gelatin count, is very different. Rideal (Rideal, 1902) states that the proportion between the two counts in the case of a London water in a year's examination was on the average one to twelve. Mathews (Mathews, 1893) in 1893, gave the following figures, the contrast between the ponds and streams, which were presumably exposed to pollution, on the one hand, and the wells, springs, and taps, on the other, being marked.

Source of Water.	Average Number of Colonies per c.c.	
	Gelatin, 20°.	Lactose-Agar, 37.5°.
Wells, springs	1664	28
Reservoirs	153	43
Ponds	296	95
Taps	242	24
Streams	273	101

According to the English Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water (1904), the ratio of the 20-degree count to the 37-degree count in good waters is

RELATION OF 20° AND 37° COUNTS IN SAMPLES OF WATER FROM APPARENTLY UNPOLLUTED SOURCES.

(WINSLOW AND NIBECKER, 1903.)

Source of Samples.	Number of Samples.	Gelatin Plates, 20°.	Litmus-lactose-agar Plates, 37°.		Dextrose Broth Tubes.			
		Average Number of Colonies.	Average Number of Colonies.	Plates Showing Red Colonies.	Number of Tubes.	Number of Tubes with Gas.	Number of Tubes with Gas 1–0.	Number of Tubes with Gas 2–1.
Cambridge supply (tap)	5	94	11	0	15	0	0	0
Wakefield and Stoneham supply (tap)	7	59	6	0	21	0	0	0
Lynn supply (tap)	6	16	3	0	18	0	0	0
Brookline supply (tap)	1	...	18	2	3	0	0	0
Plymouth supply (tap)	6	35	2	0	18	0	0	0
Peabody supply (tap)	3	141	21	0	9	2	2	0
Dedham supply (tap)	6	3,717	1	0	18	0	0	0
Newburyport supply (tap)	6	36	9	0	18	0	0	0
Salem supply (tap)	5	232	14	0	15	0	0	0
Taunton supply (tap)	4	13	2	0	12	0	0	0
Sharon (well) (tap)	3	738	46	2	9	3	0	3
Medford supply (tap)	5	524	8	0	15	0	0	0
Milton supply (tap)	2	4,700	0	0	6	0	0	0
Westerly, R. I., supply (tap)	1	...	12	0	3	0	0	0
Brooks	61	223	7	0	183	13	13	0
Driven wells	15	18	1	0	45	0	0	0
Springs	32	294	2	0	95	13	13	0
Ponds fed by brooks	15	167	9	0	45	1	1	0
Melted snow	1	...	4	0	3	0	0	0
Pools in fields	22	365	31	0	66	2	2	0
Pools in woods	22	181	3	0	65	0	0	0
Roadside pools	10	811	4	0	30	2	2	0
Stream, Blue Hill Reservation	1	...	0	0	3	0	0	0
Flow from rocks	2	47	0	0	6	0	0	0
Ponds fed by springs	6	188	2	0	18	0	0	0
Drainage from manured pasture	1	1,235	27	0	3	0	0	0
Swamps	3	269	6	0	9	5	5	0
Rain-water after twelve hours' heavy fall	7	...	2	0	21	0	0	0
Shallow well in Lynn woods	1	15	1	0	3	0	0	0
Totals	259			4	775	41	38	3

generally considerably higher than 10 to 1. "With a polluted water this ratio is approached, and frequently becomes 10 to 2, 10 to 3 or even less."

In 1903 Nibecker and one of ourselves (Winslow and Nibecker, 1903) made an examination of 259 samples of water from presumably unpolluted sources in Eastern Massachusetts, including public supplies, brooks, springs, ponds, driven wells, and pools in the fields and woods, with a view to testing the value of the body-temperature examination. In many cases the samples showed high gelatin counts, since some of the waters were exposed to surface wash from vacant land, but the average number of organisms developing on lactose agar at 37 degrees was less than 8 per c.c. The highest individual counts obtained were 95 in a meadow pool, 83 in a brook, and 74 in a barnyard well, the latter probably actually polluted. Only two samples in the whole series, one from the well above mentioned, gave any red colonies on the agar plates.

Important data as to the distribution of bacteria which will develop at high temperatures may be found in a recent paper by Gage (1906), coupled with a suggestive discussion of the general significance of bacterial ratios. The table on p. 64 shows some of the most significant results obtained by plating waters of various degrees of purity at 20, 40 and 50 degrees. We have rearranged the lines of the table so as to make the progression from more to less polluted waters a fairly regular one. The colony count at 50 degrees

AVERAGE NUMBER OF BACTERIA AND ACID-PRODUCERS DEVELOPING AT 20°, 40° AND 50° C. WITH DIFFERENT CLASSES OF WATERS.

(GAGE, 1906. REARRANGED.)

	Bacteria per c.c.			Acid-producing Bacteria.		
	20° C. 4 D.	40° C. 24 Hr.	50° C. 24 Hr.	20° C. 4 D.	40° C. 24 Hr.	50° C. 24 Hr.
Sewage . . .	2,990,000	557,500	7700	1,940,000	346,000	4400
"	1,676,000	360,000	29,500	1,032,000	283,000	24,900
Septic effluent .	485,000	126,500	410	241,000	90,000	240
Contact effluent	146,600	26,100	8300	112,400	22,700	8000
" "	389,000	59,300	8000	292,000	45,000	8000
" "	306,600	89,600	485	193,000	46,000	200
Trickling filter effluent . . .	15,500	1730	154	15,200	1360	100
" "	23,300	2030	54	16,000	1180	20
Canal water . .	16,400	112	5	6700	87	2
River water . .	16,900	207	4	2500	134	2
Settled canal water	2800	212	2	1650	66	1
Sand filter effluent (sewage) .	1640	1375	2	2360	1195	1
" " " .	35	4	0	29	2	0
" " " .	1300	130	1	345	119	0
" " " .	670	170	2	1045	154	0
Water filter effluent	32	3	1	6	1	1
" " " .	715	170	2	259	101	1
" " " .	62	1	0	16	0	0
" " " .	150	22	1	14	17	1
" " " .	64	5	1	11	3	1
Shallow well . .	1000	2	0	3	1	0
" " . .	507	72	0	82	55	0
Pond	27	1	0	8	1	0
"	71	8	0	30	5	0
Spring	49	0	0	6	0	0
"	80	2	0	8	2	0
Driven well . .	41	0	0	0	0	0

shows an even sharper differentiation than that at 40 degrees. Gage rightly concludes that "the information to be obtained by counts of bacteria and acid-producing organisms at any one of the above temperatures is greatly increased by the combination of the results obtained from counts at two or more temperatures."

In warm weather the interpretation of the body temperature count must be made less rigid than at other seasons. Recent investigations have shown that in midsummer bacteria capable of growth at 37 degrees are more abundant in normal waters than in winter and spring.

Winslow and Phelps (in a recent unpublished study) examined 86 samples from springs, wells, brooks and pools during the winter and spring months and found only 12 which showed more than 25 bacteria per c.c. and only 3 which showed more than 100 per c.c. on lactose-agar. On the other hand, of 58 samples from corresponding sources examined in summer, 16 contained more than 100 bacteria per c.c. A series of 20 pools, ponds and brooks at Mt. Desert, Maine, which were entirely free from human or animal pollution, were examined in the late summer of 1906. Only four of the 20 samples gave counts under 25 at 37 degrees, and seven of them gave counts over 100, the highest figure being 425. Similarly it has been pointed out that in tropical countries organisms capable of development at 37 degrees may thrive abundantly in normal waters.

A majority of the English Committee appointed to

consider the standardization of methods for the Bacterioscopic Examination of Water (1904), recommended the body temperature count as a standard procedure; the American Committee on Standard Methods of Water Analysis (1905) failed to adopt this method in its last report. It is to be hoped that it will see fit to do so in the future; and meanwhile individual bacteriologists will find it of much service in supplementing the 20-degree determination on gelatin. Under ordinary conditions it is clear that organisms growing at the body temperature and those fermenting lactose are not numerous in normal waters. The absolute count at 37 degrees seldom exceeds 50, and is rarely over 10 per cent of the 20-degree count, except after hot periods in the late summer; acid producers are generally entirely absent. On the other hand, the numbers on the litmus-lactose-agar plate will be likely to run into hundreds with a good proportion of red colonies when polluted waters are examined.

CHAPTER V.

THE ISOLATION OF SPECIFIC PATHOGENES FROM WATER.

THE discovery of the organisms which specifically cause infectious diseases naturally led to the hope that their isolation from polluted water might become the most convincing proof of its sanitary quality. The typhoid bacillus and the spirillum of Asiatic cholera were in this connection of paramount importance, and to the search for them many investigators devoted themselves.

In the earlier examinations of water for the typhoid bacillus an attempt was made either to use media, which especially favored the growth of the microbe sought for, or to begin with some process of "enrichment" in which the sample was incubated under conditions which would favor the growth of the pathogenic organisms while checking the development of the common water bacteria. It was apparent that the body temperature and the presence of a slight excess of free acid furnished such conditions, and most of the methods suggested rest upon these principles. Among these, one of the earliest was that of Parietti (Parietti, 1890), which consists in the addition of portions of the water to a series of broth tubes containing increasing

amounts of a solution of 4 per cent hydrochloric acid and 5 per cent phenol. From tubes in which growth occurs after twenty-four hours at 37 degrees, the organisms present may be isolated in pure cultures by some plating method and identified by subcultures.

The great difficulty with a majority of the enrichment processes is that the conditions which favor the multiplication of the typhoid bacillus are frequently suited in an even higher degree to B. coli and other intestinal organisms. Being present in almost all cases in much higher numbers than B. typhi, these germs develop abundantly, and effectually mask any disease germs originally present. In order to obviate this difficulty, Hankin (Hankin, 1899), after adding successively increasing portions of Parietti solution to tubes inoculated with the water to be tested, selected the second highest tube of the series in which growth occurred for the inoculation of a new set, finally plating as above. He believed that the chance for overgrowth in this method was somewhat decreased, but in the hands of other investigators it has not met with marked success. Klein (Thomson, 1894) in his investigations, made use of the Berkefeld filter to concentrate the organisms in the sample. Some observers have abandoned the enrichment process altogether and recommend direct plating upon phenolated gelatin or on the Elsner (Elsner, 1896) medium made by adding 10 per cent of gelatin and 1 per cent of potassium iodide to an infusion of potato whose reaction has been adjusted

to 30 on Fuller's scale. In all cases, however, the chance of success is small, as is well shown by the experiments of Laws and Andrewes (Laws and Andrewes, 1894), who entirely failed to isolate the typhoid bacillus from the sewage of London and found only two colonies of the organism on a long series of plates made from the sewage of a hospital containing forty typhoid patients. So Wathelet (Wathelet, 1895) found that of 600 colonies isolated from typhoid stools and having the appearance characteristic of B. coli and B. typhi, only 10 belonged to the latter species.

In the last five years considerable progress has been made in the development of new methods for isolating the typhoid bacillus. These fall in four distinct groups: first, the direct isolation by differential, frequently colored, media; second, enrichment methods; third, methods based on concentration of the organisms by agglutination with typhoid serum; and fourth, methods based on concentration by chemical precipitation.

In all excepting the first of these groups differential media are usually employed as a second step in the isolation. Combinations of methods have been employed in many instances, and have often been successful in the isolation of the typhoid bacillus from artificially infected emulsions of feces and waters.

Direct Isolation. — Drigalski and Conradi (Drigalski and Conradi, 1902) prepared a medium primarily for the isolation of typhoid bacilli from excreta, which may also

be applied in water bacteriology. This consists of an agar medium containing nutrose, sodium chloride, litmus, lactose, and a dye, "crystal violet"; and it is used in the form of plate cultures infected by smearing the surface after thorough cooling with a bent glass rod. The culture medium is a selective one, ordinary saprophytes failing to grow, while after fourteen to twenty-four hours at 37 degrees, colon and typhoid colonies can be readily distinguished from one another. The colon bacillus produces red, non-transparent colonies, of variable size and depth of color, while the typhoid colonies are blue or violet, transparent, and of smaller size, seldom exceeding three millimeters in diameter.

Endo (Endo, 1904) has suggested the use of a fuchsin-lactose-agar decolorized by sodium sulphite. Upon this medium B. coli produces bright red, sharply defined, round colonies in 24 hours at 37 degrees, while B. typhi gives round, colorless, transparent colonies with thin margins. This medium has been somewhat modified by Gaehtgens (Gaehtgens, 1905) by the addition of caffeine, and he found it of great service in isolating the typhoid bacillus from stools of patients suffering with the disease. No attempts were made by him to isolate the organism from polluted water.

Loeffler (Loeffler, 1903 and 1906) and Lentz and Tietz (Lentz and Tietz, 1903 and 1905) have made use of an agar medium containing malachite green. This medium is supposed to inhibit the growth of B. coli

while favoring B. typhi, and has been recommended for the isolation of the organism from feces. Dœbert (Dœbert, 1900) has shown that certain varieties of malachite green are not suited to this purpose. Nowack (Nowack, 1905) has also pointed out the same fact, and ascribed the difference to the presence of dextrin. He finds that a medium 0.8 per cent alkaline to phenolphthalein is more favorable to B. typhi and less favorable to B. coli than one neutral to litmus. With such a medium about 20 per cent of the typhoid bacilli present develop.

More recently a considerable degree of success has been attained by methods based upon the inhibitory action of caffein for B. coli.

This important fact, which was announced by Roth (Roth, 1903), has given rise to much investigation, and offers what is probably the most promising method for the isolation of the typhoid bacillus from water. Hoffman and Ficker (Hoffman and Ficker, 1904) have developed methods for the isolation of B. typhi from feces and from infected water by its use in connection with nutrose and crystal violet. For the isolation from infected water solutions are prepared as follows:

1. Ten grams of nutrose in 80 c.c. of sterilized distilled water.

2. Five grams caffein, in 20 c.c. sterilized distilled water.

3. One-tenth gram of crystal violet in 100 c.c. water. Solutions 1 and 2 are mixed by shaking together in a flask, and the mixture poured into a flask containing 900 cubic centimeters of the water to be tested. 10 c.c. of solution 3 are gradually added, and the whole thoroughly mixed by shaking and then incubated at 37 degrees for not over 12–13 hours. At the end of the incubation period loopfuls of the solution are smeared over Drigalski-Conradi plates.

By this method the B. typhi was isolated from mixtures in river water containing one typhoid bacillus to 51,867 water bacteria and colon bacilli.

A number of investigations have shown that the action of the caffein is not as markedly selective as at first claimed. Kloumann (Kloumann, 1904) obtained no better results by this method than by the Drigalski-Conradi medium alone, and Willson (Willson, 1905) found that certain strains of B. typhi were inhibited, while strains of B. coli developed feebly in the presence of 0.5 per cent of caffein.

The phenomenon of agglutination was made the basis of a method of isolating B. typhi from water by Adami and Chopin (Adami and Chopin, 1904). Two-liter samples of the water were collected in sterilized bottles (Winchester quarts), and to each was added twenty cubic centimeters of one per cent glucose broth. The sample was incubated for 18 to 24 hours at 37° C., after which ten cubic centimeter portions were withdrawn and

placed in long, narrow test tubes. To each of these tubes enough typhoid serum of known potency was added to make a regularly graded series, 1–50, 1–100, 1–150, and 1–200. The probable presence of the typhoid bacillus was manifest by the formation of flocculi within a quarter of an hour, and agglutination was complete in from two to five hours.

The tube having the greatest dilution in which agglutination was apparent was then examined by breaking off the lower end, containing the precipitate, washing the sediment two or three times with sterile water after removing the clear supernatant liquid, and allowing the bacteria to settle again. The organisms remaining were plated upon various media, and examined biochemically to determine their true character. It was found that a dilution of 1 to 60 was the highest which could be used with the organisms examined, and it is therefore probable that high dilutions (greater than 1–60) cannot be successfully used.

A study of the organisms isolated in this case was made by Klotz (1904), who found the culture to be not a typical B. typhi but a form showing certain points of similarity to both B. typhi and to B. coli, and probably intermediate between them. As this author pointed out, it is therefore evident that even when a positive result is obtained with a relatively high dilution of typhoid serum it is unwise to regard the action as absolutely specific.

Schepilewski (Schepilewski, 1903) and Altschuler (Altschuler, 1903) have also used agglutination as a means of precipitating the bacteria after enrichment cultivation in broth. The former incubated the culture at 37 degrees for 24 hours, then added a serum of high potency, allowed the mixture to stand for two to three hours and then centrifuged. The supernatant liquid was removed, and the mass of agglutinated cells broken up by shaking with glass beads and salt solution. Upon plating upon litmus lactose agar the organisms could be detected. In this way positive isolation was made from water containing 1 loopful of a broth culture in 50 liters of water. Altschuler's method of enrichment was essentially like that of Schepilewski. From the surface of the culture developed at 37 degrees, 10 c.c. were removed to a tapering tube provided with a rubber tube at the bottom. Serum was added in the proportion of one part in 50, the culture agitated to release entangled non-agglutinated bacilli and the sediment run into a tube containing 1 per cent peptone and ½ per cent salt. The agglutinated mass was broken up by shaking with sand, and the culture incubated at 37 degrees for 24 hours, and then plated on Drigalski-Conradi plates. The organism was isolated from dilute suspensions in water (150 in 1 liter) and also from the feces of a typhoid patient with which other methods gave negative results.

A number of methods for concentrating typhoid bacilli in water by chemical precipitation have been tested experi-

mentally, with some degree of promise. Vallet (Vallet, 1901) was the first to employ this principle, and made use of sodium hyposulphite and lead acetate. The mixture was centrifugalized and the precipitate dissolved in more hyposulphite. The clear solution was then plated.

Schüder (Schüder, 1903) observed that the lead salt reacted harmfully upon the bacteria, and pointed out that the hypolsuphite should be in excess. In his experiments water was allowed to stand in tall jars for 24 hours. To 2 liters of infected water, 20 c.c. of a 7.75 per cent solution of sodium hyposulphite was added, and after thorough mixing 20 c.c. of a 10 per cent solution of lead nitrate. The precipitate, after 20 to 24 hours, was treated with 14 c.c. of saturated sodium hyposulphite solution and shaken. From the clear solution 0.2 to 0.5 c.c. portions were streaked upon Drigalski-Conradi plates which were then incubated at 37 degrees for 24 hours. Ficker (Ficker, 1904) modified the process still more by using ferric sulphate, and dissolved the precipitate with neutral potassium tartrate. The final solution was then plated on Drigalski-Conradi medium. Ficker claimed that this method gives excellent results, 97–98 per cent of the typhoid bacteria being carried down with the precipitate.

Müller (Müller, 1905), after comparing different precipitation methods, adopted ferric oxychloride as the most suitable precipitant, because of its quicker and less de-

structive action. Willson (Willson, 1905) suggested the use of alum as a precipitant. He added 0.5 gr. alum per liter of water examined. The mixture was then centrifugalized, and the precipitate suspended in a small amount of water and plated on Drigalski-Conradi medium. Nieter (Nieter, 1906) made 20 parallel experiments, using very pure water infected with typhoid bacilli in varying numbers. By precipitating with ferric sulphate and sodium hydrate, centrifugalizing, and then filtering through a sterile filter he obtained results with small numbers of bacteria. Using iron oxychloride as the precipitant, he confirmed the results of Müller. By plating on malachite-green agar he was often able to get positive results when the Drigalski-Conradi medium failed.

By use of a combination of enrichment and chemical precipitation, Ditthorn and Gildemeister (Ditthorn and Gildemeister, 1906) isolated the typhoid bacillus from enormous artificial dilutions in water. In the typhoid fever epidemic in Posen, in 1906, it was found that the bile of those dying from the disease contained nearly pure cultures of typhoid bacilli. This led the authors mentioned to use bile and bile agar as enrichment media. After precipitating by Müller's method, the whole of the precipitate was added to 100 c.c. sterile ox bile and grown at 37 degrees for 24 hours, after which time 1 c.c. portions were plated. With extreme dilutions it was found desirable to incubate for 48 to 72 hours. The results were unsatisfactory in the presence of large numbers of water

bacteria. It is also pointed out that the iron oxychloride is bactericidal in 48 hours.

Drigalski (Drigalski, 1906) has suggested the separation of B. typhi from other bacteria in water through its greater motility. He succeeded in isolating typhoid bacilli from two springs by the following method: five to ten liters of water were allowed to stand for one to two days in tall milk cans at room temperature. Samples were taken from the surface and plated on litmus lactose agar (Drigalski-Conradi medium), the amount of water to be used varying with the contamination.

The most promising methods for examination of water for B. typhi may be conveniently summarized in the following tabular view adapted from Willson's paper.

TABULAR SUMMARY.

- Examination of water for Typhoid bacilli.
  - Isolation
    - (1) Filtration.
    - (2) Chemical precip.
      - Schüder's process.
      - Ficker's process.
      - Alum process.
      - Müller's process.
    - (3) Serum agglutination.
    - (4) Enrichment—Hoffman and Ficker's process.
    - (5) Cambier's process.
    - (6) Solid media
      - Gelatin (Elsner's, etc.)
      - Bile-salt agar.
      - Glucose and lactose agars.
      - Drigalski-Conradi medium.
      - Endo's medium
      - Loeffler's malachite-green agar.
  - Identification
    - Morphological and cultural characters, etc.
    - Specific reactions
      - Agglutination.
      - Pfeiffer's, etc.

Of the comparative advantages of these methods it is still too early to speak with finality. Up to the present

time the use of caffein has apparently been followed by the best results, and it seems likely that of the precipitation methods that employing the oxychloride of iron is the best. Lubenau (Lubenau, 1907) has made some interesting comparisons, using media containing malachite green and caffein and caffein alone, in which the advantage is decidedly in favor of the latter.

After this part of the process is completed the identification of the pure cultures isolated is subject to considerable uncertainty. The typhoid bacillus belongs to a large group which contains numerous varieties differing from each other by minute degrees. The inability to reproduce the disease by inoculation in available test animals, owing to their natural immunity, is a serious drawback; and the specific biochemical characters of the organism are, as it happens, mostly negative ones, as shown by comparison with B. coli, to which it is supposed to be allied.

COMPARISON OF THE CHARACTERS OF B. COLI AND B. TYPHI.

(HORROCKS, 1901.)

B. coli.	B. typhi.
(1) Surface Colonies, Gelatin Plates. — Thicker, and grow more rapidly than those of B. typhi. 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) Gelatin-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.	B. typhi.
(3) Gelatin-slope.—Thick, broad, grayish-white growth with a crenated margin.	(3) Thin, narrow, grayish-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 neutralize it.	(6) Very small amount of acid produced, requiring not more than 6 per cent of $\frac{N}{10}$ alkali to neutralize it.
(7) Neutral-red Glucose-agar.— Marked green fluorescence.	(7) No change.
(8) Glucose-gelatin and Lactose-gelatin Shake Cultures, and Glucose-agar-stab. — Marked gas formation.	(8) No gas formation.
(9) Gelatin, 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. No. II, Growth, medium neutral or faintly alkaline.	(11) No. I, no growth or change in the reaction of the medium. 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 serpent-like movement.
(14) Flagella. — Usually 1 to 3, short and brittle; sometimes 8 to 12, long and wavy.	(14) Usually 8 to 12, long and wavy.
(15) Agglutination. — As a rule, no agglutination with a dilute anti-typhoid serum.	(15) Marked agglutination with dilute anti-typhoid serum.

Of the many observers who have reported the isolation of the typhoid bacillus from water, all but the most recent are quite discredited, on account of the insufficiency of their confirmatory tests; and even the latest results

should be received with caution. Since the introduction of the Widal (Widal, 1896) reaction, founded on the fact that typhoid bacilli examined under the microscope in the diluted blood-serum of a typhoid patient lose their motility and "agglutinate" or clump together, an important aid has been furnished in the diagnosis. Yet serum tests are notably erratic, and insufficient to identify an organism without an exhaustive study of biochemical reactions. Many organisms are agglutinated by typhoid serum in a more or less dilute solution, and agglutination tests are not significant unless obtained in dilutions as great as 1–500 or 1–1000. The discovery of the Bacillus dysenteriæ of Shiga,[1] which closely resembles the typhoid bacillus, has made the identification of the latter more dubious than ever. Hiss (1904) has shown that the fermentation and agglutination reactions of the two organisms are in many respects alike, and Park and his associates (1904) have shown that there are not less than three distinct types of dysentery bacilli forming that group.

In the work so far described the typhoid organism was not isolated from polluted water, but from artificial mixtures or excreta. There are, however, a number of cases in which the organism has undoubtedly been isolated from polluted water, as by Kübler and Neufeld (Kübler and Neufeld, 1899), who examined a farmhouse

[1] For an account of the Biology of B. dysenteriæ the student is referred to an article by Dombrowsky, 1903.

well at Neumark in 1899, and Fischer and Flatau (Fischer and Flatau, 1901), who discovered an organism responding to a most exhaustive series of tests for the typhoid bacillus in a well at Rellingen in 1901. In these cases the water was directly plated upon Elsner's medium or phenolated gelatin with no preliminary process of enrichment. Willson (Willson, 1905) has summarized the instances in which the typhoid bacillus has been isolated from infected drinking water, and includes, in addition to the above-mentioned cases, the following:

1. By Lösener, in 1895, from the Berlin water supply.

2. By Conradi, in 1902, from a well at Pecs in Hungary, by use of carbol gelatin plates.

3. By Jaksch and Raŭ, in 1904, from the water supply of Prague, and also from the river Moldau, by caffeine-nutrose crystal violet agar.

4. By Ströszner, in 1904, from a well near Budapest, by the same method.

The search for the typhoid bacillus is usually suggested when an outbreak of the disease has cast strong suspicion upon some definite source of water-supply. By the time an epidemic manifests itself, however, the period of the original infection is long past, and the chances are good that any of the specific bacilli once present will have disappeared. While elaborate experiments have shown that B. typhi may persist in sterilized water for upwards of two months and in unsterilized water from three days to several weeks, the number of the organisms present is

always very rapidly reduced (Frankland, 1894). More recently, Jordan (Jordan, 1905) has demonstrated that the typhoid bacillus may be isolated from mixed cultures of B. typhi and B. coli in tap water and sewage after thirty-four days, with unchanged agglutinative powers. On the other hand, Jordan, Russell and Zeit (Jordan, Russell and Zeit, 1904), and Russell and Fuller (Russell and Fuller, 1906) have shown that in unsterilized lake water, river water, and sewage, the life of the organism may not exceed five days. Whipple and Mayer (Whipple and Mayer, 1906) have ascribed to dissolved oxygen a decided effect upon the viability of the typhoid bacillus in water, absence of oxygen tending to weaken the organism.

Epidemiological evidence confirms the results of Laws and Andrewes which teach that the number of typhoid bacilli even in polluted water is probably never very great, while the fate of Lowell and Lawrence in 1890–91 and the more recent epidemics at Butler, Pa., and Ithaca, N. Y., seem strongly to demonstrate that even a small number of virulent organisms can bring about an almost wholesale infection. Indeed, if the virulent organism were as abundant as some results would indicate (Remlinger and Schneider, 1897), the human race would long since have been exterminated. On the whole it is clear that a negative test for the typhoid bacillus means practically nothing. Since this is so, and since a positive result is always open to serious doubt, the search for the typhoid

bacillus, however desirable theoretically, cannot be regarded at present as generally profitable.

The isolation of the cholera bacillus from water can probably be accomplished with somewhat less difficulty than is encountered in the case of B. typhi. Schottelius (Schottelius, 1885) was the first to point out the necessity for growing this organism in an alkaline medium, and Loeffler (Loeffler, 1893) found that its isolation from water could be successfully accomplished by adding 10 c.c. of alkaline peptone broth to 200 c.c. of the infected water and incubating for twenty-four hours at 37 degrees, when the organism could be found at the surface of the medium.

Somewhat earlier than this Dunham (Dunham, 1887) had made a special study of the chemical reactions of the cholera bacillus and found that the organism would grow abundantly in a solution containing 1 per cent peptone and .5 per cent salt (Dunham's solution), producing the "cholera-red or nitroso-indol reaction." This medium was brought into practical use by Dunbar (Dunbar, 1892), who succeeded in isolating the organisms from the water of the Elbe in 1892, during the cholera epidemic at Hamburg.

Koch (Koch, 1893) prescribed the following method for the isolation of the organism from water:

To 100 c.c. of the water to be examined is added 1 per cent peptone and 1 per cent salt. The mixture is then incubated at 37 degrees. After intervals of ten, fifteen, and twenty hours, the solution is examined micro-

scopically for comma-shaped organisms, and agar plate cultures are made which are likewise incubated at 37 degrees. If any colonies showing the characteristic appearance of the cholera bacillus are found, these are examined microscopically, and if comma-shaped organisms are present, inoculations are made into fresh tubes to be further tested by means of the indol reaction and by inoculation into animals. The existence of other spirilla of some pathogenic power renders necessary the greatest care and caution in claiming positive isolations. That no great improvement on Koch's method has been made during the last ten years seems apparent from the statements of Kolle and Gotschlich (Kolle and Gotschlich, 1903), who employed "the peptone method with subsequent agar cultivation" in the isolation of the organisms from feces of cholera patients during the epidemic in Egypt in 1902.

Other pathogenic organisms have been isolated from waters, according to the accounts of numerous investigators, but from the sanitary point of view the typhoid and cholera bacilli are of most importance since these are manifestly the germs of disease most likely to be disseminated through this medium. For the detection of B. anthracis and other spore-forming pathogenic bacteria which may at times gain access to water from stockyards, slaughter-houses, etc., the method suggested by Frankland (Frankland, 1894) may be adopted. The water to be examined is heated to 90 degrees for two minutes and then

plated, the characteristic colonies of the anthrax organism being much more easily discerned after the destruction of the numerous non-sporing water bacteria. Again, water is sometimes the means of distributing the germs of dysentery and diarrhœa, as shown by the decrease of these diseases in Burlington, Vt. (Sedgwick, 1902), and other communities where pure water-supplies have been substituted for polluted ones. Thresh (Thresh, 1903) described an epidemic of over 1000 cases of diarrhœa with 14 deaths, which occurred in England at Chelmsford and Widford, and was undoubtedly spread by the public water-supply. A somewhat similar epidemic of dysentery occurred in Warren and Kittanning, in Pennsylvania, in 1906, as a result of contamination of the water, in this case a river-supply. It is possible that the examination of water for the B. dysenteriæ may in the future help to throw important light on its sanitary condition.

CHAPTER VI.

METHODS FOR THE ISOLATION OF THE COLON BACILLUS.

THE Bacillus coli was first isolated by Escherich (Escherich, 1884) from the feces of a cholera patient. It was subsequently found to be a normal inhabitant of the intestinal tract of man and many other animals, and to occur regularly in their excreta, and on this account it became of the highest interest and importance to sanitarians, since its presence in water-supplies was regarded as direct evidence of sewage pollution.

This organism may be described as a short, usually motile rod, with diameter generally less than one micron and exhibiting no spore formation. It forms thin irregular translucent films upon the surface of gelatin, called "grape-leaf colonies" by the Germans, produces no liquefaction, and gives a wire-nail-like growth in stick cultures. It forms a white translucent layer of characteristic appearance upon agar, produces a more or less abundant, moist, yellowish growth on potato, and turbidity and some sediment in broth; it ferments dextrose and lactose with the formation of gas of which the ratio is approximately, $\frac{H}{CO_2} = \frac{2}{1}$, as ordinarily determined; a

strong acid reaction is developed in most sugar-containing media. The organism reduces nitrates to nitrites and sometimes to ammonia. It reduces neutral red, changing its color to canary yellow with a greenish fluorescence. It grows in the Capaldi-Proskauer media, forming acid in the albumin-free medium, No. 1, and giving a neutral or alkaline reaction in the pepton-mannite medium, No. 2. It coagulates casein in litmus milk, and reduces the litmus with subsequent slow return of the color (red), and forms indol in pepton solution. Many cultures of this organism are fatal to guinea pigs when the latter are inoculated subcutaneously with one-half c.c. of a twenty-four-hour bouillon culture, and most cultures produce death when this amount is inoculated intraperitoneally. Although not a spore-forming bacillus, and in general not possessing great resistance against antiseptic substances, B. coli is less susceptible to phenol than are many other forms, especially certain water-bacteria.

A word may be added as to the fermentative powers of the colon group in other carbohydrates than dextrose and lactose. Of the monosaccharides, galactose, like dextrose, is always fermented; and among the polysaccharides, maltose and xylose are broken up as well as lactose. Inulin is not attacked. The alcohols, mannite and dulcite, are fermented by some strains and not by others. The colon group, as Smith (1893) long ago pointed out, may be divided into two distinct subtypes according to the action of the organisms upon saccharose. One subtype

forms gas and acid in saccharose media and the other does not. Winslow and Walker (1907) have recently found that those strains which ferment saccharose attack raffinose also, and point out that these two sugars which behave alike are those which lack the aldehyde grouping characteristic of dextrose and lactose. The name B. coli communior was given to the saccharose fermenting type by Durham (1901); as Ford (1903) suggests, this name should be changed to B. communior in deference to the established binomial rule of biological nomenclature. The term, B. coli, should, in strictness, apply only to those forms which fail to attack the ketonic sugars. For practical sanitary purposes, however, the distinction is unimportant. Throughout this book, therefore, both B. communior and B. coli, proper, will be considered together under the name of the colon bacillus, which is almost universally applied to both.

The litmus-lactose-agar plate (Wurtz, 1892), as noted in Chapter IV, furnishes one ready method for the isolation of B. coli from water, and it was used by Sedgwick and Mathews for the purpose as early as 1893 (Mathews, 1893). The process is based upon the fact already alluded to, that B. coli readily ferments lactose with the formation of acid. If, therefore, plates are made with agar containing both lactose and litmus, the colon colonies develop as red spots in a blue field. Since organisms other than B. coli may also develop red colonies, it is necessary to examine the red colonies further. This

is done by fishing from isolated colonies, replating and inoculating into the usual media for identification.

The plate method of isolation is recommended by the Committee on Standard Methods of Water Analysis (1905) for sewages and polluted waters, and with such sources it yields good results. For success in the use of this method it is necessary to get a sufficient dilution so that colonies may be well isolated, and to this end it is advisable that a number of different dilutions be employed, a series of plates being prepared from each. Under any conditions the detection of the colon bacillus is seriously hampered by the development of other forms. Certain observers have therefore added phenol to the agar medium, combining the effect of high temperature and an antiseptic to check the growth of water-bacteria. Copeland for this purpose added to his tubes .2 c.c. of a 2 per cent solution of phenol (Copeland, 1901). Chick (Chick, 1900) found that 1.33 parts of phenol in 1000 materially decreased the number of colon bacilli which would develop, while 1 part gave very satisfactory results, the plates showing pure cultures of B. coli. The addition of antiseptics in this way is always open to the objection that weaker strains may be killed and lost.

The test for the colon bacillus in less heavily polluted waters may be made more delicate by a preliminary enrichment of the sample by growth in a liquid medium for twenty-four hours at 37 degrees, thus greatly increasing the proportion of these organs present before plating. As

suggested in the classic researches of Theobald Smith (Smith, 1892), this method may be made approximately quantitative by the inoculation of a series of tubes with measured portions of the water. If, for example, of ten tubes inoculated each with $\frac{1}{100}$ of a cubic centimeter, four show B. coli, we may assume that some 40 of these organisms were present to the cubic centimeter. Irons (Irons, 1901), in a comparative study of various methods for the isolation of B. coli, showed that the preliminary enrichment frequently gave positive results when the results of the direct use of the agar plate were negative, and concluded that "where the amount of B. coli is small and the colony count large, the lactose plate for plating water direct is inferior to the dextrose fermentation-tube." Gage obtained similar results (Gage, 1902).

The medium ordinarily used for the preliminary enrichment is ordinary broth to which 1.0 per cent of dextrose has been added, and the reaction brought to the neutral point. Into each of a number of fermentation-tubes of this medium a measured quantity of the water to be examined is inoculated, and the culture is incubated for twenty-four hours at 37.5° C. At the end of this time the tubes are examined for gas formation. If gas is found, a small amount of the culture should be added, after suitable dilution, to litmus lactose agar and plated.

With polluted waters it will be found advantageous to plate out on the first appearance of gas (4–8 hours). It has been shown by one of us (Prescott, 1902[b]) that a very

rapid development of B. coli takes place in the first few hours after dextrose solutions are inoculated with intestinal material, and a nearly pure growth of colon bacilli often results, while other bacteria multiply more slowly. With highly polluted waters gas formation will probably begin within twelve hours, but with fewer colon bacilli present the duration must be increased. If the period of incubation be too long continued, trouble in the subsequent steps of the isolation may be encountered because of the overgrowth of B. coli by the sewage streptococci, or other forms which check the growth of the colon bacilli in the later stages of fermentation and finally kill them out.

As has already been stated, phenol has less inhibitory action upon B. coli than upon normal water-bacteria, and it was hoped that a broth containing this substance might be employed for preliminary enrichment with advantage, its inhibitory power checking the overgrowing forms, but not B. coli. This medium was used in place of dextrose broth for many of the studies made in connection with the Chicago drainage canal (Reynolds, 1902). Phenol broth consists of ordinary broth to which 0.1 per cent phenol is added, and the method of procedure is to add 1 c.c. of the water to 10 c.c. of the sterilized phenol broth and incubate at body temperature for twenty-four hours. Litmus-lactose-agar plates are then made and the examination of the red colonies carried out as described for the dextrose-broth method. It has unfortunately

proved, however, that with waters of fairly good quality the phenol interferes with the colon bacilli themselves to a serious extent. The dextrose broth furnishes a more delicate test than the carbol broth when the number of colon bacilli present is small, as is clearly shown by the following table from Irons:

PROPORTION OF POSITIVE RESULTS IN TESTS OF POLLUTED AND UNPOLLUTED WATERS BY DEXTROSE FERMENTATION-TUBE AND CARBOL-BROTH METHODS.

(IRONS, 1901.)

	Dextrose Fermentation-tube.			Carbol-broth Method.		
	+	−	?	+	−	?
Polluted waters	33	31	5	38	30	1
Relatively unpolluted waters	56	38	25	37	61	21

The English Committee, appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water (1904), recommend the use of bile-salt broth or glucose-formate broth for preliminary enrichment, and suggest that the incubation be carried out anærobically at 42° C.

The use of media containing bile salts, and even of undiluted ox-bile to which lactose has been added (Jackson, 1906), have been urged by various American bacteriologists. With sewages and heavily polluted waters the lactose-bile medium has, in fact, proved superior to

dextrose broth. When a large proportion of sewage is present the colon bacilli are fresh from the intestine and apparently able to resist the antiseptic salts. On the other hand, the large numbers of other bacteria present make the danger of overgrowths particularly great. It is possible, however, that direct plating on litmus lactose agar may prove to be preferable, even to the bile enrichment method, for waters of this class. With waters of fair quality, such as those with which we ordinarily deal in sanitary water analysis, lactose bile is open to the same objection as phenol broth, though in less degree. It inhibits not only the overgrowing forms but the weaker representatives of the B. coli group itself; and the net effect is to diminish positive results. In an examination of 176 surface waters in eastern Massachusetts, using lactose bile and dextrose broth in parallel for preliminary enrichment, the authors obtained 64 positive results by the former method against 70 by the latter. Longley and Baton (1907), from their work on Potomac water, concluded that "the value of the test with the bile lactose on unpolluted or slightly polluted water, such as we have to deal with the greater part of the time, is uniformly less than with dextrose broth, except in the larger quantities of water."

The same objection applies to other enrichment methods which involve the use of antiseptic conditions to check the development of overgrowing bacteria. Eijkman (1904) suggested incubation at 46 degrees as furnishing a more rapid differentiation between good and polluted

waters by cutting out at once organisms other than B. coli which fail to grow at this high temperature. Christian (1905), Neumann (1906), and Thomann (1907) have reported good results by the use of this method. It remains, however, to be demonstrated that high temperatures do not inhibit colon bacilli in slightly polluted waters. Nowack (1907) found that laboratory cultures of B. coli often fail to produce gas in Eijkman's medium at 46 degrees, unless large numbers are introduced. With some strains an inoculation of over a million bacteria was necessary to cause gas formation.

It appears on the whole that the safest method at present available is the dextrose broth enrichment process which alone rests on the sure basis of a great number of observed coincidences between sanitary inspection and bacteriological examination.

When it is desired to examine samples larger than 1 c.c. for B. coli it becomes necessary to modify the enrichment process by adding the nutrient material to the water instead of the reverse. For this purpose phenol-dextrose broth (consisting of broth with 10 per cent dextrose, 5 per cent peptone, and .25 per cent phenol) may be added to the sample of water to be enriched as suggested by Gage (Gage, 1901). Generally 10 c.c. of the broth is added to 100 c.c. of the water. The sample is then incubated at 37 degrees for twenty-four hours, and if at the end of that time growth has taken place, a cubic centimeter is inoculated into a dextrose tube. If this tube shows gas

formation after twenty-four hours at 37 degrees, a litmus-lactose-agar plate is made and the other diagnostic tests applied.

The Committee on Standard Methods of Water Analysis (1905) recommends that "for ordinary waters, 0.1, 1.0, and 10.0 c.c. shall be used for the colon test. For sewage and highly polluted surface-waters smaller quantities shall be used; and for ground-waters, filtered-waters, etc., the quantities shall be larger, if necessary, to obtain positive results."

Our own experience has been that it is not specially advantageous to apply the colon test in large samples, since the significance of B. coli when present in numbers less than 1 per c.c. is extremely doubtful. On the other hand, the danger of overgrowth is greatly increased in large samples and negative results may often be obtained when the organisms are really present. Hunnewell and one of us (Winslow and Hunnewell, 1902[b]) found that of 48 samples of certain polluted river waters, 18 showed B. coli when 1 c.c. was inoculated directly into dextrose broth, while in only 4 cases was a positive result obtained after preliminary treatment of 100 c.c. in carbol broth. In 153 samples from presumably unpolluted water, B. coli was found 5 times in 1 c.c. and 11 times by the examination of the larger sample. The authors, therefore, concluded as follows:

"It appears evident that the use of large samples in applying the colon test to the sanitary analysis of drink-

ing-water is not advantageous. In comparing the results of the tests in 1 c.c. and in 100 c.c., it will be noted that the proportion of lactose fermenting organisms and of colon bacilli in the unpolluted waters was more than doubled in the latter; thus waters of good quality are more likely to be condemned by the use of large samples. On the other hand, in the polluted waters a considerable proportion of the colon bacilli originally present were lost during the incubation of the large samples, so that waters of bad quality actually appeared to better advantage by the use of 100 c.c. with preliminary incubation in phenol broth."

Whipple (Whipple, 1903) notes that 2.9 per cent of some samples of water examined by him gave positive tests with .1 c.c. but not with 1 c.c., while 4.3 per cent gave positive tests with .1 c.c. or 1 c.c. and negative tests with 10 c.c. Again, in another series of samples examined, of those which gave positive tests in smaller portions 5.3 per cent were negative in 10 c.c., 4.7 per cent in 100 c.c., and 7.7 per cent in 500 c.c.

In our ordinary routine at the Institute we use one cubic centimeter sample only, inoculating five or ten tubes in duplicate with that amount. In this way we ascertain whether B. coli is generally or rarely present in one cubic centimeter of the suspected water; and this is the information of greatest practical value. If absent from one cubic centimeter the presence of the organism in ten cubic centimeters would not lead to the condemnation of the

water. If generally present in one cubic centimeter the water may be considered unsafe, whether the colon bacillus is found in smaller volumes or not. For special studies of self-purification, etc., of course fractions of the cubic centimeter must be examined. Litmus-lactose-agar plates should be made from all tubes which show any gas whatever. Fuller and Ferguson (1905) have shown that B. coli may be present even when gas formation in the enrichment tube is quite atypical. Of 43 cultures isolated by these observers at Indianapolis, 18 showed less than 20 per cent of gas after forty-eight hours in the enrichment tube, and 11 showed less than 10 per cent.

The procedure of the Committee on Standard Methods of Water Analysis (1905) calls for a forty-eight-hour incubation of the preliminary enrichment tube. Recent experience has, however, shown that a twenty-four-hour period gives approximately the same results if the production of gas rather than any specified amount of gas is the criterion of a positive test. Longley and Baton (1907) found that of 1091 enrichment tubes giving positive tests after 48 hours only 173 showed no gas in 24 hours; of these latter only two contained B. coli. The advantage of saving a day is so great as to warrant the adoption of the shorter period.

In all practical processes of examining water for B. coli one essential step is the isolation of pure cultures upon the litmus-lactose-agar plate, whether the plate be inoculated from the water direct or from a preliminary enrich-

ment culture. In the first case a measured quantity of water is added and the number of colonies of B. coli corresponds to the number of bacteria in the portion plated. In the second case, since the enrichment tube was inoculated with a known amount of water all further work is purely qualitative, and it is only necessary to obtain such a number of colonies upon the lactose plate that the isolation of a pure culture shall be easy. In practice the following procedure has been found generally successful: After the dextrose tubes have been incubated for twelve to twenty-four hours at 37 degrees, from those which show gas, one loopful is carried over to a tube containing 10 c.c. of sterile water, and of this water one loopful is taken for the inoculation of the plate. Ordinarily this will give colonies which are sufficiently well separated, but a second plate, inoculated from the dilution water with a straight needle instead of a loop, furnishes a desirable safeguard. With practice it is possible to effect a proper seeding more rapidly by barely touching the tip of a straight needle to the broth in the fermentation tube and transferring this directly to the agar. The touch must be a very light one, however, or the colonies on the plate will be too thick for proper isolation.

The litmus-lactose-agar plates made in this manner should be incubated for from twelve to twenty-four hours at the body temperature (37 degrees), at the end of which time, if B. coli is present, red colonies upon a blue field will be visible. The litmus-lactose-agar plate may be-

come blue again after forty-eight hours, owing to the formation of amines and ammonia by the action of the bacteria upon the nitrogenous matter present. If the dilution is too low, the resulting colonies will be small and imperfectly developed, making it difficult to be sure of pure cultures for the subsequent tests. A great number of colonies will also prevent the change of reaction from acid back to alkaline. Since many bacteria ferment lactose with the formation of acid, it is erroneous to regard all colonies as those of B. coli; several colonies from each plate should be isolated upon agar streaks and further studied in subculture.

In the selection of those red colonies which are to be fished from the litmus-lactose-agar plate the appearance of the growths must be closely noted. A colony of irregular contour, surrounded by a very faint area of reddening, will probably belong to some member of the B. mycoides group (Winslow and Nibecker, 1903); small, compact, bright-red colonies are characteristic of the streptococci, and Gage and Phelps (Gage and Phelps, 1903) have pointed out that of these there are two types, one of a brick-red color, and of such consistency as to be readily picked up by the needle-point, and the other smaller and of an intense vermilion color. The colonies of the colon bacillus are usually well formed, pulvinate on the surface and fusiform when growing deeper down.

If no red colonies appear on the litmus-lactose-agar plate one of three things has occurred. There may be

an organism present which forms gas in dextrose but no acid in lactose; there may be present forms which individually fail to attack lactose but growing together, symbiotically, produce gas in dextrose; or an organism originally present and capable of fermenting both sugars may have been overgrown and lost in the enrichment tube. If plates are made on the first appearance of gas the likelihood of the latter possibility will be reduced to a minimum. Neither of the first two contingencies has any sanitary significance; as we shall see later, bacteria which ferment detrose and not lactose are not specially characteristic of pollution. In any case, therefore, the absence of red colonies on the agar plate may be considered a negative result. If red colonies are present they must be subcultured and examined further.

The agar streak made from the litmus-lactose-agar plate shows after twenty-four hours certain marked characteristics. The most distinct types are two, the abundant, first translucent, later whitish and cheesy growth, covering nearly the whole surface of the agar, characteristic of B. coli and its allies, and a very faint growth, either confined strictly to the streak or made up of faint isolated colonies, dotted here and there over the surface. The latter cultures are typical of the sewage streptococci, and a microscopic examination will generally settle their status at once. Of the more luxuriant growths, some of which are stringy to the needle, many will generally prove to be atypical, and if any of the weakly fermenting forms (B.

mycoides) are present, a dull wrinkled growth will be produced.

Having submitted the sample of suspected water to a preliminary enrichment process, and having isolated pure cultures of suspicious organisms from the litmus-lactose-agar plate, the third step is the examination of the specific reactions of the organisms thus obtained. Just what characters to use in defining the "colon bacillus" is a matter of prime importance. The whole question of species among the bacteria is an extremely complex one, since around each definite species are grouped forms differing from the type in one or two of its characteristics.

As Whipple says (Whipple, 1903), "The type form of Bacillus coli is one which can be defined within reasonably narrow limits, but when the organism has been away from its natural habitat for varying periods of time, and has existed under abnormal conditions, its ability to react normally to the usual tests appears to be greatly impaired. Its power to reduce nitrates may be lost, or on the other hand may be increased; its power to produce indol may be lost, or on the other hand it may be increased; its power to coagulate milk, even, is sometimes reduced, although seldom entirely lost; its power to ferment carbohydrates may be altered so that the amount of gas obtained in a fermentation tube, as well as its ratio of H to CO_2, is quite abnormal. But in spite of all these facts, the bacillus tested may have been originally a true Bacillus coli."

It is, of course, not always certain that organisms resembling B. coli, but failing, for example, to reduce nitrates or to form indol, have been derived from typical colon bacilli by any recent process of modification. Organisms of this sort may be found which for generations breed true to their characteristics and are apparently definitely different in one property or another from the true B. coli.

The more of such atypical forms which are included the greater will be the number of positive isolations. The definition of this or any other bacterial species is more or less arbitrary; we consider as true colon bacilli those which fulfill a particular set of tests, and class as pseudo-colon organisms those which do not. If we find, having established such an arbitrary standard, that the colon bacillus, as determined by it, is found in waters known to be polluted, and not, as a rule, in those known to be free from pollution, the sanitarian can afford to ignore the theoretical question of specific values and make confident use of the practical test. In order that results may rest on a sound basis of comparable data for various waters, it is of course however essential that a standard set of reactions should be agreed upon by sanitary bacteriologists.

After a considerable period of uncertainty, in which each observer used the procedure which happened to appeal to him, the attainment of comparative results has been made possible by the establishment of standard methods of procedure by bodies of authoritative position, both in England and America. In 1904 an English Committee,

appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water, presented a series of obligatory tests and optional tests; and in 1905 the Committee on Standard Methods of Water Analysis of the American Public Health Association drew up a set of diagnostic characters for B. coli. The latter corresponds in general with the plan developed by the Massachusetts State Board of Health (Massachusetts State Board of Health, 1899) and long in use at the Massachusetts Institute of Technology.

It involves the use of the following seven tests:

DIAGNOSTIC CHARACTERS FOR B. COLI.

1. Typical morphology — non-sporing bacillus, relatively small and often quite thick.

2. Motility — when a young broth or gelatin culture is examined.

3. Fermentation of dextrose broth, with the formation of about 50 per cent of gas, of which about one-third (CO_2) is absorbed by a two per cent solution of sodium hydrate.

4. Coagulation of milk, with the production of acid, in 48 hours or more at 37° C., either spontaneously or upon boiling.

5. Non-liquefaction of gelatin.

6. Production of indol in peptone solution.

7. Reduction of nitrates.

The English standard procedure corresponds quite closely to this (Committee appointed to consider the

Standardization of Methods for the Bacterioscopic Examination of Water, 1904), although it differs from the American method in certain respects.

The American Public Health Association standard procedure, as defined above, has, in the main, proved satisfactory. In two respects, however, it needs modification.

In the first place, the requirement that motility should be demonstrated is a burdensome and needless one. Motility is a fluctuating and uncertain property and one which frequently requires repeated preliminary cultivations to make it manifest. Furthermore, non-motile colon bacilli are common in the intestine and are probably as characteristic of intestinal pollution as the motile forms. McWeeney (1904) found non-motile B. coli abundant in feces and observed cases where the organisms were motile at 20 degrees and not at 37 degrees. He quotes Stöcklin as having found 116 non-motile strains among 300 otherwise normal B. coli from feces. Evidence that non-motile bacteria, otherwise resembling B. coli, occur in unpolluted water would furnish the only basis for requiring this test as a routine procedure. No such evidence exists. The great body of data which connects the presence of B. coli with pollution includes all B. coli whether motile or not, since scarcely any bacteriologists observe this property in actual practice.

Another point in the Diagnostic Tests of the Committee on Standard Methods which requires modification in the

light of more recent knowledge is the requirement that dextrose broth shall be fermented "with the formation of about 50 per cent of gas, of which about one-third (CO_2) is absorbed by a two per cent solution of sodium hydrate." Stamm (1906) and others have pointed out that the ratio of carbon dioxide to hydrogen changes with the age of the culture. At first the proportion of the former to the latter is as two to one, and later, in the same tube, the ratio is reversed. More recently, Longley and Baton (1907), in one of the ablest and most fruitful of recent contributions to water bacteriology, have made it clear that neither of these quantitative determinations is of importance. They show, first, that the total amount of gas formed by B. coli varies widely, from 10 to 80 per cent, the mode of the curve being found not at 50 but at 35 per cent. Secondly, they show that the proportion of carbon dioxide present is a function of the total amount of gas. They find that when grown in an atmosphere of CO_2, B. coli produces a gas which consists of about 3 parts of carbon dioxide to one of hydrogen. Assuming that the gas originally formed by B. coli has always about this composition, and that the absorption of CO_2 by the medium is the chief cause of the differences observed in the gas which collects in the closed arm, the gas ratio would vary directly with the amount of total gas; the more rapidly gas is formed, the greater the proportion of CO_2 remaining unabsorbed. Calculation on this basis gives a curve very close to the observed data, and the conclusion of Longley and Baton that the

gas ratio is a function of the total quantity of gas seems us justified. The determination of the gas ratio may therefore be omitted, and any tests which show from 10 to 80 per cent gas in dextrose broth in forty-eight hours may be considered positive.

For recording the results of the various tests applied in sanitary water examination, the appended blank form has been in use at the Massachusetts Institute of Technology. On the upper part of the sheet are noted the results of the gelatin count and the litmus-agar count at 37 degrees. In the second column the number of acid-formers is placed in brackets after the total numbers. The lower part is used for the B. coli isolation.

MASSACHUSETTS INSTITUTE OF TECHNOLOGY.

Bacteriological Examination of Water.

Sample No.	Date of collection
Examined by	Hour of collection
Place of collection	Remarks

Number of Bacteria.

Gelatin-plate cultures. 48 hours.	No. colonies per c.c.	Agar-plate cultures at 37°. No. colonies per c.c. 24 hours
Average		Average
Remarks		Remarks

B. Coli Isolation.

Preliminary fermentation tube
Litmus-lactose-agar plates
Agar streak culture
Fermentation tube
Nitrate test
Milk test
Indol reaction
Gelatin-stab culture
Remarks

Opposite the words "Preliminary fermentation tube" are recorded by plus or minus signs the results of the first enrichment test in the five or ten tubes inoculated. Under the columns headed by plus signs the presence or absence of typical red colonies on the litmus-lactose-agar plate is similarly indicated. The third line serves for the results of the microscopic and macroscopic appearance of the agar streak. If this gives the proper type of growth and rod-shaped organisms are found, a dextrose fermentation tube, a nitrate tube, a milk tube, a peptone tube, and a gelatin stab are inoculated from it.

After forty-eight hours incubation at 37 degrees, the dextrose broth and milk tubes are examined and the results recorded in the proper columns by plus and minus signs. The amount of gas in the closed arm of the dextrose tube may be conveniently measured by the Frost gasometer (Frost, 1901). If a measurement of the gas ratio is desired a few centimeters of strong sodium or potassium hydrate are added and mixed with the broth by cautiously tipping the tube; a second measurement determines the amount of gas absorbed (assumed to be CO_2). The gas should first fill from one-third to two-thirds of the closed arm, and about one-third of the total amount should be absorbed.

The milk tube is tested by heating to boiling over the free flame; if coagulation occurs the test is considered positive.

After ninety-six hours incubation at 37 degrees the

peptone solution is examined for indol by adding 1 c.c. of a .02 per cent solution of sodium or potassium nitrite and 1 c.c. of a 1 to 1 solution of sulphuric acid. Both the tube and the reagents should be cooled on ice before mixing, and the tube should be left in a cool place for an hour afterward to allow time for the characteristic rose-red color of nitroso-indol to develop. At the same time (after four days) the nitrate tube is tested for nitrites by adding a drop of each of the following solutions in succession:

A.	Sulphanilic acid	.5 gram
	Acetic acid (25% sol.)	150.0 c.c.
B.	Naphthylamine chloride	.1 gram
	Distilled water	20.00 c.c.
	Acetic acid (25% sol.)	150.0 c.c.

A red or violet coloration indicates the presence of nitrites.

In making the nitrite and indol tests it is important to remember the possibility that appreciable amounts of nitrite may be present in the media — either derived from the air or from the use of impure peptone in the indol solution (Wherry, 1905). In the case of the nitrite reaction, control tubes should always be tested from the same batch of media and only a distinct red color should be considered positive.

The gelatin tube should be kept at 20 degrees for ten days. It seems undesirable in practice to prolong the test much beyond this point, although some slowly liquefying organisms are doubtless included, which would be thrown out by a longer incubation. The extent of this

PERCENTAGE OF CULTURES PASSING VARIOUS TESTS IN THE ROUTINE EXAMINATION FOR B. COLI AT THE LAWRENCE EXPERIMENT STATION OF THE MASSACHUSETTS STATE BOARD OF HEALTH.

(GAGE AND PHELPS, 1903.)

Source of Culture.	Per Cent of Preliminary Fermentations Passing Lactose-Agar Plate.	Agar Streak.					Per Cent of Cultures Inoculated into Confirmatory Media.	Per Cent of Cultures.															Per Cent of Total Cultures Called B. Coli.
		Number of Cultures Made.	Per Cent of Cultures.					Fermentation Tube.					Gelatin Stab.										
			Characteristic.	No Growth.*	Dull-wrinkled.	Viscous.		Produce Gas.	Produce Scum.	Dextrose Broth. Coagulate Milk.	Reduce Nitrates.	Produce Indol.	Not Liquefied.	Liquefied.									
													Characteristic.	Wrinkled.	Time.				Form.				
															4 Days.	7 Days.	10 Days.	14 Days.	Funnel.	Cup.	Stratiform.		
B. coli	...	...	+	0	0	0	...	+	0	+	+	+	+	0	0	0	0	0	0	0	0		
Merrimac River	...	273	86	0	13	1	236	81	13	86	82	73	69	1	21	25	28	30	10	4	16		59
Filtered water	76	1485	83	9	4	4	1236	84	3	90	76	71	83	4	4	7	9	13	5	5	3		58
Sewage, etc.	...	160	87	4	8	1	140	75	0	88	70	75	84	1	9	9	10	15	6	2	7		59
Shellfish	72	312	64	33	2	1	210	68	3	91	67	75	84	0	9	9	9	16	6	2	8		43
Spring-waters	78	53	74	0	4	23	39	56	0	67	67	59	72	0	15	18	28	28	20	0	8		41
Ice	73	79	72	16	8	0	557	67	5	88	42	88	63	7	25	25	26	30	5	4	21		48
Totals	75	2362	81	11	5	3	1908	80	4	88	74	71	80	3	8	10	13	17	6	5	6		58

* Including cultures which failed to grow on agar and streptococcus cultures, giving a very scanty, non-characteristic growth.

source of error as well as the relative importance of the various other diagnostic tests is well shown in the table (page 109) of the results obtained at Lawrence during a period of eighteen months.

It should be noted that considerable differences often appear in these biochemical reactions between tubes of the same batch of a medium, inoculated with approximately the same amount of the same culture. This has been shown very markedly for the amount of the gas and the proportion of carbon dioxide in the dextrose tube by Fuller and Johnson (Fuller and Johnson, 1899), Pennington and Küsel (Pennington and Küsel, 1900), Gage (Gage, 1902), and one of ourselves (Winslow, 1903). Variations in nitrate reduction are often even more marked, one tube, perhaps, showing a strong reaction and another none. In important cases, therefore, it is desirable to inoculate the subcultures in duplicate.

These anomalies are most frequent with cultures freshly isolated from water, and they may often be avoided, as Fuller and Johnson (1899) have shown, by subjecting the organism to a process of preliminary cultivation. For this purpose the American Public Health Association Committee recommends three successive cultivations in broth at 20 degrees, each of 24 hours duration, inoculation from the last broth tube of a gelatine plate which is incubated for 48 hours at 20 degrees, inoculation of an agar streak from one colony on the plate and incubation of this streak for 48 hours at 20 degrees.

In general routine work there will scarcely be time to carry out preliminary enrichment processes of this sort, and cultures which fail to reduce nitrates or to form indol, or which do not form sufficient acid in milk to coagulate it, must be classed as atypical colon bacilli or " Paracolon " bacilli. The interpretation of results of this kind will be discussed in Chapter IX. It may be noted in passing that the results of the test for B. coli will fall under four general heads: If the preliminary dextrose tube or the litmus-lactose-agar plate fail to show fermentation, the test is negative. If these are positive and gelatin is liquefied, we are dealing with a member of the B. cloacæ group. If, on the other hand, gelatin is not liquefied, the organism is a typical B. coli if it meets the standard requirements of the nitrate, indol, and milk tests. If not it must be classed as an allied " atypical " form.

CHAPTER VII.

SIGNIFICANCE OF THE PRESENCE OF B. COLI IN WATER.

FIFTEEN years ago the B. coli of Escherich occupied a position of very great prominence in the eyes of sanitarians. If it was not considered to be in itself a dangerously pathogenic germ, it was at least regarded as a suspiciously close relation of the typhoid organism. At this time, the alleged presence of either of these forms was quite sufficient to condemn a water-supply.

Investigation soon showed, however, that the Bacillus coli was by no means confined to the human intestine. Dyar and Keith (Dyar and Keith, 1893) found it to be the prevailing intestinal form in the cat, dog, hog, and cow. About the same time, Fremlin (Fremlin, 1893) found colon bacilli in the feces of dogs, mice, and rabbits, but not in those of rats, guinea pigs, and pigeons. Smith (Smith, 1895) recorded the presence of the organism, in almost pure cultures, in the intestines of dogs, cats, swine, and cattle; and he also found it in the organs of fowls and turkeys after death. Brotzu (Brotzu, 1895) reported B. coli and allied forms as very abundant in the intestine of the dog; and Belitzer (Belitzer, 1899) isolated typical colon bacilli from the intestinal contents of horses, cattle,

swine, and goats. Moore and Wright (Moore and Wright, 1900) recorded the finding of the colon bacillus in the horse, cow, dog, sheep, and hen, and in a later report (Moore and Wright, 1902) they noted its occurrence in swine and in some but not all the specimens of rabbits examined. In frogs it was not found. Eyre (1904) has more recently isolated typical B. coli from the intestines of mice, rats, guinea pigs, rabbits, cats, dogs, sheep, goats, horses, cows, hens, ducks, pigeons, sparrows, divers, gulls, and fish of various sorts. Houston (1904) found B. coli abundant in the feces of gulls, as might be expected from their feeding habits. Houston (1905) and other recent observers have found it impossible, even by the use of elaborate series of fermentation tests, to distinguish human B. coli from those found in animals. Savage (1906) compared colon-like organisms isolated from the intestines of swine, cattle, horses, and sheep with those of human origin in respect to their action upon lactose, dulcite, mannite, raffinose, glycerine, maltose, galactose, lævulose, saccharose, starch and cellulose; but he failed to find any general correlations between habitat and biochemical powers.

In cold-blooded animals the occurrence of B. coli is less constant. Negative results in the frog and positive results in certain fishes have just been quoted. Amyot (1902) failed to find the organism in the intestines of 23 fish representing 14 species. Johnson, on the other hand (Johnson, 1904), in the examination of the stomach

and intestines of 67 fish caught in the polluted Illinois and Mississippi Rivers, isolated B. coli 47 times. He concluded from these results that the migration of fish from a contaminated stream or lake to an unpolluted one may explain the occasional finding of B. coli in small samples, or the more regular detection of it in large volumes of the water.

Many bacteriologists have gone further and affirmed that the colon bacillus was not a form characteristic of the intestine at all, but a saprophyte having a wide distribution in nature. The first of this school, perhaps, was Kruse (Kruse, 1894), who in 1894 protested against the arbitrary conclusions drawn from the colon test as then applied. He pointed out that the characters usually observed marked not a single species but a large group of organisms. As ordinarily defined, he added, "the Bacterium coli is in no way characteristic of the feces of men or animals. Such bacteria occur everywhere, in air, in earth, and in the water, from the most different sources." Even if the relations to milk and sugar media be considered, "micro-organisms with these characteristics are also widespread." Dr. Kruse gave no experimental data on which his opinion was based. In the same year Beckmann (Beckmann, 1894) isolated a bacillus which he identified by pretty thorough tests as B. coli from the city water of Strassburg, a ground-water which he believed could by no possibility be subject to fecal contamination. Large quantities of water were used for the isolation.

Refik (Refik, 1896) recorded the constant presence of colon bacilli in water of all sorts, public supplies, wells, cisterns, and springs in the neighborhood of Constantinople, but the only characters which these "colon bacilli" exhibited in common were the "classical growth" upon potato, the possession of less than 8 cilia, and the power of active development on certain media upon which the typhoid bacillus did not grow. A more careful and significant piece of work on the same line was published by Poujol in the succeeding year. This author reported (Poujol, 1897) the isolation of B. coli from 22 out of 34 waters studied by him in relation to their use as public supplies. The waters were from various sources — springs, wells, and rivers — but all were of fair quality and many quite free from any possibility of contamination. Samples of 100 c.c. were used for analysis; in the only case in which a smaller amount was also tested, broth inoculated with 10 drops of the water and placed at 45° C. remained sterile. The author concluded that "fecal contamination can only exceptionally be invoked to explain the presence of B. coli in water. As the bacteria of the subterranean water are contributed to it from the surface of the earth by the water which filters downward, I am rather inclined to believe in a general diffusion of B. coli either on the surface of the earth, where it might be deposited with the dust of the air, or in the superficial layers of the earth, which may form one of its normal habitats." Therefore, the author

considered that caution should be exercised in condemning a water on account of the presence of B. coli, except, as he added, "for those cases where it exists in considerable quantity."

Certain Italian observers appear to have come to even less conservative conclusions. Abba (Abba, 1895) found colon bacilli constantly present in unpolluted waters near Turin. Moroni (Moroni, 1898; Moroni, 1899) reported the examination of numerous deep and shallow wells and unpolluted springs about Parma, as well as of the public water-supply of the city, and concluded that the colon bacillus was a water form and had no sanitary significance. The characters used for the identification of the species in this case were fairly exhaustive, but both Abba and Moroni used liter samples for analysis.

Levy and Bruns (Levy and Bruns, 1899) gave a new turn to the discussion by emphasizing the importance of animal inoculation, already suggested by Blachstein (Blachstein, 1893) and others. They claimed that the existence of numerous para-colon and para-typhoid organisms in air, in dust, and in unpolluted water made it impossible to decide by ordinary bacteriological methods whether true colon bacilli were present in water or not. In no case, however, did representatives of the colon group isolated by them from water kill a guinea pig, even when 1 or 2 c.c. were injected intraperitoneally. The authors, therefore, considered pathogenicity as an attribute belonging only to the true B. coli of the intestine. This paper

aroused Professor Kruse's pupil, Weissenfeld, to a publication, in which the position of the Bonn school was carried to an extreme. Weissenfeld reported (Weissenfeld, 1900) the analysis of 30 samples of water supposedly pure, and of 26 samples considered to be contaminated. In each case a single centimeter sample was first incubated in Parietti broth, and if no growth occurred, larger samples of half a liter or a liter were examined. Colon bacilli were found in all the samples, and the pathogenicity varied independently of the source of the water. The author concluded that "the so-called Bacterium coli may be found in waters from any source, good or bad, if only a sufficiently large quantity of the water be taken for analysis."

With regard to the question of pathogenicity as a diagnostic test for intestinal B. coli, there is little doubt of the correctness of Weissenfeld's conclusions. This property is so variable as to have no important value. Colon bacilli freshly isolated from the intestine are frequently non-virulent, and Savage (1903[a]) and others have shown that there is in general no correlation between pathogenic power and direct or indirect intestinal origin. On the other hand Weissenfeld's work entirely fails to show that the colon bacillus, pathogenic or non-pathogenic, is a normal inhabitant of unpolluted waters. In the first place it should be noted that the characters used by this investigator for defining the "so-called Bacterium coli" were absolutely inadequate. He classed under

that head all bacilli of medium size, which formed grape-vine-leaf colonies on gelatin and gas in sugar agar, which were more or less motile, or rarely non-motile, and which were decolorized by the Gram method. As regards coagulation of milk and formation of indol, " the bacteria isolated differed." In the second place it is difficult to see how the author could possibly have believed that his experiments proved the isolation of the colon bacillus to be " useless as an aid in the sanitary examination of water," as the title of the paper runs. Even his own work furnishes strong evidence to the contrary. In 24 of the 26 samples from bad sources, he isolated his imperfectly defined colon bacilli from 1 c.c. of the water, while in only 8 of the 30 samples of good waters could he find such organisms in that quantity.

The work of certain recent observers has suggested the possibility that the colon bacillus may live in a semi-parasitic fashion on plants as well as on animals. Of a series of 47 cultures of lactic-acid bacteria, recently examined by one of ourselves (Prescott, 1902[a]; Prescott, 1903; Prescott, 1906), 25 were found to give the reactions of B. coli. These organisms were isolated chiefly from cereals and products of milling, such as flour, bran, corn-meal, oats, barley, etc., while others were in technical use for producing the lactic fermentation. There is no evidence that any of these organisms were of intestinal origin, and yet they possessed all the characters of typical colon bacilli, even to the pathogenic action when inoculated

into guinea pigs. In Germany, Papasotiriu (Papasotiriu, 1901) was meanwhile carrying on almost exactly similar investigations to Prescott's, with identical results.

Other testimony is somewhat conflicting with regard to the occurrence of B. coli on plants. Klein and Houston (1900) reported the finding of typical colon bacilli in only 3 out of 24 samples of wheat and oats obtained from a wholesale house; rice, flour, and oatmeal bought at two different retail shops gave B. coli in all three cereals in one case and none in the other. Clark and Gage (1903) were unable to isolate B. coli from standing grains. Gordan (1904) could not find B. coli in .1 and .01 mg. samples of clean bran, but isolated it easily from that of poor quality. Winslow and Walker (1907) have recently reported the examination of 178 samples of grain and 40 samples of grasses for B. coli without success. On the other hand, Düggeli (1904) found B. coli among the bacteria occurring on the leaves of growing plants, although it was not one of the most abundant species. Barthel, too (Barthel, 1906), found B. coli widely distributed on plants from both cultivated and uncultivated regions.

These results raise the interesting questions: Is it possible that the lactic-acid bacilli and the similar forms found on plants have been indirectly derived from animal intestines, having "escaped from cultivation," as the botanists say? Or is the converse true, namely, that all colon bacilli are simply plant parasites which have found

in the warm intestinal canal, richly supplied with food, a favorable habitat?

The answer to these questions is of much theoretical interest, but need not be further considered here. The practical sanitary conclusions to be drawn are as follows:

1. Bacteria corresponding in every way to B. coli are by no means confined to animal intestines, but are widely distributed elsewhere in nature.

2. The finding of a few colon bacilli in large samples of water, or its occasional discovery in small samples, does not necessarily have any special significance.

3. The detection of B. coli in a large proportion of small samples (1 c.c. or less) examined is imperatively required as an indication of *recent* sewage pollution.

4. The *number* of colon bacilli in water rather than their *presence* should be used as a criterion of recent sewage pollution.

With these qualifications the value of the colon test was never more firmly established than it is to-day. Whether or not originally a domesticated form, it is clear that the colon bacillus finds in the intestine of the higher vertebrates an environment better suited to its growth and multiplication than any other which occurs in nature. Houston (1903[a]) records the number of B. coli per gram of normal human feces as between 100,000,000 and 1,000,000,000. It is almost certain that the only way in which large numbers of these organisms gain access to natural waters is by pollution with the domestic

industrial, and agricultural wastes of human life. If pollution has been recent, colon bacilli will be found in comparative abundance. If pollution has been *remote* the number of colon bacilli will be small, since there is good evidence that the majority of intestinal bacteria die out in water. If derived from cereals or the intestines of wild animals, the number will be insignificant except in the vicinity of great grain-fields or where the water receives refuse from grist-mills, tanneries, dairies, or lactic-acid factories.

The first recognition of the necessity for a quantitative estimation of colon bacilli in water we owe to Dr. Smith, who in 1892 (Smith, 1893[a]) outlined a plan for a study to be made by the New York Board of Health on the Mohawk and Hudson Rivers. Burri (Burri, 1895) pointed out that the use of so large a sample as a liter for examination would lead to the condemnation of many good waters. Freudenreich (Freudenreich, 1895) at the same time indicated the necessity for taking into account the *number* of colon bacilli present. He recorded the isolation of the organism from unpolluted wells, when as large a quantity of water as 100 c.c. was used, and concluded that it was entirely absent only from waters of great purity and present in large numbers only in cases of high pollution. This author also quoted Miquel as having found colon bacilli in almost every sample of drinking-water if only a sufficient portion were taken for analysis.

The practical results of the application of the colon test from this standpoint have proved of the highest value. As originally outlined by Dr. Smith, it consisted in the inoculation of a series of dextrose tubes with small portions of water, tenths or hundredths of the cubic centimeter. It was first used by Brown (Brown, 1893) in 1892 for the New York State Board of Health, and showed from 22 to 92 fecal bacteria per c.c. in the water of the Hudson River at the Albany intake, and from 3 to 49 at various points in the Mohawk River between Amsterdam and Schenectady. In some previous work at St. Louis, the colon bacilli in the Mississippi River were found to vary from 3 to 7 per c.c.

Hammerl (Hammerl, 1897) used the presence of Bacillus coli as a criterion of self-purification in the river Mur. He considered, in spite of the position taken by Kruse, that when a water contained large numbers of colon bacilli, as well as an excess of bacteria in general, it might be considered to be contaminated by human or animal excrement. As, however, the organism would naturally be present in large quantities of such a water as that of the Mur, he used no enrichment process, but made plate cultures direct; he defined the B. coli as a small bacillus, non-motile or but feebly motile, growing rapidly at 37° C., coagulating milk and forming gas in sugar media. In general, Hammerl failed to find colon bacilli in the river by this method, except immediately below the various towns situated upon it; at these

points of pollution he discovered a few colon colonies upon his plates, not more than 4 to 6 per c.c. of the water. He concluded that "the Bacterium coli, even when it is added to a stream in great numbers, under certain circumstances disappears very rapidly, so that it can no longer be detected in the examination of small portions of the water." It should be noted that Hammerl's method was much less delicate than the use of the dextrose tube for preliminary incubation.

The most important work upon the distribution of B. coli has been that carried out in England by the bacteriologists of the local government board, by Dr. Houston in particular. This investigator (Houston, 1898; Houston, 1899[a]; Houston, 1900[a]) made an elaborate series of examinations of soils from various sources to see whether the microbes considered to be characteristic of sewage could gain access to water from surface washings free from human contamination. In the three papers published on this subject the examination of 46 soils was recorded. In only 10 of the samples was B. coli found, and of these 10, 9 were obviously polluted, being derived from sewage fields, freshly manured land, or the mud-banks of sewage-polluted rivers. The author finally concluded that "as a matter of actual observation, the *relative abundance* of B. coli in pure and impure substances is so amazingly different as to lead us to suspect that not only does B. coli not flourish in nature under ordinary conditions, but that it tends to even lose its vitality and die." "In brief, I am

strongly of opinion that the presence of B. coli in any number, whether in soil or in water, implies *recent* pollution of animal sort." Pakes (Pakes, 1900) stated on the strength of an examination of "about 300 different samples of water," no particulars being published, that water from a deep well should not contain B. coli at all, but that water from other sources need not be condemned unless the organism was found in 20 c.c. or less. When colon bacilli were found only in greater quantities than 100 c.c., the water might be considered as probably safe. Horrocks (Horrocks, 1901), after a general review of English practice, concluded that "when a water-supply has been *recently* polluted with sewage, even in a dilution of one in one hundred thousand, it is quite easy to isolate the B. coli from 1 c.c. of the water." "I would say that a water which contained B. coli so sparingly 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." Chick (Chick, 1900) found 6100 colon bacilli per c.c. in the Manchester ship canal, 55 to 190 in the polluted River Severn, and numbers up to 65,000 per gram in roadside mud. On the other hand, of 38 unpolluted streams and rivulets, 31 gave no Bacillus coli and the other 7 gave 1 per c.c. or less. The Liverpool tap water, snow, rain, and hail showed no colon bacilli.

One of the first elaborate applications of the colon test was made by Jordan in the examination of the fate of the Chicago sewage in the Desplaines and Illinois Rivers.

At one time Professor Jordan was himself somewhat sceptical as to the value of the colon test, for he stated in 1890 (Jordan, 1890) that he had found, "in spring-water which was beyond any suspicion of contamination, bacteria which in form, size, growth on gelatin, potato, etc., were indistinguishable from B. coli commune." In the Chicago studies of self-purification (Jordan, 1901) the analyses were made quantitative by the examination of numerous measured samples, fractions of the cubic centimeter; and the method employed was enrichment, either in dextrose-broth fermentation tubes or in phenol broth, with subsequent plating on litmus lactose agar. The cultures isolated were tested as to their behavior in dextrose broth, peptone solution, milk, and gelatin; of the dextrose tubes made directly from the water all were considered positive which gave more than 20 per cent gas in the closed arm, with an appreciable excess of hydrogen. The results were very significant. In fresh sewage a positive result was obtained about one-third of the time in one one-hundred-thousandth of a cubic centimeter and almost constantly in one ten-thousandth of a cubic centimeter. The Illinois and Michigan canal proved almost as bad, giving positive results on seven days out of twenty-eight in dilutions of one in one hundred thousand and on twenty-eight days out of thirty-two in a dilution of one in ten thousand. At Morris, twenty-seven miles below Lockport, where the canal enters the bed of the Desplaines River, and nine miles below the entrance of the Kankakee, the

principal diluting factor, the numbers were so reduced that positive results were obtained only on eleven days out of twenty in one-thousandth of a cubic centimeter, on twenty days out of thirty in one-hundredth of a cubic centimeter, and on twenty days out of twenty-three in one-tenth of a cubic centimeter. At Averyville, one hundred and fifty-nine miles below Chicago, colon bacilli were isolated on only four days out of twenty-seven in one-tenth of a cubic centimeter, and on thirteen days out of thirty-one in one cubic centimeter. A comparison with certain neighboring rivers showed this to be about the normal value for waters of similar character, as the following table extracted from Professor Jordan's paper will show.

NUMBER OF B. COLI PRESENT IN CERTAIN RIVER WATERS.

(JORDAN, 1901.)

Source of Sample.	.1 c.c.		1 c.c.	
	No. Days Water Examined.	No. Days B. Coli Found.	No. Days Water Examined.	No. Days B. Coli Found.
Illinois River, Averyville .	27	4	31	13
Mississippi River, Grafton	34	10	35	23
Fox River	22	2	23	6
Sangamon River	25	14	27	21
Missouri River	32	13	31	21

These results harmonize rather closely with those previously recorded by Brown and Fuller, and indicate that in the larger rivers where the proportionate pollution is

not extreme, colon bacilli may be isolated in about half the 1-c.c. samples examined. Such rivers are of course inadmissible as sources of water-supply, according to modern sanitary standards, unless subjected to purification of some sort.

More recently Hunnewell and one of us (Winslow and Hunnewell, 1902[b]) examined a considerable series of normal waters for B. coli, testing 1 c.c. from each by the dextrose-broth method and a larger portion of 100 c.c. by incubation with phenol broth as described in Chapter VI. The samples were obtained from the public supplies of Taunton, Boston, Cambridge, Braintree, Brookline, Needham, and Lynn in Massachusetts, and Newport, R. I., from the Sudbury River, from the ocean, from the waters of springs bottled for the market, from ponds, pools of rain and melted snow, springs, brooks, shallow wells, and driven wells in various towns near the city of Boston. For comparison 50 samples of polluted waters from the Charles, Mystic, Neponset, and North Rivers were examined. The colon bacillus was defined as outlined in Chapter VI, and organisms which lacked the power to reduce nitrates or to form indol were classed in the "Paracolon group." The results are summarized in the following table:

PRESENCE OF B. COLI IN POLLUTED AND UNPOLLUTED WATERS.

(WINSLOW AND HUNNEWELL, 1902b.)

Unpolluted Waters.

	1 c.c.	100 c.c.
Samples examined	157	153
Dextrose broth positive	40	76
Lactose plate positive	13	31
Colon group	5	11
Paracolon group	5	5
B. cloacæ group	...	5
Streptococcus group	3	10

Polluted Waters.

	1 c.c.	100 c.c.
Samples examined	50	48
Dextrose broth positive	50	37
Lactose plate positive	50	26
Colon group	18	4
Paracolon group	6	...
Streptococcus group	25	22
B. cloacæ	1	...

As the authors pointed out, these tables indicate that bacteria capable of growth at the body temperature and fermenting dextrose and lactose are infrequently found in unpolluted waters, and colon bacilli are very rarely present. In 157 samples, typical colon bacilli were found only 5 times out of 157, in 1 c.c. Lactose fermenting organisms appeared in only 8 per cent of the normal samples and in 100 per cent of the polluted ones, in 1 c.c. Incidentally it may be pointed out that these tables well illustrate the dangers of overgrowths, particularly in large samples. It

is clear that the streptococci had killed out colon bacilli, originally present, in a large proportion of the 100-c.c. samples of polluted waters and in some of the 1-c.c. samples, since, in so many cases, gas formation was followed by the isolation of the streptococcus alone.

Clark and Gage (1903) have published the results of certain studies of Massachusetts ponds which indicate clearly the coincidence of the distribution of B. coli in single centimeter samples of surface waters, with actual sanitary conditions. They show also the slight significance of the test for this organism in larger volumes of water. Almost every source gave some positive tests in 100 c.c., while with 1-c.c. samples only those lakes appear suspicious which are, in fact, exposed to dangerous pollution.

DISTRIBUTION OF TOTAL BACTERIA AND B. COLI IN SURFACE WATERS.

(CLARK AND GAGE, 1903.)

Lake.	Population of Watershed per Square Mile.	Bacteria per c.c.	B. coli Per cent positive Tests.	
			1 c.c.	100 c.c.
1*	1400	612	13.3	33.0
2	356	319	3.5	17.2
3	116	103	0.0	0.0
4	90	170	0.0	14.0
5	62	87	0.0	9.0
6*	60	48	2.3	4.5
7*	50	66	4.6	21.0
8	47	133	0.0	9.0
9	42	131	0.0	6.7
10*	40	31	0.0	6.2
11	8	28	0.0	7.7
12	42	107	0.0	9.3

* Shores used for pleasure resorts.

Houston (1905) gives the following table which may be taken as another fair example of the distribution of B. coli in small streams and lakes. Of the two lakes studied, Loch Ericht is free from the pollution of human or domesticated animals while Loch Laggan receives some drainage from farm lands; both are of large size. The brook and river samples were collected from adjacent streams.

DISTRIBUTION OF B. COLI IN SURFACE WATERS.
(HOUSTON, 1905.)
Percentage of Samples showing B. coli in each Dilution.

Dilution.	+ .1 c.c.	+ 1.0 c.c. — .1 c.c.	+ 10 c.c. — 1. c.c.	+ 100 c.c. — 10 c.c.	Not in 100 c.c.
Brooks and River . .	7·7	53.8	34.6	3.8	...
Loch Laggan .	...	1.2	33.0	49.4	16.4
Loch Ericht. .	...	...	1.0	19.0	80.0

As an example of a heavily polluted stream, on the other hand, the following table on page 131 may be cited. It shows in a striking way the increase of B. coli in the Thames on its passage through London and its progressive purification below.

The river at the lower stations in this table was considerably diluted with sea-water, yet it showed clearly its large proportion of sewage. Normal sea-water, even in the neighborhood of the shore, shows B. coli only in large samples. Houston (1904), in another communication, reports the examination of 168 samples of sea-water near the English coast. None of the samples showed B. coli

in 1 c.c.; 97 samples gave negative results in 10 c.c.; 45 in 100 c.c., and 4 had 20 B. coli even in 1000 c.c.

B. COLI IN THE RIVER THAMES AT VARIOUS POINTS.

(HOUSTON, 1904[a].)

Percentage of positive results.

Place.	+ 10 c.c.	+10 −1 c.c.	+1 −.1 c.c.	+.1 −.01 c.c.	+.01 −.001 c.c.	+ .001 −.0001 c.c.	+ .0001 −.00001 c.c.
Sunbury . . .	...	...	...	70.6	23.5	5.9	...
Hampton . .	...	...	11.8	64.7	17.7	5.9	...
Barking . . .	...	...	...	4.2	45.8	45.8	4.2
Crossness .	...	...	...	11.1	27.7	50.0	11.1
Purfleet . . .	...	...	3.0	9.1	33.3	39.1	15.1
Grays	...	...	2.8	22.2	41.7	33.3	...
Mucking .	...	...	30.8	57.7	11.5	...	...
Chapman . .	5.0	45.0	50.	...	...	...	...
Barrow Deep .	12.0	36.0	40.	12.0	...	...	...

With ground-waters the story is the same. Even in sources of excellent quality we should expect to find, and we do sometimes find, colon bacilli in large volumes of water. Abba, Orlandi, and Rondelli (1899) showed by experiments with B. prodigiosus at Turin that when bacteria are present in great numbers on the surface of the ground, a few may penetrate for a considerable distance and ultimately reach the sources of ground-waters. The chance that disease germs could survive this process in a soil so impervious as to allow colon bacilli to appear only in large samples of water, is however infinitesimal.

An interesting contribution to the bacteriology of ground-waters was made by the Massachusetts State Board of

Health (Massachusetts State Board of Health, 1901) in connection with the examination of the spring-waters bottled for sale in the state. Ninety-nine springs were included in this study, and in almost every instance 4 samples were examined, 2 taken directly from the spring by the engineers of the board and 2 from the bottles as delivered for sale to the public. In the water of one spring B. coli was found twice, once in a sample from the spring and once in the bottled sample. This spring was situated in woodland, but was unprotected from surface drainage, and the method of filling bottles subjected it to possible contamination. In 5 other cases B. coli was found once in the sample from the spring; all were subject to pollution from dwellings or cultivated fields, and 4 of the 5 were shown to be highly contaminated, chemically. In 7 other cases B. coli was found in the bottled samples alone; 3 of these sources were of high purity, but the bottling process furnished opportunity for contamination.

Clark and Gage (1903), in the examination of 170 samples of water from tubular and curb wells of good quality used as sources of water-supply, found B. coli only five times, once in one cubic centimeter and four times in one hundred cubic centimeters. Horton (1903), from a study of ground-waters in Ohio, concluded that the presence of B. coli in wells and springs was indicative of serious pollution; of 37 waters of this class which showed B. coli, 27 had a history of typhoid fever.

Houston (1903b) makes an instructive comparison of some more or less polluted shallow wells at Chichester with deep ground-waters of high quality at Tunbridge Wells. The following table shows the value of the one-cubic-centimeter sample in discriminating between good and bad waters:

DISTRIBUTION OF B. COLI IN GOOD AND BAD WELL WATERS.

(HOUSTON, 1903b.)

Percentage of Positive Tests.

Quantity of Water.	Chichester Shallow Wells.	Tunbridge Wells, Deep Wells.
100 c.c.	90	25
10 c.c	80	6
1 c.c.	45	0
0.1 c.c.	20	0

In a subsequent investigation, Houston (1905) examined still larger samples of water from the Tunbridge Wells for B. coli: 49 samples of 100 c.c. each showed no B. coli, and 27 liter samples showed B. coli only once. Kaiser (1905) reports an interesting correlation between total numbers and B. coli in a series of 38 well waters. Of 11 wells containing over 200 bacteria per c.c. 90 per cent showed colon-like organisms in liter samples. Of 12 wells containing from 50 to 200 bacteria per c.c. 67 per cent gave colon-like organisms; of 26 wells with less than 50 bacteria per c.c., only 27 per cent showed positive results.

One of the most important applications of the colon

test is in the control of the operation of municipal water filters. It has been used for this purpose for ten years or more at Lawrence, and Fuller laid stress upon its results in his classic experiments on water purification in the Ohio valley. At Cincinnati he records the presence of colon bacilli in 60 per cent of the 1-c.c. samples from the Ohio River, while the effluent from either slow sand or mechanical filters gave positive results only half the time in samples of 50 c.c. The results of the examinations carried out at Lawrence for six years are brought together in the table below from the Annual Reports of the Massachusetts State Board of Health.

B. COLI IN MERRIMAC RIVER AND LAWRENCE FILTER EFFLUENT.

	Merrimac River, Per cent of one c.c., Samples containing B. coli.	Merrimac River, Number B. coli per c.c.	Filtered Water, Per cent of one c.c., Sample containing B. coli.
1900	99.7	87	18.1
1901	*	*	*
1902	99.0	73	4.0
1903	99.0	78	4.2
1904	100.0	73	8.0
1905	100.0	118	4.7

* Not given.

Effluents of better character are obtained by the filtration of less polluted streams, though the per cent purification effected is not so great as at Lawrence. Thus, at Harrisburg, Pa., with one cubic centimeter samples, positive tests were obtained 72 per cent of the time in the raw

Susquehanna River water and in less than 3 per cent of the samples of filtered effluent (Harrisburg, 1907). At Washington the most complete slow-sand filtration plant yet constructed has yielded the results tabulated below, for which we are indebted to the courtesy of Mr. F. F. Longley.

B. COLI IN POTOMAC RIVER AND WASHINGTON FILTER EFFLUENT.

	Dalecarlia Reservoir Inlet.			Filtered-Water Reservoir Outlet.		
	Number Tested.	Samples.		Number Tested.	Samples.	
		+ 10 C.C.	+ 1 C.C.		+ 10 C.C.	+ 1 C.C.
1906.						
February	15	5	3	24	0	0
March . .	24	12	3	27	0	0
April . . .	18	9	6	25	1	0
May . . .	25	3	1	27	0	0
June . .	26	9	8	26	0	0
July . . .	20	8	9	21	1	0
August . .	26	21	14	27	1	1
September	10	4	1	25	2	0
October . .	10	3	2	27	1	0
November .	8	3	0	25	2	0
December .	9	4	4	24	2	2
1907.						
January . .	9	5	3	26	3	3
February. .	8	2	2	23	0	0
March . .	8	7	4	26	0	0
April . . .	9	4	1	26	1	0
May . . .	23	21	15	26	0	0
June . . .	25	20	17	25	0	0
July . . .	26	11	8	26	0	0
August . .	27	13	8	27	0	0
September .	24	15	13	25	1	0

It must be remembered that in the Washington plant filtration is supplemented by thorough sedimentation, preliminary and subsequent. The entire credit for the good effluent obtained is not therefore due to the filters. At Lawrence it has been shown that removal of colon bacilli in storage reservoirs and pipe systems may be considerable. The figures obtained in 1900 at various points in the distribution system may be cited as an example.

PERCENTAGE OF SAMPLES OF WATER CONTAINING B. COLI.

Lawrence Experiment Station (Massachusetts State Board of Health, 1901.)

	Effluent of Filter.	Outlet of Reservoir.	Tap, City Hall.	Tap, Experiment Station.
In 1 c.c.	18.14	8.57	4.07	1.87
In 100 c.c. . .	38.12	23.30	15.54	15.54

In regard to the proportion of positive colon tests permissible in a filter effluent, Clark and Gage (Clark and Gage, 1900) reported some specially instructive observations made when certain of the underdrains of the Lawrence filter were relaid in the autumn of 1898. In doing this work the sand on some of the beds was seriously disturbed; and in December, after the work was completed, B. coli was found in 1 c.c. of the filtered effluent in 72 per cent of the samples examined. In January and February the organisms were found in 54 per cent and 62 per cent of the samples, respectively, while in March the

number fell to a normal value of 8 per cent. Corresponding to this excess of B. coli in the city water, there were 12 cases of typhoid fever in December, 59 cases in January, 12 in February, and 9 in March, all during the early part of the month. The authors conclude that "when filtering a river-water as polluted as that of the Merrimac, it is safe to assume that when B. coli is found only infrequently in 1 c.c. of the effluent, the typhoid germs, necessarily fewer in number and more easily removed by the filter, have been eliminated from the water."

The results of the daily tests carried out at municipal filter plants are frequently expressed in monthly or yearly averages, as in some of the cases quoted above. It must be remembered, however, that averages of this sort are accepted only by courtesy and with the implied assumption that conditions are approximately constant during the period averaged. When it is said that an acceptable effluent may show B. coli in three or four per cent of the samples tested, the statement is true only for a series of samples collected and examined at the same time. If in a given month 3 per cent of the 1-c.c. samples tested show B. coli, the effluent may or may not be safe. If on each of 20 days 3 B. coli or thereabouts were present in 100 c.c. of the water, it is probably a safe one. If on 19 days no B. coli were present, and on the twentieth day 100 c.c. showed 60 B. coli, the average result would be the same, but the water on one day was of a dangerous character. With properly managed filter plants marked variations do

not occur from day to day and average results are generally reliable. It is wholly misleading, however, to compare such results with the average examinations of an unfiltered surface water. With surface waters daily variations are the rule and a low monthly average of colon tests may include and cover up dangerous and significant high numbers at particular periods.

The general results of the studies of the colon tests which have now been carried out in great numbers all over the world may be summarized by a few further citations.

In America the fact that the number of colon bacilli in a water measures the degree of its pollution is now universally accepted. The same conclusion has been established in England by the elaborate investigations of Houston and his pupils. Savage, for example, concluded (Savage, 1902) from a study of a large number of water supplies in Wales, that even in surface waters, exposed to animal contamination from adjacent grazing grounds, B. coli is not present in 2 c.c. unless other pollution is present. In a more recent review of the whole subject, the same author (Savage, 1906) concludes that "there is no evidence or observations which have ever shown that B. coli, reasonably defined, is present in any numbers in sources which have not been exposed to some form of fecal contamination."

In Germany, Petruschky and Pusch (Petruschky and Pusch, 1903) examined a considerable series of waters

from different sources by incubating measured samples with equal amounts of nutrient broth and isolating upon agar. In 45 samples of well-waters they found B. coli 7 times in .01 c.c., 9 times in .1 c.c., and 7 times in 1 c.c. In the other 22 cases it could not be found in 1 c.c. and in 4 cases not in 100 c.c. One sample showed it only in 600 c.c. and 1 not in 750 c.c. Of 29 river-waters, only 2 failed to give positive results in .1 c.c. and 14 showed B. coli in .001 of a c.c. or less. In sewage the number varied from 1 to 1,000,000 per c.c. The authors conclude that a quantitative estimation of the B. coli content furnishes a good measure of the fecal pollution of water. Some of the best French bacteriologists have recently come to a similar conclusion. Gautié (1905) holds that the quantitative determination of B. coli is of the highest importance in water analysis; and Vincent (1905), in an excellent review of the subject, gives strong reasons for maintaining the same position. He finds B. coli absent from spring and well-waters of good quality and present in polluted water in proportion to its pollution. A number of French rivers showed numbers of B. coli varying from 1 to 110 per c.c. He concludes finally that water containing B. coli in .1 to 1.0 c.c. is unfit to drink, while if the organism is found in 1.0 to 10.0 c.c. it is of doubtful quality.

Altogether the evidence is quite conclusive that the absence of B. coli demonstrates the harmlessness of a water as far as bacteriology can prove it. That when present, its numbers form a reasonably close index of

the amount of pollution, the authors above quoted have proved beyond reasonable cavil. It may safely be said that when the colon bacillus, as defined by the tests above, is found in such abundance as to be isolated in a large proportion of cases from 1 c.c. of water, it is reasonable proof of the presence of serious pollution.

CHAPTER VIII.

PRESUMPTIVE TESTS FOR B. COLI.

THE isolation and identification of B. coli by the methods which have been described is a time-consuming and laborious operation, and one sometimes difficult to apply in the practical supervision of a water-supply.

Hence many investigators have attempted to devise tests which might be easily and quickly carried out, and which would yet give a fairly correct idea as to the existence of pollution. Such tests are spoken of as "presumptive tests."

The medium which was first urged for a rapid presumptive test was dextrose broth; and this method gained considerable acceptance five years ago. Its underlying principle is that B. coli develops rapidly in dextrose broth with gas formation of from 25 to 70 per cent of the capacity of the closed arm of the fermentation tube. Of this gas approximately one-third is carbon dioxide and two-thirds hydrogen, that is, as the gas formula is generally expressed, $\frac{H}{CO_2} = \frac{2}{1}$.

In testing a water by this method a series of samples, in suitable dilution, .001, .01, .1, 1.0, or 10 c.c., is added

directly to the dextrose-broth tubes and incubated for twenty-four hours at 37 degrees.

On measurement of the gas, if the results above given are obtained, the reaction is considered typical. If the amount of gas is between 10 and 25 per cent or more than 70 per cent, or the percentage of carbon dioxide is greater than 40, the reaction is considered atypical. If no gas forms, or less than 10 per cent, the test is called negative.

In recent years, Irons (Irons, 1901) was perhaps the first to call attention to the value of this method, stating that "when the dextrose tube yields approximately 33 per cent of CO_2, Bacillus coli communis is almost invariably present." In the next year the reliability of the fermentation test as an indication of B. coli was worked out by Gage (Gage, 1902) as given in the following table:

	1 c.c.	100 c.c.
Number of samples tested	5172	1375
Number giving preliminary fermentation	1036	474
Per cent of latter proved to contain coli	70	71

Whipple (Whipple, 1903) examined a large number of surface-water supplies by this "presumptive test" and obtained striking results, shown in the following table. The waters are arranged in six groups according to the results of sanitary inspection, Group I including waters collected from almost uninhabitated watersheds,

and Group VI waters too much polluted to be safely used for domestic purposes.

PERCENTAGE OF SAMPLES OF WATERS OF VARIOUS SANITARY GRADES GIVING POSITIVE TESTS FOR B. COLI WHEN DIFFERENT AMOUNTS WERE EXAMINED.

(WHIPPLE, 1903.)

Group.	0.1 c.c.	1.0 c.c.	10 c.c.	100 c.c.	500 c.c.
I	0.0	3.5	20.8	50.0	50.0
II	5.0	7.3	15.0	60.0	60.0
III	0.0	7.0	50.0	50.0	60.0
IV	4.0	6.8	41.7	67.0	75.0
V	5.0	13.0	75.0	100.0	100.0
VI	5.0	20.2	75.0	80.0	100.0

In view of these results Whipple suggested the following provisional scheme of interpretation:

Sanitary Quality.	Presumptive Test for Bacillus Coli.				
	0.01 c.c.	0.1 c.c.	1.0 c.c.	10.0 c.c.	100 c.c.
Safe	0	0	0	0	+
Reasonably safe	0	0	0	+	+
Questionable	0	0	+	+	+
Probably unsafe	0	+	+	+	+
Unsafe	+	+	+	+	+

It is undoubtedly true that a negative presumptive test is generally obtained with unpolluted waters. For example, in a study previously cited, Winslow and Nibecker (1903) reported that of 775 dextrose-broth tubes inoculated from 259 unpolluted sources, only 41 showed gas.

On the other hand, it is equally true that in a large proportion of cases colon bacilli are isolated from positive dextrose-broth tubes. Longley and Baton (1907) in the examination of 3553 samples of Potomac water obtained positive tests 794 times, while B. coli was actually present 529 times; 67 per cent of the presumptive tests were therefore correct. Gage (1902), in the Massachusetts work cited above, found that 70 per cent of his fermented dextrose tubes contained B. coli.

The work of recent years has made it clear, however, that both the coincidence of negative presumptive tests with the absence of B. coli and the general coincidence of positive presumptive tests with the presence of B. coli, are open to disastrous exceptions. In the study of the samples, tabulated on page 142, the presumptive test closely coincided with sanitary conditions. In the Kennebec River, too, Whipple found a close correspondence between the results of the presumptive test and the complete isolation of B. coli (Whipple, 1907). With other waters, however, discordant results have been reported. Stoughton (1905), in a study of the New York Supply, showed that B. coli could frequently be isolated when the presumptive test was negative. Fuller and Ferguson's (1905) results at Indianapolis and those of many other observers have led to the same conclusion. With heavily polluted waters the presumptive test breaks down entirely. Gas production may be absent or atypical in a large proportion of tubes inocu-

lated with water containing many streptococci or other sewage forms.

On the other hand, with some waters, positive presumptive tests may be obtained when colon bacilli are not present. According to Clark and Gage (1903) there are fifty-eight well-described species of bacteria which give the presumptive test in dextrose-broth, of which 23 are widely separated from the B. coli group. A recent unpublished investigation by Winslow and Phelps, indicates that the result of the dextrose broth test is markedly influenced by the factor of temperature. Their work consisted in the examination of 185 samples of water from 90 different sources, ponds, brooks, pools, wells and springs in five different states (Maine, New Hampshire, Massachusetts, Michigan and Virginia) at three different seasons of the year. All the waters examined were, as far as could be determined, free from specific pollution, although washings from roads or pasture-land might have had access to some of them. Most of the sources were undoubtedly unpolluted, and the examination of 119 samples for B. coli yielded only 12 positive results. The presumptive test, however, was obtained in a large proportion of the cases, and much more often in summer than in winter or spring, as indicated in the table on page 146.

DEXTROSE BROTH FERMENTATION IN 185 SAMPLES OF NORMAL WATERS AT DIFFERENT SEASONS.

(WINSLOW AND PHELPS.)

PERCENTAGE OF POSITIVE RESULTS.

	Summer 1906.	Winter.	Spring.	Summer 1907.
Framingham, Mass. . . .	87	62	23	57
Ann Arbor, Mich.	95	47	...	...
Exeter, N. H.	82	10	44	50
Richmond, Va.	...	14	14	...
Mt. Desert, Me.	95	...	...	...
All Stations	91	37	25	54

The Ann Arbor waters in this series included a number of driven wells, and the Mt. Desert sources were mountain brooks and ponds of the highest sanitary quality.

A new presumptive test has recently been suggested by Jackson (1906), which promises more satisfactory results than dextrose broth. MacConkey (1900) long ago suggested the use of media containing bile salts (sodium taurocholate) for the differentiation of B. coli and B. typhi, and bile-salts media have been used by various English observers (MacConkey, 1901; MacConkey and Hill, 1901) for the isolation of sewage bacteria. Jackson studied the action of various bile media and showed their selective inhibitory action in the striking table quoted on page 147. His important contribution to the subject, however, was the discovery that ox bile itself could be used as a culture medium, and that it was easier to prepare, cheaper and more effective than combinations of meat infusion with the purified bile salts.

SELECTIVE ACTION OF BILE SALTS.
(JACKSON, 1906.)

	Bacteria per c.c.			
	Uncontaminated Well.	Contaminated Pond.	Suspension of Feces.	Suspension of Feces.
Gelatin, 20°	920	2700	350,000	900,000
Agar, 37°	25	170	450,000	900,000
Bile agar,* 37°.	14	43	300,000	900,000
Lactose bile agar,* 37° . .	0	25	250,000	675,000
Lactose bile agar,* 37° . .	0	17	250,000	600,000
Bile agar, 37°	0	16	60,000	900,000

* Bile diluted, 1:1.

Jackson therefore suggested the use of fresh ox bile containing 1 per cent of lactose as a presumptive test instead of dextrose broth. In particular he hoped that this medium would be free to a great degree from the negative results due to overgrowths in polluted waters. He reported 275 examinations of badly contaminated waters, in which 65 per cent of the samples failed to give the dextrose-presumptive test, and only 10 per cent failed to show gas in lactose bile. In a more recent communication, Jackson (1907) reports that in the examination of 5000 samples of water at the Mt. Prospect Laboratory, the bile medium has proved uniformly satisfactory. He recommends incubation for 72 hours, results being commonly obtained, however, after 48 hours; and he considers any tube showing 25 per cent gas as positive. In a series of examinations recently carried out at the Institute of Technology, 16 per cent of the positive tubes

showed gas in 24 hours, 73 per cent after 48 hours, and the remaining 27 per cent only after 72 hours, so that the 72-hour period is frequently necessary. Sawin (1907) reports comparative results with dextrose broth and bile on different classes of waters, the most striking

COMPARATIVE PRESUMPTIVE TESTS WITH DEXTROSE BROTH AND LACTOSE BILE.

(SAWIN, 1907.)

Source.	Percentage of Samples Giving Positive Tests for B. Coli.					
	Dextrose Broth.			Lactose Bile.		
	0.1 c.c.	1.0 c.c.	10.0 c.c.	0.1 c.c.	1.0 c.c.	10.0 c.c.
1 Deep wells	0.	0.	0.	0.	0.	0.
2 Shallow wells	0.	1.0	10.0	0.	0.	6.0
3 Lake	15.0	10.0	15.0	5.0	0.	15.0
4 Lake	5.2	15.7	21.0	5.2	5.2	31.0
5 Lake	10.0	5.0	40.0	10.0	5.0	15.0
6 Lake	10.0	10.5	26.0	10.0	10.5	50.0
7 Lake	0.	10.0	5.0	00.	0.0	15.0
8 Lake	10.0	15.0	35.0	0.	5.0	30.0
Average, Nos. 3, 4, 5, 6, 7, 8	8.3	11.0	23.6	5.0	4.3	26.0
9 River	47.0	72.2	55.5	50.0	75.0	84.2
10 River	26.3	37.6	73.7	30.0	90.0	85.0
11 River	36.8	55.1	68.9	40.0	73.5	78.1
Average, Nos. 9, 10, 11	36.7	55.1	66.0	40.0	79.4	82.4
12 Brook	47.7	63.2	72.2	60.0	90.0	84.2
13 Drainage	50.0	73.7	78.9	84.2	90.0	90.0
14 Sewage	25.0	25.0	8.2	87.5	93.7	81.2
Average, Nos 12, 13, 14	40.9	53.9	53.1	77.2	91.2	85.1

of which are tabulated on page 148. It is clear that the bile medium is superior to dextrose broth for the more polluted waters.

It has been pointed out in Chapter VI that the lactose-bile medium is inferior to dextrose broth as a preliminary enrichment medium for the full isolation of B. coli, from the fact that it occasionally prevents the growth of B. coli which may be isolated by the dextrose method. As a presumptive test, however, it is far superior to dextrose broth, giving a higher proportion of positive tests with polluted waters and a lower proportion of erroneous positive tests with waters of good quality. In a recent examination of 176 surface waters in eastern Massachusetts, carried out under our direction, B. coli was isolated 70 times. The dextrose-broth test was positive 120 times, an error of 70 per cent; while the bile test, alone, was positive 78 times, an error of only 11 per cent. The tabulated results of these experiments indicate fairly the merits of the bile medium for preliminary enrichment and as a presumptive test.

PRELIMINARY AND COMPLETE RESULTS OF DEXTROSE BROTH AND BILE TESTS. 176 SURFACE WATERS.

	Preliminary Positive Results. (Gas Formation.)	Final Positive Results. (B. Coli).
Dextrose broth	120	70
Lactose bile	78	64

It is certain that the bile medium sometimes shows gas when typical B. coli are absent; it is certain that it sometimes gives entirely negative results when B. coli may be isolated in other ways. Neither of these errors is commonly of great magnitude, however, and in general the lactose bile results correspond fairly well with those obtained by the complete isolation of B. coli.

No merely presumptive test of this sort should be substituted for the complete demonstration of B. coli in the detailed sanitary study of a special source of supply. For extensive routine surveys of considerable series of sampling stations, on the other hand, the bile test offers a satisfactory approximation to the truth.

The litmus-lactose-agar plate furnishes a presumptive test of considerable value as indicated in Chapter IV, although it is probably less delicate than the fermentation methods. With polluted waters, however, comparative studies of the agar plate and the bile method are much to be desired.

Other special media have been suggested for rapid routine water analysis of which those containing "neutral red," one of the safranine dyes, have been most fully studied. Rothberger (Rothberger, 1898) first pointed out that B. coli reduces solutions of this substance, the color changing to canary-yellow accompanied by green fluorescence. Makgill (Makgill, 1901), Savage (Savage, 1901), and other English observers, as well as Braun (1906), in France, report favorable results from the use of this test,

but according to American standards, Irons (Irons, 1902) and Gage and Phelps (Gage and Phelps, 1903) conclude that the group of organisms giving a positive neutral red reaction is too large a one to give very valuable sanitary information.

Stokes (1904) urged the use of lactose broth with the addition of neutral red, and believed that the production in this medium of 30–50 per cent of gas with a $\frac{2}{1}$ gas formula and the change of neutral red to canary yellow in the closed arm of the fermentation was characteristic for B. coli.

The lactose-bile method, however, is the only rapid test whose value has yet been established by a considerable series of investigations; it seems to be the best presumptive test now available.

CHAPTER IX.

OTHER INTESTINAL BACTERIA.

It would be an obvious advantage if the evidence of sewage contamination, furnished by the presence of B. coli, could be reinforced and confirmed by the discovery in water of other forms equally characteristic of the intestinal canal. The attention of bacteriologists in England and America has been turned in this direction during the past few years; and two groups of organisms, the sewage streptococci and the anærobic spore-bearing bacilli, have been described as probably significant.

The term "sewage streptococci," as generally used, covers an ill-defined group including many cocci which do not occur in well-marked chains. Those most commonly found correspond rather closely to the type of Str. pyogenes (identical with Str. erysipelatos). They grow feebly on the surface of ordinary nutrient agar, producing faint transparent, rounded colonies, but under semi-anærobic conditions flourish better, giving a well-marked growth along the gelatin stab and only a small circumscribed film on the surface. They are favored by the presence of the sugars and ferment dextrose and lactose, with the formation of abundant acid but no gas.

They are seen under the microscope as cocci, occurring as a rule in pairs, short chains, or irregular groups. They do not show visible growth and do not form indol and nitrite in the standard peptone and nitrate solutions; most of them do not liquefy gelatin, though occasionally forms are found which possess this power. Until recently, no systematic study of the various species found in the intestine had been made and at present all cocci giving the characteristic growth on agar and strongly fermenting lactose are commonly included as "sewage streptococci."

Although the significance of the streptococci as sewage organisms is not established with the same definiteness which marks our knowledge of the colon group, these forms have been isolated so frequently from polluted sources and so rarely from normal ones that it now seems reasonable to regard their presence as indicative of pollution. Although originally reported by Laws and Andrewes (Laws and Andrewes, 1894), their importance was not emphasized until 1899 and 1900, when Houston (Houston, 1899[b], 1900[b]) laid special stress upon the fact that streptococci and staphylococci seem to be characteristic of sewage and animal waste, the former being, in his opinion, the more truly indicative of dangerous pollution, since they are "readily demonstrable in waters recently polluted and seemingly altogether absent from waters above suspicion of contamination." In six rivers, recently extensively sewage-polluted, he found streptococci in from one-tenth to one ten-thousandth of a c.c. of the water examined,

although in some cases the chemical analysis would not have indicated dangerous pollution. On the other hand, eight rivers, not extensively polluted, showed no streptococci in one-tenth of a c.c., although the chemical and the ordinary bacteriological tests gave results which would condemn the waters. Horrocks (Horrocks, 1901) found these organisms in great abundance in sewage and in waters which were known to be sewage-polluted, but which contained no traces of Bacillus coli. He found by experiment that B. coli gradually disappeared from specimens of sewage kept in the dark at the temperature of an outside veranda, while the commonest forms which persisted were varieties of streptococci and staphylococci.

In America attention was first called to these organisms by Hunnewell and one of us (Winslow and Hunnewell, 1902[a]), and the same authors later (Winslow and Hunnewell, 1902[b]) recorded the isolation of streptococci from 25 out of 50 samples of polluted waters. Gage (Gage, 1902), from the Lawrence Experiment Station, has reported the organisms present in the sewage of that city, while Prescott (1902[b]) has shown that they are abundant in fecal matter and often overgrow B. coli in a few hours when inoculations are made from such material into dextrose broth. In the recent monograph of Le Gros (Le Gros, 1902) of the many streptococci described, all without exception were isolated, either from the body or from sewage. Baker and one of us (Prescott and Baker, 1904) found these organisms present in each of 50 samples

of polluted waters. On the other hand, in the study of 259 samples of presumably unpolluted waters, by the method of direct plating, Nibecker and one of the authors (Winslow and Nibecker, 1903) found streptococci in only one sample. Gordon (1904) showed that certain streptococci are abundant in normal saliva and are found in air which has been exposed to human pollution but not in normal air. On the whole there can be no doubt of the fact that streptococci occur on the surfaces of the human and animal body more commonly than anywhere else in nature.

The isolation of these organisms either from plates or liquid cultures is easy. On the lactose-agar plate, made directly from a polluted water, the colonies of the streptococci may generally be distinguished from those of other acid-formers by their small size, compact structure, and deep-red color, which is permanent, never changing to blue at a later period of incubation. Developing somewhat slowly, however, they may be overlooked if present only in small numbers. In the dextrose-broth tube, streptococci will generally appear in abundance after a suitable period of incubation. Prescott and Baker, in the work above mentioned, found that with mixtures of B. coli and streptococci in which the initial ratios of the latter to the former varied from 1:94 to 208:1, the colon bacilli developed rapidly during the early part of the experiment, reaching a maximum after about fourteen hours, and then diminishing rapidly. The streptococci

first became apparent after ten to fifteen hours and reached their maximum after twenty to sixty hours, according to the number originally present.

Applying the same method to polluted waters, similar periodic changes were observed; pure cultures of B. coli were first obtained, then the gradual displacement of one form by the other took place, and at length the streptococci were present either in pure culture or in great predominance as shown by the accompanying tables. The samples of water were plated directly upon litmus lactose agar and the plates were incubated at 37° for twenty-four hours,

TABLE I.

RELATIVE GROWTH OF B. COLI AND SEWAGE STREPTOCOCCI FROM POLLUTED WATERS IN DEXTROSE BROTH.

(PRESCOTT AND BAKER, 1904.)

Sample Number			1	2	3	4	5	6	7	8	9	10
Red colonies developing from 1 c.c. of original sample on litmus lactose agar			4	10	9	5	8	55	35	460	1250	105
Number found, in millions per cubic centimeter, after growth in dextrose broth for various periods	11 hrs.	B. coli	0	20	68	200	185	400	130	332	420	410
		Strept.	0	0	0	0	0	0	0	0	0	0
	16 hrs.	B. coli	200	76	130	270	220	210	140	420	285	410
		Strept.	40	25	20	10	45	30	20	210	75	145
	23 hrs.	B. coli	280	150	385	370	300	570	200	405	320	300
		Strept.	140	85	280	170	300	1700	110	350	370	350
	39 hrs.	B. coli	0	0	25	110	0	210	20	24	105	
		Strept.	474	420	480	300	390	170	400	105	250	
	63 hrs.	B. coli	0	0	0	0	0	12	8	0	0	0
		Strept.	2	0	0	45	1	2	45	150	86	170
First gas noted after (hrs.).			10	10	9	9	10	8	10	6	6	8

when the red colonies were counted. At the time of plating, 1 c.c. from each sample was also inoculated into dextrose broth in fermentation tubes, which were likewise incubated at 37°. After various periods, as indicated by the table below, the tubes were shaken thoroughly and 1 c.c. of the contents withdrawn. This was diluted (generally 1–1,000,000) with sterile water, plated on litmus

TABLE II.

RELATIVE GROWTH OF B. COLI AND SEWAGE STREPTOCOCCI FROM POLLUTED WATERS IN DEXTROSE BROTH.

(PRESCOTT AND BAKER, 1904.)

Sample Number			18	19	20	21	22	23	24	25
Red colonies developing from 1 c.c. of original sample on litmus lactose agar			1	150	25	30	50	170	200	30
Number found, in millions per cubic centimeter, after growth in dextrose broth for various periods	7 hrs.	B. coli	.02	. .	. .	.01	.04	.12	.55	1.6
		Strept.	0	. .	. .	0	0	0	0	0
	17 hrs.	B. coli	266	100	88	350	510	380	330	160
		Strept.	150	0	40	140	240	128	80	220
	27 hrs.	B. coli	520	610	72	700	1000	740	100	300
		Strept.	800	860	670	1080	2500	4380	...	3900
	40 hrs.	B. coli	0	0	10	22	36	7	7	
		Strept.	252	330	260	22	66	60	52	
	52 hrs.	B. coli	10	16	38	20	70	35	10	27
		Strept.	40	16	3.8	31	41	25	10	30

lactose agar in the usual way, and incubated for twenty-four hours. The colonies of B. coli and streptococci were distinguished microscopically, and by difference in color and general characters.

The successive growth of these two intestinal groups in the same dextrose-broth tube suggests the following method for the detection of both B. coli and sewage streptococci:

Inoculate the desired quantity of water, preferably 1 c.c., into dextrose broth, in a fermentation tube, and incubate at 37 degrees. After a few hours' incubation examine the cultures for gas. Within two or three hours after gas formation is first evident, plate from the broth in litmus lactose agar, incubating for twelve to eighteen hours at 37 degrees. If at the end of this time no acid-producing colonies are found, it is probably safe to assume that there were no colon bacilli present. On the other hand, if red colonies develop, these must be further examined by the regular diagnostic tests for B. coli. After the first plating from the dextrose broth, replace the fermentation tube in the incubator and allow it to remain for twenty-four to thirty-six hours, then plate again in litmus lactose agar. This plating should give a nearly pure culture of streptococci if these organisms were originally present in the water.

The relative relation of the streptococci and the colon bacilli to sewage pollution is still somewhat uncertain. Houston (Houston, 1900) held that the former microbes imply "animal pollution of extremely recent and therefore specially dangerous kind." Horrocks (Horrocks, 1901), on the other hand, maintains, largely on the strength of certain experiments with stored sewage, that the streptococci persist after colon bacilli have disappeared and

indicate contamination with old sewage which is not necessarily dangerous. These discordant results are probably to be explained by the different media in which the viability of the bacteria was compared. It seems likely that in sewage where there is a large amount of organic food material present the streptococci may kill out the colon bacilli as they do in the fermentation tube. This would explain Horrocks' results. On the other hand, there is good evidence that the streptococci are less resistant than B. coli to the unfavorable conditions which exist in water of ordinary organic purity. In waters of potable character B. coli is frequently present without the streptococcus; and a negative test for streptococci has little significance. A positive test on the other hand furnishes valuable confirmatory evidence of pollution. This evidence is of course of special importance when, through the activity of the streptococci themselves, or from any other cause, the colon isolation has yielded an erroneous negative result.

The English Committee appointed to consider the standardization of methods for the bacterioscopic examination of water (1904), by a majority vote recommended the enumeration of streptococci as a routine procedure in sanitary water analysis, and the test deserves more careful attention than it has yet received in America.

There seems even reason to hope that the streptococci may prove of assistance in the important task of differentiating human and animal pollution, a task in which

all other tests have so far failed. Unlike the colon bacilli, streptococci from the intestines of cattle and men appear to belong to distinct types. The recognition of this fact we owe primarily to Gordon (1905), who made an elaborate study of the fermentative power of the streptococci in a long series of carbohydrate media. His work and that of Houston (Houston, 1904; Houston, 1905[a]; Houston, 1905[b]) have made it clear that the streptococci of the herbivora differ from those found in the human body in their low fermentative power. In a recent review of the genus, Andrewes and Horder (1906) describe the type characteristic of the herbivora under the name, Str. equinus, and define it by its failure to ferment lactose, raffinose, inulin or mannite, or to reduce neutral red. Five other types are described from the human mouth and intestine; all of them ferment lactose, and most reduce neutral red and ferment raffinose. The commonest intestinal form clots milk, reduces neutral red and ferments saccharose, salicin, coniferin, and mannite. The specific types of the genus streptococcus, grade into each other by almost imperceptible degrees, and streptococci fermenting lactose and raffinose and reducing neutral red are sometimes found in bovine feces. The fact that Str. equinus is the commonest form among herbivora and a rarer form in man will however prove an important contribution to water bacteriology, if confirmed by other observers.

The English bacteriologists have ascribed much importance as indicators of sewage pollution to another group

of organisms, the anærobic spore-forming bacilli, of which the B. sporogenes is a type. This form was isolated by Klein (Klein, 1898; Klein, 1899) in 1895, in the course of an epidemic of diarrhœa at St. Bartholomew's Hospital, and described under the name of B. enteritidis sporogenes; it is closely related to the B. aerogenes capsulatus of Welch (Welch and Nuttall, 1892).

Klein's procedure for isolating the B. sporogenes is simple in the case of highly polluted waters. A portion of the sample to be examined is added to a tube of sterile milk, which is then heated to 80° C. for ten minutes to destroy vegetative cells. The milk is next cooled and incubated under anærobic conditions, which may be accomplished most conveniently by Wright's method. A tight plug of cotton is forced a quarter way down the test tube, the space above is loosely filled with pyrogallic acid, a few drops of a strong solution of caustic potash are added, and the tube is tightly closed with a rubber stopper. After eighteen to thirty-six hours at 37 degrees the appearance of the tube will be characteristic if the B. sporogenes is present. "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 colorless, 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 B. sporogenes when isolated in pure culture on glucose agar is a stout rod. It liquefies gelatin, forming in this medium large oval spores. It is strongly pathogenic for guinea pigs, by which character it is distinguished from the B. butyricus of Botkin.

The researches of Klein and Houston (Klein and Houston, 1898, 1899) have shown that the B. sporogenes occurs in English sewage in numbers varying from 30 to 2200 per c.c. and that it is often absent in considerable volumes of pure water. In Boston sewage it may usually be isolated from .01 or .001 of a c.c. (Winslow and Belcher, 1904).

Since this organism is not present in very large numbers, even in sewage, the test of a water supply must be made with large samples, and the concentration of at least 2000 c.c. of water by filtration through a Pasteur filter is recommended by Horrocks as a necessary prelude (Horrocks, 1901). Since the spores of an anærobic bacillus may persist for an indefinite period in polluted waters, their presence need not necessarily indicate recent or dangerous pollution. On the whole, it does not appear that the practical application of the anærobic test will ever be a wide one.

Besides the streptococci and the anærobic spore formers, various intestinal bacilli, more or less closely allied to the

B. coli group, have received attention from the English bacteriologists. A large number of species and races have been described which are intermediate in their properties between B. typhi and B. coli, all being non-spore-forming, non-liquefying rods, which produce a more or less characteristic growth on solid media. Durham (1898) divided these forms into three main divisions, grouped, respectively, about B. typhi, B. enteritidis and B. coli. Organisms of the first division ferment neither dextrose, lactose nor saccharose; those of the second ferment dextrose but not lactose; and those of the B. coli division form gas in both these sugars. The relationship of the commonest species is indicated in tabular form below:

BACTERIA OF THE COLON-TYPHOID GROUP.

Species.	Dextrose.		Lactose.	
	Gas Formation.	Acid Production.	Gas Formation.	Acid Production.
B. alcaligenes	None	None	None	None
B. typhi	None	Slight	None	Slight
B. dysenteriæ	None	Distinct	None	Slight
B. enteritidis	Active	Strong	None	Slight
Paratyphoid bacilli . . .	Active	Strong	None	Slight
Hog cholera bacillus . . .	Active	Strong	None	Slight
B. coli	Active	Strong	Active	Strong

In the typhoid division, B. alcaligenes and B. dysenteriæ are the best known forms, besides B. typhi itself.

B. alcaligenes stands at the lower end of the whole series in fermentative power. B. typhi forms a slight initial acidity in milk and a slight acidity in dextrose broth, while the reaction of B. alcaligenes in sugar media is always alkaline. B. dysenteriæ, on the other hand, differs from B. typhi in the direction of the B. enteritidis group, producing a well-marked acid reaction, but no gas in dextrose media. B. typhi and B. dysenteriæ are, of course, also distinguished by their specific serum reactions. Neither B. alcaligenes nor any other member of this group (except the disease-producers themselves, when clearly identified by agglutination tests) has any well-established sanitary significance. Non-acid-forming bacteria of this general type are frequently found in feces, but they are also found in other habitats, and comparative data are entirely lacking to show that they are more abundant, proportionately, in polluted than in normal waters.

The second great division of the colon-typhoid bacteria is the hog cholera group, or the Gärtner group, as Durham (1898) called it. As defined by him, it differed from the typhoid group by gas formation in dextrose, and from the colon group by the production of a final alkaline reaction in milk. It includes the Gärtner bacillus (B. enteritidis), the hog cholera bacillus (B. choleræ suis), and the paratyphoid bacilli. Some of these forms, the paratyphoid bacilli, for example, and B. enteritidis (isolated in cases of meat poisoning), produce intestinal

disease in man. Unless, however, specific pathogenes can be identified by serum reactions, the non-lactose-fermenting forms have no clear significance in water analysis. They occur in the intestine, though not apparently in great abundance. Houston (1904) attempted to isolate bacteria fermenting dextrose but not lactose from normal human stools; but out of 257 colonies studied only six failed to form acid and gas in lactose media. Organisms of this character may also be found in unpolluted water, as shown by the occurrence of positive dextrose-broth tests followed by negative litmus-lactose-agar plates (see Chapter VIII).

Organisms belonging to the colon group itself, and producing gas and acid in both dextrose and lactose media, are much more clearly related to sewage pollution. Numerous investigations of normal waters have shown that bacteria possessing these two properties are rarely found where pollution is absent, while the commonest intestinal forms exhibit this dual fermentative power. The mass of evidence establishing excretal origin is stronger in the case of B. coli than for any of its allies. B. communior, which differs in possessing the power of fermenting saccharose, is, however, almost invariably included with B. coli in practical work. The significance of those forms which differ from B. coli by lacking one or all of the properties of motility, of indol formation, or of nitrate reduction, or by failing to coagulate milk in the standard time, is somewhat less clear. B. aerogenes

is a well-defined species of this group, which differs from B. coli in lacking motility, in possessing a capsule, in forming a somewhat heavier growth on media than B. coli, and in failing to form indol. This and other allied forms are sometimes called "atypical B. coli," or "paracolon bacilli," and Vincent gives them the picturesque name, "microbic satellites of B. coli."

Sometimes, as pointed out in Chapter VI, these organisms are merely weakened strains of B. coli which have lost certain powers through exposure to unfavorable environment. The results obtained by Peckham (1897) suggest that the indol reaction in particular is highly variable. By successive daily transfers in peptone broth she was able to increase the amount of indol produced by normal B. coli, and by a longer continuance of the same process to again weaken and abolish the power of forming it. Gas formation too was slackened in the cultures grown for too many transfers in the same medium. Horrocks (1903) found that B. coli kept in unsterilized well waters and tap waters and in sterilized sewage and Thames water for two to three months, showed only a feeble indol production and a delayed action on milk and neutral red. Even the fermentative powers which distinguish the B coli and the B. enteritidis groups may be modified by environmental conditions. Twort (1907) reports that by continued cultivation in sugar media he was able to develop fermentative power in certain members of the Gärtner group which lacked such powers before.

It is certain that in the intestine these atypical forms are less common than the colon bacillus itself, bearing to it indeed very much the relation implied in Vincent's term, satellites. Houston (1903[a]) examined in detail 101 cultures of coli-like microbes isolated from feces, and found that 72 per cent of the cultures were typical in all respects, while 11 per cent more differed only in being non-motile. The remaining 17 per cent were atypical, reacting abnormally to milk, indol, neutral red, litmus whey or Capaldi and Proskauer's medium. In a later investigation, Houston (1904) made a careful study of the distribution of the atypical forms in feces, sewage, polluted water, and the filtered water-supplies of London. According to his ingenious system of nomenclature, "fl" indicates an organism which produces green fluorescence in neutral red broth; "ag," one which forms acid and gas in lactose media; "in," one which produces indol; and "ac," one which acidifies and clots litmus milk. The combination of all these properties gives "Flaginac," or typical B. coli; "aginac," is a form which fails to reduce neutral red; "flagac," one which fails to form indol, etc. "Flaginac" B. coli form the great majority of coli-like microbes in feces, but Houston found that in filtered water they are outnumbered by atypical forms, of which he recognized thirty-five distinct types.

On the whole, all the evidence tends to the assumption that the atypical forms, or "paracolon bacilli," generally represent weakened strains from the intestinal B. coli

stock. As Savage says, "we know that nearly all the coli-like organisms in feces are quite typical B. coli, that in sewage a good many atypical varieties are present, and that in contaminated water and soil the proportion present is still larger." The presence of these forms in water must, in the light of present knowledge, be considered suspicious, though not an indisputable evidence of contamination. The only safe rule, when atypical forms are found without standard B. coli, is to secure another sample for examination. Fortunately this condition rarely occurs, since typical colon bacilli are generally found in duplicate samples when the atypical forms are present.

B. cloacæ, which differs from B. coli in the liquefaction of gelatin, stands in much the same position as the atypical forms of the colon bacillus. We have seen that organisms which ferment dextrose and lactose are rarely found in normal waters, and this form must, therefore, be regarded with suspicion. Detailed studies of its distribution are necessary, however, before its presence can be given the same weight as that of the colon bacillus.

There are numerous other sewage bacteria whose presence is more or less characteristic of polluted waters. Organisms of the Proteus group are sometimes present, exhibiting marked morphological variations, from the coccus form to long twisted threads, and forming on gelatin irregular amœboid colonies with filiform processes extending into the surrounding gelatin. The B. subtilis group of strongly ærobic spore-forming bacilli, giving a

brown wrinkled parchment-like growth on agar, and moss-like liquefying colonies on gelatin, is usually represented; but this form is more characteristic of decaying vegetation in the surface layers of the soil than it is of sewage. Vincent (1907) and other French observers consider the determination of the total number of anærobic bacteria as significant since the decomposition of organic matter is accompanied by anærobic growth. It is not claimed, however, that bacteria of this type are characteristic of animal more than of vegetable decompositions, and the total anærobic count apparently adds nothing of importance to the information gained by the ordinary gelatin plate method. The property of liquefaction was formerly believed to be of significance, inasmuch as the liquefying bacteria were regarded as indicative of pollution. This position is, however, no longer tenable, since many bacteria, typical of the purest waters, may cause liquefaction.

As Savage says in summing up this question: "The number of different species of organisms in sewage is very great, and it is highly probable that many of them occur in all specimens of ordinary sewage; but, except for B. coli, streptocci, and B. enteritidis sporogenes, their presence has not been ascertained with sufficient constancy, nor has their numerical occurrence been sufficiently investigated to make them of value as indicators of sewage pollution." (Savage, 1906.)

CHAPTER X.

THE SIGNIFICANCE AND APPLICABILITY OF THE BACTERIOLOGICAL EXAMINATION.

THE first attempt of the expert called in to pronounce upon the character of a potable water should be to make a thorough sanitary inspection of the pond, stream, well, or spring from which it is derived. Study of the possible sources of pollution on a watershed, of the direction and velocity of currents above and below ground, of the character of soil and the liability to contamination by surface-wash are conceded to yield evidence of the greatest value. Often, however, an opinion is desired as to the quality of water sent from a distance without the opportunity of examining its surroundings; and even when sanitary inspection can be made, its results are by no means conclusive. If house or barnyard drainage or sewage is actually seen to enter a water used for drinking purposes it is obviously unnecessary to carry out delicate chemical or bacteriological tests to detect pollution. On the other hand, no reconnoissance can show certainly whether unpurified drainage from a cesspool does or does not reach a given well; whether sewage discharged into a lake does or does not find its way to a neighboring

intake; whether pollution of a stream has or has not been removed by a certain period of flow. Evidence upon these points must be obtained from a careful study of the characteristics of the water in question, and this study can be carried out along two lines, chemical and bacteriological.

A chemical examination of water for sanitary purposes is mainly useful in throwing light upon one point — the amount of decomposing organic matter present. It also gives an historical picture which may be of some value or suggestiveness. Humus-like substances may be abundant in surface-waters quite free from harmful pollution, but these are stable compounds. Easily decomposable bodies, on the other hand, must obviously have been recently introduced into the water and mark a transitional state. "The state of change is the state of danger," as Dr. T. M. Drown once phrased it. Sometimes the organic matter has been washed in by rain from the surface of the ground, sometimes it has been introduced in the more concentrated form of sewage. In any case, it is a warning of possible pollution, and the determination of free ammonia, nitrites, carbonaceous matter, as shown by "oxygen consumed," and dissolved oxygen yield important evidence as to the sanitary quality of a water.

Furthermore, nitrates, the final products of the oxidation of organic matter, and the chlorine introduced as common salt into all water which has been in contact with the wastes of human life, furnish additional informa-

tion as to the antecedents of a sample. The results of the chlorine determination are indeed perhaps more clear than those of any other part of the analysis, for chlorine and sewage pollution vary together, due allowance being made for the proximity of the sea and other geological and meteorological factors. Unfortunately, it is only past history and not present conditions which these latter tests reveal, for in a ground-water completely purified from a sanitary standpoint such soluble constituents remain, of course, unchanged. Thus, in the last resort, it is upon the presence and amount of decomposing organic matter in the water that the opinion of the chemist must be based.

The decomposition of organic matter may be measured either by the material decomposed or by the number of organisms engaged in carrying out the process of decomposition. The latter method has the advantage of far greater delicacy, since the bacteria respond by enormous multiplication to very slight increase in their food-supply, and thus it comes about that the standard gelatin-plate count at 20 degrees roughly corresponds, in not too heavily polluted waters, to the free ammonia and "oxygen consumed," as revealed by chemical analysis. If low numbers of bacteria are found, the evidence is highly reassuring, for it is seldom that water could be contaminated under natural conditions without the direct addition of foreign bacteria or of organic matter which would condition a rapid multiplication of those already

present. The bacteriologist in such cases can declare the innocence of the water with justifiable certainty. When high numbers are found the interpretation is less simple, since they may exceptionally be due to the multiplication of certain peculiar water forms. Large counts, however, under ordinary conditions, when including a normal variety of forms indicate the presence of an excess of organic matter derived in all probability either from sewage or from the fresh washings of the surface of the ground. In either case danger is indicated.

A still closer measure of polluting material may be obtained from the numbers of colonies which develop on litmus lactose agar at 37 degrees, since organisms which thrive at the body temperature, and particularly those which ferment lactose, are characteristic of the intestinal tract and occur but rarely in normal waters.

Gage (Gage, 1907) has shown that by counts at 20, 30, 40, and 50° C., information may be quickly obtained which is of great assistance in judging the character of the water.

"Modern methods of bacterial examination of water, consisting usually of determinations of the numbers of bacteria by means of plates incubated at room temperature, and of tests for the presence or absence of one or two specific types, occasionally lead to an erroneous interpretation of the quality of a water, owing to the fact that they do not yield adequate data by which abnormal and inaccurate results may be separated from those which are

truly indicative of purity or pollution. Furthermore, as several days must elapse before the bacterial tests can be completed, the results when obtained may have passed their usefulness. If, however, we can so modify our procedure that the varied character of the bacteria in waters of different classes may be quickly and accurately recognized, the value of bacterial water analysis will be enormously increased. Much of this information may be obtained by the use of selective media, selective temperatures, or by a proper combination of the two.

"By the use of litmus lactose agar in place of agar or gelatin we obtain similar counts of total bacteria, and in addition are able to separate those bacteria into two groups, which do and do not produce acid fermentation of lactose, and the numbers of the two classes of bacteria so obtained indicate more completely the character of the water than would the numbers of either class alone. By incubating our plates at temperatures of 30 or 40° C. we are able to obtain counts in twelve to eighteen hours, which counts, while smaller than those on plates incubated for a longer period at a lower temperature, appear to be fully as significant. If we increase our number of determinations by incubating duplicate plates at two or more temperatures, the various results and the ratios between them furnish a check upon one another in addition to increasing the available data upon which to base an interpretation.

"The distinction between polluted waters and waters of

good quality is more sharply marked by counts at 30° C. than is the case with counts at 20° C. Determinations at this temperature appear to be especially applicable to the control of water filters, since the relative quality of the raw and filtered waters and the expression of the removal of bacteria by those filters are practically identical with determinations made at 20° C., and, in addition, they become available within a few hours after the sample is collected." (Gage, 1907.)

Finally, the search for the Bacillus coli furnishes the most satisfactory of all single tests for fecal contamination. This organism is preëminently a denizen of the alimentary canal and may be isolated with ease from waters to which even a small proportion of sewage has been added. On the other hand, it is never found in abundance in waters of good sanitary quality, and its numbers form an excellent index of the value of waters of an intermediate grade. The streptococci appear to be forms of a similar significance useful as yielding a certain amount of confirmatory evidence. The full bacteriological analysis should then consist of three parts — the gelatin-plate count, as an estimate of the amount of organic decomposition in process; the total count, and the count of red colonies, on litmus lactose agar, as a measure of the organisms which form acids and thrive at the body temperature; and the study of a series of dextrose-broth tubes for the isolation of colon bacilli and streptococci. The simple examination of the lactose-bile tube or the count on

the litmus-lactose-agar plate serve for what Whipple has well called presumptive tests.

The results of the bacteriological examination have, in several respects, a peculiar and unique significance. First, this examination is the most *direct* method of sanitary water analysis. The occurrence of nitrites or free ammonia in a small fraction of one part per million, or of chlorine in several parts per million, do not in themselves render a water objectionable or dangerous. They merely serve as indicators to show that germ-containing and germ-sustaining organic matter is present. By a determination of the chlorine and study of the relations of carbon and nitrogen, it is possible to determine with some degree of accuracy whether this organic matter is of plant or animal origin, and hence to rate its objectionable or dangerous character. By the bacteriological examination, on the other hand, we are able to determine directly whether particular kinds of organisms characteristic of sewage are, or are not, actually present in the water. What we dread in drinking-water is the presence of pathogenic bacteria, mainly from the intestinal tract of man, and it is quite certain that the related non-pathogenic bacteria from the same source will behave more nearly as these disease germs do than will any chemical compounds. In the second place, the bacteriological methods are superior in *delicacy* to any others. Klein and Houston (1898) showed by experiment with dilutions of sewage that the colon test was from ten to one hundred times as

sensitive as the methods of chemical analysis; and studies of the self-purification of streams have confirmed their results on a practical scale. Thus in the Sudbury River it was found that while the chemical evidences of pollution persisted for six miles beyond the point of entrance, the bacteria introduced could be detected for four miles further (Woodman, Winslow, and Hansen, 1902).

The statement is sometimes made that while bacteriological methods may be more delicate for the detection of pollution in surface-waters, contamination in ground-waters may best be discovered by the chemical analysis. That such is not the case has been well shown by Whipple (Whipple, 1903), who cites the following two instances in which the presumptive test revealed contamination not shown by the chemical analysis:

"A certain driven-well station was located in swampy land along the shores of a stream, and the tops of the wells were so placed that they were occasionally flooded at times of high water. The water in the stream was objectionable from the sanitary standpoint. The wells themselves were more than 100 feet deep; they penetrated a clay bed and yielded what may be termed artesian water. Tests for the presence of Bacillus coli had invariably given negative results, as might be naturally expected. Suddenly, however, the tests became positive and so continued for several days. On investigation it was found that some of the wells had been taken up to

be cleaned, and that the workmen in resinking them had used the water of the brook for washing them down. This allowed some of the brook-water to enter the system. It was also found that at the same time the water in the brook had been high, and because of the lack of packing in certain joints at the top of the wells the brook-water leaked into the suction main. The remedy was obvious and was immediately applied, after which the tests for Bacillus coli once more became negative. During all this time the chemical analysis of the water was not sufficiently abnormal to attract attention. On another occasion a water-supply taken from a small pond fed by springs, and which was practically a large open well, began to give positive tests for Bacillus coli, and on examination it was found that a gate which kept out the water of a brook which had been formerly connected with the pond was open at the bottom, although it was supposed to have been shut, thus admitting a contaminated surface-water to the supply." Whipple also calls attention to the report on the Chemical and Bacteriological Examination of Chichester Well-waters by Houston (Houston, 1901), in which the results of chemical and bacteriological examinations of thirty wells were compared. It was found that the bacteriological results were in general concordant and satisfactory. The wells which were highest in the number of bacteria showed also the greatest amount of pollution, as indicated by the numbers of B. coli, B. sporogenes, and streptococci. On the other hand,

the chlorine and the albuminoid ammonia showed no correspondence with the bacteriological results.

Vincent (Vincent, 1905) cites an interesting case of the detection of progressive pollution of a ground-water by bacteriological methods. The well of a military camp in Algeria showed 200 bacteria per c.c. before the arrival of a regiment of troops. Its subsequent history is indicated in the table below.

PROGRESSIVE POLLUTION OF A WELL.

(VINCENT, 1905.)

	Bacteria per c.c.	Bacillus Coli per c.c.
Before arrival of troops	200	0
6 days after arrival	770	0
14 days after arrival	4240	1
41 days after arrival	6960	2
60 days after arrival	14,900	10

Thirdly, negative tests for Bacillus coli and low bacterial counts may be interpreted as proofs of the good quality of water, with a *certainty* not attainable by any other method of analysis. Many a surface-water with reasonably low chlorine and ammonias has caused epidemics of typhoid fever; but it is impossible, under any natural conditions, that a water could contain the typhoid bacillus without giving clear evidence of pollution in the dextrose-broth tube or on the lactose-agar plate.

In the examination of springs, especially those used for domestic supplies at country houses, the authors have

found that the bacteriological examination offers a more delicate and a more certain index of the quality than may be obtained by chemical analysis. In a number of instances, springs located in pastures have become slightly polluted by animals, but to so small an extent that the chemical examination gave no indication of trouble. The bacteria, however, increased greatly in number, and colon bacilli could be readily isolated from 75 per cent of the 1-c.c. samples of a long series used in making the presumptive test. A single case may suffice as an illustration. This was a spring located on a hill in Hopkinton, Mass.

The chemical analysis was as follows:

Color	None
Turbidity	None
Sediment	None
Odor (hot)	None
Odor (cold)	None
	Parts per Million.
Total solids	33.0000
Loss on ignition	7.0000
Fixed residue	26.0000
Hardness	11.0000
Chlorine	10.0000
Nitrogen as —	
albuminoid ammonia	0.0000
free	0.0000
nitrites	0.0000
nitrates	0.0000

The bacteriological examination showed a total count of 375 bacteria per c.c. and a 37-degree count of 350 per c.c.

The presumptive tests for Bacillus coli showed that gas-producing organisms were present in a majority of 1-c.c. samples, and typical colon bacilli were isolated. In this case the contamination was brought about by cattle gaining access to the area immediately surrounding the spring; but the same conditions might easily have led to infection from human beings.

Similar results have been reported by Savage and Bulstrode (Savage, 1906) in the examination of the water-supply of Bridgend.

It seems to the writers that the real application of chemistry begins where that of bacteriology ends. When pollution is so gross that its existence is obvious and only its amount needs to be determined, the bacteriological tests will not serve, on account of their excessive delicacy. In studying the heavy pollution of small streams, the treatment of trades wastes, and the purification of sewage, the relations of nitrogenous compounds and of oxygen compounds are of prime importance. In other words, when pollution is to be avoided, because the decomposition of chemical substances causes a nuisance, it must be studied by chemical methods. When the danger is sanitary and comes only from the presence of bacteria, bacteriological methods furnish the best index of pollution.

In the study of certain special problems the paramount importance of bacteriology is generally recognized. The distribution of sewage in large bodies of water into which it has been discharged may thus best be traced on account

of the ready response of the bacterial counts to slight proportions of sewage, particularly since the ease and rapidity with which the technique of plating can be carried out make it possible to examine a large series of samples with a minimum of time and trouble. The course of the sewage carried out by the tide from the outlet of the South Metropolitan District of Boston was studied in this way by E. P. Osgood in 1897, and mapped out by its high bacterial content with greater accuracy than could be attained by any other method. Some very remarkable facts have been developed by similar studies as to the persistence of separate streams of water in immediate contact with each other. Heider showed that the sewage of Vienna, after its discharge into the Danube River, flowed along the right bank of the stream, preserving its own bacterial characteristics and not mixing perfectly with the water of the river for a distance of more than twenty-four miles (Heider, 1893). Jordan (Jordan, 1900), in studying the self-purification of the sewage discharged from the great Chicago drainage canal, found by bacteriological analyses that the Des Plaines and the Kankakee Rivers could both be distinguished flowing along in the bed of the Illinois, the two streams being in contact, yet each maintaining its own individuality. Finally, the quickness with which slight changes in the character of a water are marked by fluctuations in bacterial numbers renders the bacteriological methods invaluable for the daily supervision of surface

supplies or of the effluents from municipal filtration plants.

In the commoner case, when normal values obtained by such routine analyses are not at hand, the problem of the interpretation of any sanitary analysis is a more difficult one. The conditions which surround a source of water-supply may be constantly changing. No engineer can measure the flow of a stream in July and deduce the amount of water which will pass in February; yet the July gauging has its own value and significance. So a single analysis of any sort is not sufficient for all past and future time. If it gives a correct picture of the hygienic condition of the water at the moment of examination it has fulfilled its task, and this the bacteriological analysis can do. The evidence furnished by inspection and by chemical analysis should be sought for and welcomed whenever it can be obtained, yet we are of the opinion that, on account of their directness, their delicacy, and their certainty, the bacteriological methods should least of all be omitted, and, if necessary, they alone may furnish conclusive testimony as to the safety of a potable water.

CHAPTER XI.

BACTERIOLOGY OF SEWAGE AND SEWAGE EFFLUENTS.

THE first object of modern sewage disposal is the oxidation of putrescible organic matter. Chemical, rather than bacterial, purification is the prime requisite; and chemical tests therefore serve best as criteria of the results obtained. Bacteria are the agents in the process of sewage purification; but the most generally useful measure of the work accomplished is the chemical oxidation attained. "To employ a simile, it is a case of the saw and the two-foot rule — the saw will do the cutting, but the rule will measure the work cut." (W. J. Dibdin.)

In certain cases, however, bacterial as well as chemical purity must be effected, in view of special local requirements. The sewage from a contagious disease hospital, for example, should be freed from infectious material as a factor of safety. Sewage discharged into a body of water adapted for bathing may well be so treated as to protect those using the water. In the case of seaboard cities where sewage effluents are likely to contaminate oyster beds and other layings of edible shellfish the problem assumes great importance. Where bacterially im-

pure effluents are discharged into streams used for sources of water-supply the town taking water may protect itself by filtration. It should so protect itself, at any rate, from the pollution necessarily incident to surface-waters; and, unless the bacterial condition of a stream or lake is made very materially worse by the discharge of sewage effluents, it is fair that the responsibility of purification should rest on the water works, rather than on the sewage purification plant. Shellfish, on the other hand, cannot be purified. Either pollution must be prevented, or the industry abandoned. Under such circumstances sanitary authorities may rightly demand, as they have demanded at Baltimore, that bacteria, as well as putrescible organic matter, shall be removed in sewage treatment. Under such circumstances the bacterial control of purification plants is as essential as in the case of water filters.

In England, considerable attention has been devoted to this subject and numerous methods have been recommended as furnishing valuable criteria of the bacterial quality of sewage effluents. Houston (1902[b]), for example, suggests various tests involving the use of litmus milk, pepton solution, gelatin tubes, and neutral-red broth, as well as the inoculation of animals. He considers the determination of the numbers of B. coli and B. sporogenes as of greatest moment, while the identification of streptococci is of value in certain cases, and the enumeration of liquefying bacteria, spore-forming ærobes, thermophilic bacteria, and hydrogen sulphide producing

bacteria is of subsidiary importance. Rideal (1906) has recently commended a somewhat less extensive series of tests, including ærobic and anærobic counts, both at 20 and 37 degrees, with the determination of the number of liquefiers and the number of spore formers. The results attained do not seem to warrant any such elaborate procedure. As far as the authors are aware, the determination of liquefying bacteria, anærobic bacteria and thermophilic bacteria does not add any information of material importance to that obtained from the total count. Some test for specific sewage organisms is of course desirable. Here again, however, the determination of B. sporogenes and sewage streptococci tells the observer little more than can be learned from the routine use of the colon test. In the United States the practice of sewage bacteriologists is crystallizing around the total count and the estimation of B. coli. In the absence of evidence as to the specific value of other data, the routine control of filter plants may well be limited to these two determinations.

The total count of bacteria should be made, as in the case of waters, on gelatin at 20 degrees. Determinations carried out in duplicate on agar at 37 degrees add additional information of considerable value. The ratio of the 37-degree count to the 20-degree count varies with different sewages. At Boston the body temperature count is 70 to 80 per cent of the total count; at Lawrence it appears to be proportionately much lower (Gage, 1906). In using either medium, it is well to add lactose and lit-

mus and note the number of red colonies, as a check on the enumeration of B. coli.

The determination of the number of colon bacilli in sewage and effluents should furnish an integral part of bacteriological sewage analysis since it is important to know whether the decrease of intestinal bacteria in the process of purification is proportional to the reduction of total bacteria. The State Sewerage Commission of New Jersey has adopted this procedure in its supervision of the disposal plants in that state; and the results seem amply commensurate with the labor involved. As in the case of polluted waters the enumeration of B. coli may be carried out, either by the study of the red colonies which appear on litmus-lactose-agar plates inoculated with the sample directly, or by the use of a preliminary enrichment process. The complete identification of B. coli seems unnecessarily tedious, however, where the organisms are present in such abundance. Some approximate presumptive method is indicated here, if anywhere; and the experience with polluted water, reviewed in Chapter VIII, points to the Jackson bile medium as the most promising one. More than a year's experience at the Sewage Experiment Station of the Massachusetts Institute of Technology has shown that this presumptive test in general yields good results. As pointed out above, a 72-hour incubation period at 37 degrees is required. All tubes showing 20 per cent gas at the end of this time may be considered positive tests for B. coli without serious error.

The total number of bacteria and the number of colon bacilli naturally vary widely in the sewages of different cities and towns. European sewages, being more concentrated, show as a rule higher numbers than are found in America. Results compiled from various sources show from one to five million bacteria in the sewages of Essen, Berlin, Charlottenburg, Leeds, Exeter, Chorley, and Oxford, two to ten millions in the sewages of London, Walton and W. Derby, and over ten millions in the sewages of Paris, Ballater and Belfast (Winslow, 1905). The number of colon bacilli in English sewages varies from 50,000 to 750,000. In American sewages, on the other hand, bacteria are somewhat less numerous. At Lawrence the determinations made from 1894 to 1901 showed on the average 2,800,000 bacteria per c.c. At Worcester, Eddy reported 3,712,000 in 1901 (Eddy, 1902); at Ames, Iowa, Walker (1901) found 1,248,256 in the same year. At Columbus, Johnson (1905) reports an average of 3,600,000 bacteria per c.c.; the individual numbers varied from 320,000 to 27,000,000. The number of colon bacilli varied from 50,000 to 1,000,000 and averaged 500,000. Day samples of Boston sewage collected three times a week, from October, 1906, to April, 1907, showed an average of 1,200,000 bacteria per c.c. In the summer months numbers are notably higher than at other seasons in many sewages. Thus in 1903, Boston sewage contained 2,995,000 bacteria in July, 4,263,600 in August, 11,487,500 in September, 3,693,000 in October,

587,100 in November, and 712,000 in December (Winslow, 1905). There is also a marked diurnal variation in the bacterial content of sewage, since the flow contains a smaller proportion of intestinal matter at night than at other times. For example, a series of hourly samples at the Sewage Experiment Station of the Massachusetts Institute of Technology showed the following results:

BACTERIA IN BOSTON SEWAGE — AVERAGES FOR EACH 4-HOUR PERIOD. AUGUST 13–14, 1903.

(WINSLOW AND PHELPS, 1905.)

Period.	Bacteria per c.c.
7.30–11.30 A.M.	1,800,000
11.30 A.M.–3.30 P.M.	3,200,000
3.30–7.30 P.M.	4,600,000
7.30–11.30 P.M.	3,500,000
11.30 P.M.–3.30 A.M.	1,000,000
3.30–7.30 A.M.	400,000

It is evident that many published results of bacterial examinations of sewage are in excess of the true values, since they refer in most cases to day samples only.

The bacterial content of sewage effluents varies widely according to the process of purification adopted and the efficiency of the particular plant. The only process which yields a notably purified effluent from the bacteriological standpoint is that of filtration through sand. Processes of this type when operated with care may give a bacterial purification well over 99 per cent. The average numbers obtained from the outdoor experimental filters at Lawrence (each $\frac{1}{200}$ acre) in 1905 are tabulated below.

BACTERIA IN SEWAGE AND SAND FILTER EFFLUENTS AT LAWRENCE.

(CLARK, 1906.)

	Bacteria per c.c.		
Applied sewage	1,206,000	Filter 5C	2,906
Filter 1	8,315	Filter 6	10,896
Filter 2	1,059	Filter 9A	4,585
Filter 4	587	Filter 10	1,743
Filter 5B	19,200		

The Septic Tank and Intermittent Sand Filter at Iowa State College gave the following bacterial results in 1899 and 1900, the averages representing daily examinations of the sand effluent and weekly examinations of crude and septic sewage.

BACTERIA IN SEWAGE SEPTIC EFFLUENT AND SAND FILTER EFFLUENT AT IOWA STATE COLLEGE.

(WALKER, 1901.)

	Bacteria per c.c.	Monthly Averages.	
Month.	Sewage.	Septic Effluent.	Sand Effluent.
August, 1899	2,392,600	1,388,300	2,246
September	8,815,000	3,245,000	3,660
October	6,064,800	4,941,000	4,320
November	4,537,333	3,014,000	2,261
December	816,333	848,000	2,319
January, 1900	848,000	726,000	830
February	345,533	233,810	3,451
March	132,125	112,500	2,480
April	2,121,000	1,392,800	13,263
May	1,021,000	783,300	3,077
June	1,318,100	1,391,300	2,359
July	3,908,700	4,578,333	2,270
August	403,118	215,700	546
September	1,181,533	383,733	850

Such high efficiencies as these two tables indicate can scarcely be expected even with the sand process under the actual working conditions of a municipal plant. At Vineland, N. J., for example, the intermittent filters show a reduction of 90 to 95 per cent in total bacteria and a somewhat higher reduction of B. coli. The results of three examinations made in 1906 are given below.

BACTERIA IN SEWAGE AND SAND FILTER EFFLUENT AT VINELAND, N. J.

(N. J. STATE SEWERAGE COMMISSION, 1907.)

Date.	Bacteria per c.c.		B. Coli in	
	Sewage.	Effluent.	Sewage.	Effluent.
March 2	480,000	20,000	.0001 c.c.	.01 c.c.
July 26 . . .	496,000	61,000	.0001 c.c.	.001 c.c.
July 26 . . .	511,000	38,000	.00001 c.c.	.001 c.c.

At Columbus the experimental sand filters effected an average reduction of 87 per cent in total bacteria and of 98.5 per cent in colon bacilli. The number of B. coli remaining in the effluent varied from 500 to 10,000 per c.c. (Johnson, 1905).

McGowan, Houston, and Kershaw (1904) report the following figures for English sewage farms.

ANALYSIS OF EFFLUENTS FROM SEWAGE FARMS.

(McGOWAN, HOUSTON, AND KERSHAW, 1904.)

Place.	Bacteria per c.c.				B. Coli per c.c.
	Gelatin, 20°.		Agar, 37°.		
	Number.	Per Cent Removal.	Number.	Per Cent Removal.	
Aldershot .	183,266	99	37,308	99	1000–10,000
Altrincham	363,400	97	7,275	99	100–1000
Cambridge .	711,476	94	78,327	94	1000–10,000
Croydon . .	1,413,200	95	112,000	97	1000–10,000
Leicester .	532,777	95	70,500	95	1000–10,000
S. Norwood	778,322	98	35,157	99	100–1,000
Rugby . .	637,133	97	81,526	97	1000–10,000

The newer bacterial processes, contact beds, and trickling filters naturally show a much less satisfactory bacterial removal than sand filtration beds. In the Columbus experiments, Johnson (1905) found from one to two million bacteria in the effluents of contact beds and from 750,000 to 1,900,000 in the effluents from trickling filters. The average percentage reduction effected by seven contact beds and six trickling filters is shown below.

REDUCTION OF BACTERIA AT COLUMBUS, OHIO.

(JOHNSON, 1905.)

Contact Beds.	Per Cent Reduction.	Trickling Filters.	Per Cent Reduction.
Primary A	60	A	74
Primary B	43	B	70
Primary C	33	C	70
Primary D	33	D	69
Primary E	0	E	46
Secondary A	38	F	21
Secondary B	39	...	...

Thumm and Pritzkow (1903), at the Berlin Experiment Station, obtained the results tabulated below.

BACTERIA IN SEWAGE, CONTACT EFFLUENT, AND SAND EFFLUENT AT BERLIN.

	Bacteria per c.c.
Crude sewage	16,900,000
Primary contact effluent (coarse coke)	12,400,000
Secondary contact effluent (fine coke)	5,600,000
Tertiary sand effluent	1,100,000
Primary contact effluent (fine coke)	7,400,000
Secondary sand effluent	1,800,000

At the experiment station of La Madeleine, in Lille, Calmette (1907) reports 5,000,000 bacteria per c.c. in the crude sewage, 2,900,000 in the second contact effluent and 800,000 in the effluent from the trickling bed. Of 20,000 B. coli per c.c. applied to the filters, the contact system delivered 4000 and the trickling bed 2000 per c.c.

The average results of examinations made three times

a week at the Sewage Experiment Station of the Massachusetts Institute of Technology, during two different periods, were as follows:

BACTERIA IN SEWAGE, SEPTIC EFFLUENT, AND TRICKLING EFFLUENTS AT BOSTON.

(WINSLOW AND PHELPS, 1907.)

	Bacteria per c.c.				B. Coli. Positive Tests in .000001 c.c.[1]
	July–Sept., 1906.		Oct., 1906–April, 1907.		July–Sept., 1906.
	No.	Per Cent Reduction.	No.	Per Cent Reduction.	Per Cent.
Sewage . . .	1,300,000	. . .	1,200,000	. . .	65
Septic effluent	1,650,000	Inc.	750,000	38	66
Effluent from trickling bed	750,000	42	200,000	83	35
Septic tank and trickling bed	750,000	42	180,000	85	35

[1] Jackson bile test.

The contact beds, as operated on a practical scale in England, show considerably higher numbers. At London the Barking and Crossness beds yielded effluents containing one to five million bacteria per c.c., of which 100,000 to 600,000 were B. coli.

There are few plants of the newer types now in operation in the United States, and fewer still are controlled by bacteriological examinations. At Plainfield, N. J.,

however, the combination of septic tank and double contact beds produces a bacterial purification of 80 to 90 per cent as measured by total numbers. The following table shows the results of four examinations made in 1906.

BACTERIA IN SEWAGE, SEPTIC EFFLUENT, AND CONTACT EFFLUENT AT PLAINFIELD, N. J.

(N. J. STATE SEWERAGE COMMISSION, 1907.)

Date.	Bacteria per c.c.			B. Coli in —		
	Sewage.	Septic Effluent.	Secondary Contact Effluent.	Sewage.	Septic Effluent.	Secondary Contact Effluent.
				c.c.	c.c.	c.c.
July 9 .	2,295,200	659,200	591,300	.00001	.00001	.0001
July 9 .	2,043,300	555,000	172,600	.000001	.0001	.0001
Aug. 9.	1,371,700	989,700	186,700	.00001	.0001	.0001
Aug. 9.	1,655,000	...	338,000	.000001	...	.00001

It is obvious that effluents of this character cannot be considered satisfactory from the standpoint of bacterial purification. As Houston concluded, after a careful review of the subject, "The different kinds of bacteria and their relative abundance appear to be very much the same in the effluents as in the crude sewage. Thus, as regards undesirable bacteria, the effluents frequently contain nearly as many B. coli, proteus-like germs, spores of B. enteritidis sporogenes and streptococci, as crude sewage. In no case, seemingly, has the reduction of these objectionable bacteria been so marked as to be very

material from the point of view of the epidemiologist" (Houston, 1902[a]).

Experimental studies with specific bacteria have confirmed these conclusions. Houston (1904[b]) found that B. pyocyaneus appeared in the effluent of a trickling bed ten minutes after application to the top and continued to be discharged for ten days. In septic tanks and contact beds, the same germ persisted for ten days. Rideal (1906) quotes experiments by Pickard at Exeter, which show that typhoid bacilli may persist for two weeks in a septic tank and that contact bed treatment only effects a 90 per cent removal of these organisms.

Where bacterial purity is required some special process of disinfection must be combined with the contact bed or the trickling filter. For this purpose treatment with chloride of lime or other chemicals is rapidly gaining ground as an important adjunct to bacterial disposal plants; and in connection with this process bacteriological control is an essential.

Rideal (1906) first showed at Guildford that 30 parts of available chlorine per million would reduce the number of bacteria in crude sewage from several millions to 50,000, while 50 parts would reduce their number to 20 per c.c. Colon bacilli were reduced from one million per c.c. to less than one per c.c. by 30 parts of chlorine. In septic effluent 25 to 44 parts of chlorine per million reduced B. coli from two and a half to four and a half million per c.c. to less than one per c.c. With contact

effluents smaller amounts of chlorine proved efficient. The primary effluent required 20 parts per million, the secondary effluent 10.6 parts per million and the tertiary effluent 2.5 parts per million to reduce the number of B. coli so that this organism could not be isolated in 5 c.c.

In this country Phelps and Carpenter (1906) demonstrated the practical usefulness of bleaching powder disinfection, at the Sewage Experiment Station of the Massachusetts Institute of Technology. As indicated in the table below, smaller amounts of chlorine than were used by Rideal will give good results with more dilute American sewages.

BACTERIA IN TRICKLING FILTER EFFLUENT BEFORE AND AFTER TREATMENT WITH CHLORIDE OF LIME (5 PARTS PER MILLION AVAILABLE CHLORINE).

(PHELPS AND CARPENTER, 1906.)

Date.	Bacteria per c.c.		B. Coli, Jackson Bile Test.	
	Before.	After.	Before .000001 c.c.	After 1.0 c.c.
1906.				
August 11....	270,000	69	+ o	+ o
13....	630,000	41	o o	+ o
14....	135,000	406	+ +	+ o
15....	230,000	21	o o	o o
16....	250,000	37	+ o	o o
18....	110,000	40	o o	+ o
20....	90,000	54	+ o	o o
21....	220,000	22	o o	o o
23....	...	...	+ o	o o
Average.....	240,000	86	33%	22%
Average removal	99.96 per cent.		99.993 per cent.	

The success of chemical disinfection varies with the character of the sewage or effluent treated since the organic matter present consumes a certain amount of the disinfectant and renders it inoperative. Discordant results are therefore reported from different sources.

An important series of experiments carried out in Ohio by Kellerman, Pratt, and Kimberly (1907) showed good results with sand filter effluents and contact effluents. Septic sewage, on the other hand, required large amounts of chlorine to produce a reasonable bacterial reduction. The following table, on page 199, shows the results obtained at Marion, Ohio.

In Germany, on the other hand, Schumacher (1905), Kranepuhl (1907), and Kurpjuweit (1907) found larger amounts of chlorine necessary, in the neighborhood of 60 parts per million parts of sewage. Their tests were somewhat severe, however, the criterion of success being the absence of B. coli in a large proportion of liter samples.

The science of sewage bacteriology is in its infancy; and it is difficult to give any general rules for the interpretation of bacteriological examinations designed to indicate whether disposal plants are successful or not. Houston stated provisionally that the 20° count should be under 100,000, and the 37° count under 10,000, while B. coli should be absent from .001 c.c. and B. sporogenes from .1 c.c. (Houston, 1902[b]). This standard seems to us far too lenient. Either organic purity alone is necessary, as at many sewage disposal plants, or a

BACTERIA IN SEPTIC EFFLUENT, CONTACT EFFLUENT, AND SAND EFFLUENT AT MARION, O., BEFORE AND AFTER TREATMENT WITH CALCIUM HYPOCHLORITE.

(KELLERMAN, PRATT, AND KIMBERLY, 1907.)

Date.	Effluent.	Average Available Chlorine. Parts per Million.	Bacteria per c.c.			
			20° C.		37° C. Total Count.	
			Untreated.	Treated.	Untreated.	Treated.
1907.						
Apr. 11	Septic	4.3	850,000	1,100,000	1,200,000	240,000
Apr. 12	Septic	6.2	4,400,000	550,000	850,000	260,000
Apr. 15	Septic	7.6	600,000	400,000	450,000	190,000
Apr. 28	Contact	2.9	110,000	2,500	...	...
Apr. 29	Contact	5.0	65,000	1,600	73,000	370
Apr. 3	Contact	4.4	500,000	800	160,000	400
Mar. 21	Sand	3.8	49,000	570	9,800	150
Mar. 22	Sand	3.0	56,000	140	7,000	60
Mar. 26	Sand	1.5	70,000	4,000	20,000	160

Date.	Effluent.	Average Available Chlorine. Parts per Million.	Bacteria per c.c.			
			37° C. Red Colonies.		B. Coli.	
			Untreated.	Treated.	Untreated.	Treated.
1907.						
Apr. 11	Septic	4.3	55,000	7,400	...	...
Apr. 12	Septic	6.2	60,000	15,000	...	...
Apr. 15	Septic	7.6	100,000	51,000	...	...
Apr. 28	Contact	2.9	...	...	20,000	Not in .5
Apr. 29	Contact	5.0	10,000	0	15,000	" " .5
Apr. 3	Contact	4.4	21,000	3	20,000	" " 1.0
Mar. 21	Sand	3.8	1,300	0	1,000	" " 1.0
Mar. 22	Sand	3.0	800	0	2,000	" " 1.0
Mar. 26	Sand	1.5	4,000	1	2,000	In 1.0

higher grade of purity than this should be attained. It seems wisest at the present time to avoid fixing any general standards of purity for sewage effluents. Each case should be judged intelligently on its own merits. In general, however, where bacterial purification is indicated at all, it seems fair to demand that the effluent should be of such a quality as not to increase materially the bacterial content of the body of water into which it is discharged.

Before leaving the subject of sewage bacteriology, brief reference must be made to the importance of bacteriological studies in relation to the processes of sewage purification which bring about the removal of the organic matter itself. Nothing is more necessary to the development of the present art of sewage disposal than knowledge of the micro-organisms concerned and of the conditions which favor their activity; but such knowledge is woefully deficient. Something is known of the nitrifying organisms long ago discovered by Winogradsky. Such recent work as that of Schultz-Schultzenstein (1903), Boullanger and Massol (1903), and Calmette (1905), has cleared up many points concerning these forms; but much remains to be done. In regard to the reducing action of bacteria in the septic tank and contact bed we are almost wholly in the dark. Septic tanks work well with some sewages and badly with others; and the presence or absence of the right bacteria is probably largely responsible for the different results. In some cases, as at

Plainfield, N. J., the seeding of a tank with cesspool contents has produced a material improvement in septic action.

Knowledge of the kinds of bacteria involved would make it possible to substitute scientific control for such empiricism and might well lead to improved methods of a more intensive character than are yet available. The work already done upon a laboratory scale furnishes promise of such results. The student who wishes to follow out this line of investigation will find a good summary of what is already known of the hydrolysis and denitrification of nitrogenous bodies and the decomposition of cellulose and other carbohydrates in Rideal's "Sewage, and the Bacterial Purification of Sewage" (1906).

Gage (1905) has made a suggestive study of the bacteria which carry on the reducing changes in sewage which deserves the study of all who are interested in the more theoretical aspects of sewage treatment. His method consisted in plating sewages and effluents, isolating typical cultures and determining their power to decompose peptone and nitrates with the production of ammonia and free nitrogen. The rate of gelatin liquefaction, the amount of nitrate reduced, the amount of free ammonia formed, and the amount of nitrogen liberated were quantitatively determined for each culture thus isolated.

The numerical values obtained, multiplied by the number of bacteria, apparently of the same type, observed in the plates, gave coefficients of the liquefying, denitrifying,

ammonifying, and nitrogen liberating power of the effluent; and these coefficients may be considered as measures, for a given sample, of the tendency of the bacterial flora to set up certain changes. The results of further studies made by Clark and Gage (1905), on sewages and on sand, contact, and trickling effluents, show that there may be important differences between various sewages in this respect which must render their purification more or less easy. They indicate that the effluents obtained from intermittent sand filters in cold weather contain larger numbers of ammonifying and denitrifying bacteria than appear at other seasons, which may help to explain the poorly nitrified effluents obtained in the winter season. Along these and similar lines research work in sewage bacteriology promises to be fruitful of results.

APPENDIX.

MEDIA MAKING.

Extract from the Report of the Committee on Standard Methods of Water Analysis, 1905.

In view of the marked influence upon bacteriological reactions of variations in culture media caused by differences both in ingredients and in technique of preparation, it is necessary that uniform methods be used in order to obtain comparable data. In specifying the various ingredients used in culture media it is the intention of the committee that they shall be uniform in quality, but it is not the intention that the recommendations as to ingredients and technical manipulations shall stand in the way of true progress as to improvements. When, however, improved or modified methods are used, the variations from the standard methods shall be plainly set forth together with the reasons for the modifications.

INGREDIENTS.

Distilled water shall be used in the preparation of standard culture media.

Infusions of fresh lean meat, and not meat extract, shall be used as the basis of various media.

Sodium chloride shall not be added to any culture medium herein specified.

Pepton shall be that of Witte (dry from meat).

Gelatin shall be the best French brand, so-called. It shall be as free as possible from acids and other impurities, and shall be of such a character that a 10 per cent solution prepared in the usual way shall not soften when kept at a temperature of 25° C.

Commercial agar in threads shall be of as high a grade as can be obtained. Agar may be purified by washing.

The various sugars, such as dextrose, lactose, and saccharose, shall be as nearly as possible the chemically pure compounds designated. Unusual effort to obtain such sugars is considered to be necessary.

Glycerine shall be double distilled.

In place of litmus, azolitmin* shall be used as a 1 per cent aqueous solution.

Of the various other ingredients used, nearly all of which are of a mineral nature, special effort shall be made to see that they are chemically pure products within the full meaning of this expression.

STERILIZATION.

Of the two available methods of sterilization, the intermittent method at a temperature of 100° C. is considered on the whole to be preferable. The higher temperatures

* Preferably Kahlbaum's (authors).

of the autoclave facilitate chemical reactions and changes which in some cases are undesirable.

When the latter method is used, media contained in ordinary receptacles shall be sterilized by exposure in an autoclave at a temperature of 120° C. (15 pounds pressure) for five minutes. Where media are sterilized in large bulk, the period of heating shall be extended to 12 minutes. It is preferable, however, to sterilize media in reasonably small containers (500 to 700 c.c.).

In intermittent sterilization, media shall be placed on each of three successive days in streaming steam for 30 minutes after the steam fills the sterilizer.

REACTION.

Phenolphthalein shall be the standard indicator used in obtaining the reaction of all media. Turmeric paper possesses similar properties, and its use is advised where phenolphthalein is not available.

Titrations and adjustment of reactions shall be made as follows:

Put 5 c.c. of the medium to be tested into 45 c.c. distilled water. Boil briskly one minute. Add 1 c.c. of phenolphthalein solution (5 g. of commercial salt in one liter of 50 per cent alcohol.) Titrate while hot (preferably while boiling) with $\frac{N}{20}$ caustic soda. A faint but distinct pink color marks the true end-point. This distinct pink color may be more precisely described as a

combination of 25 per cent of red (wave length approximately 658) with 75 per cent of white as shown by the disks of the color top, described under Record of Tints and Shades of Apparent Color. In practice titration is continued until the pink color of alkaline phenolphthalein matches that of the fused disks.

All reactions shall be expressed with reference to the phenolphthalein neutral point and shall be stated in percentages of normal acid or alkaline solutions required to neutralize them. Alkaline media shall be recorded with the minus (−) sign before the percentage of normal acid needed for their neutralization, and acid media with the plus (+) sign before the percentage of normal alkaline solution necessary for their neutralization.

The standard reaction of culture media shall be + 1.0 per cent. If it differs from 1 per cent by more than 0.2 per cent it should be readjusted.

Wherever reactions other than the standard above given are used it shall be clearly stated in all results of bacterial work, and the reasons therefor also stated.

STORAGE OF MEDIA.

It is recognized by the committee that it is desirable to prepare media in large quantities in order to guard against discrepancies in composition; but, all things considered, the complications resulting from the varying amounts of heating incident to withdrawing portions from time to time and tubing it, are believed to more

than offset this advantage. Consequently, when possible, media shall be put at once into tubes and placed in cold storage.

To guard against changes due to evaporation all media not used promptly shall be stored in a moist atmosphere, preferably in an ice-box, or else the flask shall be sealed by dipping the cotton plug in paraffin.

NUTRIENT BROTH.

Nutrient broth shall be prepared as follows: Infuse 500 g. chopped lean meat 24 hours with 1000 c.c. distilled water in refrigerator. Restore loss by evaporation. Strain infusion through cotton flannel. Add one per cent pepton. Warm on water bath, stirring until the pepton is dissolved. Heat over boiling water (or steam) bath 30 minutes. Restore loss by evaporation. Titrate. Adjust reaction to + 1 per cent by adding normal hydrochloric acid or normal sodium hydrate, as required. Boil two minutes over free flame, constantly stirring. Restore loss by evaporation. Filter through absorbent cotton and cotton flannel, passing the liquid through until clear. Titrate and record final reaction. Tube, using 10 c.c. in each tube. Sterilize.

SUGAR BROTHS.

Sugar broths shall be prepared in the same general manner as the standard nutrient broth, with the addition of one per cent of dextrose, lactose, saccharose or other sugar; or the sugar may be added to the completed

nutrient broth just before sterilizing. Except in the case of dextrose broth it is important that the muscle-sugar in the meat infusion be removed by inoculating with B. coli.

The reaction of sugar broths shall be neutral to phenolphthalein.

Sterilization shall be done in streaming steam in the case of all sugar broths to prevent inversion of the sugar.

For the routine work of testing samples of water for B. coli, especially large volumes of water are to be mixed with broths of such strength as to make the resulting mixture one of normal strength. Liebig's Beef Extract may be substituted for beef infusion in the preparation of dextrose broth only: three grams of the beef extract for each liter of broth.

NUTRIENT GELATIN AND AGAR.

Nutrient gelatin and agar shall be prepared as follows:

	Gelatin.	Agar.
1.		Boil 15 g. thread agar in 500 c.c. water for half an hour and make up weight to 500 g. or digest for 10 minutes in the autoclave at 110° C. Let this cool to about 60° C.
2.	Infuse 500 g. lean meat 24 hours with 1000 c.c. of distilled water in refrigerator.	Infuse 500 g. lean meat 24 hours with 500 c.c. of distilled water in refrigerator.
3.	Make up any loss by evaporation.	
4.	Strain infusion through cotton flannel.	
5.	Weigh filtered infusion.	
6.	Add one per cent Witte's pepton and 10 per cent gold label sheet gelatin.	Add two per cent of Witte's pepton.

	Gelatin.	Agar.
7.	Warm on water bath, stirring till pepton and gelatin are dissolved and not allowing the temperature to rise above 60° C.	
8.		To 500 g. of the meat infusion add 500 c.c. of the three per cent agar, keeping the temperature below 60° C.
9.	Heat over boiling water (or steam) bath for 30 minutes.	
10.	Restore loss by evaporation.	
11.	Titrate, after boiling one minute to expel carbonic acid.	
12.	Adjust reaction to + 1.0 per cent by adding normal hydrochloric acid or sodium hydrate as required.	
13.	Boil two minutes over free flame, constantly stirring.	
14.	Make up loss by evaporation.	
15.	Filter through absorbent cotton and cotton flannel, passing the filtrate through the filter until clear.	
16.	Titrate and record the final reaction.	
17.	Tube, using 10 c.c. of medium in each tube.	
18.	Sterilize five minutes in the autoclave at 120 degrees, or for 30 minutes in streaming steam on three successive days. Put the gelatin at once into ice-water till solidified.	
19.	Store in the ice-chest in a moist atmosphere, to prevent evaporation.	

LACTOSE (OR DEXTROSE) LITMUS AGAR.

Lactose or dextrose litmus agar shall be prepared in the same manner as nutrient agar, with the addition of one per cent of lactose (or dextrose) to the medium just before sterilization. The reaction shall be made neutral to phenolphthalein.

If the medium is to be used in tubes the sterilized azolitmin solution shall not be added until just before the final sterilization.

If the medium is to be used in Petri dishes the sterilized azolitmin solution shall not be added to the medium until it is ready to be poured into the dishes.

MILK.

The milk to be used as a culture medium shall be as fresh as possible, "Certified Milk" being ordinarily the best obtainable in city laboratories. It shall be placed in a refrigerator over night to allow the cream to rise and the suspended matter to settle. The skimmed milk shall be siphoned off into a flask for use. It will be found more convenient, however, to allow the milk to stand in a separating funnel. Should the milk be too acid the reaction shall be corrected to + 1 per cent by the addition of normal sodium hydrate. It is then ready to be tubed and sterilized. Litmus milk shall be prepared as above, with the addition of sterile 1 per cent azolitmin. As it is impossible to make each lot of litmus milk with the same shade of color, it is recommended that a control tube be always exposed with the inoculated tubes for purposes of comparison.

NITRATE BROTH.

Dissolve one gram pepton in one liter of tap water, and add 0.2 grams of nitrite-free potassium nitrate.

It is convenient to prepare a stock solution of potas-

sium nitrate by dissolving four grams of solid nitrate in 100 c.c. of distilled water and use five c.c. of this solution in the above formula. Ten c.c. of the medium thus prepared shall be placed in a test tube and sterilized in the usual way.

BROTH FOR INDOL TEST.

Standard broth may be used for the indol test if precautions are taken to remove the muscle sugar, by inoculating the beef infusion with B. coli before making the broth.

Pepton solution (one per cent pepton in water), however, is preferred by some for use in the indol test, and is considered generally to be satisfactory. Sodium nitrite (0.01 per cent) shall be added in all cases.

APPARATUS.

Few definite requirements need be made respecting apparatus. The quality of the glass shall be such as not to be easily acted upon by the reagents used, and all glassware shall be scrupulously clean when used. When necessary it shall be sterilized by dry heat for one hour at about 150° C. A slight browning of the cotton stoppers is a good index of proper exposure.

In some operations, as, for example, the determination of the thermal death point, it is necessary to use test tubes of a definite size and thickness. For this purpose the standard size culture tube shall be 15 cm. long, 1.6 cm.

in diameter, and of medium weight. Tubes to be filled with gelatin for quantitative work may be those described as 6 in. × ⅝ in. "heavy."

The standard loop for making transfers shall be prepared as follows:

Bend the end of a piece of No. 27 platinum wire about 10 cm. long over a bit of No. 10 wire, and fasten the loop thus formed into a glass rod to serve as a handle. A loopful of culture shall be interpreted as meaning all the fluid that the loop can hold. That is, the fluid shall form a bi-convex body and shall not be simply a film covering the space in the loop.

The standard fermentation tube shall be a tube 1.5 cm. in diameter, bent at an acute angle, closed at one end and provided with a bulb at the other which is large enough to receive all the liquid contained in the closed portion. The length of the closed end of the tube shall be about 8 cm.

INCUBATION.

There shall be two standard temperatures of incubation for special work, namely, 20° C. and 37° C., the first corresponding to ordinary room temperature, the second to blood heat. The temperature of the incubators shall not be allowed to vary from these two standards by more than 1° C. in either direction.

The atmosphere of the incubator shall be kept moist, preferably near the point of saturation. The incubator

shall be ventilated so as to insure a reasonably good circulation of air in order to prevent the accumulation in the incubator of gases which might be prejudicial to the development of the bacteria.

No definite period of incubation can be prescribed which will be suitable for all the work of species determination, but in reporting results the period used shall always be stated and form a part of the report. General statements as to the necessary periods will be found in connection with the principal tests.

PRELIMINARY CULTIVATION.

It is impossible to control completely the original vitality of bacteria when ready for cultivation, because in most cases the conditions for their optimum growth are not known. Experience, however, has shown that, when bacteria are submitted to a period of preliminary cultivation or rejuvenation in nutrient broth and transfers of young cultures made from one tube to another at frequent intervals, the result is to put the bacteria into a condition where subsequent cultures give greater uniformity in their characteristics than where this procedure is not followed. The following shall be considered as the standard procedure for this preliminary cultivation, and all bacteria shall be so treated before proceeding to the detailed tests.

Procedure. — Make a transfer from an agar culture of the bacterium to be tested into a tube of nutrient broth,

and incubate for 24 hours at 20° C. Transfer from this culture to a second tube of broth, and again incubate for 24 hours at 20° C. Transfer from this second culture to a third tube of broth and incubate again for 24 hours at 20° C. From this third broth culture make a gelatin plate and incubate for 48 hours at 20° C. (This is to prevent working with a possible mixed culture due to accidental contamination.) From one of the colonies on the gelatin plate transfer to a tube of slanted agar, incubate at 20 degrees for 48 hours, and use this culture for making subsequent inoculations in the various media.

ADDITIONAL FORMULÆ.

LOEFFLER'S BLOOD SERUM.

This medium consists of 3 parts blood serum and 1 part of 1 per cent broth with reaction + 0.8. The serum is obtained from fresh beeves' blood, which is collected in sterile jars and allowed to stand for 24 hours in the refrigerator for coagulation. The serum is then drawn off, filtered, and mixed with the dextrose broth in the proportion above indicated. Hill finds that filtering the serum through the coagulum obtained after adjusting the reaction of the broth, gives a filtrate which is clear and almost colorless (Hill, 1899).

Tubes are filled with the mixture, placed in trays so that the desired slant is obtained, and carefully heated in a Koch coagulator containing cold water in the water-jacket. This water is brought to a boil and kept boiling

for three hours. Repeating this process on three successive days solidifies the serum so that it may be subsequently sterilized in flowing stream for twenty minutes on three successive days.

PHENOL BROTH.

To 1000 c.c. water add separately, and in the following order, 100 grams dextrose, 50 grams pepton, 1.0 grams phenol. Heat until all constituents are dissolved, boil for fifteen minutes, and sterilize for fifteen minutes at 110–120° C.

NEUTRAL RED BROTH.

To neutral broth is added 0.5 per cent dextrose and 1 per cent of a 0.5 per cent aqueous solution of Grübler's neutral red. Sterilize at 100°.

MACCONKEY'S MEDIA.

A. Agar.

Agar	1.5 grams
Sodium taurocholate (pure)	0.5 gram
Pepton	2.0 grams
Water	100.0 c.c.

This is boiled, clarified, and filtered as usual, then 1.0 gram lactose is added, and the medium tubed and sterilized for three successive days at 100°.

B. Broth.

Sodium taurocholate (pure)	0.5 gram
Pepton	2.0 grams
Glucose	0.5 gram
Water	100.0 grams

Boil, filter, and add sufficient neutral litmus, fill fermentation tubes, and sterilize at 100 degrees.

Litmus Solution. — To one-half pound of litmus cubes add enough water to more than cover; boil, and decant off the solution. Repeat this operation with successive small quantities of water until from 3 to 4 liters of water have been used and the cubes are well exhausted of coloring matter. Pour the decantations together and allow them to settle over night. Siphon off the clear solution. Concentrate to about 1 liter and make the solution decidedly acid with glacial acetic acid. Boil down to about one-half liter and make exactly neutral with caustic soda or potash. To test for the neutral point, place one drop of the solution in a test tube. One drop of $\frac{N}{20}$ HCl should turn the drop red, while one drop of $\frac{N}{20}$ NaOH should turn it blue. Filter the solution and sterilize at 110° C. This solution should be added to the media just before use in the proportion of about $\frac{1}{4}$ c.c. to 5 c.c. of medium.

For the methods of preparation of other special culture media the student is referred to the papers published by the investigators who have originated the media in question.

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