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WATER BACTERIOLOGY

WATER BACTERIOLOGY

With Special Reference to

Sanitary Water Analysis

SIXTH EDITION

BY

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PRINTED IN THE UNITED STATES OF AMERICA

Dedicated to

WILLIAM THOMPSON SEDGWICK

BY THREE OF HIS PUPILS

AS A TOKEN OF GRATEFUL AFFECTION

PREFACE TO SIXTH EDITION

This book first appeared (under the title *Elements of Water Bacteriology, with Special Reference to Sanitary Water Analysis*) in 1904. At that time, the techniques available for the sanitary analysis of water were just beginning to crystallize. They had been intensively studied in the laboratories of the Massachusetts Institute of Technology; and the *Elements of Water Bacteriology* was originally prepared as a textbook for the students in that institution.

Since that time, the evolution of the science has been rapid and continuous, particularly under the stimulation of the Committee on Standard Methods of the American Public Health Association. No other textbook on this subject has appeared in the United States and four successively revised editions of the *Elements of Water Bacteriology* were called for in 1908, 1913, 1924, and 1931.

Further revision was obviously indicated by the advances of the past fourteen years. Since the original authors are at present working in other fields and are no longer in close contact with the day-by-day applications of water bacteriology, they have sought a collaborator who could speak with authority on this subject. They have been fortunate in securing the coöperation of Mr. M. H. McCrady, Chief of the Division of Laboratories of the Ministry of Health and Social Welfare of the Province of Quebec. For the alterations and additions to the present volume, Mr. McCrady has been primarily responsible. The changes made have involved almost complete rewriting of the last (1931) edition.

In view of the important development in recent years of our knowledge of the ecology and physiology of the forms of bacterial life indigenous to natural waters, it should be emphasized that the present volume does not deal with this significant phase of biology. Its scope — as it has been from the first — is limited to the public health phases of the subject, the distribution and behavior and significance of those organisms introduced into water from extraneous sources and having sanitary significance.

The Standard Methods of Water Analysis of the American Public Health Association have, of course, been accepted as basic. The present volume is intended to provide the historical and philo-

sophical background for adequate comprehension of the meaning of those methods; it is intended to be a textbook for the student, and a source of reference for the laboratory worker who applies the techniques involved and the investigator who, in the future, will further improve and extend those techniques.

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

October, 1945

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S. C. P.
C.-E. A. W.
M. H. M.

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 the Massachusetts Institute of Technology. For workers in the 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 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

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CHAPTER I

THE BACTERIA IN NATURAL WATERS

Scope of the Subject. The bacteriology of water may be considered from two viewpoints, one of which is essentially that of natural history, the other that which involves its significance for man's welfare. The first deals with the distribution and physiology of bacteria that are indigenous to water — the true water bacteria. The second concerns the study of the occurrence and physiology of bacteria commonly found in water but not indigenous to that medium. These are organisms which have come from extraneous sources such as soil, decomposing plant and animal structures, and sewage; they include bacteria that may be pathogenic to man if ingested, or that may be associated with pathogenic organisms.

Throughout the past half-century the public health aspect of water bacteriology has received by far the greater share of attention, and it was not until lately that bacteria indigenous to water claimed the interest of any considerable number of investigators. In recent years rapid progress has been made in the latter field, particularly in the ecology of the bacterial flora of fresh-water lakes and sea water, including a large flora of sessile and other forms found on submerged surfaces. This has resulted in the development of a phase of water bacteriology with aims and techniques different in many respects from those of the more specialized sanitary bacteriology of water. These two divisions are far from being mutually exclusive, however, and very frequently the results obtained by the field naturalist in water bacteriology are applicable to the problems of the sanitarian, and vice versa.

Since we are here concerned particularly with the public health aspect of the subject, the pages that follow will treat mainly of the sanitary significance of bacteria in water.

Bacteria and Their Nutritive Relations. 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 are most other living things, but in the soil itself, and in the air above, and in the waters under the earth.

Like other organisms the bacteria are sensitive to external

conditions, and respond quickly to slight changes in their environment. Temperature, moisture, hydrogen-ion concentration 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. On the other hand, as a class, they lack 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, very widely. Fischer (1900) divided the whole group into three great subdivisions according to the nature of their metabolism. The prototrophic forms are characterized by minimal nutrient requirements, and include organisms like the nitrifying bacteria which require no complex organic compounds at all, but derive their nourishment from carbon dioxide or carbonates, nitrites, and phosphates, or from inorganic ammonium salts. A second group, metatrophic bacteria, includes those types 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 coliform bacteria, which normally occur on the surface of the plant or animal body, and feed upon the fluids or on the extraneous material collected upon its surface and which may, under certain circumstances, invade the tissues themselves and produce disease.

The vast majority of bacteria belong to the second, or metatrophic group, and live as saprophytes on dead organic matter wherever it may occur in nature, particularly in that diffuse layer of decomposing plant and animal material which we call the humus, or surface layer of the soil. 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 matter is particularly abundant in the surface layers of the soil. Here, therefore, the bacteria are most numerous; and in air and

water their numbers vary according to the extent of contact with the living earth.

Classification of Waters. Natural waters, then, 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, *stored waters* in lakes and large ponds where storage has reduced bacterial numbers and produced a state of comparative purity and stability; and fourth, the *ground waters* from which previous contamination has been even more completely removed by filtration through the deeper layers of the soil.

Bacterial Content of Various Waters. Even rain and snow, the sources of our potable waters, are by no means free from germs; they contain them in numbers which vary 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 from bacteria, so that a number of sterile plates may often be obtained when 1-ml. samples of such rain water are examined. These results are in harmony with the observations of Tissandier (reported by Duclaux, 1898), who found that the dust in the air amounted to 23 mg. per cu. m. in Paris and 4 mg. in the open country. After a rainfall these figures were reduced to 6 mg. and 0.25 mg. respectively.

With regard to what may be considered normal values for rain it is difficult to give satisfactory figures. Those obtained by Miquel (1886) during the period 1883 to 1886 showed on the average 4.3 bacteria per milliliter in the country (Montsouris) and 19 per ml. in Paris. Snow contains rather higher numbers than rain. Janowski (1888) found in freshly fallen snow from 34 to 463 bacteria per milliliter of snow water. Modern methods of examination would probably show somewhat higher numbers.

As soon as the rain drop 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 milliliter; furthermore, the amounts of organic and mineral matter 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 from the flowing water

by sedimentation; many soil bacteria, for which water is an unfavorable medium, gradually perish; and in general a new 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 milliliter. Moreover, heavy rains which introduce wash from the surrounding watershed may at any time upset whatever equilibrium exists, and surface waters are likely to show sudden fluctuations in their bacterial content.

Seasonal Variation of Bacteria in Surface Waters. Sharp variations in bacterial content are particularly apt to occur in the spring and fall as a result of the rain and melting snow at those seasons. The high numbers shown for various rivers in Table 1 illustrate this point.

TABLE 1. SEASONAL VARIATIONS IN BACTERIAL CONTENT OF RIVER WATERS
Bacteria per Milliliter, Monthly Averages

River	Year	Jan.	Feb.	March	April	May	June
Thames	1926*	8,315	8,085	3,387	2,731	2,400	1,077
Lea	1926*	10,335	21,730	5,487	27,810	2,528	3,636
New	1926*	8,140	6,035	1,374	2,562	1,555	968
Ohio	1918†	9,690	165,200	54,500	6,760	5,300	3,240
Kentucky	1915‡	8,400	17,700	20,000	900	3,240	35,000
Missouri	1919§	12,000	20,600	29,600	31,100	30,000	31,200
Detroit	1943–2‖	14,000	4,700	10,500	1,100	700	51
River	Year	July	Aug.	Sept.	Oct.	Nov.	Dec.
Thames	1926*	1,376	700	1,149	1,015	6,027	2,455
Lea	1926*	3,100	1,857	5,600	1,453	44,153	2,994
New	1926*	1,141	986	1,514	888	5,307	1,816
Ohio	1918†	1,310	1,680	2,580	1,800	6,260	34,650
Kentucky	1915‡	86,700	55,400	56,000	13,700	9,280	24,400
Missouri	1919§	30,800	29,600	26,900	28,400	29,400	17,700
Detroit	1943–2‖	60	40	43	200	1,700	5,500

* Houston, 1926. † Cincinnati, 1918. ‡ At mouth; Frost and Streeter, 1924.
§ Kansas City, 1919. ‖ Figures obtained through the courtesy of W. M. Wallace.

The rainfall is the main factor which causes these 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 superficial impurities which can be washed away have been removed, it may dilute the stream with comparatively pure water. What the net effect of rain may be depends, therefore, on the

characteristics of the soil. A river of fairly good quality shows its highest numbers in rainy periods. In a highly polluted stream, on the other hand, the constant influx of sewage overbalances occasional contributions of surface contamination. Thus Gage (1906) showed (Table 2) 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.

TABLE 2. VARIATIONS IN BACTERIAL CONTENT, MERRIMAC RIVER
(Gage, 1906)

Flow of Stream (Cubic feet per second per square mile of watershed)	Bacteria per ml.		*B. coli** per ml.	
	Canal	Intake	Canal	Intake
Less than 1	7,500	10,800	66	88
1–2	6,800	6,200	50	51
2–4	3,600	5,600	29	39
Over 4	3,400	3,100	16	29

* The *Bacillus coli* of the earlier water bacteriologists corresponds approximately to our present species *Escherichia coli*.

The contrast between the two classes of rivers is well brought out in a study of the Lahn and the Wieseck, by Kisskalt (1906). Table 3, compiled from his data, gives an excellent idea of the total numbers of bacteria and their seasonal fluctuations in a stream of fair quality (the Lahn) and a highly polluted one (the Wieseck). In the former, the bacterial numbers are highest when rain brings surface pollution; in the latter, when the sewage constantly present is least diluted.

TABLE 3. MONTHLY VARIATIONS OF BACTERIA IN A NORMAL AND IN A POLLUTED STREAM

Bacteria per Milliliter
(Kisskalt, 1906)

	Lahn	Wieseck		Lahn	Wieseck
1904			1904		
July	318	104,000	December*	1,220	21,200
July	132	156,800	1905		
August	840	98,400	January*	3,668	29,920
October*	1,235	28,400	February*	5,380	11,900
October*	420	58,000	March*	1,210	8,250
November	2,340	39,200	April*	4,925	5,910
November*	1,740	52,000	May	570	14,800
December*	780	28,600	June	686	50,180

* Rain or high water due to previous thaw.

That this contrast may be observed also in a single river, which receives large increments of sewage at points along its course so spaced as to permit extensive self-purification of the stream in the intervals between, is shown by Table 4, prepared from data obtained by Frost and Streeter (1924) in their studies of the Ohio River. For

TABLE 4. MONTHLY VARIATIONS OF BACTERIA IN THE OHIO RIVER

Bacteria per Milliliter, Monthly Averages, 1916

(Frost and Streeter, 1924)

Month	Above Cincinnati	Below Cincinnati	Above Louisville
January	29,400	25,200	35,500
February	20,500	14,300	29,200
March	33,800	28,200	32,200
April	9,080	11,100	9,900
May	3,010	26,000	6,300
June	5,850	19,800	12,200
July	914	64,700	3,600
August	3,440	47,400	2,700
September	858	170,100	700
October	999	194,400	833
November	783	144,800	2,500
December	13,500	34,600	25,400

over a hundred miles above the city of Cincinnati comparatively little pollution is entering the Ohio River and self-purification has considerably reduced the bacterial content of the water by the time it reaches that city. The seasonal fluctuations in the number of bacteria in the river just above Cincinnati therefore simulate those of a relatively unpolluted stream. At a point below the city, however, the effect of the pollution from a sewered population of a half-million is so dominant that the normal fluctuations in bacterial content are almost completely masked. For a distance of about 120 miles below Cincinnati, until the river reaches Louisville, self-purification again plays its rôle, with the result that at the latter city evidence of the contribution of sewage from Cincinnati has almost disappeared and once more the normal bacterial fluctuations are evident.

Effect of Storage upon Bacteria in Water. 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 notable reduction in numbers occurs. Table 5 shows the very rapid decrease in the bacterial count of the water of Lake Windermere, an English lake, at various distances from the mouth of the River Brathay which flows into one end of the lake, as reported by Taylor (1940).

Although the lake is less than one-half mile wide, the number of bacteria dropped very sharply a short distance from the river's mouth. Similar observations have been described by Fred, Wilson, and Davenport (1924), and others.

TABLE 5. NUMBERS OF BACTERIA AT VARIOUS DISTANCES FROM THE MOUTH OF THE RIVER BRATHAY, WINDERMERE, AT DEPTH OF 1 METER

(Taylor, 1940)

Distance from River Mouth (meters)	Bacteria* per ml.	Distance from River Mouth (meters)	Bacteria* per ml.
22 July, 1938		2 November, 1939	
0	14,300	River	20,200
67	15,260	0	18,500
133	12,120	191	620
536	4,720	351	1,060
757	4,400	542	960
30 November, 1938		670	960
2,800	4,300	861	650
3,200	3,900	990	700
4,000	4,150	1,212	640
4,800	4,180	1,467	570
5,300	3,420	1,690	550
6,000	3,260	4,180	580

* On special agar, 20° C., 15 days.

The striking effect produced upon the water of the Potomac River by its successive passage through the three reservoirs of the Washington water supply is shown by the figures in Table 6 furnished by Mr. F. F. Longley, Engineer then in charge of the Washington filter plant.

TABLE 6. REDUCTION OF BACTERIA IN WASHINGTON RESERVOIRS

Bacteria per Milliliter, Monthly Average, 1907

	Potomac River	Dalecarlia Reservoir	Georgetown Reservoir	Washington City Reservoir
January	4,400	2,400	2,200	950
February	1,000	950	1,000	750
March	11,500	8,300	7,200	3,600
April	3,700	2,100	1,400	475
May	750	350	325	130
June	2,300	950	600	100
July	2,700	600	350	160
August	3,000	275	425	80
September	6,200	....	1,900	230

The still more striking results obtained at London are indicated in Table 7.

TABLE 7. AVERAGE REDUCTION OF BACTERIA BY STORAGE AT LONDON
(Houston, 1926)

Water	Bacteria per ml.	
	Gelatin 20°	Agar 37°
Raw Thames River	3,429	171
Do. stored at Staines	2,377	29
Do. stored at Lambeth	303	8
Do. stored at Chelsea	341	16
Do. stored at Southwark	187	15

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 of Boston contained on the average during the eleven years, 1893–1903, 220 bacteria per milliliter, 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 shown in Table 8 for the Chestnut Hill Reservoir of the Metropolitan Water Works (Boston). The averages are based on weekly analyses covering the eleven years, 1893–1903. The marked increase in April and

TABLE 8. MONTHLY VARIATIONS IN BACTERIAL CONTENT OF CHESTNUT HILL RESERVOIR, 1893–1903

Month	Bacteria per ml.	Month	Bacteria per ml.
January	82	July	82
February	73	August	95
March	71	September	**134**
April	**123**	October	89
May	69	November	103
June	73	December	96

September is the notable feature of these analyses. It 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 large reservoir, lake, or pond should not ordinarily be more than a few hundred per milliliter and may often be under a hundred. The 20° counts, shown in Table 9, of samples from twenty eastern Canadian lakes, large and small, recently examined by the Quebec Ministry of Health, are typical of natural waters of this class.

TABLE 9. BACTERIA IN EASTERN CANADIAN LAKES

Lake	Bacteria per ml.	Coliforms per 100 ml.	Lake	Bacteria per ml.	Coliforms per 100 ml.
1	9	0	11	130	2
2	13	0	12	200	9
3	31	2	13	240	27
4	39	0	14	300	7
5	46	0	15	350	5
6	55	15	16	500	7
7	80	8	17	550	1
8	110	3	18	650	0
9	110	6	19	650	11
10	120	0	20	850	2

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 in Table 10 at various points on the high seas between Portugal and Brazil. Near the European coast numbers were much higher.

TABLE 10. BACTERIA IN THE ATLANTIC OCEAN

Bacteria per Milliliter

(Otto and Neumann, 1904)

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

ZoBell (1938) at La Jolla, California, found bacteria in the sea to vary in number from less than one to only a few hundred per milliliter. Drew (1912) reported high numbers of bacteria in surface sea water off the Bahamas, ranging from 13,000 to 16,000, falling off below 200 fathoms (in the cold bottom waters at 10° C. or below) to 0 to 17.

ZoBell, Anderson, and Smith (1937) examined the water of Great Salt Lake when its salinity was 27.6 per cent and its temperature 1.2° C. The average count of six samples on agar made up with distilled water or sea water was only about 6 per ml., and on agar made up with 75 per cent lake water, 167. The colonies required 2 to 3 weeks to develop and the majority were very small.

The bacterial content of natural ice is usually very low unless it is formed from rather heavily polluted water or from polluted water trapped on the surface of ice already formed. Recently examined samples of ice from Quebec rivers gave the results shown in Table 11 which are typical of the average marketed product.

TABLE 11. BACTERIA IN NATURAL ICE

Ice	Bacteria per ml.	Coliforms per 100 ml.	Ice	Bacteria per ml.	Coliforms per 100 ml.
1	1	0	9	16	0
2	2	0	10	16	0
3	4	0	11	16	0
4	4	1	12	18	0
5	10	1	13	18	0
6	14	0	14	19	0
7	14	0	15	19	0
8	16	0	16	26	0

Factors Influencing the Diminution of Bacteria in Surface Waters. 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. Sedimentation, the activity of other microörganisms, light, temperature, food supply, and perhaps more obscure conditions such as osmotic pressure appear to be the most important.

The subsidence of bacteria, either by virtue of their own specific gravity, or as the result of changes in reaction or 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.

The share of other organisms, especially predatory protozoa, in hastening the reduction of bacteria in water is well recognized. Huntemüller (1905), after infecting water containing flagellate protozoa with typhoid organisms, 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 bacteria 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. Stokvis and Swellengrebel (1911) showed that ciliated infusoria may also consume large numbers of bacteria if the incubation temperature and the oxygen supply are favorable, and Hörhammer (1911) reported that certain crustacea such as Cyclops may devour considerable quantities of typhoid bacteria when present in masses from cultures, stained with methylene blue, and suspended in water.

The importance of protozoa in reducing the bacterial content of highly polluted waters has been very clearly shown by Purdy and Butterfield (1918). These observers found that bacterial numbers remained fairly constant in a sewage culture containing no protozoa, that protozoa (*Paramoecia*) died out rapidly in bacteria-free sewage, and that when both bacteria and protozoa were present the former increased rapidly at first but from 2 to 6 days later the bacterial numbers declined as those of the protozoa increased.

The dilution of sewage or heavily polluted water with relatively unpolluted water produces, at times, a rather unexpected effect upon the bacterial density. In their studies of the natural purification of the Ohio River, Frost and Streeter (1924) noticed an increase in the number of bacteria in the water during the first 10 to 15 hours of flow below certain points of major pollution; and Streeter (1931) observed that the addition of water from a tributary to a more heavily polluted stream frequently resulted in an increase, instead of a decrease, in the bacterial density of the mixture. These peculiar effects have been noticed in other river pollution studies, and Hoskins

and Butterfield (1933) give an excellent example of the latter which is here reproduced in slightly modified form, as Table 12.

TABLE 12. EFFECT OF DILUTING A POLLUTED WATER (ILLINOIS RIVER) WITH A RELATIVELY UNPOLLUTED WATER (KANKAKEE RIVER)

(Hoskins and Butterfield, 1933)

Month	Percentage of Dilution of Illinois by Kankakee	Percentage of Change in Pollution Density from Upper to Lower Station as Measured by:			
		Gelatin count 20° C.	Agar count 37° C.	Coliform organisms	5-Day oxygen demand
1921					
October	11.3	− 23.2	− 21.3	...	0
November	33.0	+ 65.7	+109	+126	+50.7
December	35.6	+156	+241	+ 83.5	+60.0
1922					
January	35.6	+118	+110	+ 39.6	+74.0
February	31.6	+ 19	+ 85.6	+ 22.1	+39.1
March	49.5	+157	+118	+242	+83.5
April	65.5*	− 19.8	− 16.9	+ 30.2	− 4.3
May	36.0*	− 45.2	+ 6.9	− 7.5	0
June	19.0	+ 59.2	+ 65.8	+ 53.7	+36.4
July	9.0	− 48.9	− 51.4	− 26.9	−27.3
August	7.8	− 32.3	− 35.8	+ 60.2	0

* Period of excessive flood stages and unusual conditions of flow throughout the river system.

An explanation of the above phenomena, both of which appear to be largely the result of dilution, may perhaps be found in the suggestion of Butterfield and Purdy (1931) that for a given set of conditions a maximum bacterial population can be supported, and if for any reason the population falls below this level multiplication of the bacteria follows. The results of experimental studies of polluted water by Hoskins and Butterfield (1933) and of sewage by Heukelekian (1933) furnish further evidence that bacterial multiplication is an important factor in the readjustment of the biological balance disturbed by dilution of a polluted water. Hoskins and Butterfield (1933) suggest that normally protozoa and other destructive agencies keep the bacterial density of such a water below its limiting level, but dilution so reduces this density that the bacterial concentration needed by the protozoa present is no longer available and the bacteria, therefore, are provided with an opportunity to augment their number; and since the latter can multiply much faster than the protozoa, a marked increase in the bacterial density may result before a biological balance

is restored. It is probable, as Streeter (1931) has pointed out, that disintegration of aggregates and bacterial clumps is also partly responsible for the larger numbers of bacteria observed in sewage and polluted waters subsequent to their dilution. After this initial increase in the bacterial population has reached its maximum, however, a progressive decline sets in, and available evidence indicates that the bacteria-consuming plankton usually present plays no small part in determining the rate of the decline.

Another factor of potential significance in the reduction of bacterial numbers in water is the bacteriophage of d'Hérelle (1922). In fact, the first clear record of bacteriophage action, according to d'Hérelle, is the observation of Hankin (1896) that the waters of certain Indian rivers exert a peculiar antiseptic action. Thus the water of the Jumna below Agra contains over 100,000 bacteria per milliliter whereas at a distance of 5 km. the numbers fall to less than 100. The antiseptic power of these waters is destroyed by boiling as indicated by the results of the experiment with cholera vibrios shown in Table 13.

TABLE 13. VIBRIOS IN INDIAN WATER

	Unheated Water	Boiled Water		Unheated Water	Boiled Water
After 0 hours	2,500	5,000	After 4 hours	0	6,000
After 1 hour	1,500	4,000	After 25 hours	0	10,000
After 2 hours	1,000	6,000	After 49 hours	0	36,000
After 3 hours	500	10,000			

Somewhat similar observations were made by Eliava relative to the water of the Konra River at Tiflis and his results are particularly suggestive of bacteriophage action since the water vibrios in this instance grew actively in peptone solution at first and were then killed, as shown by repeated examinations.

Bilouet (1926) made the interesting observation that from the water of the Deule River a bacteriophage lytic for intestinal bacteria could be derived.

Certain bacteriologists have held that the toxic waste products of the bacteria themselves may render water unfit for their own development. Horrocks (1901), Garré (1887), and von Freudenreich (1888) showed that an "antagonism" exists when bacteria are grown in artificial culture media; as a result the substratum which has supported the growth of one form may be rendered antiseptic to another. Frost (1904) exhaustively studied the phenomenon of antagonism by exposing typhoid organisms 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. Although it is difficult to believe that under ordinary conditions poisons are produced with sufficient power to render a stream or lake specifically toxic for any particular type of bacteria, it seems to be established that in polluted waters such effects may be produced. It appears 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 water of greater purity. Horrocks (1899), too, found that freshly isolated typhoid bacteria were still alive in sterile sewage after 60 days, but they disappeared in 5 days when *Escherichia coli* was also present. These phenomena may be due, however, to a struggle for oxygen, or for food, or to other causes, as well as to the assumed presence of highly toxic bacterial products, of which there is still but limited independent evidence.

Many investigations conducted since the pioneer researches of Downes and Blunt (1877) have confirmed the results reported by them. Their results showed that direct sunlight is fatal to most bacteria in the vegetative state and even to spores if the exposure is sufficiently long, and that 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 (1893) found that under certain conditions the bactericidal power of sunlight extended to a depth of about three meters before it became imperceptible. On the other hand, Procaccini (1893) found that when sunlight was passed vertically through 60 cm. of drain water the lower layers contained nearly as many bacteria after 3 hours' treatment as before the exposure. The middle and upper portions showed a great reduction in numbers, however. Taylor (1940) detected no significant difference between the bacterial counts of samples taken at the surface of a lake and of those taken at a depth of 1 meter, and concluded that the intensity of sunlight was not particularly responsible for the variations he observed in the bacterial counts in waters of the English lakes.

Few studies have been made of the effect of light on bacteria in flowing water. Jordan (1900) 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 observed a considerable reduction of the bacteria in the Isar at Pullach after the period of diurnal insolation, as shown by Table 14.

TABLE 14. EXAMINATIONS OF THE ISAR AT PULLACH
(Rapp, 1903)

Carried out September 26, 1898, no rain having fallen for three weeks:

Water Temperature	Air Temperature	Time of the Experiment	Bacteria per ml.
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

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

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, described in Chapter III, will show how delicately the bacteria respond to comparatively slight changes in their food supply. Wheeler (1906) found that typhoid organisms would persist in almost undiminished numbers in sterilized water from a polluted well containing considerable organic matter if kept in the dark at 20°, whereas in purer water or in the light they died out in 2 to 6 weeks. A part of the favorable effect of organic materials is probably due to protective colloid effects (Winslow and Brooke, 1927).

Whipple and Mayer (1906*a*, *b*) called attention to another important factor in the general problem. They reported that typhoid and coliform organisms survive in water better under an atmosphere of air than under an atmosphere of hydrogen; Hinds (1916), however, reports directly opposite results for *E. coli* with a somewhat different technique. The significant fact to be derived from these studies is that for certain types of bacteria the oxygen tension in the water or in the atmosphere may have a considerable effect on the rate of growth or period of survival.

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 (0.005 *N* HCl) is fatal to typhoid organisms, whereas ten times as much acid is required for sterilization when 1 per cent of peptone is present to

check the dissociation of the hydrogen. In Hazen and Whipple's study of the Allegheny, Monongahela, and Ohio rivers at Pittsburgh the antiseptic effect of acid wastes was strikingly shown (*Engineering News*, 1912). In connection with the important sanitary problem presented by the control of "soft drinks" it should be noted that carbonated beverages exert a distinct antiseptic action, which is markedly accentuated in those which also contain citric or lactic acids (Koser and Skinner, 1922). This distinctly antiseptic or disinfecting action of carbon dioxide has been observed by numerous investigators. But it should be recalled that a certain minimum amount of carbon dioxide is essential for bacterial life (Valley and Rettger, 1927; Plastridge and Rettger, 1929).

The effect of various mineral salts upon bacterial viability was exhaustively reviewed by Falk (1923). Oikawa (1926) showed that water passing through soils may absorb or dissolve mineral salts with consequent effect on the bacteria contained in the water. Jackson (1922) reported total counts of only a few hundred thousand bacteria per milliliter and *E. coli* counts between 100 and 200 per ml. at points on the Naugatuck River where the pollution was so great that much larger numbers would have been expected. Copper wastes from brass factories were chiefly responsible for this condition.

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 to life in the earth, or on 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 in ordinary potable waters. As 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."

Effect of Temperature upon Bacteria in Water. The effect of temperature upon the survival of bacteria in water differs according to the primary condition of food supply which has just been discussed. This interrelation between thermal conditions, food supply, and bacterial survival varies with different kinds of organisms and constitutes an important factor in their seasonal distribution in natural

waters and soil. When bacteria are in a medium in which they are able to grow and multiply, warmth, within reasonable limits, of course, favors their development. At times this may be true even of certain intestinal bacteria in water. Thus, at Harrisburg, Pa., a series of *E. 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 2 days and the temperature of the water in the reservoir was nearly at blood heat (Harrisburg, 1907). Clemesha (1912*a*), in India, made an exhaustive study of this multiplication of coliform-like microbes in warm waters and showed that it is confined to certain particular types within that group. For most intestinal bacteria the conditions necessary for growth and multiplication do not obtain in water and an entirely different temperature effect is manifest. When a bacterium cannot multiply, the only vital activity which can take place is a katabolic wasting away, which soon proves destructive, and the higher the temperature the more rapidly the fatal result is reached. In winter a frog lives at the bottom of a pond, breathing only through its skin and eating not at all, but as soon as the tem-

TABLE 15. EFFECT OF TEMPERATURE ON SURVIVAL OF TYPHOID BACTERIA IN WATER

(Houston, 1911)

Temperature	Percentage of Typhoid Organisms Surviving after 1 week	Period of Final Disappearance of Organisms
0° C.	46	9 weeks
5° C.	14	7 weeks
10° C.	0.07	5 weeks
18° C.	0.04	4 weeks

perature rises it must eat and breathe through its lungs or perish. It is quite true that even in ice 40 per cent of typhoid bacteria perish in 3 hours, and 98 per cent in 2 weeks (Sedgwick and Winslow, 1902). Later work has shown, however, that they die in spite of the cold, not on account of it, and that the decrease is more rapid at higher temperatures, unless of course food supply and other conditions permit multiplication. Houston (1911) furnished a very clear demonstration of this temperature effect by storing typhoid bacteria in water; his results are shown in Table 15.

Ruediger (1911) showed that coliform organisms were far more abundant in the Red Lake River during the winter when the river was covered with ice than in summer, although the volume of the river and the amount of sewage pollution were about the same. Typhoid

bacilli in celloidin dialyzers floated down the river showed only 2.5 and 3.5 per cent surviving in 2 days and 0.51, 0.89, 2.2, and 3.2 per cent surviving in 3 days when the river was not frozen; dialyzers suspended through the ice in colder weather showed 6.1, 10.5, 17.7, 46.8, and 62.9 per cent surviving in five different experiments after 2 days, 31 per cent in 3 days, 19 per cent in 7 days, and 2.5 per cent in 14 days. Ruediger attributed this greater persistence at low temperatures to the absence of poisonous waste products of other organisms and to protection from the light; but there can be little doubt that it was mainly a result of the general preservative effect of cold. From an epidemiological standpoint the conclusion that disease germs perish quickly in warm waters is amply confirmed. Most outbreaks of typhoid fever due to polluted water occur in cold weather and this is, in part at least, due to the greater persistence of typhoid bacteria at low temperatures.

Relation between Time of Storage and Self-purification. 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. In a comprehensive study of the self-purification of the Potomac River, the investigators of the U. S. Public Health Service found that the relation between time and the degree of purification effected is so close that it may be stated mathematically. Professor Phelps expresses this in the formula $\log \frac{n_1}{n_2} = kt$, where t is the time and n_1 and n_2 the respective numbers of bacteria; k, of course, will vary with the temperature and the other conditions discussed above. This formula expresses the general relationships involved; but the rate of reduction does not exactly follow the curve of a monomolecular reaction (see Winslow and Falk, 1923; Falk and Winslow, 1926; for a fuller analysis of the whole problem, Winslow, 1929). For practical purposes the logarithmic rate of reduction is a sufficiently close approximation to the truth.

Cohen (1922), in an exhaustive study of the viability of coliform and typhoid bacteria in water, found that the temperature coefficients for these two organisms are distinctly different, their relative resistance being as 67 is to 1 at 0° C. and as 8 to 1 at 30°. The viability of *Eberthella typhosa* in water falls rapidly at *p*H values more acid than 5.0 or more alkaline than 6.4. For *E. coli* the zone of optimum *p*H is wider and it centers about absolute neutrality.

The absolute time required to make a polluted water safe will obviously vary with the value of k in the formula given above.

Jordan, Russell, and Zeit (1904), in an important series of experiments, added typhoid bacteria 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 3 days except in a single instance. Russell and Fuller (1906) confirmed these general results; they found that typhoid organisms would live for 10 days in the unsterilized water of Lake Mendota, but they could be isolated only after 5 days when immersed in sewage. Other observers record much greater viability for typhoid bacteria; but experiments leading to such conclusions deal only with the maximum survival period for a few out of great numbers of germs introduced into water or mud, and entirely ignore the quantitative aspects. When the proportion of the original bacteria surviving is considered, the period necessary to bring about a reasonably safe condition is found to be short. Houston (1908) showed that, when water is artificially infected with typhoid organisms and stored, 99.9 per cent of the disease germs perish in 1 week, although some may persist for 1 to 9 weeks.

In later experiments Houston (1911) found that "uncultivated" typhoid bacteria added to the water directly from the urinary sediment of a disease carrier perished much more rapidly than the laboratory strains; they usually disappeared entirely after 1 week and always after 3. On a number of occasions Houston gave dramatic expression to his confidence in these negative laboratory findings by drinking half-pint portions of water which a few weeks previously had contained millions of typhoid organisms. There is sufficient practical epidemiological evidence, such as that offered in the Chicago Drainage Canal case and in the lawsuit over the condition of the water supply of Jersey City, to confirm the general conclusion that any water which has been stored for 4 weeks is practically safe.

Bacteria in Ground Waters. In general it has been shown 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 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 also much of their food material. The numbers of bacteria in the soil itself decrease rapidly as one passes downward.

Kabrhel (1906) found several million per gram in surface samples of woodland soil, a few thousands or tens of thousands half a meter below, and usually only hundreds in 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 is adopted, slow-growing types develop and considerable numbers may be obtained.

For convenience ground waters may be divided into three groups: 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 aerial 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 per milliliter. Maschek (1887) found 36 wells out of 48 examined in Leitmeritz which had a bacterial content of over 500 per ml. Fischer (Horrocks, 1901) reported 120 wells in Kiel which gave over 500 bacteria per milliliter and only 51 with less than that number.

In the examination of 147 shallow farmyard wells by one of us (S.C.P.) it was found that 124 of the wells which contained no *E. coli*, and were therefore probably free from fecal pollution, averaged 190 bacteria per milliliter, whereas 23 wells which gave positive tests for *E. coli* averaged 570 per ml. The distribution of the two series of samples according to the number of bacteria present is indicated in Table 16. Very similar results were reported for shallow wells used as farm water supplies in Minnesota by Kellerman and Whittaker (1909), although the general quality of the wells examined was considerably below that of the series recorded in Table 16.

TABLE 16. BACTERIA IN SHALLOW FARMYARD WELLS

Percentage of Samples in Each Group

Bacteria per ml.:	0	1–10	11–20	21–50	51–100	101–500	501–1,000	1,001–2,000	2,001–3,000
Series I. *E. coli* absent	3	16	14	16	11	31	5	4	..
Series II. *E. coli* present	..	..	5	..	10	57	10	14	5

The 20° counts obtained from two groups of shallow well samples recently collected in Quebec Province are shown in Table 17. One group of 43 wells contained no coliforms in volumes of 110.5 ml.,

whereas a like volume of each of the other 93 wells contained these organisms. The paucity of low counts in shallow well samples containing coliform organisms and the occasional finding of very large numbers of bacteria in samples free from coliforms are shown very clearly in Tables 16 and 17.

TABLE 17. BACTERIA IN SHALLOW WELLS

Percentage of Samples in Each Group

Bacteria per ml.:	0	1–10	11–20	21–50	51–100	101–500	501–1,000	1,001–2,000	Over 2,000
Coliforms absent	..	19	7	9	7	21	19	2	16
Coliforms present	..	..	..	2	2	18	19	12	46

The characteristics of the soil must always be given due consideration. Coarse, rocky soils with fissures and large voids obviously offer poor barriers to distribution, and wells in such regions may be polluted, whereas in compact or sandy soils which are fairly uniform such a condition would not result. Rainfall and the height of the ground water are also significant. Thus Stiles, Crohurst, and Thompson (1927) reported that *Escherichia* organisms were recovered from ground water, i.e., from shallow driven wells, at distances up to 232 feet from an experimental trench into which uranin plus excretal pollution was placed. Chemical pollution (uranin) was recovered up to 450 feet from the same trench; both uranin and the *Escherichia* bacteria traveled in only one direction, that of ground-water flow. Wet weather (high ground water) was found to be conducive to extension of pollution; dry weather was inhibitive. *Escherichia* organisms tended to localize at or near the ground-water table. As pollution travels, it does not appear to expand laterally with the trench as the apex of a truncate cone, but contracts to narrower breadth. If the direction of ground water is unknown, distance represents the great factor of safety.

Caldwell and Parr (1937) studied the flow through unconsolidated soils of ground water polluted by bored-hole latrines. The velocity of flow of the ground water through the various soil strata was from 3 to 8 feet per day, which was considerably greater than that of about 1 foot per day reported by Stiles, Crohurst, and Thompson for the flow through the sands of their experimental fields. After fecal matter had been added to the water in the latrine which extended through the 5 feet of ground water, there was apparently little lag caused by filtration, for the bacterial pollution (*E. coli*) traveled at

almost the same velocity as that of the ground water during the first 2 or 3 days. Thereafter, its progress was slower and although the excreta of an average family were added each day to the latrine's contents, *E. coli* did not appear in shallow driven wells 25 to 35 feet distant until 2 months later and then only for a brief period before regression of the pollution caused by clogging of the soil by fecal particles and biological growths. This clogging retarded and finally almost stopped the flow from the latrine, the bacterial pollution thereafter retreating steadily until it was not detectable at a distance of 5 feet from the latrine. These investigators did not notice any tendency of the bacteria to localize at or near the water table, but it should be observed that the velocity of ground-water flow was only 3 feet per day through the upper soil, whereas it was 5 feet and 8 feet through the middle and lower soils respectively, and the bacteria seemed to travel farthest in the ground-water streams of greatest velocity. In other words, the vertical distribution of the pollution at any point was determined by the arrangement of the soil strata. The results agreed with those of other investigators, however, relative to the direction of flow of the bacterial pollution: it traveled only in the same direction as that of the ground water, and in a narrow band which gradually broadened from the 15-inch diameter of the latrine to a maximum of about 3 feet at a distance of 15 feet from the latrine, and then narrowed gradually to its apex at the maximum distance of about 35 feet. In these experiments evidence of chemical pollution was found 85 feet but not 100 feet from the latrine.

Apparently the distance that pollution will travel in ground water through the soil depends upon a number of factors, chief of which are the volume of polluting material and its physical make-up, the permeability of the soil, the hydraulic gradient of the ground water, and climatic conditions. The direction in which the pollution will extend is that of the ground-water flow and, as Stiles, Crohurst, and Thompson emphasized, if the direction of the ground-water flow is unknown, distance represents the principal factor of safety.

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 (1901) of 37 springs which were practically unpolluted and had less than 1 ppm. excess of chlorine over the normal, 54 samples were examined; they gave an average of 41 bacteria per milliliter. Only 6 samples showed figures over 50.

Deep, "driven", or "tubular" wells, if carefully constructed, should ordinarily be free from all surface-water contamination and

should show low bacterial counts. The results shown in Table 18, which were obtained by Houston in the examination of a series of deep wells of high quality at Tunbridge Wells, are fairly typical. Fifteen

TABLE 18. BACTERIAL CONTENT OF DEEP WELL WATERS

Bacteria per Milliliter

(Houston, 1903)

36	6	9	4	1
16	17	4	3	12
2	4	10	5	2

driven wells located in the neighborhood of Boston, examined by two of us (S.C.P. and C.-E.A.W.) in 1903, showed at the end of 48 hours an average of only 18 colonies per milliliter; and the results of certain examinations of other wells and springs, by the same authors, are given in Table 19.

TABLE 19. BACTERIA IN DEEP WELL AND SPRING WATERS

Bacteria per Milliliter

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, the number of bacteria is small; and the peculiar character of the organisms present is often manifested by slow development at room temperature (frequently no growth until the third day), entire absence of liquefying colonies, and an abundance of chromogenic species.

The Types of Bacteria Found in Water. As a rule the majority of the bacteria isolated on ordinary culture media from water belong to a few fairly well-marked groups (the Franklands, 1894; Ward, 1897; Fuller and Johnson, 1899; Jordan, 1903).

The more obvious of these groups are (*a*) the fluorescent bacteria; (*b*) the chromogenic bacteria, including violet, red, and yellow forms; (*c*) organisms of the coliform group; (*d*) organisms of the *Proteus* group; (*e*) non-gas-forming, non-chromogenic, non-spore-forming rods which do not produce *Proteus* colonies and may or may not acidify milk and liquefy gelatin; (*f*) spore formers of the *Bacillus subtilis* type; and (*g*) white, yellow, and pink cocci.

CHAPTER II

THE COLLECTION OF SAMPLES FOR BACTERIOLOGICAL EXAMINATION OF WATER

The Objective of Sampling. The principal objective of sampling a water for bacteriological examination consists, first, in securing a sample that is representative of the supply, and, second, in preserving the bacterial content of the sample, as nearly as possible, in its original state until the water is examined. Rarely is it feasible to attain this objective completely; even to approach it will often tax the judgment, the ingenuity, and the resourcefulness of the person collecting the sample. Since, however, the bacteriological results obtained may be worthless and perhaps even misleading unless the sample *when examined* is reasonably representative of the supply, a special effort should be made to select a fair sample, to collect it properly, and to prevent an excessive alteration of its bacterial content until it reaches the laboratory. The importance of proper collection of samples cannot be overemphasized.

Sample Bottles. Samples of water for bacteriological examination should be collected in clean, sterile, glass-stoppered bottles of good quality. Wide-mouth bottles of at least 100-ml. capacity are preferable.

They should be cleaned thoroughly before using, by treatment with sulphuric acid and potassium bichromate, rinsed in clean water, dried by draining, and sterilized by heat at 170° C. for at least 1 hour, or by steam at 15 pounds pressure for 15 minutes. If the bottle is not to be used immediately, the neck and stopper should be protected from dust or other contamination by wrapping with tin or aluminum foil, or a paper substitute, before sterilization.

For sampling water which has been chlorinated and which therefore may contain residual chlorine, from 0.02 to 0.05 gram of powdered sodium thiosulphate should be placed in each bottle and the whole sterilized by dry heat. If this precaution is not observed, the continued action of any residual chlorine present may reduce the number of viable bacteria in the sample before it can be examined; this was well demonstrated by Mallman and Carey (1933).

Collection of Representative Samples. The greatest care must be taken not only to collect a fair sample, but also to protect it from any contamination that might alter its bacterial content. Neither the

fingers nor anything other than the water should be permitted to touch the inside of the neck of the sample bottle or the cone of the stopper, as the water may thereby be seriously contaminated and rendered unfit for examination. It is well known that bacteria are found abundantly upon the skin; Winslow (1903*b*) showed that even *E. coli* is frequently present on the hands. If possible, when taking the sample, the stopper with its foil cover in place should be held between the fingers of one hand while the bottle is held in position with the other; but if this is impracticable, the stopper with its foil cover may be placed, upside down, on a clean, dry surface.

In order to obtain a fair sample, special precautions must be observed, 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 5 minutes if from a tap in regular use, or for a longer period if the water has been standing in the house-service system. In the small pipes, changes in bacterial content are apt to occur; certain species will die and others will multiply. If there is reason to suspect that the tap is dirty, soiled by kitchen waste, or otherwise contaminated, it must be flamed until well heated by means of a burning wad of cloth or cotton saturated with alcohol or kerosene; the use of alcohol is preferable, because from this fuel no soot will be deposited on the tap.

If a sample is to be taken from a pump, similar precautions are necessary. The pump should be in continuous operation for at least 5 minutes, and preferably for half an hour before the sample is taken, in order to avoid excessively high numbers of bacteria due to their multiplication within the well and pump: the bacterial condition of the water as it passes through the ground is what we wish to determine (except when surface wash may enter a well as discussed in a later paragraph). Thus Heraeus (1896), in a well water which had been little used during the preceding 36 hours, found 5,000 organisms per milliliter; 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, as shown in Table 20.

TABLE 20. EFFECT OF PUMPING ON THE BACTERIAL CONTENT OF WELL WATER

Well water after continuous pumping for 15 minutes	458
Well water after continuous pumping for many hours	140
Well water later	68
Well water after continuous pumping for 15 minutes	578
Well water after continuous pumping for many hours	179
Well water later	73

After a proper interval of pumping the sample of a well water may be collected from the petcock 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 source are often great; if the flooring about the pump is not tight, as happens so often, continued pumping may wash in an unusual amount of surface pollution.

Shallow wells not provided with a pump may be sampled by means of a bottle with a clean cord attached and wound around it, the whole wrapped in paper and sterilized by dry heat. If preferred, the cord with a slip knot at one end may be wrapped and sterilized separately and attached to the bottle as required.

When sampling surface waters, particular care must be exercised 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 grasp the bottle near its base and plunge it, open mouth downward, to a depth of a foot or so, the hand and bottle describing a wide arc as they rapidly pass into and out of the water; by this procedure water that has been contaminated by the hand is prevented from entering the bottle. Whenever any current exists, the mouth of the bottle should be directed against it in order that it may carry away any bacteria from the fingers or the holding device. In rapidly flowing streams it is necessary only to hold the bottle at the surface with the mouth pointed upstream.

As many surface and well waters are deleteriously affected by admixture of surface drainage after a long-continued rain or a heavy downpour, it is frequently advisable, in order to determine the quality of a supply when it is at its worst, to sample the water during or after a period of particularly unfavorable weather.

For taking samples at moderate depths — from 2 to 15 feet — a sampling rod is an extremely useful aid. It consists of a rigid or jointed rod of almost any sturdy material, from bamboo to steel, provided with a right-angled base to which the sample bottle is fastened. When the bottle is lowered by means of the rod to the desired sampling point, the stopper can be jerked out by pulling an attached cord; or a mechanism, actuated by a spring, which will remove the stopper and replace it after the bottle has been filled, can be fixed near the lower extremity of the rod. An excellent design of this type of rod, which was used for collecting samples of water over oyster beds, is described by Lenert (1933).

Water samples from greater depths may be collected by means of apparatus the essentials of which 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. A number of such devices have been employed. An early design is that described by Abbott (1899); a good form was devised by Hill and Ellms (1898). More recently Mortimer (1940) developed an apparatus for taking samples at depths of 0 to 60 meters which includes several ingenious and practical features, not the least of which is an inverted U-tube at the top of the sample bottle which, while the water sample is being collected, traps a bubble of air that prevents entrance of water from lesser depths while the bottle is being raised to the surface. A variety of mechanical aids, used by the U. S. Public Health Service in its Ohio River Pollution Survey, were described by Carnahan (1941). He includes devices for sampling water, mud and bottom deposits, and also a portable sample-hoisting equipment.

Miquel and Cambier (1902) and other authors recommend the use of an evacuated glass bulb with a fused capillary tube which can be broken off at the desired moment. Wilson (1920) described a valuable form of deep water sampler operating on the same principle. Another interesting apparatus, for use at great depths in the sea, is that devised by Drew (1912).

A series of samples can be collected at regular intervals of time from a water pipe or a flowing stream by means of a novel automatic, electrically controlled sampler designed by Matheson (1937*a*); this type of equipment promises to be of particular utility in filtration plants, where frequent sampling is necessary for close control of the product.

Ice may be sampled by splitting a large piece with a flamed chisel and chipping away from the exposed, uncontaminated interior small fragments that may be caught in sterile bottles or Petri dishes. The dishes are placed in the 37° C. incubator for a short time to melt the ice, and the resulting water samples are combined to constitute the sample to be examined. Greenfield (1916*a*) has described for sampling ice a special apparatus the principle of which is based on that of a coal sampler. Whatever the method of sampling employed, it is advisable to transport one or more large pieces of the ice to the laboratory where sampling facilities are readily available; blocks of ice, if well packed in dry sawdust, can usually be shipped for moderate distances with little loss from melting. It should be noted that examination of the body of water from which the ice has formed frequently furnishes more information regarding the probable sanitary

quality of the ice supply than examination of a few random samples of the ice.

Sample bottles should not be filled completely; an air space of about 1 inch should be left in the bottle to facilitate the thorough mixing of the sample by shaking which is necessary immediately before its examination.

Unless the supply to be examined is sampled at frequent intervals, more than one sample should be collected in order that results reasonably representative of the quality of the water may be assured.

Storage of Samples. Because of the marked effect of storage in bottles upon the bacterial content of water, examination of samples in the field, immediately after their collection, usually yields more reliable results than examination some hours later. If there is available a well-trained water bacteriologist who is thoroughly cognizant of the hazards of contamination to which bacterial cultures prepared in the open are exposed, and if weather conditions are favorable, the inherent advantages of field examination may be realized. Several ingenious field kits for such work have been devised, one very good form of which, described by Van Buskirk (1912), permits the use of the plate method of examination. The opportunity for air contamination in the open is, however, always great, and the numerous difficulties and inconveniences usually encountered upon attempting bacteriological work outside a proper laboratory have militated against the general practice of examination in the field. A temporary, sheltered, local laboratory, if reasonably well equipped, may enable results to be obtained that are practically as satisfactory as those secured at the site of collection of the sample, provided it is not far removed from the points of sampling; but such temporary laboratories are too often of the makeshift variety. On the whole, the authors are of the opinion that, unless a well-equipped mobile laboratory is available, results from examination in a permanent laboratory are to be preferred to those obtained in the field, especially if the laboratory can be reached within 12 hours or so after the sample has been collected.

If the water sample cannot be examined immediately after it has been collected, means must be provided to maintain it, as nearly as possible, in its original state, in regard to bacterial content, until the examination can be made. Satisfactory preservation is possible, however, only when the period of storage is limited. As soon as the sample 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 samples the rise in numbers is gradual; in others it is very rapid. The Franklands (1894) record the instance of a deep well water in which the bacteria increased from 7 to 495,000 in 3 days. On the basis of his research, Miquel (1891) 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 the decrease, after the numbers of bacteria have reached a maximum, is likewise rapid and steady. To what extent protection from light, increase in temperature, and destruction of higher microörganisms are 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.

TABLE 21. BACTERIAL CHANGES IN WATER DURING STORAGE

(Whipple, 1901)

Sample	Initial Temperature (° C.)	Temp. of Incubation of Sample (° C.)	Bacteria per ml.				
			Initial	After 3 hr.	After 6 hr.	After 24 hr.	After 48 hr.
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*
K	6.7	20.0	430	345	405	.....	750,000*
L	23.2	23.0	510	340	230	8,000	20,000
M	23.2	2.5	525	300	220	380	2,200

* 0.0005 per cent peptone added to the water.

Whipple (1901) exhaustively studied the details of this multiplication of bacteria in stored waters and showed (see Table 21) that there is first a slight reduction in the number present, which lasts perhaps for 6 hours; then follows the great increase noted by earlier observers. It is probable that there is a constant increase of the typical water bacteria which is overbalanced at first by a reduction in other forms for which the environment is unsuitable.

Wolffhügel and Riedel (1886) suggested that such multiplication was dependent upon the air supply, because the bacteria in vessels closed with rubber stoppers multiplied less rapidly than the bacteria in containers plugged with cotton. ZoBell and Stadler (1940), however, demonstrated that multiplication and respiration of aerobic bacteria are independent of the oxygen tension, at least within the investigated range of 0.30 to 36.0 milligrams per liter. Whipple (1901) 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 Table 22, that the size of the bottle

TABLE 22. EFFECT OF SIZE OF VESSEL UPON THE MULTIPLICATION OF WATER BACTERIA DURING STORAGE

(Whipple, 1901)

Sample	Bottle	Temp. of Incubation (° C.)	Bacteria per ml.					
			Initial*	After 3 hr.	After 6 hr.	After 12 hr.	After 24 hr.	After 48 hr.
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

* Average of five plates.

markedly influenced the growth. A similar effect of the size of receptacle upon bacterial activity in sea water was remarked by ZoBell and Anderson (1936), and in lake water by Stark, Stadler, and McCoy (1938).

Kohn (1906) suggested that the solution of certain constituents of glass which favor bacterial growth was one cause of this phenomenon, but ZoBell (1943) reported comparable effects whether the glass was of Pyrex or of various kinds of soft glass. The latter investigator concluded that the principal cause of increased bacterial multiplication in a sample bottle presenting a relatively large solid surface is the adsorption and concentration of food upon this surface, which

renders the food more available to bacteria than when it is dispersed in low concentration throughout the water. Stark, Stadler, and McCoy (1938) confirmed this conclusion; they found in their investigations of lake water that a film of organic matter forms first upon an exposed solid surface and that bacterial multiplication then proceeds apace in this favorable environment. Further confirmation of this explanation is provided by the well-attested fact that the effect of receptacle size upon bacterial activity is noticeable only when the concentration of nutrient materials in the sample is very low; no effect of relative volumes of receptacle and sample is observed when the water contains an excessive amount of food for the bacteria present.

Icing of Samples. Whipple's table showed that multiplication during storage was greater at a higher temperature; this is a well-recognized general fact. In order to obviate the abnormal results of storage increase it is therefore obvious that samples must not only be examined shortly after collection but that they must also be kept cool during their necessary storage. If fairly pure waters are placed upon ice and kept at temperatures between 6° and 10° C., there will usually be little material increase in their bacterial content in 12 hours.

TABLE 23. EFFECT OF STORAGE AND ICING UPON 184 SAMPLES OF POLLUTED WELL WATER

(Caldwell and Parr, 1933*a*)

Icing	Storage Average (hr.)	Final Water Temperature Average (° C.)	1 Samples Coliform +	2 Samples *E. coli* +	3 Number of Colonies on Endo Pour Plates at 37° C.	4 Number of Colonies on Endo Pour Plates at 37° C.
					Red	Others
None	0.0	19.9	124*	93*	8,960*	44,670* (16)†
Iced	4.5	10.0	80.6%	59.1%	20.0%	58.9 (16)
None	4.5	25.7	56.4	49.5	17.0	77.6 (18)
Iced	27.8	17.1	38.7	43.0	10.8	73.6 (17)
None	27.8	26.9	34.7	37.5	12.8	272.9 (92)

* Numerical data for unstored samples; data for others expressed in percentage of these.
† Number in ( ) indicates plates with counts greater than 1,000, not further estimated.

The effect of storage and icing upon the sensitive intestinal bacteria found in more polluted waters, however, varies with the type of organism present. This fact is clearly demonstrated in Table 23, which gives results obtained by Caldwell and Parr (1933*a*) upon

examination of waters from experimental pipe-wells situated near latrines. Although the majority of the bacteria growing at 37° C. (column 4 of the table) follow the general rule by multiplying even in samples stored for long periods at room temperature, organisms of the very important coliform group die off more or less rapidly (columns 1, 2, and 3) regardless of the temperature. Icing of samples, however, generally retards the death rate of coliform organisms and prevents rapid multiplication both of bacteria growing at 20° C. and of those growing at 37° C. Webster and Raghavachari (1935), comparing the coliform results from a variety of iced and uniced Madras waters which had been in transit for 20 to 48 hours, found that of 104 duplicate samples 19 iced portions yielded "true coli" when the uniced portion did not, whereas the reverse occurred in only 1 sample; and of 75 triplicate samples, only 5 of the portions examined at the point of collection yielded these organisms when the iced portion gave negative results (the organisms were recovered from one of the uniced portions of these latter samples). They concluded that "a properly chilled sample is almost as satisfactory as a sample examined on the spot."

Icing is therefore essential, particularly during the first 12 hours of storage. Even iced samples should not be held too long, however; Jordan (1900) found that the bacterial counts of three samples of river water packed in ice for 48 hours fell off from 535,000 to 54,500, from 412,000 to 50,000, and from 329,000 to 73,000, respectively. Consequently, it is advisable to adhere as closely as possible to the Standard Methods requirement that the interval between sampling and examination should not exceed 12 hours for relatively pure waters and 6 hours for impure waters, and that the temperature of the sample should be kept between 6° and 10° C. throughout the period of storage.

There has been evidence, during the past decade, of a tendency to ignore the necessity of icing water samples that are to be examined only for the presence of coliform organisms, even to the extent of sending uniced samples long distances by mail. The authors believe that this tendency is to be deprecated; errors resulting from neglect of icing are almost sure to be on the wrong side of safety and to lead eventually to pronouncing as safe waters that are frankly dangerous. Caldwell and Parr's conclusion that "It should be emphasized that the practice [of examining uniced samples] derives sanction from expediency and does not conform in general to the conditions best for analysis," and Webster and Raghavachari's dictum that "an unchilled sample cannot be accepted as a substitute for a fresh or chilled sample," reflect the considered opinion of the great majority

of experienced water bacteriologists both in the Americas and abroad. Nor can cold weather always be relied upon to keep sample shipments at a low temperature; a sojourn of only a few hours in a warm express or mail car may effect a radical change in the bacterial content of the sample; even during the colder months, therefore, water samples that are not to be examined within an hour or so should be iced.

The cases ordinarily employed for transporting iced samples consist essentially of a box, usually insulated, which is divided into two watertight sections, one to hold the sample bottles in their dustproof containers and the other to hold broken ice. In the more effective designs, provision is made for completely, or almost completely, surrounding the bottle compartment with ice. It is advisable to put an ample quantity of clean, dry sawdust in the ice compartment to absorb the water from the melting ice; if possible, this should be in place when the case leaves the laboratory, for dry sawdust is not always readily available in the field. Care must be taken, of course, to prevent contamination of the samples by the sawdust or ice.

Information Concerning the Sample. The importance of furnishing complete information respecting the source of the sample cannot be too greatly stressed, because without such information a satisfactory interpretation of the results obtained from examination of the water is generally impossible. The person collecting the sample should prepare, usually while still in the field, detailed notes fully describing the water supply, its constructional features, and its surroundings. When sampling a well or spring, these data should include a full description of its construction; the provision, if any, for excluding surface drainage; the nature of the ground surface, with particular reference to slopes; the probable direction of the ground-water flow; the proximity of water courses; the character of the soil and rock; the existence of possible sources of pollution such as privies, cesspools, drainage ditches, barnyards, etc.; the observed effect of rains upon the appearance of the water; and the exact point of collection of the sample.

In sampling a surface supply, complete details should be furnished relative to the waterworks and its surroundings; the existence of any sewer outlets or other sources of pollution; and again the exact point at which the sample was taken.

Information regarding any recent rainfall, the health of the family or community using the water (particularly with reference to incidence of enteric disease, past or present), and the date and hour of sampling should also be recorded. A detailed sketch, showing the supply and its surroundings, with all distances between the supply

and possible sources of pollution clearly indicated, will prove well worth the effort expended in its preparation.

Finally, particular care should be exercised to ensure that each sample is plainly marked so that it can be readily identified upon its arrival at the laboratory. The use of gummed labels on the bottles is inadvisable, for a trace of moisture may cause them to become detached. If permanently numbered bottles or bottles with frosted labels are not available, attached tags, or cloth or paper covers tied over the stoppers, may be marked. The same mark or number is also placed upon the information sheet; the latter should always accompany its corresponding sample, for the analytical procedure employed by the laboratory in its examination may depend upon the source of the sample and its surroundings.

CHAPTER III

DETERMINATION OF THE NUMBER OF ORGANISMS DEVELOPING AT ROOM TEMPERATURE

The Influence of the Medium on Bacterial Counts. The customary methods employed to determine the number of bacteria in water do not reveal the total bacterial content, but only a small fraction of it; this becomes apparent when we consider the large number of organisms, nitrifying bacteria, strict anaerobes, 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 nitrites and ammonium compounds are unable to develop in the presence of the considerable concentration of organic matter contained in ordinary laboratory media. Winslow (1905), in the examination of sewage and sewage effluents, found 20 to 70 times as many bacteria by microscopic enumeration as by the gelatin plate count. Similar ratios of direct counts to plate counts, not only of sewage but also of natural waters, have been reported by many investigators. Upon submerging glass slides in lake water and examining the bacteria appearing on them after various intervals, Henrici (1933) and Henrici and Johnson (1935) discovered a great many species which do not grow on ordinary media. ZoBell and Allen (1935), using the same method, found millions of bacteria on 2 square inches of slide which had been submerged for 48 hours in sea water that gave plate counts of only 100 to 350 per ml., and they estimated that some 40 to 50 species are regularly represented in sea water, the majority of which grow in sea-water media but not in media made with fresh water. Other special media enable counts to be obtained, even from fresh waters, that are much greater than those yielded by the ordinary methods. The Nährstoff Heyden (albumose) agar, for example, was once strongly advocated by Hesse and Niedner (1898) and other German bacteriologists upon such grounds. In this country Gage and Phelps (1902) showed that the numbers obtained by the ordinary procedure were only from 5 to 50 per cent of those found when using the Nährstoff agar. Müller (1900) observed that the larger counts obtained by plating on this special medium were

due to the fact that it specially favored the more prototrophic forms among the water bacteria themselves. He found, for example, that water which normally showed six times as many bacteria on Nährstoff agar as on nutrient gelatin might give a Nährstoff-gelatin ratio of 20 to 30 after it had been standing for some time in the supply pipes. In Table 24 results reported by Gage and Phelps

TABLE 24. PERCENTAGES OF BACTERIA DEVELOPING ON REGULAR AGAR AS COMPARED WITH NÄHRSTOFF AGAR FOR DIFFERENT WATERS
(Gage and Phelps, 1902)

Class of Water	Days of Incubation						
	2	3	4	5	6	7	8
REGULAR AGAR							
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

(1902) show strikingly the different regular-agar to Nährstoff-agar ratios from waters of various qualities.

Obviously the effect of using such a medium as Nährstoff agar is to increase disproportionately the bacterial counts obtained from pure waters and thus to diminish the difference in bacterial content between normal and contaminated sources. A like effect results from the use of the direct count, the special media, and the submerged-slide method frequently employed in ecological studies of bacteria: the additional bacteria revealed by these means may be of importance to the economy of natural waters, but their recognition in a sample of water contributes little to an estimate of its sanitary quality. For such an estimate the usual methods are fairly adequate. Within limits, it is of no great importance that one method permits the growth of more bacteria than another. When the quantitative analysis is being used as a measure of sewage pollution the essential point is that the section of the total bacterial flora which is obtained should be thoroughly

representative of that portion of it which is most significant — the group of the quickly growing, rich-food-loving sewage forms. In this respect, meat-peptone-agar and its gelatin counterpart are excellent. They are well adapted to the growth of intestinal and putrefactive bacteria and therefore serve best the prime object of bacteriological water examination.

The exact composition of the medium employed is, however, of the greatest importance in controlling the number of bacteria which will develop. Table 25, taken from Gage and Phelps (1902), shows the

TABLE 25. PERCENTAGES OF BACTERIA DEVELOPING ON MEDIA OF DIFFERENT COMPOSITIONS

(Gage and Phelps, 1902)

Medium	Days of Incubation							
	2	3	4	5	6	7	8	9
Nährstoff agar	19	60	78	85	95	99	99	100
Nährstoff peptone agar	10	22	26	28	30	30	30	30
Peptone 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 glycerol 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
Peptone 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
Närhstoff gelatin	5	6	9	11	13	13	13	13

considerable difference which may be caused by the presence or absence of meat infusion, peptone, etc., in media of generally similar character.

Much slighter variations than these are significant. Whipple (1902) showed that not only the particular kind of gelatin used, but also its exact physical condition as affected by sterilization and other previous treatments, will materially affect the results obtained. Gage and Adams (1904) found marked differences in counts resulting from the use of the two commercial peptones best known at that time. A long series of waters plated on agar made up with Merck's and Witte's peptones, respectively, showed the average relative results given in Table 26.

Gage and Adams also showed that the composition of the water used in preparing the medium 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.

TABLE 26. AVERAGE RELATIVE NUMBER OF BACTERIA ON PEPTONE AGAR WITH DIFFERENT PEPTONES
(Gage and Adams, 1904)

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

The reaction of the medium was found as early as 1891 to be important. Reinsch (1891) showed that the addition of 0.01 gram of sodium carbonate to the liter increased sixfold the number of bacteria developing. Fuller (1895), and Sedgwick and one of us (Sedgwick and Prescott, 1895*b*), 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. Fortunately, the classic work of Clark and his associates (Clark and Lubs, 1915*b*; Clark, 1920) has provided bacteriologists with procedures that enable them to control, readily and accurately, the hydrogen-ion concentration of all culture media used in water work.

The conditions of incubation also markedly affect the bacterial count. It is well known that very different temperatures may be required by different bacteria for maximal rate of multiplication. It was shown long ago by Whipple (1899) and others that the number of bacteria developing in plate cultures is to a certain extent dependent upon the presence of abundant oxygen and moisture. Thus, when the number of bacteria developing in a moist chamber was reckoned as 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. The length of the period of incubation is particularly important. Table 27, from Miquel and Cambier (1902), shows clearly the effect upon the plate count of extending the time of incubation. However, in the main, the characteristic water bacteria develop slowly, sewage bacteria almost without exception being rapid growers. The longer period of

incubation is, therefore, not only inconvenient, but also undesirable, since it obscures the difference between good and bad waters.

TABLE 27. 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	1,000
8 days	780		

The first requisite in a procedure for water analysis is, then, 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 initiative of Wyatt Johnston, and the work of G. W. Fuller, G. C. Whipple, and other members of the Committee on Standard Methods of the American Public Health Association, and the assistance in later years from members of corresponding committees of the American Water Works Association have placed the art of quantitative water analysis in this country in a very satisfactory state by contrast with the varying practices which in earlier times prevailed in England and Germany. The first reports on this subject were made in 1895, 1896, and 1897 (Committee of Bacteriologists, 1898). A permanent Committee on Standard Methods of Water Analysis was then formed; it reported in 1901 (Fuller, 1902) and submitted, in 1904, its final report (Committee on Standard Methods of Water Analysis, 1905), which later came to be known as the First Edition of Standard Methods of Water Analysis. Further reports were presented in editions published in 1912 (Committee on Standard Methods of Water Analysis, 1912), in 1917, 1920, and 1923. The responsibility for the preparation of the 1925, 1933, and 1936 editions of Standard Methods of Water Analysis was shared by committees of the American Public Health Association and the American Water Works Association. These reports recommend in considerable detail a standard routine procedure for the quantitative and qualitative bacteriological examination of water for sanitary

purposes; they have had a far-reaching effect in simplifying and unifying the methods for water analysis used on this continent.

Similar results followed from the work of an English committee (Committee Appointed to Consider the Standardization of Methods for the Bacterioscopic Examination of Water, 1904) which, however, did not consider in great detail the preparation of media, and more recently from that of an Office Committee of the Ministry of Health, assisted by Sir Alexander Houston of the Metropolitan Water Board and other eminent British authorities, which resulted in the publication of the report, The Bacteriological Examination of Water Supplies (Committee, Ministry of Health, 1934), a revised edition of which appeared in 1939. This report gives detailed directions for the bacteriological examination of water, discusses the interpretation of results, describes the preparation of the recommended media, and constitutes, for all practical purposes, the English equivalent of the bacteriological sections of the American Standard Methods of Water Analysis.

The latest report of the Committee on Standard Methods for the Examination of Water and Sewage (1944) will be adhered to in this and succeeding chapters unless otherwise specifically stated.

Standard Procedure for Quantitative Determination of Bacteria in Water. 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 incubating it 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 three stages: plating, incubating, and counting.

Plating. The bottle containing the sample of water is first shaken 25 times in order to distribute the bacteria fairly evenly throughout the sample. If the number of bacteria present is probably not greater than 300 per ml., 0.5 or 1 ml. is then withdrawn with a sterile pipet and delivered into a sterile Petri dish of 100-mm. diameter. To this is added 10 ml. of standard culture medium (nutrient gelatin at a temperature of about 30° C., or nutrient agar, or tryptone-glucose-extract agar at about 42° C.). If it is suspected that the number of bacteria per milliliter may exceed 600, dilution is necessary. This is best accomplished by adding 1 ml. of the water in question to 9, 99, or 999, etc., ml. of sterile tap water or the special phosphate water recommended in Standard Methods, according to the extent of dilution required. The diluted sample is then shaken 25 times and 0.5 or 1 ml. is taken for the enumeration. In order to determine the number of

bacteria originally present it is only necessary to multiply the observed count by the factor 10, 20, 100, or 1,000, etc.

If numerous dilution blanks of 99 ml., or 90 ml., are required at frequent intervals, a dilution-bottle filler described by Prickett (1935) is useful. It fills two dilution bottles at a time by the movement of a lever, and since the measuring cylinders are refilled from a continuous flow of water while the bottles are being replaced, a series of dilution blanks can be prepared in one quarter of the time taken when a buret is employed.

When a sample of water from an unknown source is to be examined it is generally desirable to prepare two or more plates with each of the above dilutions; those which will probably give 30 to 300 colonies on the plate after incubation should be selected as the ones on which to rely for the count. Replicate plates not only furnish an average count that is more accurate than the count from a single plate, but also often permit a count to be obtained despite the loss of an occasional plate on account of breakage, the presence of spreading colonies, or other mischance. 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. Not more than 20 minutes should elapse between the preparation of dilutions and the final pouring of plates; otherwise an appreciable alteration in the number of bacteria may occur. The plates are allowed to stand until the medium has solidified; in warm climates it may be necessary to place them on a cooling table (a metal box-like apparatus through which cold water is circulated), or in a refrigerator for a short time. Finally, when the medium has *thoroughly* "set" or hardened, the plates are placed in the incubator.

It was formerly customary to mix the water with the medium 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 the medium is poured into the plate the mouth of the tube should be flamed to remove any possibility of contamination.

The usual method of determining the number of bacteria in water for sanitary purposes in Germany, England, and the United States was originally by the use of gelatin plates with a 2-day incubation period at 20° C. The 1905 Standard Methods recommended this procedure, which was generally adopted. The 1913 Standard Methods, however, suggested the use of agar with a 1-day period at

37° C. in order to obtain quicker results and an indication of the presence of bacteria more nearly related to pathogenic types. The comparative value of the two methods has been well discussed by Whipple (1913). Each determination yields results which are of significance in estimating the sanitary quality of a water supply; and neither can wholly supersede the other. The 1923 and subsequent editions of Standard Methods wisely included both the 20° C. and the 37° C. counts and the use of the two sets of plates will, in general, be found advantageous.

In the experience of the authors it has been found that the use of porous tops for Petri dishes is objectionable because of the rapid drying at the surface and for other reasons. Although it is always recommended that incubation shall take place in a well-ventilated incubator in an atmosphere practically saturated with moisture, this condition is difficult to maintain constantly, and if it is not met the numbers obtained in porous-topped plates are likely to be misleading. Inverting the plates during incubation will restrict the formation of "spreaders" due to condensed water vapor, but in many surface waters rapidly growing types of bacteria, with a tendency to form spreading or multiple colonies ("archipelagoes"), may be found. Care in enumeration of such colonies is essential. In routine examinations the competent observer soon acquires a knowledge of what may be regarded as the normal character of the flora of a particular water, and he may resort to modification of the degree of dilution in order to secure numerical data from which to derive his final or average figures.

Another procedure for obtaining an estimate of the number of bacteria in water is that which employs the Esmarch or roll tube. This method has been used occasionally in field work in order to dispense with the necessity of transporting plates. It consists in placing 1 ml. of sample in a tube of melted gelatin or agar and then rotating the tube until the mixture has solidified in a thin layer on the inner wall. After incubation, the colonies that have developed are counted. Certain disadvantages inherent in this procedure are obvious — uneven thickness of medium in parts of the same tube as well as in different tubes, loss of time involved in "rolling" the tube, difficulty in counting, etc. — but it can give satisfactory results in some kinds of work, as Wilson (1922) has shown. It possesses the merit of simplicity and may prove useful in an emergency when plates cannot conveniently be employed.

The 20° count will be discussed in this chapter and the body temperature count will be considered in Chapter V.

Composition of the Medium. Only the strictest adherence to standard methods of preparing culture media can ensure comparable results; the ordinary media should then, for all practical sanitary work, be made up with distilled water, beef extract, peptone, and other ingredients, in exact accordance with the directions given in Standard Methods. A distinct step forward was taken many years ago when beef extract was substituted for the time-consuming and inconstant meat infusion previously used in preparing plating media. At present, Bacto-beef extract, Bacto-peptone, Bacto-tryptone, or brands giving equivalent results are specified for use in nutrient gelatin, nutrient agar or tryptone-glucose-extract agar (T.G.E. agar), the three plating media recommended by the Standard Methods Committee. The spissitude of the gelatin must be such that a 10 per cent standard medium will have a melting point of 25° C. or over; and, in order to allow for contained moisture, 120 grams of the undried market product as stored in the laboratory cupboard is used per liter of distilled water. Similarly, 15 grams of the undried market agar per liter is specified. The use of dehydrated culture media, such as the Bacto products, is permitted provided they give results equivalent to those obtained from freshly prepared media.

The reaction specified for nutrient gelatin and nutrient agar is a *p*H of 6.4 to 7.0; that for T.G.E. agar is 6.6 to 7.0. The option of employing the latter medium in place of standard nutrient agar is offered by the Committee on Standard Methods (1944) so that laboratories engaged in the examination of both water and milk need not stock the two media, for T.G.E. agar is the standard plating medium employed in milk work. Experimental data reported by Mallmann and Breed (1941) and by Howard, Lochhead, and McCrady (1942) indicated that T.G.E. agar gives practically the same results as nutrient agar with water samples, and that the colonies are larger and more easily counted on plates containing T.G.E. agar. It is to be observed that T.G.E agar for water examination does *not* contain the skim milk which is required in this medium for milk work.

Although nutrient gelatin was formerly very widely employed for plating water samples, many inconveniences attend its use: gelatin plates are difficult to handle in warm weather, they are frequently spoiled by rapidly liquefying organisms, and the medium is more costly than nutrient agar. For these reasons the 1925 edition of Standard Methods recommended nutrient agar as an alternative to nutrient gelatin for 20° C. counts, with the result that before long the agar medium almost entirely displaced the other; at the present time

nutrient agar is very generally employed for both the 20° C. and the 37° C. counts.

Incubation at 20° C. Incubation should take place in a dark, well-ventilated chamber where the temperature is kept between 19° and 21° C., and where the atmosphere is practically saturated with moisture. The presence of pans of water in the incubator and the exercise of care to avoid packing the piles of plates so close together that proper circulation of air is prevented will assist in satisfying these requirements.

In American and German practice, plates made for sanitary water analysis are counted at the end of 48 hours (Standard Methods specifies a period of 48 ± 3 hours). The English Committee (1904) fixed the time at 72 hours, and the revised edition of the Report of the Committee of the Ministry of Health (1939) still recommends a 3-day period of incubation of nutrient agar plates at 20° to 22° C. The French Service de Contrôle des Eaux of the Paris water supply, which includes several control laboratories, uses a gelatin medium and incubates the plates at 20° C. for 15 days. Theoretically, it does not matter what period of incubation is used, provided it is standardized for comparison of results. Practically, our short period has great advantages.

Counting. The number of bacteria is determined by counting, with the aid of a lens magnifying about 1½ diameters, the colonies developed upon the plate. Proper lighting conditions are extremely important when counting plates. For example, colonies readily seen with certain combinations of reflected and transmitted light may become practically invisible when viewed by reflected light alone. It is important, therefore, in order to obtain comparable results, that plates be counted under standard conditions of illumination. Various counting devices have been proposed, notably those devised by Ayers (1911), Graf (1929), and Wilson *et al.* (1935); more recently Archambault, Curot, and McCrady (1937) described an instrument known as the Quebec Colony Counter. The Committee on Standard Methods (1944) requires that illumination equivalent to that provided by this colony counter be employed.

For counting gelatin plates, Ayers (1911) suggested the use of a metal box with glass top and bottom, through which cold water is circulated. When inserted between the Petri dish and the ruled counting plate, this can obviously be adapted to practically any counting device.

It is generally accepted that the number of colonies on a plate should be between 30 and 300; and Standard Methods specifies that if plates with colony numbers within this range are available, other

plates should be discarded. When, however, the same amount of sample has been placed in two or more plates, only one of which gives a count between 30 and 300, all plates containing that amount of sample are counted and the counts are averaged. Counts of less than 30 from 1 ml. of sample must, of course, be taken as observed.

Uniform methods of counting certain types of colony should be practiced. When colonies occur in chains, each chain should be considered a single colony, for each is probably the result of fortuitous disintegration of a group of organisms caused by the mixing of sample and medium, and to count each colony in a chain is frequently impossible. Each spreader should likewise be counted as one colony, but if there is evidence that the spreader has repressed the formation of other colonies, or if the spreader covers more than one half of the plate, the plate should be discarded. Occasionally a plate may be encountered which is not badly crowded but which contains numerous colonies of very similar appearance; the record of the count of such a plate should include a note to this effect, for these colonies probably represent water bacteria of no sanitary significance that have multiplied in the sample bottle or in the water from which the sample was taken. The presence of colonies of chromogenic organisms should likewise be recorded, for they are occasionally found in ground waters, such as springs, of good quality, and only in exceptional circumstances do they suggest contamination. As a rule it is the plate containing a variety of colonies of different sizes and shapes, and presenting a disagreeable appearance and an unpleasant odor, that is most suggestive of pollution, for such colony variety, appearance, and odor are usually associated with the bacterial flora of sewage and surface wash.

In order to avoid fictitious accuracy and yet give due weight to the precision of the plating method, the Standard Methods Committee recommends that the number of bacteria per milliliter be recorded to only two significant figures. A count of 175 is recorded as 180, a count of 174 as 170, and a count of 55 as 55. Since only a fraction of the total number of bacteria in a sample is usually represented by the plate count, the gelatin and agar counts at 19° to 21° C. are officially designated as the "standard gelatin plate count" and the "20° C. standard agar plate count" respectively.

A method of expressing results by the use of a bacterial index, i.e., the logarithm of the observed count, has been suggested by Wells and Wells (1922). This may advantageously be used in studies on sewage or on fluids where the numbers are extremely high, but it offers no obvious advantage, in our opinion, in the study of waters which may

be considered available for public supply or for drinking purposes. For such waters the direct counts present the facts which are used in interpretation of sanitary values.

Accuracy of Plate Counts. Plate counts deviate from the average count of colonies that should be produced by bacteria or clumps of bacteria because of errors that derive from a variety of sources. Since an understanding of the limitations of the accuracy of counts is essential to a proper interpretation of their significance, the more serious of these errors and some of the means employed to restrict them are considered in the brief discussion which follows.

THE PLATING ERROR. This error is the combined effect of all the errors encountered during the course of the quantitative examination except those associated with the preparation of dilutions. Among the various errors that contribute to the plating error, the following are the more important:

(*a*) Error due to faulty technique and lack of precision of pipets and other apparatus used in the plating procedure. Strict adherence to the details of standardized methods, verification of the precision of apparatus, and above all, exercise of special care when counting plates, should keep this error within reasonable limits.

(*b*) Error caused by variation in the degree of disintegration, during the plating, of clumps of bacteria in one portion of the sample compared with that in another portion. This error may assume very serious proportions when a sewage or heavily polluted water containing many large aggregations of bacteria is examined; a single large clump may be so broken up by pipeting or by mixing the sample portion with the medium that a comparatively high count may be obtained from the plate containing these organisms. Numerous replicate plates will usually furnish a reasonably precise count of the bacteria in such waters despite the hazard of this source of error.

(*c*) Error due to variation in the numbers of bacteria that lose their viability during varying intervals required to complete the plating. Hastening the plating procedure should limit this error; Standard Methods, it will be remembered, specifies that plating be completed within 20 minutes after dilutions have been prepared.

(*d*) Error caused by colonies overcrowding the plate. Overcrowding not only makes counting difficult, but also often results in the repression of certain bacteria by the toxic substances produced by their neighbors. The latter effect is frequently apparent in the small size of the colonies on a crowded plate. Wilson (1922), employing pure cultures and roll tubes, similar to Esmarch tubes, with a plating surface equivalent to about one half that of a 100-mm.

Petri dish, found the effect of overcrowding perceptible when the colonies numbered approximately 50 per tube, which is equivalent to 90 to 100 per plate. Water samples, however, at times contain organisms that produce rather large colonies and therefore overcrowd plates even when the count is much less than 100. Reduction of the quantity of sample plated, although an obvious solution of the difficulty, cannot be carried too far, because if the number of colonies is very small the error of sampling due to uneven distribution of the bacteria in the sample may seriously diminish the accuracy of the count. Consequently, a compromise must be effected between measures to avoid overcrowding, on one hand, and measures designed to limit the error of sampling by increasing the count, on the other.

Wilson (1922) found that counts of *Salmonella suipestifer* on a plating surface equivalent to one half that of the ordinary plate could be increased to 400 before the effects of overcrowding became serious; he considered that roll-tube counts within the range of 200 to 400 were the most satisfactory. Stein (1918), however, in a study of counts from water and sewage samples, concluded that the highest counts were obtained when the colonies numbered 200 to 400 per 100-mm. plate, only half the number recommended by Wilson. The explanation of the discrepancy is very probably the difference in type and size of the colonies counted. Experience and expediency have induced bacteriologists engaged in the examination of water to accept as satisfactory the lower range of 30 to 300 colonies per plate. Both American and English bacteriologists favor this density, although it is recognized that the higher counts within the range, e.g., 100 to 300, are usually the more accurate.

Another method of avoiding crowded plates and securing fairly accurate counts is to use replicate plates with higher dilutions of the sample. The effect of this procedure is simply to distribute a very high count among several low-count plates. It is generally agreed that 3 to 5 replicate plates with counts from each of 100 to 400 furnish fairly satisfactory average counts, although Stein recommended the use of 3 to 10 such plates for accurate work with sewage. Again, however, mainly because they do not require precision of a very high order, bacteriologists engaged in the control of water supplies usually employ a less precise method, using only duplicate or triplicate plates.

(*e*) Error of distribution. This is the error due to irregular distribution of the bacteria in the different portions of the sample. Despite the use of a perfect technique, differing numbers of bacteria will be picked up by repeated pipeting of equal portions of water from the sample bottle or dilution tube.

Since plate counts, as Eisenhart and Wilson (1943) point out, may be considered as samples from a Poisson series — the law of small probabilities — the percentage standard deviation of the mean of replicate counts should, theoretically, be given by the simple formula $\pm \frac{100}{\sqrt{T}}$ where T is an observed total count from a number of plates.

It will be noticed that this percentage standard deviation varies inversely as the square root of the total count; if, for example, the total count is 1,600, the percentage standard deviation is ±2.5 per cent, only one half of that of a total count of 400. Unfortunately, it is not possible to determine experimentally the distribution error (as here defined) of plate counts because, in a plating experiment, this error cannot be dissociated from all the other errors (due to faulty techniques, etc.) to which the plate count is subject.

Many studies have been made, however, to estimate by means of experiment the magnitude of the complex *plating error,* which includes all the errors heretofore mentioned. Jennison (1937), who employs the designation " distribution error " for this error, plated a pure culture of *E. coli,* employing 3 to 5 plates giving counts of 100 to 400 colonies per plate, and found the percentage standard deviation of the *mean* counts to vary from ±0.5 to ±8.3, the average being ±4.0 to ±5.0. Anderson and Stuart (1935) reported the standard deviations of *individual* plate counts of pure cultures shown in Table 28. The

TABLE 28

Number of Plates	Mean Count	Percentage Standard Deviation
150	35.67	15.01
195	45.37	14.92
199	79.74	11.48

increase in the percentage standard deviation (loss of precision) as the counts decreased is well illustrated by these results. They also show, when compared with Jennison's results, the loss of precision resulting from the use of individual counts instead of average counts from replicate plates.

THE DILUTION ERROR. If dilutions are employed, the count is subject to an additional error resulting from various errors in pipeting, and in measuring the volumes of the dilution blanks required to reach the desired dilution. The dilution errors to be expected when various stages of dilution and certain tolerances of pipets and dilution blanks are used have been calculated by Jennison and Wadsworth (1940). They found that the dilution error is of the same order of magnitude

as the plating error of counts from 3 to 5 replicate plates. In fact, the percentage standard deviation due to dilution is approximately ±2.8 for each stage when the tolerance of pipet and blanks is 2.0 per cent, the tolerance permitted by Standard Methods. Evidently the dilution error cannot be ignored, especially if several stages of dilution are employed; it is also evident that the *fewest possible stages* should be used to prepare the desired dilution. Jennison and Wadsworth (1940) indicate further that the *total error* of a plate count obtained through the use of dilutions can be calculated by means of a formula (employing different notation): $E = \pm \sqrt{P^2 + D^2}$, where E is the total error expressed as percentage standard deviation, and P and D are the plating and dilution errors respectively, similarly expressed. If, for example, the plating error is ±10.0, and four stages of dilution are employed with an error of $4 \times \pm 2.8$, the total error is, roughly, $\pm \sqrt{(10.0)^2 + (11.2)^2} = \pm 15.0$. In this particular example it will be noticed that the use of four stages of dilution increased the total error by 50 per cent. Given this standard deviation of 15.0 per cent and a mean count of, say, 300, the odds are about 19 to 1 that the *true* mean lies within the range $300 \pm 2 \times 0.15 \times 300$, or 210 to 390 (since, by definition, plus and minus twice the standard deviation represents approximately a probability of 95 per cent, or odds of 19 to 1); and it may be concluded that it is unlikely that the true mean lies outside this range.

It is interesting to compare certain of the above estimates of the errors of plate counts with estimates made by Stein (1918) from a study of repeated examinations of water and sewage samples. He concluded that the variations to be expected in *individual* plate counts might be summarized as follows:

1. For careful and accurate work (200 colonies per plate):

Standard deviation	±12 per cent
Deviation 1 in 10 times	±25 per cent

2. For ordinary routine work (200 colonies per plate):

Standard deviation	±25 per cent
Deviation 1 in 10 times	±50 per cent

Anderson and Stuart found their individual counts of about 80 to have a percentage standard deviation of ±11.48. Jennison's estimate of ±4.0 to ±5.0 for averages of 3 to 5 plates should probably be doubled to obtain the error of his individual counts. If to each of these estimates is added a dilution error (for apparently Stein included dilution errors in his estimates and the others did not) it is evident

that there is little difference between the percentage standard deviations of plate counts reported by these observers.

It may be concluded, therefore, that plating errors expressed as percentage standard deviations of individual counts of 30 to 300 will usually vary from 0 to ±20 per cent if the examination is carefully made; with this error may be combined a dilution error of approximately ±2.8 per cent for each dilution stage. No doubt the plating error (standard deviation) will be in the neighborhood of ±10.0 per cent when counts of 100 to 300 are obtained from ordinary waters. It may be nearer ±20.0 per cent when the count from such waters is lower, and very large variations must be expected in individual counts from heavily polluted waters or sewage in the examination of which several dilutions are required. Repeated examination of the original sample and the use of replicate plates are therefore indicated if an accurate estimate of the plate count of a sample of this last class of waters is desired; ordinarily, however, the bacterial content of such waters varies so greatly from time to time that a less thorough examination of a number of samples is usually preferable to an intensive examination of one or a few samples.

It is evident from this discussion that no significance can be attached to minor differences between the individual plate counts obtained in the course of routine water examination. In fact, it is not an unusual laboratory experience to find counts of 30 and 50, or 150 and 200, or 350 and 450, on duplicate plates; and occasionally much larger differences are observed. If great accuracy of count is essential, it is nearly always possible, through the use of numerous plates and repeated examination, to secure practically any precision desired. In ordinary sanitary work, however, differences of ±25 per cent in results have no important influence on practical interpretation of the results obtained, since the range between the counts of safe and unsafe waters is enormously greater.

CHAPTER IV

THE INTERPRETATION OF COUNTS OF BACTERIA DEVELOPING ON GELATIN OR AGAR PLATES AT 20° C.

Standards for Potable Water. The information furnished by quantitative bacteriology regarding 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 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 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 (1891) published a table according to which water with less than 10 bacteria per milliliter was "excessively pure," with 10 to 100 bacteria "very pure," with 100 to 1,000 bacteria "pure," with 1,000 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 designations applied to waters of the last two classes, but many bacteriologists have placed the standard of purity much higher. The limits set by various German observers range, for example, from 50 to 300. Even in the very early years of water examinations Sternberg (1892), in a conservative fashion, 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 1,000 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 excite

suspicion in a spring might be quite normal for a lake; only figures in excess of those common to unpolluted waters of the same type give an indication of danger. Furthermore, the bacteriological tests are far more delicate than any others at our command; very minute additions of food material cause 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 Chapter II, 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 to 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; in the second place, only well-marked differences in numbers should be considered significant.

In the early days of the science, discussion ran high respecting the interpretation of bacteriological analysis, and particularly with regard to the relation of bacterial numbers to the organic matter present in a water. Different observers obtained inconsistent results, and Bolton (1886) concluded that there was no interrelation whatever between the organic pollution of a water and its bacterial content. 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, however, require a definite amount of food of their own kind.

Kohn (1906) determined the minimal nutrient material requisite for certain bacteria and found that they could develop in the presence of 198×10^{-10} to 198×10^{-13} per cent of glucose, 66×10^{-13} to 66×10^{-17} per cent of ammonium sulphate, and 66×10^{-13} to 66×10^{-19} per cent of ammonium phosphate. Similar 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 nature of the contamination vary. 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.

Bacterial Content of Surface Waters. In judging a surface water the student will be aided by reference to the figures given for certain normal sources in Chapter I. Although no fixed standard can be set, it is evident from the large amount of data which has accumulated that fresh samples of unpolluted surface waters under normal conditions will ordinarily give results not exceeding 300 or 400 organisms per milliliter, and often much below these figures. Flowing streams will generally show somewhat higher counts than lakes or reservoirs. Numbers much higher than those mentioned 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 kind 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; their numbers rise to an extraordinary height near the point of pollution and fall off below, as the stream suffers dilution and the sewage organisms perish. Miquel (1886) recorded 300 bacteria per milliliter in the water of the Seine at Choisy, above Paris; 1,200 at Bercy in the vicinity of the city, and 200,000 at St. Denis after the entrance of the drainage of Paris. Prausnitz (1890) found 531 bacteria per milliliter in the Isar above Munich, 227,369 near the entrance of the principal sewer, 9,111 at a place 13 kilometers below the city, and 2,378 at Freising, 20 kilometers farther down. Jordan (1900), in his study of the fate of the sewage of Chicago, found 1,245,000 bacteria per milliliter in the drainage canal at Bridgeport, 650,000 at Lockport, 29 miles below, and numbers steadily decreasing to 3,660 at Averyville, 159 miles from the point of original pollution. Below Averyville the sewage of Peoria entered and the numbers rose to 758,000 at Wesley City and decreased to 4,800 in 123 miles of flow to Kampsville. Frost and Streeter (1924) reported an average of 858 bacteria per milliliter in

the Ohio River above Cincinnati during the low river stage of September, 1916, an increase to 170,100 below Cincinnati, and then a drop to 700 just above Louisville, 123 miles farther down the river. Brezina (1906) found 1,900 bacteria per milliliter in the Danube River above, and 110,000 at the mouth of, the Danube 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) recorded from 1,000 to 46,000 bacteria per milliliter 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 milliliter 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.

Bacterial Content of Ground Waters. In ground waters it has been shown 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 48 hours counts measured only in units or tens, as recorded in Chapter I, are often obtained. 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 this event, however, the appearance of the plates at once indicates peculiar conditions, for the colonies are mostly of one kind and distinct from any of the sewage species. Thus, Dunham (1889) reported that the mixed water from a series of driven wells showed 2 bacteria per milliliter, whereas another well, situated just like the others, contained 5,000, all belonging to a single species common in the air. Except in such peculiar cases as this, high numbers in a ground water suggest contamination.

Since the bacterial counts of unpolluted shallow or dug wells, on the other hand, may vary from a few per milliliter to hundreds or thousands, only very low or very high figures are of particular significance. Very low counts are especially reassuring because a polluted water usually contains a fairly large number of bacteria, and a very high count indicates either contamination or the presence of organic material sufficient to support multiplication of bacteria in the well. The latter condition often prevails in very dense soils such as clay, where the movement of the ground water is very restricted and where the multiplication and death of bacteria and other organisms gradually result in an accumulation of organic matter which supports a large population of one or a few bacterial types.

Such a water is not necessarily unsafe; in fact, a considerable proportion of our rural population is entirely dependent upon this type of supply. If the other results of the sanitary analysis of the water are satisfactory and if the sanitary survey reveals no possibility of pollution, the high bacterial count alone may not justify condemnation of the well.

Bacteria in Filtered Waters. The process of slow sand filtration for the purification of unprotected surface water is essentially similar to the action which takes place in nature when rain soaks through the ground to appear in wells and springs. 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 more 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 a good standard for measuring the efficiency of such filtration plants. At Lawrence, in 1905, Clark found an average of 12,700 bacteria per milliliter in the raw water of the Merrimac River and the number present in the filtered water was only 70 (Massachusetts State Board of Health, 1906). When the number of bacteria in the applied water is smaller it is difficult to obtain so high a percentage efficiency. At Washington, for example, the prolonged sedimentation formerly employed generally reduced the bacterial numbers to less than a thousand and it was almost impossible to secure a 99 per cent removal. The actual numbers of bacteria in the effluent were, however, much lower than at Lawrence. The monthly average results obtained for a year at these two plants are shown in Table 29.

Rapid sand, or mechanical, filtration of water gives results very like those obtained by slow sand treatment. The average monthly counts of the applied and effluent waters at the mechanical filter plant of Harrisburg are included in Table 29 for comparison with the figures recorded at Washington and Lawrence. It must be remembered that these results were obtained in the early years of water treatment before chlorination had come into general use; the filter effluent constituted the final water.

The bacteria in the effluent of a filter do not all come through the filter with the water which is being treated. A substantial proportion (where purification is high, a large proportion) of these bacteria are contributed by growths of metatrophic forms in the lower layers of the filter and in the underdrains. The contribution from the underdrains will be lower in winter than in summer, and with increasing

TABLE 29. REMOVAL OF BACTERIA BY NATURAL SAND FILTERS AND MECHANICAL FILTERS

Bacteria per Milliliter in Applied Water and Effluent, Monthly Averages

Month	Washington, 1906		Lawrence, 1905		Harrisburg, 1906	
	Applied Water	Effluent	Applied Water	Effluent	Applied Water	Effluent
January	1,500	39	14,200	110	9,510	104
February	550	16	14,800	55	21,228	298
March	650	19	10,300	55	31,326	75
April	400	22	3,600	170	39,905	42
May	65	17	1,900	12	6,187	86
June	220	17	9,600	9	2,903	31
July	160	26	3,900	55	685	10
August	190	14	19,500	37	1,637	5
September	130	14	13,500	44	836	12
October	275	16	39,800	110	7,575	63
November	220	12	8,700	70	26,224	236
December	700	45	.....	...	37,525	163

rates of filtration the number of bacteria coming through with the water will steadily increase while the number (per milliliter of effluent) contributed by the underdrains will steadily decrease (Hazen, 1900).

In a study of the efficiencies during the year 1923–24 of 10 rapid sand plants treating Ohio River water, the monthly average 20° counts of which varied from 455 to 70,500, Streeter (1925) found percentages of the raw water bacteria and of the influent water bacteria remaining after each stage of purification as shown in Table 30.

TABLE 30. AVERAGE PURIFICATION EFFICIENCIES OF 10 OHIO RIVER PLANTS

20° Agar Counts

	Settled Water	Applied Water	Filter Effluent	
			Unchlorinated	Chlorinated
Percentage of raw water bacteria	33.0	10.8	4.1	0.72
Percentage of influent water bacteria	33.0	26.3	38.3	18.8

Through the judicious use of coagulants, chlorine, and other chemical and physical aids, many large modern purification plants are delivering water with extremely low bacterial counts. The Detroit Springwells Filtration Plant, for example, obtained monthly average

results in the year 1942–43 from treatment of the Detroit River water as shown in Table 31.

TABLE 31. WATER TREATMENT AT DETROIT, 1942–43*

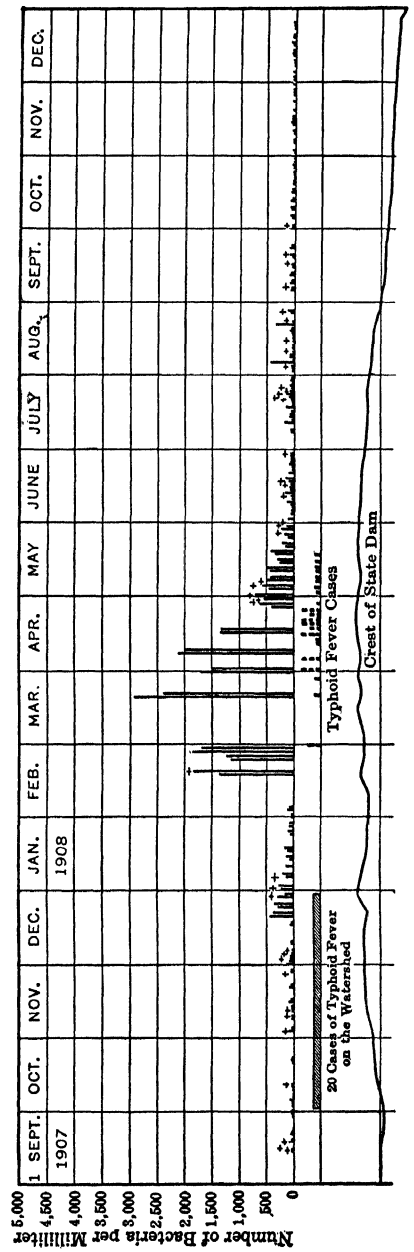
Monthly Averages, 20° Agar Counts

Month	Raw	Applied	Filtered	Tap
1942				
July	65	15	36	1
August	40	4	5	0
September	59	2	2	0
October	561	4	1	0
November	3,360	7	1	0
December	14,081	13	1	1
1943				
January	29,332	54	4	2
February	24,281	46	6	4
March	27,979	47	7	6
April	7,113	96	2	2
May	3,098	38	4	4
June	463	20	4	3

* These data are used through the courtesy of Mr. W. M. Wallace, Superintendent of Filtration and Sewage Treatment.

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. The operation, even of small plants not provided with local laboratory facilities, is usually supervised with the aid of bacteriological examinations by state or other agencies. According to Weibel (1942) the population served by more than 5,000 community treatment plants in continental United States in 1940–41 was about 74,300,000, or 56 per cent of the total population; no doubt a large proportion of these plants were subject to some measure of bacteriological control. In the same way the condition of an unpurified surface supply may be determined by frequent bacteriological examination and warnings of danger may be issued to the public (this has been done in many cities). In general, any regular determination of variations from a normal standard furnishes ideal conditions for the bacteriological methods. The detection by 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.

The results presented in the diagram on page 58 offer an interesting example of the value of the total count of bacteria as compared with the estimation of *E. coli*. This chart, prepared by Mr. J. W. Acker-



Courtesy of J. Walter Ackerman

Changes in Total Count and *E. coli* Content in Lake Owasco during the Year of the Water Epidemic of Typhoid Fever at Auburn, N. Y.

man, Engineer of the Auburn, N. Y., Water Board, shows by the vertical lines the total count and by the crosses the presence of *E. coli* in the water supply of Auburn drawn from Lake Owasco. In the year indicated in this diagram, and in other years, before and after, the total count of bacteria rose sharply at the time of the spring thaw, whereas *E. coli* was on the whole more abundant during the summer. The explanation of this phenomenon probably is that a certain proportion of coliform organisms is always contributed by the small brooks which enter the reservoir from agricultural land. At the time of the spring thaws, which for the most part wash an open farming country, the normal contribution of coliforms from the fields is obscured by the rain and melting snow, whereas only the rise in total count registers the fact that contaminating material of all sorts is being washed into the lake. With this contaminating material, for the most part of a harmless nature, human excreta are washed in from certain points on the watershed, and in the spring of 1908 these excreta contained specific typhoid infection and an epidemic in Auburn was the result.

In this instance, then, the total count of bacteria was a more accurate index of danger than the *E. coli* content. It will be noted that the increase in numbers of bacteria was not only very great but also that it came with striking suddenness, and persisted for several weeks, when the counts again resumed normal magnitude. Overgrowth of coliforms, rather than their absence, may occur under such conditions. This interesting and unusual example in no way invalidates the general soundness of the coliform test as our most certain method of demonstrating water pollution.

CHAPTER V

DETERMINATION OF THE NUMBER OF ORGANISMS DEVELOPING AT BODY TEMPERATURE

Relation between Counts Made at 20° and 37°. The count of colonies upon the gelatin or agar plate incubated for 48 hours at 20° C. measures, as pointed out earlier, the number of 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 contaminated with wash from 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 body, 37° C., which altogether checks the growth of the majority of normal soil and water forms. The determination of the number of organisms growing at body temperature may throw light, then, on the presence of direct sewage pollution, since the bacteria from the alimentary canal flourish under such conditions, whereas most of those derived from other sources do not. Savage classified the bacteria which may be found in water under three headings: normal inhabitants, like *Pseudomonas fluorescens;* unobjectionable aliens (from soil), like *Bacillus mycoides;* and objectionable aliens (from excreta), like *E. coli.* Many of the first two groups are unable to grow at 37°. This criterion is not an absolute one. Savage (1906) reported an experiment in which unpolluted soil, which had not been manured or cultivated for at least 3 years, was added to tap water, with the result that a 20° count of 76 was increased to 1,970 and a 37° count of 3 was raised to 1,630. In this instance 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°, therefore, helps to distinguish contamination by wash of the soil of a virgin woodland from pollution by excreta, since in the former the proportion of blood temperature organisms is much smaller than in the latter. Further-

more, 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. For example, water from a spring of good quality was shipped to the laboratory from a considerable distance: gelatin plates showed 4,200 bacteria per milliliter, but agar plates at 37° were sterile.

Significance of the 37° Count. A majority of the English Committee Appointed to Consider the Standardization of Methods for Water Examination (1904) recommended the body temperature count as a standard procedure. The American Committee on Standard Methods, in its 1905 report, did not recommend this method even for alternative use. In its 1912 report, however, it substituted the 37° for the 20° count, which was dropped entirely. As pointed out in Chapter III, this course seemed unwise, and it was formally condemned at the meeting of the Laboratory Section of the American Public Health Association in September, 1912, by the passage of a vote declaring that " ordinary routine examinations of water for sanitary purposes, and in the control of purification plants for the present, should include the determination of the number of bacteria developing at 20 degrees and 37 degrees." By this action the body temperature count was properly placed on a par with the 20° count as an integral part of sanitary bacteriological water examination and it has held this position through all the later editions of Standard Methods. In fact, the advantages and convenience attending the use of the 37° count have made it such a popular method of estimating bacterial numbers that it is often employed alone for this purpose.

The body temperature count must, of course, be made upon agar plates. The medium and procedure are much the same as those already described for the routine quantitative bacteriological examination in Chapter III.

The period of incubation ordinarily adopted for body temperature counts is 24 hours. Lederer and Bachmann (1911) found that 48-hour 37° counts of sewage effluents may be 2 to 6 times as high as those obtained in 24 hours. The 2-day period is used at present in British practice. American bacteriologists, however, preferring to avoid loss of time in securing the counts, have favored the use of the shorter incubation period, and Standard Methods specifies that agar plates for the 37° count be incubated for 24 hours.

When using agar plates at 37° 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, as mentioned earlier, by the use of 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). Special care must be taken, however, when such covers are used, to keep the atmosphere in the incubator nearly saturated with moisture or errors will be introduced by the excessive evaporation from the medium used.

In the earlier years of water bacteriology additional evidence relative to the quality of a water sample was sought by adding a sugar and sterile litmus to the agar medium and observing the fermenting powers of the organisms present, as first suggested by Wurtz (1892) for differentiation of *E. coli* from *E. typhosa*. Since the more numerous intestinal organisms, the coliforms and streptococci, ferment lactose with the production of acid, and since the great majority of the commoner saprophytic bacteria are unable to decompose this sugar, the number and proportion of distinctly red, or acid-producing, colonies appearing on the litmus lactose agar plate in 24 hours at 37° C. was considered of significance. Faintly colored colonies were usually disregarded because certain bacteria of the hay bacillus group and a few other soil types produce a trace of acid from lactose. It is true that a large proportion, 25 per cent or more, of the colonies on a litmus lactose agar plate of a sewage or a heavily polluted water may consist of strong fermenters, and that in unpolluted waters the number and proportion of such organisms is usually very small. The difficulty of sharply differentiating typical from atypical red colonies, however, and the frequent appearance of numerous red colonies on plates of many relatively unpolluted surface waters, especially during the warm summer months, have militated against the general use of this method of examination, and no mention of it is found in the later editions of Standard Methods. The similar use of MacConkey agar in Britain is likewise on the decline, and both editions of the British equivalent of Standard Methods have recommended the use of plain nutrient agar for the 37° count.

Although litmus lactose agar is not very satisfactory, a lactose agar plate with an improved indicator such as Bacto-neutral-red furnishes at little extra cost a fairly satisfactory additional means of detecting *change* in the bacterial population of waters. If employed as a medium supplementary to the plain agar of Standard Methods it should find many useful applications.

Very high counts on agar at 37° C. are usually obtained from

sewage and polluted waters. Ellms and Wirts (1943) report an average count of 1,960,000 per ml. during the year 1940 from the raw sewage at the Easterly Sewage Treatment Plant of Cleveland, Ohio; the 20° count was 2,700,000. At Buffalo, N. Y., the monthly average 37° count of the raw sewage in 1938–39 was found by Symons and Simpson (1941) to vary from 86,800 to 2,020,000. In 1916 the Ohio River just above the city of Cincinnati gave monthly average 37° counts of 477 to 3,090, whereas at a sampling station immediately below that city the counts were 3,300 to 216,000 (Frost and Streeter, 1924). The city of St. Louis takes its raw water supply from the Mississippi and the Missouri rivers; the 37° counts of these raw waters during the year 1942–43 varied from 600 to 110,000 per ml. and from 200 to 270,000 per ml. respectively, and averaged 25,200 and 26,800 per ml. respectively (Report of Water Commissioner, St. Louis, 1943).

In relatively unpolluted waters, not only is the absolute number of organisms developing at body temperature less, but also the ratio of the 20° to the 37° count is usually very different. Frost and Streeter (1924) found the ratio of the 20° gelatin count to the 37° agar count of the Ohio River water above Cincinnati to average about 6.3 during the years 1914–16 combined, and below, after the sewage of the city was received, to average about 2.7. At the upper station, soil wash presumably contributed a larger proportion of the total number of bacteria than it did at the lower sampling point.

TABLE 32. RELATION OF 20° AND 37° COUNTS IN DRILLED OR DRIVEN WELL WATERS CONTAINING NO COLIFORM ORGANISMS IN 110 ML.

Well	Bacterial Count		Well	Bacterial Count	
	Agar 20°	Agar 37°		Agar 20°	Agar 37°
1	2	2	13	600	2
2	425	38	14	85	4
3	70	12	15	28	4
4	95	2	16	180	4
5	48	20	17	90	2
6	170	6	18	325	8
7	65	46	19	32	10
8	650	60	20	20	10
9	6	4	21	140	65
10	120	10	22	40	26
11	0	4	23	0	2
12	14	0	24	8,000*	0
			Average	139	14

* High count not included in average.

The average 20° count of 24 driven or drilled wells which were recently examined by the Quebec Ministry of Health and in 110 ml. of each of which no coliform organisms were found, was 139 per ml., whereas the average 37° count was 14. The detailed results are shown in Table 32. It will be observed that in these well waters which, according to the indication furnished by the absence from them of coliform organisms, were probably of good quality, the 37° count is usually very low or less than 10 per cent of the 20° count. These relations are typical of good waters, although exceptions occur with some frequency. Tanner (1916), in a series of 4,379 water samples, found 412 which gave higher numbers on agar at 37° than on gelatin at 20°, most of them being shallow wells or treated waters. The other 3,967 samples showed an average ratio of the 20° count to the 37° count of 2.4, the ratio being low for wells and treated waters and high for surface waters, just the reverse of what might have been expected.

The distribution of 37° counts obtained upon the examination of 136 Quebec shallow wells is given in Table 33. Only about one fifth

TABLE 33. BACTERIAL CONTENT OF SHALLOW WELLS

Percentage of Samples in Each Group

Number of Wells	Coliforms per 100 ml.	Bacteria per ml. on Agar, 37° C.								
		0	1–10	11–20	21–50	51–100	101–500	501–1,000	1,000–2,000	Over 2,000
43	0	7	39	21	12	12	5	..	2	2
25	1–10	..	24	16	12	16	24	4	..	4
68	Over 10	..	7	6	6	3	40	12	10	16

of the samples containing no coliforms yielded 37° counts in excess of 50 per ml., and a like proportion of the waters containing more than 10 coliforms per 100 ml. gave body temperature counts of less than 50. The intermediate group with 1 to 10 coliforms per 100 ml. were approximately equally divided: half with counts under 50 per ml. and half with higher counts.

Significance of High Temperature Counts. Important data relative to the distribution of bacteria which will develop at high temperatures may be found in a paper by Gage (1906), coupled with a suggestive discussion of the general significance of bacterial ratios. Table 34 shows some of the most significant results obtained by plating waters of various degrees of purity at 20°, 40°, and 50°. The lines of the table have been rearranged to make the progression from more to less polluted waters fairly regular. The colony count at 50°

shows an even sharper differentiation than that at 40°. Gage rightly concluded that " the information to be obtained by counts of bacteria

TABLE 34. AVERAGE NUMBER OF BACTERIA AND ACID PRODUCERS DEVELOPING AT 20°, 40°, AND 50° C., WITH DIFFERENT CLASSES OF WATERS

(Gage, 1906, Rearranged)

	Bacteria per ml.			Acid-producing Bacteria		
	20° C. 4 days	40° C. 24 hr.	50° C. 24 hr.	20° C. 4 days	40° C. 24 hr.	50° C. 24 hr.
Sewage	2,990,000	557,500	7,700	1,940,000	346,000	4,400
"	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	8,300	112,400	22,700	8,000
" "	389,000	59,300	8,000	292,000	45,000	8,000
" "	306,000	89,600	485	193,000	46,000	200
Trickling filter effluent	15,500	1,730	154	15,200	1,360	100
Do.	23,300	2,030	54	16,000	1,180	20
Canal water	16,400	112	5	6,700	87	2
River water	16,900	207	4	2,500	134	2
Settled canal water	2,800	212	2	1,650	66	1
Sand filter effluent (sewage)	1,640	1,375	2	2,360	1,195	1
Do.	35	4	0	29	2	0
Do.	1,300	130	1	345	119	0
Do.	670	170	2	1,045	154	0
Water filter effluent	32	3	1	6	1	1
Do.	715	170	2	259	101	1
Do.	62	1	0	16	0	0
Do.	150	22	1	14	17	1
Do.	64	5	1	11	3	1
Shallow well	1,000	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

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 less rigid than at other seasons. Numerous investigations have shown that in midsummer bacteria capable of growth at 37° are more abundant in normal waters than in winter and spring. Race

(1916) points out that the ratio of the 37° count to the 20° count varies directly with the outdoor temperature to which the natural waters are subjected, the ratio being high in summer and fall and decreasing in winter and spring.

Winslow and Phelps 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 milliliter and only 3 which showed more than 100 per ml. on lactose agar. On the other hand, of 58 samples from corresponding sources examined in summer, 16 contained more than 100 bacteria per ml. A series of 20 pools, ponds, and brooks at Mt. Desert, Me., which were entirely free from human or animal pollution, were examined in the late summer of 1906. Only 4 of the 20 samples gave counts under 25 at 37°, and 7 of them gave counts over 100, the highest figure being 425.

Of the series of 20 relatively unpolluted Eastern Canadian lake waters mentioned in Chapter I (page 9), the samples of which were collected in the warmer months, only 3 showed a ratio of 20° count to 37° count of more than 10, and the 37° count of 5 of the lakes was approximately equal to or greater than the 20° count.

This seasonal variation is brought out very clearly by the 1942–43 data given in Table 35 for the raw water at the Detroit Water Works

TABLE 35. 20° AND 37° COUNTS OF RAW WATER AT DETROIT FILTRATION PLANT

1942				1943			
Month	Bacteria per ml.		Ratio	Month	Bacteria per ml.		Ratio
	Agar 20°	Agar 37°			Agar 20°	Agar 37°	
July	60	9	7	January	14,000	32	438
August	40	8	5	February	4,700	15	313
September	43	10	4	March	10,500	55	191
October	200	9	22	April	1,100	18	61
November	1,700	15	113	May	700	46	15
December	5,500	17	324	June	51	17	3

Park Filtration Plant which are furnished through the courtesy of Mr. W. M. Wallace, Superintendent of Filtration and Sewage Treatment at Detroit. It may be added, in passing, that both the 20° and the 37° counts of the finished water averaged, for the year, only 2 per ml.

A special case in which the ratio between the 20° and the 37° count fails to be significant is that of a water which has been treated with

chlorine. Most of the bacteria which survive chlorine treatment are, of course, spore formers, many of them belonging to the hay bacillus group, and it happens that most of these spore formers can grow at body temperature. Thus, it is common to get counts as high at 37° as at 20° with such waters, although the absolute numbers are generally small. This point is illustrated in Tables 36 and 37, which

TABLE 36. COMPARATIVE EFFECTS OF CHLORINE DISINFECTION UPON 20° AND 37° COUNTS, MERRIMAC RIVER WATER, AT LAWRENCE, MASS.
(Clark and Gage, 1909)

Sample	Bacteria per ml.			
	Untreated Water		Treated Water	
	20°	37°	20°	37°
A	3,400	30	12	4
B	28,900	130	4	4
C	14,000	75	35	47
D	3,700	81	43	62

show the results of experimental treatment of Merrimac River water at the Lawrence Experiment Station and of swimming pool water at the University of Wisconsin.

TABLE 37. COMPARATIVE EFFECTS OF CHLORINE DISINFECTION UPON 20° AND 37° COUNTS, SWIMMING POOL WATER AT UNIVERSITY OF WISCONSIN
(Tully, 1912)

Sample	Bacteria per ml.			
	Untreated Water		Treated Water	
	20°	37°	20°	37°
A	275	16	0	1
B	445	480	4	5
C	920	483	8	8
D	5,630	680	4	2
E	19,100	1,140	30	45
F	24,000	1,190	130	120
G	10,000	1,080	14	27
H	1,700	690	15	9
I	2,570	780	12	30
J	2,800	560	27	66

The organisms found in water after efficient chlorination will, of course, in general be of innocuous types except possibly when *Clostridium welchii* forms are present. Smeeton (1917) examined 105 organisms isolated on aerobic plates from chlorinated water and found

that 89 were aerobic spore formers (*B. subtilis* being most abundant), 11 chromogenic non-spore-forming water bacteria, and 5 yellow cocci.

TABLE 38. 37° COUNTS AT WASHINGTON FILTER PLANTS, 1942–43*

RAPID SAND PLANT

Month	Raw	After Sedimentation	Applied†	Filtered	Finished‡
July	1,589	1,400	200	2.2	1.7
August	5,509	2,729	184	2.2	2.1
September	1,549	1,061	146	6.4	1.6
October	5,106	3,035	128	2.1	1.8
November	441	321	260	3.1	2.5
December	2,262	1,789	173	2.8	2.4
January	966	835	43	4.1	3.2
February	980	543	68	5.0	4.3
March	683	616	42	1.3	1.4
April	1,530	884	37	1.7	1.4
May	2,312	1,571	42	2.3	2.1
June	1,966	1,305	116	6.2	4.2

SLOW SAND PLANT

			Applied After Sedimentation§	Filtered	Finished‖
July	...	...	329	4.8	2.5
August	...	...	210	5.0	2.3
September	...	...	189	4.1	1.7
October	...	...	170	2.6	1.5
November	...	...	110	2.1	1.5
December	...	...	40	1.7	1.4
January	...	...	209	2.1	1.5
February	...	...	167	2.1	1.7
March	...	...	43	2.3	1.5
April	...	...	34	2.8	1.5
May	...	...	50	2.5	1.6
June	...	...	197	3.4	1.8

* These data are used through the courtesy of Mr. C. J. Lauter, Chemical Engineer of the Washington Filtration Plants.

† Alum-coagulated, prior to chlor-ammoniation. ‡ Post-chlor-ammoniated and *p*H corrected by lime. § Alum-treated from Nov. 17, during period of low turbidity. ‖ Chlor-ammoniated.

Under ordinary conditions it is clear that organisms which grow at body temperature (and those fermenting lactose) are not numerous in normal waters. The absolute count is usually under 50, and generally less than 10 per cent of the 20° count except during the

warmer months of the year. On the other hand the body temperature count will probably run into hundreds and approach much more closely the 20° count when polluted waters are examined.

Choice of Counts for Purification Plant Control. Filter plants vary in the choice of counts employed for measuring their bacterial efficiency; some use the 20° count, some the 37° count, and others use both. If the raw water is not heavily polluted, the 20° count is usually favored because the number of bacteria growing at 37° C. is frequently so low that the efficiency measured by the reduction in this count is subject to considerable error. If, however, the 37° count of the raw water is sufficiently high, the bacterial efficiency of the purification is often measured by the 37° count alone. In Table 38 are shown the results obtained in 1942–43 by the use of this count in determining the effect of the various processes of purification employed at Washington, D. C. Two plants, a rapid sand and a slow sand, filter the water of the Potomac River after its sedimentation in reservoirs; the filter effluents are then treated as indicated in the table.

Table 39, of results obtained at the Howard Bend Filtration Plant of St. Louis, Mo., which takes its raw supply from the Missouri River, shows the use of the 37° count as an aid in the control of a

TABLE 39. 37° COUNTS OBTAINED AT HOWARD BEND (ST. LOUIS) PURIFICATION PLANT*

Bacteria per Milliliter

Month	River Water	Coagulated Water	Settled Water	Influent Water	Effluent Water	Clear Water Basin	Water to Mains
1942							
April	20,000	1,400	150	24	6	5	3
May	40,000	1,600	150	22	6	5	3
June	39,500	1,600	220	35	11	9	4
July	59,000	2,200	300	43	12	10	3
August	43,500	2,300	425	36	9	6	3
September	57,000	2,600	350	46	14	11	4
October	26,500	1,200	180	19	4	3	2
November	7,000	450	75	12	3	3	1
December	5,700	550	105	16	4	3	2
1943							
January	3,500	500	150	35	4	4	3
February	13,000	1,900	300	75	13	4	9
March	6,800	1,100	180	46	8	7	5
Average	26,790	1,500	215	34	8	6	4

* Annual Report of Water Commissioner, 1943.

plant handling a raw water with a much greater bacterial content. This plant employs pre-sedimentation to remove the heavier sediment, coagulation with lime and iron sulphate, sedimentation, secondary coagulation and sedimentation with aluminum sulphate and sterilization with ammonium sulphate and chlorine, rapid filtration, and post-chlorination.

CHAPTER VI

COLIFORM ORGANISMS AND METHODS FOR THEIR DETECTION

I. The Coliform Group and the Presumptive Test

The Coliform Group of Bacteria. The *Bacterium coli-commune* (corresponding approximately to *Escherichia coli*) was first isolated by Escherich (1885) 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; for this reason it became of the greatest interest and importance to sanitarians, since its presence in water supplies was regarded as direct evidence of sewage pollution.

Specific disease germs are difficult to isolate from water unless they are fairly numerous, despite advances in methods of isolation; and water may of course be grossly polluted with sewage without any specific disease germs whatever being present. All sewage-polluted water, however, is potentially dangerous because, where fecal matter exists, disease germs are at any time likely to appear. A test for fecal matter as distinguished from infected material is, therefore, essential; for such a test the coliform group of bacteria is especially well suited. The organisms of this group are practically never dangerous in themselves, but as indices of the probable presence of disease germs from patients or carriers they are peculiarly significant.

The coliform group of bacteria, as defined by Breed and Norton (1937) and as generally regarded by water bacteriologists, includes "all lactose-fermenting aerobic bacteria used as a measure of pollution of water." Standard Methods defines this group in the following terms: "It is recommended that the coliform group be considered as including all aerobic and facultative anaerobic gram-negative non-spore-forming bacilli which ferment lactose with gas formation."

The coliform organisms have long been regarded as members of the coliform-typhoid-dysentery series, a collection of bacteria clearly allied to each other in certain respects and including the coliform, typhoid, paratyphoid, and dysentery groups of organisms.

They all ferment glucose but, as a rule, only the coliforms ferment

lactose rapidly with production of acid and gas. These lactose fermenters are the characteristic organisms of the intestine and, since it is a test for excremental contamination that we need, the coliform group is commonly used as the most satisfactory index of water pollution.

The Standard Methods definition of coliform organisms places them, according to the classification formulated by Bergey *et al.* (1939), in Family X, *Enterobacteriaceae,* of Order I, *Eubacteriales,* of the Class *Schizomycetes.* This family is described as follows: "Gram-negative rods widely distributed in nature. Many animal parasites and some plant parasites causing blights and soft rots. Grow well on artificial media. All species attack carbohydrates forming acid, or acid and visible gas (H_2 present). All produce nitrites from nitrates. When motile, the flagella are peritrichous."

Bergey *et al.* divide this family into five tribes, the key to which is given below:

FAMILY X. *Enterobacteriaceae*

I. Ferment dextrose [glucose] and lactose with the formation of acid and visible gas. Usually do not liquefy gelatin.
Tribe I. *Eschericheae.*

II. Plant parasites causing blights and soft rots. Ferment dextrose [glucose] and lactose with formation of acid, or acid and visible gas. Usually attack pectin.
Tribe II. *Erwineae.*

III. Chromogens producing a red pigment. Ferment dextrose [glucose] and lactose with formation of acid, or acid and a small amount of visible gas. Liquefy gelatin.
Tribe III. *Serrateae.*

IV. Ferment dextrose [glucose] but not lactose with formation of acid and visible gas. Usually liquefy gelatin.
Tribe IV. *Proteae.*

V. Ferment dextrose [glucose] with formation of acid, or acid and visible gas. Some ferment lactose with the formation of acid, but never visible gas. Usually do not liquefy gelatin.
Tribe V. *Salmonelleae.*

Tribe V, *Salmonelleae,* includes the typhoid (*Eberthella*), paratyphoid (*Salmonella*), and dysentery (*Shigella*) groups of organisms.

The very great majority of coliform organisms, as defined by Standard Methods, are members of the tribe *Eschericheae;* but occasional bacteria of the tribes *Erwineae, Serrateae,* and others are

also included in the group, as the descriptions given in the above key suggest. It appears, however, that relatively few organisms of these two latter tribes, which comprise certain plant pathogens, and water, soil, and food bacteria, produce visible gas from lactose within 48 hours at 37° C., the incubation time and temperature usually employed when testing for the presence of coliform organisms; it is therefore probable that only a very small proportion of the coliforms found in water supplies are members of these two tribes. The results of Elrod (1942) are of interest in this connection: of fifty coliform organisms isolated from soil, grain, feces, etc., not one possessed the ability to macerate vegetable tissue which was characteristic of his *Erwinia* cultures; but there is no doubt that plant pathogens are occasionally identified as coliforms.

Bergey *et al.* divide the tribe *Eschericheae* into three genera and ten species, with the habitats indicated in the following table:

TRIBE I. *Eschericheae*

	Habitat
Genus I. *Escherichia.*	
1. *Escherichia coli.*	Normal inhabitant of the intestine of man and all vertebrates. Widely distributed in nature. Frequently causes infections of the genito-urinary tract. Invades the circulation in agonal stages of diseases.
2. *Escherichia freundii.*	Normally found in soil and water and to a varying degree in the intestinal canal of man and animals. Widely distributed in nature.
Genus. II. *Aerobacter.*	
1. *Aerobacter aerogenes.*	Normally found on grains and plants and to a varying degree in the intestinal canal of man and animals. Widely distributed in nature.
2. *Aerobacter cloacae.*	Found in human and animal feces, sewage, soil, and water.
Genus III. *Klebsiella.*	
1. *Klebsiella pneumoniae.*	Associated with pneumonia and other inflammations of the respiratory tract.
2–6. Five other *Klebsiella* species, all associated with pathogenic conditions in man or animals.	

The first two genera, *Escherichia* and *Aerobacter,* include practically all the coliforms belonging to this tribe. Some so-called coliforms, however, may be members of the genus *Klebsiella,* for

despite a measure of success that has attended more recent efforts to distinguish between *Klebsiella* and the other two genera, clean-cut differentiation is still often impossible, according to Osterman and Rettger (1941) who, from a study of numerous encapsulated organisms from various localized infections and from grains, sewage, and soil, concluded: "There is at present no single test or group of tests which will differentiate many of the Friedlander [*K. pneumoniae*] cultures from those of the coli-aerogenes [*Escherichia-Aerobacter*] group." Because of the difficulty in differentiating these two groups, and because the sanitary significance of the presence in water of pathogenic *Klebsiella* forms may be considered at least equal to that of other organisms of this tribe, water bacteriologists ordinarily make no effort to distinguish between coliform-like *Klebsiella* types and the more common coliforms. For all practical purposes, therefore, coliform organisms may be considered members of an *Escherichia-Aerobacter* group, a series of bacterial types, each differing imperceptibly or almost imperceptibly from its neighbors, extending from the species *E. coli* at one end of the series to the species *A. cloacae* at the other. Although organisms representing additional genera such as *Erwinia, Serratia, Klebsiella,* and others may be included among the bacteria identified as coliform organisms, there is no evidence to indicate that their occasional presence *significantly* affects the results of the coliform test. We shall return later to a consideration of the differences between the various species of the coliform group and shall consider for the present the principles involved in the detection of the group as a whole (non-spore-forming, aerobic, gram-negative bacilli, fermenting glucose and lactose with acid and gas production).

Estimation of the Number of Coliform Organisms in Water. Since the means employed at present for the estimation of the density of coliforms in water are the result of a slow but progressive evolution from earlier methods, a knowledge of these and of the intermediate developments which have led to our present techniques is essential to a satisfactory understanding of the *raison d'être* of the latter. In the sections which follow, therefore, our discussion of coliform procedures will include an historical account of the numerous investigations and consequent official pronouncements that have finally resulted in our modern Standard Methods for coliform enumeration.

The most obvious and distinctive characteristic of the coliform group as a whole is the fermentation of lactose with the production of acid and gas, and both these products have been used for its identification. The litmus lactose agar plate, discussed in Chapter V, furnishes one method for estimating the number of coliform organisms

in water, and it was used by Sedgwick and Mathews for the purpose as early as 1892 (Mathews, 1893). If plates are made with agar containing both lactose and litmus, the coliform organisms ferment the lactose with the production of acid, and their colonies develop as red spots in a blue field. Since other organisms (notably the streptococci) may also develop red colonies, it is necessary to examine them further. This is done by fishing from isolated colonies, replating, and inoculating into other media for identification. Success in the use of the plate method depends upon getting well-isolated colonies; consequently it is advisable to employ a number of different dilutions of the water sample and prepare a series of plates from each. The plate method of estimation was recommended by the 1913 Standard Methods for sewages and polluted waters in which coliform organisms were present in 1 ml. or less. It was suggested that litmus lactose agar in Petri dishes with porous covers be used and that these plate cultures be incubated at 40° instead of 37° C. Subsequently, the use of the agar plate for direct determination of the coliform density in water and sewage was omitted from Standard Methods because of the limited quantity of sample that could conveniently be examined by direct plating and the difficulty experienced in recognizing coliform colonies. More recent editions of Standard Methods have again presented the direct plating method, but mainly as an essential feature of tentative procedures for estimating the relative numbers of different types of coliform organisms in water. We shall review these procedures in Chapter VIII.

The test for coliforms may be made not only more delicate but also more practicable by preliminary cultivation of the sample in a favorable liquid medium for 24 to 48 hours at 37° C. By this means comparatively large quantities of sample can be conveniently examined, and the proportion of coliform organisms present may be greatly increased before plating. As suggested in the classic researches of Theobald Smith (1893*a*), this method can be made quantitative by planting measured portions of sample in a series of tubes. If, for example, 10 tubes are planted each with 0.01 ml., and 3 show the presence of coliforms, we may assume that some 30 of these organisms were present in 1 ml. Irons (1901), in a comparative study of various methods for the isolation of coliforms, was one of the first to show that preliminary enrichment frequently yielded positive indications when results from direct plating in agar were negative.

The medium most commonly used on this continent prior to 1906 for preliminary enrichment was ordinary nutrient broth to which 1

per cent of glucose had been added; the reaction was then brought to the neutral point of phenolphthalein. Into each of a number of fermentation tubes of this medium a measured quantity of the water to be examined was planted, and the culture was incubated for 24 to 48 hours. If gas was formed, a small amount of the culture was added to a suitable solid medium for isolation and identification of the organisms present. Although the liquid and solid media used in this procedure have been changed from time to time and various refinements introduced, the principles of the method have been retained, and after nearly a half century they are still employed in our most reliable procedure for estimating the coliform density of water supplies.

It is to be observed, however, that even at the present time, this procedure requires a considerable expenditure of time, labor, and material: the enrichment culture must be incubated for 1 to 2 days; the agar plate used for isolation of bacteria from this culture is incubated for 1 day; as we shall see later, colonies fished from the plate are transferred to lactose broth and agar slants, and these are incubated for 1 to 2 days; finally, a gram-stained slide must be prepared from the agar slant culture and examined microscopically. If gas is formed in the secondary lactose broth and only gram-negative coliform-like rods appear in the slide preparation, the identification of coliforms is considered complete; but if sporing organisms are found, a further attempt must be made to isolate coliform organisms not associated with spore formers. The whole examination, therefore, may require from 3 to 5 days, and sometimes longer.

The Presumptive Test for Coliform Organisms. The delay in securing final results, which was caused by the use of preliminary enrichment, and the labor involved in isolating and identifying bacteria from the enrichment culture soon led to attempts to simplify the procedure. Experience with the glucose broth fermentation tube indicated that a fair conception of the sanitary quality of a water might be obtained from the results of this test alone without the further process of isolating specific organisms. It appeared that a rather definite proportion of tubes showing a characteristic fermentation proved upon further examination to contain coliforms, and it was therefore suggested that the glucose broth test be used as a rapid " presumptive " test.

In earlier years, Irons (1901) was perhaps the first to call attention to the value of this method, stating that when the glucose tube yields approximately 33 per cent of carbon dioxide, *E. coli* is almost invariably present. In the next year the reliability of the fermentation

test as an indication of coliforms was worked out by Gage (1902), as given in Table 40.

TABLE 40

	1 ml.	100 ml.
Number of samples tested	5,172	1,375
Number giving preliminary fermentation	1,036	474
Percentage of latter proved to contain coliforms	70	71

Whipple (1903) examined a large number of surface-water supplies by this presumptive test and obtained striking results, as shown in Table 41. The waters are arranged in six groups according to the results of sanitary inspection; group I includes waters collected from almost uninhabited watersheds, and group VI waters too much polluted to be safely used for domestic purposes.

TABLE 41: PERCENTAGE OF SAMPLES OF WATERS OF VARIOUS SANITARY GRADES GIVING POSITIVE TESTS FOR *E. coli* WHEN DIFFERENT AMOUNTS WERE EXAMINED

(Whipple, 1903)

Group	0.1 ml.	1.0 ml.	10 ml.	100 ml.	500 ml.
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

It is undoubtedly true that a negative presumptive test is generally obtained with unpolluted waters. For example, Winslow and Nibecker (1903) reported that of 775 glucose 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 tests coliform organisms may be isolated from positive glucose broth tubes. The work of later years made it clear, however, that the general coincidence of positive glucose presumptive tests with the presence of coliforms is subject to disastrous exceptions. An unpublished investigation by Winslow and Phelps indicated that the results of the glucose broth test are markedly influenced by the factor of temperature. Their work comprised 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 pollu-

tion, 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 coliforms yielded only 12 positive results. The presumptive test, however, was positive with a large proportion of the samples, and much more often in summer than in winter or spring, as indicated in Table 42. The Ann

TABLE 42. GLUCOSE BROTH FERMENTATION IN 185 SAMPLES OF NORMAL WATERS AT DIFFERENT SEASONS

Percentage of Positive Results

(Winslow and Phelps)

	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

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.

Phelps and Hammond (1909) in this country, Fromme (1910) in Germany, Houston (1911) in England, and Clemesha (1912*a*, *b*) in India all reported similar results which made it clear that organisms fermenting glucose but not lactose are fairly abundant in stored and relatively pure waters, particularly in warm weather.

It was obvious, therefore, that many bacteria of little or no sanitary significance were able to ferment glucose; consequently, the more selective lactose was gradually substituted for glucose in media employed for presumptive tests. The 1913 Standard Methods used lactose in a medium containing peptone and bile, but in 1917 lactose broth was specified as the presumptive medium. This broth contained 0.3 per cent beef extract, 0.5 per cent peptone, and 1 per cent lactose, and the reaction was adjusted to make it neutral to phenolphthalein. Fermentation tubes of the medium, in which graduated quantities of the water samples were planted, were incubated at 37° C. for 48 hours and, if more than 10 per cent of gas was formed within 24 hours in the closed arm (or inverted vial) of the tube, the presumptive test was considered positive. If no gas was formed, the test was negative. Any other result, such as less than 10 per cent of

gas in 24 hours, was considered a doubtful one which required further investigation, including an attempt to isolate and identify any coliform organisms that might be present; in the parlance of the water bacteriologist, it was necessary to attempt "confirmation of the doubtful presumptive."

The 1923 Standard Methods lowered the proportion of lactose in the lactose broth to 0.5 per cent, but otherwise the presumptive test was left practically the same as in the 1917 edition. Burling and Levine (1918) had shown that in either glucose or lactose broth the concentration of carbohydrate should be limited to 0.5 per cent because in a higher concentration *E. coli* particularly may be killed by the acidity produced and may therefore be lost. Hasseltine (1917) had confirmed the suspicion expressed in the First Edition of Standard Methods of the existence of another source of error: the occasional conversion of some of the lactose to glucose by long exposure of the complete medium to heat during the process of sterilization. The presence of glucose in the lactose broth would, of course, defeat the purpose of its substitution by lactose. In order to eliminate this source of error, Hasseltine suggested sterilization of the lactose separately in aqueous solution, its addition to the previously sterilized broth, and final sterilization of the mixture after distribution in sterile tubes at 100° C. for 30 minutes. This method of preparation, or addition of sterile lactose solution to sterile tubes of broth, or exposure of the complete medium to heat for only 30 minutes, including the usual period of 15 minutes at 15 pounds pressure, was soon generally adopted.

In the earlier days of the coliform test much stress was laid upon the exact amount of gas ultimately produced, 25 to 70 per cent of the capacity of the closed arm being considered evidence of the presence of coliforms. The unreliability of quantitative gas results after a long incubation period, however, was demonstrated by Fuller and Ferguson (1905), Longley and Baton (1907), and others. The many factors which influence the volume of gas formed in the fermentation tube were made clear by Browne and Ackman (1917). They found that the amount of gas varies not only with the temperature, the time of incubation, and the initial reaction of the culture medium, but also with the particular medium and the length of the inverted vial used for collecting the gas. Even when all conditions are apparently constant, wide variations occur in the quantity of gas formed in individual tubes.

Prompt formation of gas, on the other hand, seems to be of very real importance. Levine (1920) found that presumptive tests in which

10 per cent or more of gas appeared in 24 hours generally were confirmed (98 per cent); if less than 10 per cent of gas was formed in 24 hours, 91 per cent were confirmed; if no gas appeared in 24 hours but over 10 per cent in 48 hours, 73 per cent were confirmed; and if there was less than 10 per cent of gas after 48 hours, only 45 per cent were confirmed. These figures were obtained from untreated supplies; a much lower proportion of positive presumptive tests from chlorinated supplies was confirmed because of the frequent presence of spore-forming, lactose-fermenting bacteria in such waters.

In recent editions of Standard Methods the formation in lactose broth (lactose 0.5, peptone 0.5, beef extract 0.3 per cent) of gas *in any amount* within 24 hours at 37° C. is considered a positive presumptive result; absence of gas after 48 hours is considered a negative result; appearance of gas in the second 24-hour period is considered a doubtful result and an attempt must be made to confirm it.

General Significance of Presumptive Test Results. Even when the lactose broth test, as described above, is used, a substantial proportion of positive presumptive tests may be obtained which are not

TABLE 43

Station	No. of Samples	Average Coliforms per ml.	Average Fermenting Non-coliforms per ml.	Non-coliforms per 100 Coliforms	Percentage of Fermenting Organisms Confirmed as Coliforms
Giesboro Point	770	295	24.1	8.2	92.4
Fort Foote	789	254	9.7	3.8	96.3
Fort Washington	778	123	6.2	5.0	95.2
Mt. Vernon to Whitestone Point	851	102	8.5	8.3	92.3
Indian Head	241	123	15.7	12.8	88.6
Possum Point	212	66.2	13.8	20.8	82.7
Maryland Point	691	1.44	0.75	52.1	65.7
Pope's Creek	740	0.19	0.33	174.0	36.5
Lower Cedar Point	476	0.14	0.16	114.0	46.7
All below Lower Cedar Point	2,261	0.052	0.057	110.0	47.7

due to organisms of the coliform group. The commonest bacterial forms which give rise to such spurious presumptive results are the anaerobic spore formers of the *C. welchii* type. Like the coliform organisms themselves these bacteria are characteristic intestinal organisms and their use as possible indicators of pollution will be

discussed in Chapter X. Being spore formers, however, it is clear that they may survive for a long time in water and their significance must therefore be very different from that of the coliforms. The proportion of positive presumptive tests associated with the presence of *C. welchii* rather than coliform bacteria varies widely with different waters and, in general, is greatest where fresh pollution, as opposed to past pollution, is least. Frost (1916) found that only 1 or 2 per cent of the positive presumptive tests of polluted river waters were due to anaerobes but that with treated waters the proportion might rise to 13 or 14 per cent. Cumming (1916) reported the exceedingly interesting results presented in Table 43 which indicate the progressively decreasing percentage of gas-positives confirmed as one proceeds down the Potomac River and as self-purification of the stream takes place.

Levine (1921*a*) reported a series of water examinations made while on army service in France; the results, given in Table 44, show clearly the very slight value of the presumptive test with chlorinated waters.

Upon examination of the St. John River by the International Joint Commission on Boundary Waters it was found that anaerobic spore

TABLE 44. CORRELATION OF RATE OF GAS PRODUCTION WITH CONFIRMATION OF THE PRESUMPTIVE TEST FOR COLIFORM GROUP IN LACTOSE BROTH

Rate of Gas Production	Untreated Supplies		Chlorinated Supplies	
	Number of Tubes Showing Gas	Percentage of Gas Tubes Confirmed	Number of Tubes Showing Gas	Percentage of Gas Tubes Confirmed
Rapid (10 per cent or more in 24 hours)	684	97.7	57	44.0
Moderate (less than 10 per cent in 24 hours)	193	91.2	15	20.0
Slow (no gas in 24 hours; 10 per cent or more in 48 hours)	276	73.2	156	6.4
Very slow (no gas in 24 hours; less than 10 per cent in 48 hours)	33	45.5	26	0.0

formers were for some reason so common that of 444 positive presumptive tests 305, or 69 per cent, were due to organisms of this type.

A second, though much less common, cause of spurious presumptive tests is the presence of spore-bearing aerobic forms, allied in many respects to *B. subtilis* but capable of forming gas in lactose and other carbohydrate media. Koser and Shinn (1927) found these bacteria

in 23 of 52 samples of soil from cultivated fields and pastures; some samples contained as many as 1,000 per gram. Apparently the most frequently encountered organism of this type is *Bacillus aerosporus.* These organisms were first described by Meyer (1918) and were isolated on eight different occasions from the tap water of Newport, Ky. They were later reported by Ewing (1919) in the city water supply of Baltimore, by Perry and Monfort (1921) and Hinman and Levine (1922) in chlorinated surface waters in Iowa, and by Norton and Weight (1924) and Greer (1928) in Lake Michigan water.

Furthermore, it was shown by Sears and Putnam (1923) that a positive presumptive test in the absence of coliform group organisms may also be due to the symbiotic action of two different organisms neither of which alone can form gas from the original carbohydrate present. With lactose, for example, these investigators observed vigorous gas production in the presence of *Streptococcus fecalis* and *Salmonella schottmülleri.* In general this phenomenon was observed with pairs of organisms which included one capable of forming acid from the primary carbohydrate, and another capable of forming gas from other substances. Media in which the first (acid-forming) organism has been grown alone, and which are then sterilized and inoculated with the second organism, fail to exhibit this phenomenon; consequently, Sears and Putnam concluded that the symbiotic action depends upon simultaneous utilization of certain intermediate products of metabolism. Leitch (1925), employing combinations of non-lactose-fermenting organisms isolated from water, soil, and sewage, confirmed these findings and reported further that 10 out of 14 recorded combinations produced 10 per cent or more gas from lactose broth. Upon isolating, from gas-positive lactose broth presumptives, organisms that were apparently representative of the non-lactose-fermenting flora of each sample and then recombining them in lactose broth, he frequently found as much gas produced as in the original presumptive tube and sometimes more. In every instance one of the organisms fermented lactose with the production of acid and another produced gas from glucose. The acid formers were usually gram-positive, non-motile organisms, chiefly streptococci or staphylococci; the gas formers were always bacilli, some gram-negative, motile organisms, failing to produce indole or ferment sucrose; others fermented sucrose, produced indole, and resembled the organisms of the *Proteus* group. Leitch concluded that these bacteria which may produce gas from lactose through symbiotic action are widely distributed in nature. Holman and Meekinson (1926) used combinations of various pathogenic and other organisms

for investigating this phenomenon, confirmed the essential requirement that one organism must be an acid former and another a gas former, and found that a number of factors, such as the stage of growth of the bacteria and their functional activity, the hydrogen-ion concentration of the medium and its regulation by bacterial products, and the relative time of acid and gas production, markedly influenced the result of what they preferred to designate " synergism " for the reason that this term merely tells what happens without implying, as does the term symbiosis, that mutual advantage is derived from the coöperation of the organisms.

Finally, it was shown by Prescott and Baker (1904), Jordan *et al.* (1904), Greer and Nyhan (1928), Norton and Barnes (1928), and others that a variety of organisms, either separately or associated, may so overgrow or affect coliform organisms as to prevent their isolation. Hutchison, Weaver, and Scherago (1943) reported isolation of microörganisms antagonistic to *E. coli* from about 17 per cent of 72 water samples; none was isolated from 7 samples of cistern water. *Pseudomonas, Actinomyces, Sarcina, Micrococcus,* and other bacteria were included among the antagonists. When water samples inoculated with these organisms and *E. coli* in the proportion of 1,000–2,000 to 10 respectively per milliliter were stored at room temperature for 24 hours, 11 of 12 antagonists reduced the number of coliforms, 3 killing all of them in the samples.

These four types of interference are probably responsible for the great majority of the failures of the presumptive test to indicate the presence of coliforms when they are actually present in the water sample and for the many more numerous spurious positive results that are encountered in the examination of treated waters and the better class of natural waters such as springs, wells, and lakes. This interference presents a truly formidable impediment to the development of a rapid and efficient presumptive test for the presence of coliform organisms, as evidenced by the remarkable fact that despite innumerable attempts during the past fifty years to devise such a test the only accepted procedure at the present time is the test for gas in lactose or lauryl broth within 24 hours of incubation; and, because of the doubtful significance of a positive result even with this test, Standard Methods very properly restricts its use to the examination of sewage, sewage effluent (except when chlorinated), and polluted water known to be unfit for drinking; it may also be employed in the examination of raw water in purification plants *provided* the test has been shown, by comparison with more precise methods, to be applicable; obviously, an excessive number of false

positive presumptive results from the raw water might lead to an exaggerated conception of the efficiency of the purification process. On the other hand, several of the presumptive media that have been proposed yield fairly satisfactory results and may well be used in supplementary, unofficial tests in order to obtain in a short time, when desired, an approximate estimate of the coliform density of a water. The value of various media in such tests will be considered in the course of the following discussion relative to their usefulness as enrichment media.

Relative Value of Various Enrichment Media. Whether the incubation of a water sample in broth culture is used as a presumptive test or merely as a preliminary to the isolation and identification of the organisms concerned, it is important to use a medium that will, as far as possible, prove favorable to the coliform group and unfavorable to all other organisms. We have seen that in this respect lactose broth is superior to glucose broth, which it has now superseded. It is by no means certain, however, that some other media cannot be found which are even better than lactose broth, and it is pertinent to consider in some detail the progress which has been made along this line.

In the past a great variety of enrichment media have been studied from this viewpoint. Among these may be mentioned the following: phenol broth (Irons, 1901; Reynolds, 1902); the Eijkman test, involving incubation at 46° C. (Eijkman, 1904; Thomann, 1907; Fromme, 1910); media containing neutral red (Rothberger, 1898; Makgill, 1901; Savage, 1901; Gage and Phelps, 1903; Stokes, 1904; Braun, 1906); aesculin media (Harrison and van der Leck, 1909; Hale and Melia, 1911); and liver broth (Jackson and Muer, 1911). None of these media has achieved any general popularity among American bacteriologists.

Much attention, however, has been devoted to the use of media containing bile or bile salts. MacConkey (1900) long ago suggested the use of bile salt (sodium taurocholate) in media for the differentiation of *E. coli* and *E. typhosa*; and MacConkey (1901) and MacConkey and Hill (1901) employed similar media for detection of fecal contamination. Since then, bile salt has remained an essential ingredient of the enrichment, or primary media used in Britain for detection of coliform organisms in water, sewage, and other materials; in fact, excepting the substitution of lactose for glucose as in American practice and the addition of neutral red to indicate acid formation and to permit the production of fluorescence by certain types of coliforms, the most popular British enrichment

medium in use at the present time is practically identical with that proposed by MacConkey. Apparently, however, no specifications for the bile salt employed have ever been generally accepted; many years ago Houston (1925) stated that after World War I the bile salt available was much less efficacious than that previously supplied, and McCrady (1939*a*) reported that he found marked variations in the characteristics of media prepared with various American bile salts. Perhaps it was because of the difficulty in obtaining bile salt of uniform composition that the use of MacConkey broth never became popular on this continent. Recent studies of the use of this medium in the examination of American waters will be reviewed later in this chapter.

American interest in the MacConkey type of medium was first directed, principally through the initiative of Jackson (1906), to the use of ox bile, a product fairly uniform and readily available. Jackson studied the action of various bile media; their selective inhibitory action is strikingly shown in Table 45. In accordance

TABLE 45. SELECTIVE ACTION OF BILE SALTS

(Jackson, 1906)

	Bacteria per ml.			
	Uncontaminated Well	Contaminated Pond	Suspension of Feces	Suspension of Feces
Gelatin, 20°	920	2,700	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.

with Jackson's suggestion, the 1913 Standard Methods specified the use of fresh ox bile containing 1 per cent each of peptone and lactose. Like most media containing ingredients intended to inhibit undesired organisms, lactose bile was found to inhibit also certain representatives of the coliform group. Jackson maintained that the coliforms unable to grow in bile were attenuated forms of remote intestinal origin and that in tests of sewage and contaminated waters lactose bile was far superior to other media. But these conclusions were contested by Jordan (1913) and Cumming (1916). Jordan found that lactose bile inhibited a large proportion of viable coliform cells and that freshly isolated strains were inhibited as well as old strains;

Cumming reported that, with sewage, lactose bile yielded only 25 per cent as many positive results as lactose broth, whereas with river water the proportion was increased to 50 to 70 per cent. Hale (Levine, 1921*a*), on the other hand, found lactose bile much superior to lactose broth.

Much of the confusion that existed in earlier years regarding the value of bile enrichment media was no doubt due, as Levine (1922) pointed out, to the variation in the proportions of bile used by different observers. Jordan and Cumming employed whole bile (equivalent to 10 per cent dried bile), but Hale used only 5 per cent dried bile. Levine found that 1 to 2 per cent dried bile inhibits most spore formers, both aerobic and anaerobic, and accelerates the growth of coliform organisms; higher concentrations, however, he found to be distinctly inhibitory to the coliform group itself.

A lively controversy over the relative merits of lactose broth and lactose bile as enrichment media persisted for several years, but finally it became clear that lactose broth permitted the detection of the greater proportion of the coliform organisms present. In the 1917 Standard Methods, lactose bile was replaced by lactose broth, and since then the latter has held its position as the most generally accepted standard enrichment medium.

The Use of Inhibitive Dyes in Enrichment Media. A new and highly promising suggestion for the improvement of the presumptive test was made by Hall and Ellefson (1918) who, following the remarkable work of Churchman on the bacteriostatic action of dyes, showed that the addition of gentian violet to lactose broth eliminated a large proportion of the anaerobic gas formers. The same authors (1919) later made a careful study of the inhibitive effect of gentian violet upon coliforms and other gas formers and found that increasing proportions of gentian violet in enrichment media tend to yield a larger and larger proportion of confirmations of presumptive tests but, as might be expected, also tend to diminish the absolute number of isolations by inhibiting the weaker strains of coliforms. A concentration of about 1 part gentian violet in 9,000 parts of lactose broth gives practically 100 per cent presumptive tests confirmed, but on the other hand the total proportion of samples yielding coliform organisms begins to fall off at concentrations of gentian violet above 1 part in 100,000. Gentian violet was found to be much less inhibitive in glucose broth than in lactose broth.

Bronfenbrenner, Schlesinger, and Soletsky (1920) reported rosolic acid to be a good differential antiseptic for checking the development of gram-positive organisms while permitting almost all gram-negative

organisms to grow readily. Even *Shigella dysenteriae,* which is inhibited by brilliant green and crystal violet, is resistant to rosolic acid. Finally Muer and Harris (1920) reported that brilliant green in a dilution of 1 part in 30,000 checks the development of *C. welchii* in a bile medium whereas coliforms grow readily in the presence of 1 part in 350.

Winslow and Dolloff (1922), in a comparative study of several triphenylmethane dyes which have been recommended by various investigators, determined the limiting concentration of each dye in lactose broth and in a lactose bile medium. The latter contained 5 per cent of sodium cholate and was distinctly more favorable to the development of coliform group organisms than lactose broth. All of the three dyes tested, rosolic acid, gentian violet, and brilliant green, had about the same toxicity for *E. coli* in lactose bile, one part in 1,000 proving inhibitive. Rosolic acid behaved in exactly the same way in lactose broth. Gentian violet on the other hand was from 5 to 50 times as toxic in lactose broth as in the bile medium, and brilliant green from 200 to 1,000 times as toxic. *A. aerogenes* was inhibited by one part of brilliant green in 100,000 parts of lactose broth and one *E. coli* strain by one part in a million.

Brilliant-green Lactose Bile. Because of the numerous conflicting opinions expressed by various observers regarding the relative merit of certain enrichment media, it became increasingly evident that the coliform floras of different waters were not reacting in the same manner when planted in a given enrichment medium, and that an adequate conception of the general applicability of a medium was to be secured only through the collaboration of a large number of laboratories working with a variety of waters. One of us (M. H. M.) undertook, for Committee No. 1 on Standard Methods of Water Analysis of the American Water Works Association, to conduct a large-scale study of the brilliant-green lactose bile medium proposed by Muer and Harris (1920), as a part of the first of many such coöperative investigations of media which have been carried out by American water bacteriologists and which have proved a very satisfactory means of evaluating the general usefulness of various media and methods.

Brilliant-green bile, containing 5 per cent oxgall (dried), 1 per cent peptone, 1 per cent lactose, and 0.01 per cent brilliant green, was expected to be particularly effective in eliminating interference due to lactose-fermenting spore formers of both the aerobic and anaerobic types. As a matter of fact, this medium and its successor, brilliant-green bile 2 per cent (containing 2 per cent dried oxgall) have proved

their value in this respect. The bile appears to inhibit completely the aerobic, and the brilliant green and bile to inhibit almost completely the anaerobic spore formers, as Muer and Harris claimed. It is very seldom that interference by either type of organism is detected in routine work with these media, although several observers have shown that pure cultures of *C. welchii* often grow in the 2 per cent medium.

Some twenty laboratories situated in the United States and Canada collaborated in a comparison of brilliant-green lactose bile and lactose broth, both prepared and distributed in dehydrated form. An incubation period of 72 hours at 37° C. was used with the bile in accordance with the recommendation of its proponents, and 48 hours with the lactose broth. A brief summary of the results reported (Dunham, McCrady, and Jordan, 1925) is given in Table 46. The

TABLE 46. COMPARISON OF LACTOSE BROTH AND BRILLIANT-GREEN LACTOSE BILE

Medium	1923 Series		
	Tubes Planted	Gas+	Coliform Isolations
Lactose broth	1,653	61%	25.6%
Brilliant-green bile	1,635	22%	20.4%
	1924 Series		
Lactose broth	7,354	36%	18.9%
Brilliant-green bile	7,354	21%	17.1%

ratio of coliform isolations from lactose broth to those from brilliant-green bile was 100 : 80 in the first series of tests and 100 : 90 in the second series. Although brilliant-green bile appeared to inhibit the growth of some coliform strains, the collaborating laboratories all reported that it practically eliminated evident interference by spore-forming bacteria. Results of additional comparisons of brilliant-green bile with lactose broth by Howard and Thompson (1925) and others confirmed these indications.

Encouraged by these reports, the Committee sought to improve the medium. Dunham and Schoenlein (1926) made a careful study to determine the optimum bile : brilliant-green ratio and recommended the following formula for brilliant-green lactose bile which is still preferred by most water bacteriologists:

BRILLIANT-GREEN LACTOSE BILE, 2 PER CENT

(" B.G.B., 2 per cent ")

Difco Product	Per Cent
Dried oxgall	2.0
Peptone	1.0
Lactose	1.0
Brilliant-green	0.133
Final *p*H	7.1–7.4

This medium, as well as the 5 per cent bile medium employed in the previous study, was distributed to 15 collaborating laboratories for another comparison with lactose broth. The results reported by Jordan (1927), who conducted this study, showed that coliform organisms were recovered from 95.2 per cent of the 663 " B.G.B., 2 per cent " tubes in which gas had formed in 48 hours at 37° C., but that the number of coliform recoveries from these was only 80.5 per cent of the coliform recoveries from lactose broth gas-positives. Moreover, when the reports from the collaborating laboratories were considered separately, the bile media compared even more unfavorably with lactose broth. Table 47, a recalculation of Jordan's results

TABLE 47. PERCENTAGE OF COLIFORM ISOLATIONS OBTAINED THROUGH THE USE OF VARIOUS ENRICHMENT MEDIA

(Jordan, 1927, and Butterfield, 1933*b*)

Results Reported from:	Standard Lactose Broth	Brilliant-green Bile, 5%	Brilliant-green Bile, 2%
Omaha	100	65.9	70.4
Columbus	100	73.5	86.7
Grand Rapids	100	90.5	90.5
Cincinnati	100	50.0	56.3
Toronto	100	80.2	85.1
Indianapolis	100	68.2	81.1
Washington	100	75.0	90.0
Fort Worth	100	45.4	66.7
Lansing	100	86.0	84.0
Montreal	100	76.2	80.9
Chicago	100	65.7	88.5
Minneapolis	100	70.5	95.0
Tucson	100	70.4	81.8
Sacramento	100	88.5	82.8
Detroit	100	90.9	90.9
Average	100	71.6	80.5

by Butterfield (1933*b*), shows the wide variations in the efficiencies of the bile media reported by the different laboratories.

There was one very favorable feature of the results reported, however: not a single instance was recorded of failure to recover coliforms because of the presence of spores, although a total of 1,193 bile cultures was examined. Nevertheless it was evident that considerable inhibition of coliform organisms and a certain lack of specificity had been exhibited by both of the bile media. The B.G.B., 2 per cent, gave somewhat better results than the 5 per cent bile but, as shown in the table, the recovery of coliforms through its use was not generally very satisfactory.

After this work was done, a number of other observers reported their results in comparing B.G.B., 2 per cent, and lactose broth. Horwood and Heifetz (1934) and France (1936) found the bile medium to yield excellent results — even better than those obtained through the use of lactose broth. On the other hand, Butterfield (1933*b*) reported that the bile permitted only one third of the coliform recoveries secured with lactose broth as the enrichment medium; and Parr and Caldwell (1933) observed a 23 per cent decrease, upon substitution of the bile for the lactose broth, in the number of samples of latrine-polluted well waters from which coliform organisms could be isolated.

It finally became clear that even the new brilliant-green bile was not generally acceptable as a substitute for lactose broth either as a standard presumptive medium or as a standard enrichment medium. Since it was found to be fairly satisfactory with many waters, however, its usefulness in supplementary presumptive tests for the purpose of quickly obtaining an indication of serious pollution was generally recognized. Furthermore, as we shall see later, B.G.B., 2 per cent, has proved of very great value in a rapid confirmation test that has greatly simplified the work of confirming positive indications furnished by lactose broth enrichment.

Other Enrichment Media. The failure of brilliant-green bile to supplant lactose broth was followed by renewed attempts to find a more satisfactory presumptive and enrichment medium. Some of these efforts resulted in the development of media of considerable merit. Dominick and Lauter (1929) proposed an "M.B.-B.C.P." broth containing methylene blue and bromocresol purple, the purpose of which was to inhibit the growth of *Aerobacter, C. welchii,* and gelatin-liquefying organisms that interfered with the detection of *E. coli* in the control of filter plant operation. The medium was rather heavily buffered, as had been advocated by Thompson (1927) and Janzig and Montank (1928). An early change of color from blue to yellow, attributed to adsorption of methylene blue by *E. coli*

and production of acid, was said to furnish a preliminary warning of the presence of *Escherichia* forms; the purpose of the buffer was to permit organisms of this genus to utilize rapidly the available lactose without lowering the *p*H of the culture below 5.6–5.7, and to leave insufficient carbohydrate for fermentation by other coliforms. Leahy, Freeman, and Katsampes (1931) reported results apparently favoring the new medium, although their figures indicated a coliform recovery from lactose broth which was 12.9 per cent greater than that from the M.B.-B.C.P. medium. Favorable results were also reported by McCants (1931) and Nolte and Kramer (1933); and Horwood and Heifetz (1934) reported 339 coliform recoveries from the new broth compared with only 306 from lactose broth. On the other hand, Howard (1932) compared this medium with brilliant-green bile, 2 per cent, and lactose broth in the examination of raw Lake Ontario water, filtered, and chlorinated city water, and concluded that the M.B.-B.C.P. medium possessed little advantage over the brilliant-green bile; it was slower in its action than either of the other two media and was more difficult to prepare; practically the same number of coliform recoveries were obtained, however, with each of the three media compared. Butterfield (1933*b*) reported securing only 7 recoveries with the new medium and 31 with lactose broth; Evans and Bahlman (1931), working with settled Ohio River water, obtained 42 coliform recoveries from the new medium and 79 from lactose broth; and in the examination of raw brook water France (1936) isolated coliforms 673 times from the new, and 861 times from the standard medium.

Another presumptive and enrichment medium, a buffered broth containing crystal violet, was advanced by Salle (1930) as a substitute for lactose broth. Salle recalled the investigations of Browning, Gilmour, and Mackie (1913) which had shown that, although the diamidotriphenyl-methane group of dyes represented by brilliant green, and the triamidotriphenyl-methane group of which crystal violet is a representative, are both strongly bactericidal toward gram-positive organisms, the latter group is considerably less toxic to coliform organisms; Salle's experiments confirmed this finding in respect to crystal violet and brilliant green. Comparing crystal-violet broth and lactose broth in the examination of 101 water samples, Salle found 33 lactose broth gas-positives from which coliforms were not recovered, but only 1 crystal-violet gas-positive failed to be confirmed; and a larger number of coliform recoveries were obtained from the crystal-violet broth. Stark and England (1933), however, observed that gas production by a large number of *Esch-*

erichia and *Aerobacter* cultures was much slower in this medium than in the standard medium, and some of the cultures produced no gas at all in crystal-violet broth within 48 hours of incubation. Stark and England opposed the substitution of the proposed medium for lactose broth.

Fuchsin lactose broth was developed by Ritter (1932) and Schreiner. It is a very simple medium consisting of lactose broth to which 0.0015 per cent of fuchsin is added. Ritter reported that coliform organisms were recovered from 94.53 per cent of 33,532 fuchsin broth gas-positives; of a special series of 533 samples only 7 fuchsin positives failed to yield coliforms whereas 119 lactose broth positives failed in this respect. D. M. Taylor (1940), upon comparison of this medium with brilliant-green bile, 2 per cent, and lactose broth in the examination of 100-ml. portions of sample, found that although coliforms were isolated from 89.2 per cent of fuchsin positives, the total number of coliform recoveries from this medium and from lactose broth was 668 and 701 respectively. Similar experience was reported by Farrell (1936) when employing these media in the examination of a small series of well waters. In addition, the results obtained by a large group of American and Canadian laboratories, reported by McCrady (1937), showed that coliforms were recovered from only 70.5 per cent of 2,903 fuchsin broth gas-positives; and of 258 fuchsin broth positives from 192 samples of finished waters, only 21 per cent were found to contain coliform organisms.

Stark and England (1935) developed a different type of medium containing, instead of a dye, sodium ricinoleate, a surface-tension depressant, for restraining the multiplication of spore formers and other interfering organisms. It also contains sodium formate which accelerates the growth of coliform organisms and, as a result of its destruction, provides a buffer effect that prevents an excessive hydrogen-ion concentration fatal to some strains of coliforms. There appears to be little doubt that this medium effectively inhibits the growth of spore formers, but it appears also that a number of non-coliform organisms possess the ability to produce gas from sodium formate and it is probably for this reason that failure to recover coliforms from a rather large proportion of formate ricinoleate gas-positives was reported by Ruchhoft (1935*b*), Farrell (1936), and McCrady (1937). This medium seems so effective in restraining the multiplication of spore formers, however, that it is employed in a supplementary Standard Methods test for eliminating interference by these organisms when a culture is shown by microscopic examination to contain them.

Hajna and Perry (1943) proposed the use of a buffered tryptose bile salt broth, designated as " EC " (*E. coli*), medium for enrichment. In their preliminary studies, and in a further evaluation of the medium by Perry and Hajna (1944) with the aid of a number of collaborating laboratories, very satisfactory results were secured. It appears to be an effective medium and the results of further experimentation with it may prove interesting. The employment of " EC " broth in an Eijkman type of test will be described in Chapter VIII.

Recent speculation regarding the utility of MacConkey broth with American waters induced Farrell (1937) to compare this medium, Raghavachari's modification of it, and lactose broth in the examination of 94 surface and well waters; approximately the same number of coliform recoveries were obtained from each of these media. In 1937 the Committee on Standard Methods of the American Public Health Association enlisted the aid of four laboratories situated in the United States and Canada for the purpose of comparing MacConkey broth, prepared with ingredients recommended by British authorities, with standard lactose broth. A summary of the results obtained by each laboratory is shown in Table 48, which is taken from the report by McCrady (1939*a*).

TABLE 48. COMPARISON OF MACCONKEY BROTH AND LACTOSE BROTH

Laboratory	No. of Samples Producing Gas in One or Both Media	MacConkey Broth			Lactose Broth		
		Tubes Gas+	Coliform Isolations		Tubes Gas+	Coliform Isolations	
			No.	Per Cent		No.	Per Cent
New York City	96	217	131	60	367	132	36
Toronto	30	134	82	61	137	92	67
Maryland State	22	94	92	98	124	92	74
Quebec Province	73	406	326	80	442	327	74
Total		851	631	74	1,070	643	63

It is clear that at least three of the four collaborating laboratories did not find the production of gas in MacConkey broth to be a very reliable indication of the presence of coliforms, for only 74 per cent of the 851 gas-positives were shown to contain these organisms. On the other hand, approximately the same number of coliform recoveries were obtained as when lactose broth was used, although the MacConkey gas-positives numbered 20 per cent less than the lactose broth gas-positives. Comparison of these results with those obtained in the course of a similar study of fuchsin broth suggests that the

latter is perhaps preferable to MacConkey broth. It is interesting to note that Atkinson and Wood (1938), employing MacConkey and lactose broths and confirming gas-positives in accordance with American methods except in the use of MacConkey agar for isolation, secured the following results from an examination of 34 untreated Australian waters: with MacConkey broth 174 coliform recoveries from 216 gas-positives, and with lactose broth 247 from 305 gas-positives. Coliforms were recovered from 81 per cent of the gas-positives of each medium but lactose broth permitted 42 per cent more coliform recoveries than did the MacConkey medium. Atkinson and Wood also reported that the coliform count resulting from the use of lactose broth was greater in 27 of the 34 samples examined, and they stated that, since the use of MacConkey broth without confirmation was not suitable for the examination of Victorian waters, the method adopted for water analysis was practically that of the (American) Standard Methods (1933).

Lauryl Sulphate Tryptose Broth. It will be recalled that the principal ingredient employed to inhibit spore-forming organisms both in MacConkey broth and in American bile media, viz., bile salt or oxgall, is a surface-tension depressant. Sodium ricinoleate, used in formate ricinoleate broth, is likewise a surface-tension depressant. Larson, Cantwell, and Hartzell (1919), employing a specially prepared castor-oil soap as a depressant, found that when the surface tension of broth was depressed below 45 dynes, *B. subtilis* grew down in the medium instead of forming a pellicle at the surface, and spore formation was repressed or entirely prevented. They also observed that surface tensions below 46 dynes inhibited the growth of *Bacillus anthracis*. Frobisher (1926) suggested several methods of employing surface-tension depression for differentiation of bacterial species. The results of the experimental work of Gibbs, Batchelor, and Sickles (1926) and others suggest that many of the effects attributed to surface-tension depression are in fact due to the specific chemical action of the depressants employed. In any event, whether the effect is physical or chemical, it is generally agreed that coliform bacteria as well as many related enteric disease organisms are little affected by many surface-tension depressants; but spore formers, especially those of the aerobic type such as *B. aerosporus,* are very sensitive to them or to the surface-tension depression caused by them. Koser and Shinn (1927), for example, found that various combinations of 2 per cent oxgall, peptone, and lactose, with and without brilliant green all prevented the growth of this type of organism; the observed inhibition was evidently caused by the oxgall. The fact that so

many different surface-tension depressants appear to inhibit these particular spore formers suggests that their effect, in this instance, is at least partly due to lowering of the surface tension rather than entirely to their chemical action.

Cowles (1938) proposed the use in lactose broth of another depressant, sodium lauryl sulphate, one of the alkyl sulphates used in many industries as wetting agents; favorable results from its use in lactose broth were reported by some observers, but one of us (M. H. M.) did not find it very satisfactory. In the meantime, Darby and Mallmann (1939) had been studying the effect upon pure coliform cultures of varying concentrations of certain ingredients in broth media and had developed a phosphate-buffered lactose medium in which tryptose, a peptone found by them to be superior to ordinary peptone in promoting the reproduction of coliform organisms, was employed. These authors stated that in this medium the reproduction of coliforms was accelerated during the lag and early logarithmic growth phases, and the stationary phase was considerably shorter than in lactose broth. Subsequently, Mallmann and Darby (1941) added to this medium sodium lauryl sulphate in the form of Duponal W.A. Paste (found to be one of the least toxic of several similar preparations) in a concentration of 0.1 per cent, and reported marked success in recovering coliform organisms from gas-positive enrichment tubes of this new medium. Interfering organisms seemed to be completely inhibited.

The Committee on Standard Methods of the American Public Health Association undertook a coöperative study of a slight modification of this medium, using Duponal W.A. Flakes instead of the Paste because of their apparently greater stability. The medium employed was the following:

LAURYL SULPHATE TRYPTOSE BROTH

	Grams
Bacto-tryptose	20.
Bacto-lactose	5.
Potassium phosphate dibasic (K_2HPO_4)	2.75
Potassium phosphate monobasic (KH_2PO_4)	2.75
Sodium chloride	5.
Sodium lauryl sulphate (Duponal W.A. Flakes)	0.10

The ingredients were dissolved in 1 liter of cold distilled water, distributed in tubes and autoclaved 15 minutes at 15 pounds pressure. The final *p*H was approximately 6.8.

Seventeen laboratories collaborated in this study. The results of immediate interest, as reported by McCrady (1943), are shown in

Table 49. It will be observed that from 3,415 sample portions planted in lactose broth and lauryl broth, gas was formed in 54.8 per cent of the former and 47.7 per cent of the latter, the reduction in number of gas-positives due to the use of lauryl broth being 13.1 per cent. The number of coliform recoveries was 1,117 from lactose

TABLE 49. EFFECT OF SUBSTITUTION OF LAURYL SULPHATE TRYPTOSE BROTH FOR LACTOSE BROTH IN THE EXAMINATION OF 604 WATER SAMPLES

Enrichment Medium	No. of Sample Portions Planted	Portions Gas-positive		Gas-positives Completely Confirmed Using Secondary			
				Lactose Broth		Lauryl Broth	
		No.	Per Cent of Sample Portions	No.	Per Cent of Sample Portions	No.	Per Cent of Sample Portions
Lactose broth	3,415	1,873	54.8	1,117	32.7	1,142	33.4
Lauryl broth	3,415	1,628	47.7	1,222	35.8	1,261	36.9

broth and 1,222 from lauryl broth; the use of the new medium resulted in a recovery increase of 9.4 per cent. Upon employing lauryl instead of lactose broth to test the ability of organisms fished from the confirmatory agar plate to produce gas from lactose, the increase in coliform recovery was raised to 12.9 per cent. By substituting the new medium for the old, therefore, coliform recovery was increased by approximately 13 per cent and the number of gas-positives to be confirmed was decreased by about the same proportion.

The comparative coliform recoveries obtained through the use of the two broths in the examination of different types of water supplies are shown in Table 50; for more details see Table 64. A striking feature of this table is the results obtained with finished waters: although the number of lauryl broth gas-positives was only 43 per cent of the number of lactose broth gas-positives, 6 per cent more coliform recoveries were obtained from them. Since coliforms were isolated from only 77.5 per cent of the lauryl gas-positives, whereas 61.0 per cent of the lactose broth gas-positives were shown to contain these organisms, the new medium apparently may not be employed as a presumptive medium without confirmation. The favorable results obtained from its use as an enrichment broth, however, led to its acceptance by the Committee on Standard Methods (1944) as a medium that may be employed instead of lactose broth, if desired, in the examination of all waters except final filtered,

treated, and filtered-treated waters, and also in the examination of these if it has been shown to yield a coliform recovery from such waters as great as that obtained through the use of standard lactose broth.

TABLE 50. COMPARATIVE COLIFORM RECOVERY FROM LACTOSE BROTH AND LAURYL BROTH

(McCrady, 1943)

Type of Water	Coliform Isolations		Lauryl Broth Gas-positives in Terms of Percentage of Lactose Broth Gas-positives
	Lactose Broth Per Cent	Lauryl Broth Per Cent	
Raw	100	105	96
Unfinished	100	106	42
Finished	100	106	43
Swimming pools	100	113	68
Wells and springs	100	117	108
Sea water	100	125	122
Miscellaneous	100	137	102

Thus, of all the various presumptive and enrichment media that have been proposed to replace lactose broth, none has proved satisfactory for a presumptive test without confirmation; only one, lauryl broth, has been accepted in recent years by a Standard Methods Committee as an alternative enrichment medium that may be substituted for lactose broth, and its use is approved unconditionally only in the examination of certain types of water. Some of these media, however, particularly brilliant-green bile, 2 per cent, and fuchsin broth, should find useful applications in unofficial supplementary presumptive tests for rapid approximate estimation of water quality, especially in purification plant control.

It is to be noticed that the Committee on Standard Methods (1944) arbitrarily limits the incubation of enrichment cultures to 48 $\pm$ 3 hours at 35° to 37° C.; although a somewhat greater recovery of coliform organisms may be obtained by extending the time of incubation, the additional evidence thus secured is not considered sufficiently important to justify further delay in arriving at final results.

CHAPTER VII

COLIFORM ORGANISMS AND METHODS FOR THEIR DETECTION

II. Confirmation of the Presence of Coliforms

Confirmation of the Enrichment Gas-positive. When the positive presumptive test alone is not to be relied upon as definite proof of the presence of coliform organisms (and it has been shown that the application of this short-cut test is severely restricted) confirmation of the indication it furnishes of the probable presence of coliforms is necessary. In other words, further proof that these organisms are present is required.

Two degrees of confirmation are recognized by Standard Methods and employed in practice: " complete confirmation," an attempt to isolate coliform organisms from the enrichment culture and completely identify them; and " confirmation," an attempt to secure, by means of procedures requiring less time and effort than complete confirmation, such proof of the presence or absence of coliforms in the enrichment culture as may be considered sufficient for practical control of water supply quality. It is the latter type of confirmation that will be considered in this and the sections immediately following.

Before discussing in detail the various methods of confirmation employed, it is important to emphasize the necessity of practicing confirmation as soon as possible after the *first trace* of gas has appeared in the lactose broth or lauryl broth enrichment tube. The most important member of the coliform group, *E. coli,* usually ferments lactose with comparatively rapid production of acid, the average *p*H of lactose broth dropping within 24 hours of incubation to less than 5.0, according to Ruchhoft, Kallas, and Chinn (1931), who studied 39 strains of this species; the average *p*H of 158 coliforms of different species after 48 hours varied from 4.3 to 5.7, the average being 4.75. Exposure of coliforms to high hydrogen-ion concentrations and other products of bacterial metabolism, however, may so reduce their viability that they cannot be recovered from the enrichment culture. For example, Norton and Barnes (1928) found that *C. welchii,* when associated in a culture with coliforms producing a limiting *p*H of 5.0 to 5.3 in 48 hours, may lower the *p*H to 4.2 to 4.3.

In such cultures recovery of coliforms was uncertain even after 24 hours of incubation; and after 48 hours coliform organisms were never recovered when the *p*H was 4.2 to 4.3 and not always when it was 4.4 to 4.6. Enrichment tubes should be confirmed, therefore, immediately after the appearance of gas, preferably not later than 22 to 26 hours after being placed in the incubator, for in this early period coliforms, if present, are usually abundant, and interference from other organisms is unlikely to affect the results seriously.

Confirmation by the Plate Method. One reliable method of confirming the positive enrichment tube is to plate in a solid medium a drop of the greatly diluted enrichment culture; or still better, to spread or " streak " a minute portion of the enrichment culture over the surface of a hardened agar plate. Since it is the *surface* colonies of certain species of coliforms that are the more readily recognized, the latter method is almost universally employed for plating.

In order to effect a proper seeding of the solidified agar plate, a straight needle slightly curved at the tip should be used. The enrichment tube should be shaken gently or rotated to mix the contents, and inclined so that any membrane or scum of undesired bacteria at the surface of the liquid culture is moved to one side. The end of the flamed needle is then inserted in the liquid culture to a depth of about one millimeter, withdrawn, and immediately passed lightly back and forth on the surface of the hardened agar in the plate, only the curved section of the needle touching the agar. Care must be taken to avoid returning to the trail made by the needle, for the purpose is to leave on the agar surface a constantly diminishing trace of the culture. A fault often committed by the inexperienced is that of using plates in which the agar has not thoroughly solidified; the result is frequently a scratched or torn agar surface with practically all the culture from the needle wiped off and left in a few spots to form colonies containing mixtures of bacteria. Another fault very commonly committed is that of spreading too much of the culture on the agar surface; in this event, an overcrowded plate is obtained on which the colonies are not only largely composed of mixtures of organisms but are also not characteristic in appearance because they have not been permitted to develop normally. For generally successful isolation of coliforms it is absolutely essential that a few colonies be separated by at least 0.5 cm. from other colonies. Another cause of overcrowded plates is neglect to make sure that the agar surface is reasonably dry. If a wet agar surface is streaked, the culture on the needle is more or less evenly distributed over the plate and no resulting colonies may be well separated. By tilting the cover of the Petri dish and slightly

exposing the agar surface for a few minutes after pouring the hot melted agar, the excess of vapor is permitted to escape instead of condensing on the agar or interior of the dish.

After the plate has been streaked, it should be inverted and incubated for 22 to 26 hours at 37° C. and then examined for the presence of colonies typical of the species *E. coli.* If such colonies are present, the enrichment gas-positive is said to be confirmed. If only colonies not typical of this species are found, two or more of those considered most likely to be coliform colonies must be fished from the plate and further examined by the complete confirmation procedure to be described in a succeeding section. If, however, no colonies have developed on the plate, one or more of several things have occurred: coliform or other lactose-fermenting organisms originally present in the enrichment culture may have been destroyed or so reduced in number by antagonistic bacteria such as *C. welchii* that none was contained in the minute portion of enrichment culture transferred to the plate; *C. welchii* or some other organism which does not grow on aerobic plates may have produced the gas; or the organisms responsible for the gas production may have grown exceedingly slowly on the agar. In this last event, further incubation of the plate for a day or two may result in the appearance of colonies of coliforms, but because of the questionable significance of the presence of such slowly growing organisms, and in order to avoid unnecessary delay in the examination, Standard Methods has arbitrarily limited the incubation of plates to 1 day.

Failure of any *coliform* colonies to appear on the plate may be due to one or more of a number of circumstances in addition to those just mentioned: symbiosis or synergism may have been the cause of the gas production in the enrichment tube; other bacteria may have overgrown coliforms originally present, so outnumbering them that if a few were transferred to the plate they were masked by their associates; coliform organisms originally present may have been destroyed or reduced in number by antagonistic bacteria such as *Pseudomonas aeruginosa* or streptococci, as well as *C. welchii,* particularly if confirmation was not attempted until after 40 to 48 hours. Greer and Nyhan (1928), from their experiments with mixtures of coliforms and certain interfering organisms in lactose broth, concluded that the order of frequency with which they are most likely to be isolated from gas-positive cultures is as follows: after 24 hours of incubation, *E. coli, C. welchii, P. aeruginosa, S. fecalis, B. aerosporus;* after 48 hours, *S. fecalis, P. aeruginosa, C. welchii, E. coli, B. aerosporus.*

On the other hand, although it is true that colonies very typical of *E. coli* colonies may prove to be of some other organism, the frequency of this type of error is comparatively small, usually less than 10 per cent if a good plating medium is employed; furthermore, since this error leads to an overestimate of the coliform density of the water sample, it is on the side of safety.

Recognition of Coliform Colonies. The appearance of coliform colonies varies according to the kind of solid medium upon which the organisms are grown. The first differential medium generally accepted in America for the isolation of coliform bacteria was litmus lactose agar which has already been discussed in Chapter V. On this medium *Escherichia* organisms usually produce dark red colonies; *Aerobacter* colonies are red, pink, blue, or white. Because numerous organisms other than coliforms develop pink or red colonies on this agar, because this medium favors the growth of spreaders which may overgrow coliform colonies, and because the rapid diffusion of acid produced by lactose-fermenting bacteria often causes large areas of the plate to be colored red, litmus lactose agar was soon superseded by other solid media. Meyer (1917) suggested the use of 3 per cent agar to limit the overgrowth of coliforms by spreaders. This stiff agar gave results nearly, but not quite, as good as those obtained with Endo medium.

Endo Agar. The Endo medium now employed in some laboratories is the result of minor modifications of the original medium that Endo (1904) first proposed for isolation of typhoid organisms. It consists essentially of a stiff nutrient agar containing lactose, and an indicator, composed of basic fuchsin and sodium sulphite, which is particularly sensitive to certain products of lactose fermentation. The virtue of the medium lies in this indicator, the action of which, however, is still debatable. Endo attributed the red color of coliform colonies on his agar to acid formation by these bacteria; but acid does not produce the deep red color and sheen typical of *Escherichia* colonies on Endo medium. Other observers believed the reaction to be similar to that of Schiff's reagent with aldehyde but the two apparently are not identical. DeBord (1917) considered the reaction to be caused by the combined action of acid and aldehyde; for in his experiments neither alone could produce the characteristic color effect. Margolena and Hansen (1933) concluded from their study of the reaction that it was best explained by the theory of Neuberg and Nord (1919), viz., the sulphite traps small amounts of acetaldehyde, an intermediate product of fermentation, as it is produced by the coliform organisms, and the medium then acts as a reagent to indicate the presence of the

accumulated product; consequently the sulphite must be present in the medium while the organism is growing for, if it is not, the acetaldehyde is rapidly oxidized and no reaction is obtained upon subsequent addition of the indicator. Evidently, if this theory is correct, the ratio of sulphite to fuchsin should be rather important, because an excess of the former is needed to serve as the trapping agent and yet too great an excess may prevent the color reaction. To judge from the variety of ratios proposed by Kendall and Day (1911), Hasseltine (1917), Levine (1918*b*, 1921*a*), Harris (1925), and others, considerable difficulty was experienced in arriving at a generally acceptable formula. Conn and Darrow (1934) attributed this difficulty to the variation in the solubility and other characteristics of different lots of fuchsin and suggested a ratio of sulphite to fuchsin of 12.5:1, obtained by adding to each 100 ml. of base medium of *p*H 7.4, 1 ml. of 1 per cent basic fuchsin in 95 per cent alcohol, and 0.125 g. of anhydrous sodium sulphite dissolved in 5 ml. of distilled water. They stated that the use of 1 per cent fuchsin solution instead of 10 per cent, or saturated, solution eliminates the difficulty caused by variation in the solubility of different fuchsins, that an excess of alcohol prevents the formation of an insoluble brown precipitate upon addition of sulphite to some lots of the dye, and that the ratio of 12.5:1 assures a satisfactory proportion regardless of the variation in quality of commercial fuchsins.

Two formulas for the preparation of Endo agar are presented in Standard Methods; one is a 3 per cent agar containing 1 per cent each of peptone and lactose, 0.5 per cent beef extract. To each 100 ml. is added 1 ml. of a 3 per cent solution of basic fuchsin in 95 per cent alcohol and 0.125 g. of anhydrous sodium sulphite dissolved in 5 ml. of distilled water; this gives a sulphite-fuchsin ratio of about 4.2:1. The other medium contains the same proportions of peptone and lactose, but is buffered with 0.35 per cent dipotassium phosphate (K_2HPO_4), and to each 100 ml. of medium is added 0.5 ml. of filtered basic fuchsin (pararosaniline) and 0.25 g. of anhydrous sodium sulphite, the ratio of sulphite to fuchsin being about 5:1. Evidently, according to the results of Conn and Darrow mentioned above, care should be exercised to choose lots of fuchsin which will give satisfactory results in these Standard Methods formulas.

On properly prepared Endo agar *Escherichia* usually produces dark red, flat, button-like colonies 1 to 3 mm. in diameter, with a distinct metallic sheen and reddening of the adjacent medium; *Aerobacter* colonies rarely possess a sheen, are usually larger, more convex, and vary in color from pink to red. A serious fault of Endo agar is that

it permits rapid diffusion of the red color from coliform-like colonies which reduces the facility with which some coliform colonies may be distinguished from those of other bacteria. Another disadvantage of this medium is its instability; light, air, and heat redden it in a short time. For best results, therefore, it must be freshly prepared.

Kahn (1918*a*, 1918*b*, 1919) suggested the use of Endo agar slants instead of plates, with both stab and surface inoculation; coliform organisms produce more typical colonies on the slant where the thickness of the medium is greater than that of the plated medium and they form gas in the stab, whereas anaerobes neither grow on the surface nor produce gas in the stab. Although there is an obvious convenience in using tubes instead of plates, there is also the probability of overcrowding the slant with colonies because of its comparatively limited area unless very large tubes are employed; and of course it is impossible to be sure that the formation of gas in the stab and the production of any particular colony on the surface are due to the same type of organism.

Levine Eosin Methylene Blue Agar (E.M.B. Agar). This medium, developed by Levine (1918*a*), is a modification of an agar first proposed by Holt-Harris and Teague (1916) for the isolation of enteric disease organisms. The formula of this very popular E.M.B. agar is given below.

LEVINE EOSIN METHYLENE BLUE AGAR

Agar (undried)	15 g.
Peptone (Difco)	10 g.
Dipotassium phosphate (K_2HPO_4)	2 g.
Lactose	10 g.
Eosin, yellowish (2 per cent aqueous solution)	20 ml.
Methylene blue (0.5 per cent aqueous solution)	13 ml.
Distilled water	1,000 ml.

Neither adjustment of the reaction nor filtration of the medium is necessary. Autoclave at 15 pounds pressure for 15 minutes. The medium is decolorized during sterilization, but upon cooling, the color is restored.

Levine (1943) pointed out that his original formula specified a ratio of eosin to methylene blue (actual dye contents) of about 5.8:1; but after World War I many of the lots of methylene blue on the market contained so much more of the dye than did the previously used Grübler products that it was necessary to alter the formula; and after certification of methylene blue by the Commission on Standardization of Biological Stains, which made available a product of fairly

constant dye content, the proportions of the two dyes given in the above formula were found to produce the best results.

This medium was included in the 1923 Standard Methods and still remains the solid medium most widely employed for isolation of coliform organisms. Care must be taken, if the Difco dehydrated product is used, to distinguish between Levine eosin methylene blue agar and Bacto eosin methylene blue agar, for the latter is another modification of Holt-Harris and Teague's medium especially adapted for use in the isolation of organisms of the typhoid-dysentery group and is not so suitable for coliform isolation as the Levine medium.

A description of coliform colonies as they appear on E.M.B. agar, given by Levine (1921*a*), is reproduced in Table 51. Another, and

TABLE 51. DIFFERENTIATION OF *Escherichia* AND *Aerobacter* ON EOSIN METHYLENE BLUE AGAR

(Levine, 1921*a*)

	Escherichia	*Aerobacter*
Size	Well-isolated colonies are 2–3 mm. in diameter.	Well-isolated colonies are larger than *Escherichia*, usually 4–6 mm. in diameter or more.
Confluence	Neighboring colonies show little tendency to run together.	Neighboring colonies run together quickly.
Elevation	Colonies slightly raised, surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance by transmitted light	Dark, almost black centers which extend more than ¾ across the diameter of colony; internal structure of central dark portion difficult to discern.	Centers deep brown, not so dark as *Escherichia* and smaller in proportion to the rest of the colony. Internal structure often striated in young colonies.
Appearance by reflected light	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen.	Much lighter than *Escherichia*. Metallic sheen not observed except occasionally in depressed center when such is present.

particularly useful, description of the various colonies appearing on E.M.B. agar is that of Howard and Thompson (1925), who also reported the frequency with which the different types of colonies isolated by them fermented lactose, formed indole, etc. The colony descriptions and the figures showing the proportion of each type which fermented lactose are reproduced in modified form in Table 52. Practically all these descriptions will be readily recognized by those who have had experience in fishing colonies from E.M.B. plates.

TABLE 52. COLONIES ON EOSIN METHYLENE BLUE AGAR

(Howard and Thompson, 1925)

DESCRIPTION OF COLONIES IN ORDER OF RESEMBLANCE TO LEVINE'S DESCRIPTION

A Well-isolated colony, 2–3 mm., dark center, purple to black, flat or slightly concave, with distinct greenish metallic sheen.

A-1 Well-isolated colony, 2–3 mm., dark center, purple to black, distinctly flat surface, with greenish metallic sheen.

A-2 Well-isolated colony, 2–3 mm., dark center, purple to black, distinctly flat surface, with greenish metallic sheen, but with irregular edge.

A-3 Well-isolated colony considerably larger than A-2, purple to black, flattened surface, with dull greenish metallic sheen, forming a characteristic dry wrinkled surface.

B Well-isolated colony, 2–3 mm., dark center, purple to black, flat or slightly concave, but with complete absence of sheen.

B-1 Same as B, excepting distinctly flat surface.

B-2 Same as B-1, except that colony forms irregular edge. The dry wrinkled growth was sometimes noticed on agar slant.

D Colony not so well isolated as type A. Has a bright wine color (pale purple) without sheen.

E Large colony, 3–5 mm., with purple to brown center, usually has no sheen and markedly convex (*A. aerogenes?*).

F Small pink colony, 1–2 mm., markedly convex, no sheen.

F-1 Pale colony with purple center, 1–2 mm., well isolated, no sheen.

G Very small colonies almost like pin pricks, with brilliant greenish metallic sheen.

G-1 Includes all other unclassified groupings.

FERMENTATION OF LACTOSE BY ORGANISMS OF THE ABOVE COLONY TYPES

Type of Colony	A	A-1	A-2	A-3	B	B-1	B-2
Number isolated	317	321	114	1	118	66	7
Number positive	284	304	110	1	105	61	7
Number negative	33	17	4	0	13	5	0
Percentage positive	89.6	94.7	96.5	100	89.0	92.4	100
Percentage negative	10.4	5.3	3.5	0	11.0	7.6	0
Type of Colony	D	E	F	F-1	G	G-1	
Number isolated	23	85	15	35	6	125	
Number positive	11	79	5	21	1	22	
Number negative	12	6	10	14	5	103	
Percentage positive	47.8	93.0	33.3	60.0	17.6	16.7	
Percentage negative	52.2	7.0	66.7	40.0	82.4	83.3	

The mode of action of E.M.B. agar is considered by Wynne, Rode, and Hayward (1942) to depend upon two factors: the formation by eosin and methylene blue of a methylene blue eosinate which acts as an acidic dye, and the production by bacteria of a low *p*H which

enables the cells of the colony to take up the acidic dye. Colonies of bacteria that do not ferment lactose are colorless because their cells, in the alkaline environment produced by them, do not take up the stain. The methylene blue eosinate was found to be composed of one molecule of each of the dyes. Levine (1943) showed that a sugar-free base is necessary for the preparation of the medium if differentiation of coliform types is desired, because addition of glucose causes all coliform colonies to take on a metallic sheen; and that close adherence to the absolute amounts of dyes specified in the formula is essential to success in the use of the medium.

Other Solid Media. Numerous other agar media have been proposed for coliform isolation. Skinner and Murray (1924), experiencing difficulty caused by growth of spreading colonies of the *B. mycoides* and *Bacillus cereus* types when employing E.M.B. agar in the examination of soil and muddy waters, solved the problem to their satisfaction by adding crystal violet in the proportion of 1 : 100,000. Using this modification of Levine's medium, they succeeded not only in restraining the growth of spreaders but also in enhancing the differential characteristics of *Escherichia* and *Aerobacter* colonies.

Another solid medium, a buffered lactose agar containing erythrosin, methylene blue, and bromocresol purple, was suggested by Salle (1930) as an improvement of a similar medium previously proposed by him. In this agar, the combination of methylene blue and erythrosin gives a metallic sheen to *E. coli* colonies, and the acidity produced by this species of organism changes the color of the bromocresol purple from purple to orange, whereas *Aerobacter* colonies are unaffected in either respect.

MacConkey agar, which is the MacConkey enrichment medium to which 2 per cent of agar is added, remains after nearly a half century the unofficial standard medium in Britain. At the usual *p*H 7.5, the color of the medium is a claret red, but coliform colonies change the color to a darker red and are often surrounded by a haze of precipitated bile, the result of the action of the acids produced by the fermentation of lactose upon the bile salt and of subsequent adsorption of the neutral red indicator. MacConkey agar has been used only to a limited extent on this side of the Atlantic.

Reliability of Identification by Appearance of Colonies on Solid Media. Reports of the proportion of colonies on solid media correctly identified vary considerably. Levine (1921*b*) stated that the results of water examinations made in the Army laboratories in France showed that 94 per cent of the colonies on E.M.B. agar that were

labeled *Escherichia* and 85 per cent of those labeled *Aerobacter* proved to be correctly identified.

Howard and Thompson (1925), in a study of more than 1,200 colonies developed on E.M.B agar, found that 8.7 per cent of the colonies designated as coliforms failed to ferment lactose; and 12 absolutely typical colonies, which grew on Endo and MacConkey agars as well, failed to ferment any of six different sugars including glucose and lactose; of a group of 85 colonies considered to be *Aerobacter,* 7 per cent failed to ferment lactose. These observers concluded that although E.M.B. agar is one of the best media for use in coliform isolation, further examination of the organisms is essential if reliable results are required.

Greer *et al.* (1928) compared Endo and E.M.B. agars and obtained results which indicated a marked superiority of the latter medium. Some of these results are shown in Table 53. A comparison of Endo

TABLE 53. COMPARISON OF E.M.B. AGAR AND ENDO AGAR

(Greer, Noble, Nyhan, and O'Neil, 1928)

	E.M.B. Agar	Endo Agar
Total plates	1,723	1,723
Total growths	632	1,285
Growth, but secondary lactose not fermented	29	270
Raw water		
Coliforms	462	337
B. aerosporus	14	263
Treated water		
Coliforms	37	31
B. aerosporus	1	257
Swimming pools		
Coliforms	85	78
B. aerosporus	0	11

and E.M.B. agar was also made by Ruchhoft, Kallas, Chinn, and Coulter (1931) who concluded from the examination of more than 1,000 parallel platings that growth of *Aerobacter* and differentiation of *Aerobacter* from *Escherichia* colonies are more readily obtained by using E.M.B. agar than by using Endo agar, but the two media yield practically the same results if only isolation of coliform organisms is required.

Ruchhoft *et al.* reported also the results of a comparison of E.M.B. agar and the crystal-violet modification of this medium proposed by Skinner and Murray (1924) which indicated little advantage in using the modified medium for examining the waters of the Chicago region.

It will be recalled, however, that the crystal-violet E.M.B. agar was designed for a specific purpose and apparently with success.

Hoak (1935) examined a number of raw, settled, and filtered waters, using the crystal-violet agar of Salle (1930) but, as was to be expected, found many coliform colonies on this agar to present no indication of their identity, and for this reason concluded that it was less satisfactory for coliform differentiation than E.M.B. agar.

Little comparative work with MacConkey agar has been done on this continent in recent years, but Atkinson and Wood (1938), in the course of an examination of Australian waters, isolated coliforms from 296 of 614 enrichment gas-positives with the aid of E.M.B. agar, and 277 through the use of MacConkey agar although mixed growths appearing on the latter agar were transferred to fermentation tubes and again plated.

There is little doubt that E.M.B. agar is one of the best, if not the best, of solid media proposed for the isolation of coliform organisms. Perhaps, as Mallmann and Darby (1941) suggest, this agar may not permit the growth of some strains of coliforms; but it allows the great majority of them to grow, it is remarkably selective, and it furnishes a means of tentative differentiation of certain species that is often surprisingly accurate. Nevertheless, despite its undoubted value, *absolute* reliance cannot be placed upon its indications. Occasionally a colony possessing all the characteristics of *E. coli* colonies may prove to be a colony of *Aerobacter* and vice versa; much more often a colony which appears to be typical of *Aerobacter* colonies is found to contain no coliform organisms at all. The relative frequency of such errors in identification usually increases as the quality of the water samples examined improves: the proportion of mistakes made when attempting to identify coliform-like colonies of bacteria from springs and dug wells of doubtful quality, for example, is ordinarily much greater than that encountered in the examination of more seriously polluted brooks and rivers. All the solid media of which we have had experience, however, possess to a greater or less degree this same defect of uncertain differentiation of coliforms from non-coliforms, and of one coliform species from another. It is not surprising that this should be so; since, by definition, coliform organisms are among those which can produce gas from lactose, and since a solid medium offers no means of determining whether the organisms of a particular colony possess this ability, indications of the presence of other products resulting from lactose fermentation must be relied upon for colony differentiation, and unfortunately these are at times inadequate.

Standard Methods, in accepting for confirmation identification by

appearance alone of colonies typical of *E. coli*, recognizes the accuracy with which a good plating medium indicates the presence of this species of coliform. We have seen that such identification is subject to an error which is usually considerably less than 10 per cent and which is on the safe side. In requiring complete identification of two or more colonies most likely to be coliforms, if none typical of *E. coli* is present, Standard Methods recognizes also the inability of any present plating medium to indicate with satisfactory accuracy the presence of coliform species other than *E. coli*. Since the chief merit of the plate method of confirmation lies in its differentiation of *E. coli* from other bacteria, it is obviously best adapted to the examination of rather highly polluted waters and those unfinished waters in which *E. coli* organisms are particularly numerous. If, however, colonies of this species are rarely found on the plates and complete confirmation of atypical colonies is frequently necessary, confirmation by means of liquid media will be found much more satisfactory. Before describing this method, however, it is convenient to consider the procedure known as the completed test.

The Completed Test. This term is used to designate the procedure employed in the attempt to discover and completely identify coliform organisms in colonies produced on Endo or E.M.B. agar plates that have been streaked from gas-positive fermentation tubes. The test consists of three parts: selection of a coliform-like colony from those on the plate and inoculation of a trace of it into a lactose broth fermentation tube and upon a nutrient agar slant, both of which are incubated for 48 hours at 37° C.; examination of the lactose broth to determine whether the lactose has been fermented with the production of gas; and, if gas has been formed in the lactose broth, examination of the agar slant to determine whether the inoculum has grown under the aerobic conditions provided, and microscopic examination of a gram-stained slide preparation of the agar slant culture to determine whether gram-negative, coliform-like rods are present and spore-forming or other organisms are absent.

Selection of the Colony to be Examined. The appearance of coliform colonies on Endo and E.M.B. agar has already been described in the discussion of simple confirmation by the plate method. If a colony typical of *E. coli* is present on the plate, the center or the raised portion of it is barely touched with a needle and a trace of the colony is transferred to a lactose broth tube and an agar slant. The objective in fishing a colony is to obtain, if possible, a pure culture, and to avoid a mixed culture. If material from near the edge of the colony is fished, growth, invisible to the naked eye, of some other

organism may be picked up by the needle. A colony should never be scooped up from the plate, for a large proportion of mixed cultures is certain to result.

When making the transfer, the interior wall of the lactose broth tube should be touched with the end of the needle at the surface of the lactose broth; then by gently rubbing the end of the needle against the glass at this point a slight turbidity in the broth will indicate that the transfer has been successfully performed; if the needle is simply dipped into the broth, some mucoid growths may not be transferred.

Immediately upon withdrawal from the lactose broth tube the needle is passed to the agar slant which is inoculated in the usual manner. A second fishing from the colony in order to obtain material for the agar slant inoculation should be avoided at all cost, for organisms picked up by the second fishing may differ entirely from those obtained by the first fishing.

If no colonies typical of *E. coli* are present on the plate, two of those colonies considered to resemble most closely colonies of coliforms should be chosen and each transferred to lactose broth and an agar slant as described above. The descriptions of colonies by Howard, and Levine, mentioned in the discussion of confirmation, will assist the beginner to recognize coliform-like colonies, but only study and replating of isolated organisms, coupled with long experience, will enable him to become efficient in colony selection.

One point cannot be emphasized too strongly: the necessity of fishing only discrete colonies separated by at least 0.5 cm. from other colonies. If for some reason or other there are no such colonies, a special effort must be made to obtain a pure culture, although the chance of achieving this is greatly reduced. Even when conditions are apparently most favorable for successful fishing of pure cultures, mixed cultures of coliforms and non-coliforms are obtained with a frequency which depends upon the bacterial flora of the water sample. As a rule this mischance probably does not occur very often, but it may be encountered frequently when non-coliform organisms growing slowly on the plating agar are so numerous that many coliform colonies are partly composed of invisible growths of such organisms, or when non-coliform mucoid bacteria that adhere to coliform organisms are prevalent in the enrichment tube. Ruchhoft, Kallas, Chinn, and Coulter (1931) upon microscopic examination of 13,222 gram-stained preparations of cultures from routine plates, reported 5.5 per cent to contain spore-forming organisms, 9.0 per cent to be mixed cultures without spores, and 14.5 per cent to be probably mixed cultures; and

upon a second microscopic examination of a series of 539 agar slant cultures some days after the first was made, the proportion of the series that was considered to be mixed cultures was increased from 29.3 to 39.8 per cent. Although this must be considered an extreme case it is not surprising that, as Ruchhoft and his colleagues point out, when spreading hundreds of thousands of organisms over the limited agar surface touched by the needle more than one type of bacteria should be deposited at a certain point if the enrichment culture contains different types in comparable numbers.

The lactose broth tubes inoculated from the plate should be incubated for 24 to 48 hours and examined from time to time. If any gas whatever, even a tiny bubble, has been formed the culture is considered to possess the ability to produce gas from lactose. Thereupon, a microscopic slide preparation of the corresponding agar slant culture is made and gram-stained. Although many variations of Gram's staining method have been proposed, a number of which have been reviewed by Hucker and Conn (1923, 1927), that most commonly employed is the Hucker modification described in Standard Methods. When preparing the slides care should be taken to avoid thick smears; a thin, evenly distributed smear will be found much more satisfactory. It is advisable to control the action of the stain from time to time by placing at one end of the slide a drop of each of two suspensions, one of typically gram-negative *E. coli,* the other of typically gram-positive staphylococci; comparison of these two stained controls will reveal whether the stain is functioning properly.

Spore-forming organisms vary considerably in the speed with which they form spores in the agar slant cultures; some produce spores within a short time, others not until 4 days or more; but since they are usually gram-positive and of greater size than coliform organisms, they are generally easily recognized as, at least, non-coliform bacteria. Spores may be central, excentric, terminal, or subterminal, and may be slightly larger than the width of the cell as in *C. welchii,* or much larger, as in *B. aerosporus.* If spores have been formed rapidly, free spores may be present: usually comparatively large, refractile bodies, generally oval in shape. The Medical Research Committee (1919) of England has given good descriptions of a number of lactose-fermenting anaerobic spore formers and Koser and Shinn (1927) have described in detail the aerobic spore formers, often found in water, which are chiefly derived from the soil.

If spore formers are detected in the agar slant culture it is still possible that coliform organisms may also be present; consequently, a further search for them should be made. To this end a large loopful

of the agar slant culture is transferred to formate ricinoleate broth, which is particularly efficacious in preventing the multiplication of spore formers. If, after 48 hours of incubation, gas appears in this medium a trace of the culture is plated out on agar and the completed test made to determine whether coliform organisms are actually present. If no gas appears after 48 hours, however, it is concluded that the gas in the lactose broth enrichment culture was produced by a spore former and coliform organisms are considered to have been absent.

The Use of Liquid Media in the Confirmed Test and the Completed Test. The labor and expense involved in the confirmation of enrichment gas-positives by the plate method and its supplement, the completed test, to say nothing of the skill and experience needed in such work, were for years an impassable barrier to further progress toward thoroughly adequate laboratory control of the great majority of our water supplies. Recognizing the practical difficulties in the way of general application of the confirmation procedure, Standard Methods long required confirmation of only one gas-positive enrichment tube of the series employed: that gas-positive tube containing the smallest quantity of water sample. It was obvious, however, that the results of confirmation of this one tube were hopelessly inadequate to serve as an indication of the coliform density of the sample, for of several gas-positive tubes all *except the one* in which the smallest quantity of sample had been planted might contain coliforms. Particularly in the examination of natural waters of fair quality, in which non-coliform lactose-fermenting organisms might outnumber the coliforms and produce gas in enrichment tubes inoculated with the smaller portions of sample, this confirmation procedure frequently failed to supply the information required.

Moreover, since success with the plate method of confirmation depends very much upon the facility with which the organisms present produce characteristic colonies and upon the ability of the technician to recognize and differentiate them, failure to isolate coliforms from a considerable proportion of enrichment tubes containing only *Aerobacter* forms, for example, is only to be expected; and it is not surprising that two technicians fishing colonies from the same plate may occasionally secure entirely different results.

It was dissatisfaction with this laborious and unsatisfactory method of confirming enrichment gas-positives which yielded colonies atypical of *E. coli*, on the one hand, and recognition of the need of more frequent examination of water supplies, on the other, that led to the intensive search for improved presumptive and enrichment media

which has been described. The great amount of effort expended in this search resulted in the development of no reliable presumptive test, but of an enrichment medium, lauryl broth, which promises to surpass standard lactose broth in general utility. There was another result, however, of far-reaching importance. In the course of the first study of brilliant-green bile by Committee No. 1 of the American Water Works Association, Jordan (Dunham, McCrady, and Jordan, 1925) suggested an innovation in method which proved to be as effective as it was simple; and which, because of its simplicity, renders possible that greater frequency of examination of water supplies which is essential to proper control of their sanitary quality.

This suggestion derived from the following consideration. Since lactose broth appeared to be the enrichment medium which permitted the maximum recovery of coliform organisms, its continued use seemed unavoidable; but it was conceivable that coliform organisms sensitive to a slightly inhibitive, selective medium such as brilliant-green bile might so adapt themselves to artificial media while multiplying in simple lactose broth as to survive and produce gas when later subcultured in the more unfavorable, selective medium. If satisfactory confirmation of enrichment positives could be effected in this manner, a number of advantages would be realized: (1) a great saving of labor and material, for transfer of a loopful of enrichment culture to a small tube of selective medium would require a minimum of effort, glassware, and medium; (2) marked economy of skilled technical assistance, for relatively untrained technicians could be quickly taught to make the transfers properly; (3) marked improvement in uniformity of results secured by different technicians, for two transfers from the same enrichment tube should nearly always yield identical results; (4) a great reduction in the labor and material expended in isolating and identifying coliform organisms, if this additional step is required, by application of the completed test to the gas-positive subculture; for the inhibitive properties of the selective medium might be depended upon to "screen out" the majority of negative tubes and reduce considerably the number of tubes to be completely examined.

Jordan's first extensive series of results obtained with B.G.B., 5 per cent, in this confirmation procedure was very encouraging, as the summary in Table 54 shows. Considering the bile subculture procedure as a method for confirmation, the presence of coliforms was indicated (by gas formation) in 594 of the water sample portions examined, or 10.4 per cent more than the 539 portions from which coliform organisms were actually isolated by means of complete

confirmation of the lactose broth gas-positives. But when the subculture was employed as an exclusion test to screen out false lactose broth gas-positives, practically the same number of coliform isolations were obtained from 594 gas-positive bile subcultures as from 2,506 lactose broth enrichment gas-positives; the use of the subculture thus reduced the labor of isolation and identification of coliform organisms by about 76 per cent. The results obtained from filtered-treated water are particularly striking: the number of coliform isolations (8) secured from the examination of 8 bile subcultures is approximately that obtained from the completed tests of 760 lactose broth positives.

TABLE 54

	Gas+ Lactose Broth	Coliform Isolations	Gas+ B.G.B. Subculture	Coliform Isolations
Filtered water	774	270	293	271
Filtered water chlorinated	760	9	8	8
Tap water	972	260	293	257
Total	2,506	539	594	536

The large group of laboratories that collaborated in a comparison of 2 per cent and 5 per cent brilliant-green bile as presumptive media, the results of which were discussed on page 89, also studied the utility of these two media in the new confirmation procedure. A condensed summary of their results as reported by Jordan (1927), including for comparison those obtained from the use of the bile media in presumptive tests, is given in Table 55. As liquid confirma-

TABLE 55. USE OF BRILLIANT-GREEN BILE IN CONFIRMATION TESTS

(Jordan, 1927)

Media Employed	Gas+ 48 hr.	Coliform Isolations	
		Number	Per Cent
Lactose broth	...	784	....
Brilliant-green bile 2 per cent	598	562	94.0
Brilliant-green bile 5 per cent	663	631	95.2
Lactose broth followed by brilliant-green bile 2 per cent	762	740	97.1
Lactose broth followed by brilliant-green bile 5 per cent	762	744	97.6

tory media, each of the bile media indicated the presence of coliforms in 762 of the lactose broth enrichment gas-positives, or 97 per cent of those found by isolation and identification procedures to contain these organisms; and as media for use in eliminating false lactose broth

gas-positives, they permitted the recovery of coliform organisms from 740 to 744 of the 762 bile gas-positives, or about 97 per cent of the latter and about 95 per cent of the coliform isolations (784) obtained by complete confirmation of all the lactose broth gas-positives. Although the different collaborating laboratories reported coliform recoveries from the 2 per cent bile subcultures varying from 71 to 100 per cent of those from the lactose broth gas-positives, the recovery usually was 90 per cent or better. Incidentally, the presence of spores in cultures of organisms isolated from the bile subculture gas-positive caused rejection of only 3 of the latter.

It is interesting to observe that Richey (1941), fourteen years later, obtained similar results, shown in Table 56, from a comparison of

TABLE 56. COMPARISON OF 2 PER CENT AND 5 PER CENT BRILLIANT-GREEN BILE AS CONFIRMATORY MEDIA

(Richey, 1941)

Media Employed	Gas+ 48 hr.	Coliform Isolations	
		Number	Per Cent
Lactose broth	441	317	71.9
Lactose broth followed by brilliant-green bile 2 per cent	358	326	91.6
Lactose broth followed by brilliant-green bile 5 per cent	358	335	93.5

2 per cent and 5 per cent brilliant-green bile in the examination of 103 Mississippi waters, most of which were from deep and surface wells. Evidently Richey also found very little difference between the two bile media, whether used for confirmation or for exclusion of false enrichment gas-positives; and he, too, observed very little interference from spore formers in these media, for none of the 5 per cent bile and only two of the 2 per cent bile subcultures were found to contain this type of organism.

The results reported by Jordan, followed by similarly favorable reports from Howard (1932), Norton (1933), and others, led to the inclusion of brilliant-green bile, 2 per cent, in the 1933 Standard Methods as a liquid confirmatory medium for use in the examination of *unfinished* waters, the formation of 10 per cent or more gas in the bile after 48 hours of incubation to be recorded as an indication of the presence of coliform organisms. Additional favorable results from this procedure were subsequently reported by France (1936) and Mallmann and Hepler (1936).

Comparison of Various Liquid Media for Use in Confirmation. Naturally, following the successful use of bile media for confirmation

of enrichment gas-positives, attention was directed to the possibility that other liquid media, which had been proposed for presumptive tests, might prove to be satisfactory in the rôle of confirmatory media. Ruchhoft (1935*b*) compared brilliant-green bile, 2 per cent, formate ricinoleate broth, and MacConkey broth as confirmatory media in the examination of two series of Chicago chlorinated waters, one before and one after neutralizing any remaining residual chlorine. The results are shown in Table 57. As Ruchhoft observed, these results

TABLE 57. COMPARISON OF LIQUID CONFIRMATORY MEDIA

(Ruchhoft, 1935*b*)

	Gas+ (Any Amount)	Coliform Isolations	
		Number	Per Cent
Unneutralized samples			
Primary lactose broth	315	65	21
Primary lactose broth + brilliant-green bile, 2 per cent	62	56	90
Primary lactose broth + formate ricinoleate	100	71	71
Primary lactose broth + MacConkey broth	61	50	82
Neutralized samples			
Primary lactose broth	97	45	46
Primary lactose broth + brilliant-green bile, 2 per cent	34	26	77
Primary lactose broth + formate ricinoleate	86	45	52
Primary lactose broth + MacConkey broth	28	16	57

show that neutralizing of remaining residual chlorine permits the recovery of coliforms from numerous sample portions which would otherwise yield negative results; it also permits the growth of an increased proportion of non-coliform organisms in formate ricinoleate broth. Other features of this series of data are the poor results obtained with MacConkey (and brilliant-green bile) subcultures, the low proportion of formate ricinoleate gas-positive subcultures from which coliform organisms were isolated, and the satisfactory recovery of coliforms through the use of this medium.

Upon closer examination of his results, Ruchhoft found that many more coliform isolations were obtained by using four confirmatory liquid media and completely confirming all gas-positives than by employing any one of them. The proportion of the lactose broth positives found to contain coliforms (by the use of the *four* media) which was shown by complete confirmation of the gas-positives of each confirmatory medium to contain them, is shown in Table 58.

Evidently the Standard Method employing the completed test, as well as the other confirmatory methods, failed to recover coliforms from some 20 per cent or more of the lactose broth gas-positives in which, by one means or another, the organisms were demonstrated to be present. It had long been admitted that the Standard Method of complete confirmation was not perfect, but here was a striking example of its deficiencies.

TABLE 58. PROPORTION OF SAMPLES CONTAINING COLIFORMS DETECTED BY VARIOUS CONFIRMATION PROCEDURES

(Ruchhoft, 1935*b*)

Procedure	Unneutralized Samples Per Cent	Neutralized Samples Per Cent
Completed test (Standard Method)	74.7	79.0
Formate ricinoleate + completed test	81.7	79.0
Brilliant-green bile + completed test	64.4	45.6
MacConkey broth + completed test	57.5	28.1

The 1936 Standard Methods permitted the use of brilliant-green bile, 2 per cent, crystal-violet broth, M.B.-B.C.P. broth, formate ricinoleate, and fuchsin broth as confirmatory media. Formation of any amount of gas within 24 hours was to indicate the presence of coliform organisms, provided "prior examinations have established a satisfactory correlation of the Confirmed Test with the Completed Test." In order to determine the relative worth of these various confirmatory media and perhaps eventually reduce their number, a comprehensive study was undertaken by the Committee on Standard Methods with the collaboration of 21 state, provincial, and city laboratories situated in the United States and Canada. Five methods of confirmation of the lactose broth enrichment gas-positives were employed: the usual plate method of confirmation followed by the completed test, and confirmation by transferring a loopful of the enrichment culture to a fermentation tube of each of several confirmatory media; furthermore, if gas appeared in any of the latter, the subculture was subjected to the completed test to determine the presence or absence of coliform organisms. Table 59, taken from the report by McCrady (1937), presents the aggregate results of this study. Calculation shows that if the 3,628 lactose broth gas-positives from which coliforms were isolated by any of the five confirmatory procedures is taken as 100 per cent, *gas production* in brilliant-green bile, crystal-violet broth, and fuchsin broth indicated the presence of coliforms in 96.1, 103.1, and 104.9 per cent of them, respectively; the table indicates that from these confirmatory gas-positives almost as

many coliform isolations were obtained as through the use of the usual standard completed test.

A particularly interesting feature of the results is shown in column 3 of the table: from 504 of the lactose broth gas-positives, coliforms were not recovered by the usual completed test, but they were recovered from one or more of the confirmatory media in which they

TABLE 59. RESULTS OF FIVE DIFFERENT METHODS OF CONFIRMATION OF 6,043 LACTOSE BROTH GAS-POSITIVES OBTAINED FROM 1,213 SAMPLES

Confirmation Procedure	1	2		3		4	
	Gas-positives	Coliforms Isolated		Coliforms Isolated from Corresponding Lactose Broth Gas-positives by Other Procedures		Sum of Columns 2 and 3; Tubes Containing or Probably Containing Coliforms	
	No.	No.	Per Cent of Gas-positives	No.	Per Cent of Gas-positives	No.	Per Cent of Gas-positives
SMC*	6,043	3,124 (3)	51.7	504	8.3	3,628	60.0
BG (+SMC)	3,488	3,074 (8)	88.1	225	6.5	3,299	94.6
CV (+SMC)	3,742	3,101 (4)	82.9	289	7.7	3,390	90.6
F (+SMC)	3,804	3,072 (6)	80.8	275	7.2	3,347	88.0
FR (=SMC)	4,097 (6)	3,199 (11)	78.1	324	7.9	3,523	86.0

The first figure of column 4 indicates that the number of lactose broth primary tubes from which, by one or more methods, coliform organisms were isolated = 3,628.

The figures in parentheses (which are included in the adjoining totals) represent the number of missing tubes, incomplete results, etc., which are considered positive in order to avoid loss of the remaining data obtained from these particular samples; a study of these data suggests that the error thus introduced is very probably negligible.

* The symbol SMC signifies the Standard Methods completed test procedure; BG, CV, F, and FR signify the use of brilliant-green bile, crystal-violet broth, fuchsin broth, and formate ricinoleate broth, respectively, as liquid comfirmatory media.

had produced gas. In other words, the usual standard method failed to detect coliforms in about 14 per cent of the lactose broth gas-positives which were shown by other methods to contain them. It will be recalled that a corresponding failure of about 20 per cent was reported by Ruchhoft when he was working with the difficult Chicago chlorinated water.

To compare the results obtained from the individual samples by means of the five confirmatory procedures, the device of the "cumulative error" was employed: the difference between the number of positives indicated by each method of confirmation and the number of sample portions from which, by means of any of the five confirmatory methods, coliforms were actually isolated, was computed for each sample; all positive differences were summed to give a total designated the "positive cumulative error," and all negative differences were similarly summed to give the "negative cumulative error." For example, if by any method or methods coliforms were isolated

from 5 portions of a water sample, and gas appeared in 7 of the crystal-violet confirmatory tubes representing as many water sample portions, the error due to the use of the crystal-violet confirmatory method was recorded as 2+, and all such results were added together to obtain the positive cumulative error of the crystal-violet method. Naturally, there can be no *positive* cumulative error referable to the usual *standard* procedure because lactose broth gas-positives are not recorded as indicating the presence of coliforms unless these organisms have been isolated from them. Comparison of these cumulative errors affords a fairly adequate indication of the relative precision of the various methods of confirmation; the larger the positive cumulative error, the greater is the number of waters that may be unjustly suspected; the larger the negative error, the greater is the number of unsafe waters that may escape detection. Table 60 shows the

TABLE 60. CUMULATIVE ERRORS OF RESULTS FROM FIVE CONFIRMATORY PROCEDURES AS PERCENTAGES OF TOTAL OF SAMPLE PORTIONS CONTAINING COLIFORM ORGANISMS (ISOLATED BY 1 OR MORE OF 5 METHODS)

Water Type	Number of Samples	Number of Laboratories	SMC	BG		CV		F		FR		Total Coliforms Isolated by 1 or More of 5 Methods
			−	+	−	+	−	+	−	+	−	
All types*	1,213	21	13.9	4.4	8.2	8.4	5.2	11.1	6.3	14.6	1.7	3,628
Streams	150	15	11.5	3.5	4.4	5.0	2.5	4.9	3.3	6.6	0.4	793
Unfinished	193	9	20.7	3.4	7.6	10.3	5.9	18.0	3.8	20.9	1.9	473
Finished	306	13	29.2	9.4	25.5	49.1	19.8	108.5	12.3	76.4	17.0	106
Springs	69	11	23.1	6.9	18.8	11.5	6.9	9.2	9.6	20.0	2.3	260
Wells	308	16	10.0	3.8	7.8	5.1	4.7	4.8	7.6	9.7	1.6	1,595
Lakes	110	5	16.6	1.3	7.6	4.9	8.5	6.7	5.8	9.0	0.0	223
Sea water	6	2	17.1	2.9	2.9	0.0	2.9	0.0	5.7	5.7	2.9	35
Reservoirs and pools	11	1	1.14	5.7	11.4	0.0	17.2	31.4	2.9	31.4	0.0	35

* Includes 60 unclassified.

cumulative errors of the confirmed test results obtained from the various types of water examined. To judge from the figures of this table, the brilliant-green bile method of confirmation yielded results better than those obtained with the other methods of confirmation, for its positive cumulative error ranged only from 1.3 to 9.4 per cent. The negative cumulative error of the standard method ranged from 10.0 to 29.2 per cent. The positive errors of the methods employing crystal-violet, fuchsin, and formate ricinoleate broths were usually excessive, especially in the examination of unfinished and finished waters.

Table 61 presents the results that were obtained with border-line

samples, or samples which were indicated, by one or more confirmatory methods, to contain small numbers of coliform organisms. It shows, for example, that a grand total of 436 samples which yielded lactose broth gas-positives were found to contain no coliforms; but 18 of these samples were indicated by formation of gas in brilliant-green bile confirmatory tests to contain 2 coliforms per 100 ml. of sample, 38 samples were indicated by crystal-violet broth to contain this coliform density, 56 by fuchsin broth and 37 by formate ricinoleate broth. Seven were indicated by brilliant-green bile to contain more than 2 coliforms per 100 ml. of sample, whereas formate ricinoleate indicated 45 samples to contain this coliform density. On the other hand, of 415 samples shown to contain more than 2 coliforms per 100 ml., brilliant-green bile indicated 14 of these to be free from coliforms; the standard completed test indicated 18 samples to be of this category; but the other confirmatory media yielded considerably better results in this respect.

TABLE 61. CERTAIN DISCREPANCIES IN RESULTS OBTAINED BY VARIOUS CONFIRMATORY METHODS WHEN ONE OR MORE METHODS INDICATED ABSENCE OF COLIFORM ORGANISMS FROM SAMPLE

Type of Samples and Number of Laboratories Reporting	Coliform Density by All Methods = 0 per 100 ml.									Coliform Density by All Methods = > 2 per 100 ml.					
	Number of Samples with LBP Gas-positives	Coliforms = 2/100 ml. by Gas Only in				Coliforms = > 2/100 ml. by Gas Only in				Number of Samples with LBP Gas-positives	Coliforms = 0 per 100 ml. by				
											Gas Only in				SMC Completed
		BG	CV	F	FR	BG	CV	F	FR		BG	CV	F	FR	
T, F, or FT waters, 13 laboratories	283	3	17	33	16	0	6	19	25	92	4	1	1	0	8
Natural waters, 10 laboratories	108	7	14	15	16	1	11	6	10	271	10	3	7	0	10
Totals	391	10	31	48	32	1	17	25	35	363	14	4	8	0	18
		2.6%													
Natural waters, Ontario — Toronto	45	8	7	8	5	6	6	7	10	52	0	0	0	0	0
Grand Total	436	18	38	56	37	7	23	32	45	415	14	4	8	0	18

This work was soon followed by similar studies of various media for use in confirmatory tests. Table 62 shows the results, reported by McCrady (1939*a*), of a study by 4 laboratories of MacConkey broth used as an enrichment, or primary medium, and of brilliant-green bile used as a confirmatory medium in the examination of waters from a variety of sources.

D. M. Taylor (1940) compared brilliant-green bile, 2 per cent, and

fuchsin broth as confirmatory media in the examination of 100-ml. portions of Minnesota surface and ground waters, with results that indicated the fuchsin broth to be practically the equivalent of the bile medium except with respect to the cumulative errors: the positive and negative cumulative errors of the bile confirmatory procedure were 4.63 and 9.41 per cent, respectively; those of the fuchsin broth procedure, 7.99 and 10.83 per cent. It should be noted that Ritter (1938) has suggested an increase in the proportion of fuchsin to

TABLE 62. RESULTS OF FIVE DIFFERENT METHODS OF EXAMINATION OF WATER SAMPLES

	No. of Samples Producing Gas in One or Both Primary Media	MacConkey Broth Primary Tubes			Lactose Broth Primary Tubes				
		Gas+	Coliform Isolations		Gas+	Coliform Isolations		Brilliant-green Confirmation	
			No.	Per Cent				Gas	Coliform Isolations
New York City	96	217	131	60	367	132	(12)*	141	115 (6)
Toronto	30	134	82	61	137	92	(8)	127	98 (2)
Maryland	22	94	92	98	124	92	(1)	87	86 (1)
Quebec	73	406	326	80	442	327	(34)	359	335 (8)
		851	631	74	1,070	643	(55)	714	634 (17)

* Figures in parentheses indicate the number of tubes showing gas from which no coliforms were isolated, but from the corresponding lactose broth primary of which coliforms were isolated by the other method. Thus, the indication (6), at the end of the first line of the table, signifies that 6 of the 141 gas-positive brilliant-green confirmatory tubes failed to yield coliforms upon attempted isolation, but coliforms were isolated directly from the corresponding 6 lactose broth primary tubes.

0.0025 per cent in fuchsin broth used for confirmation; upon replacing the fuchsin broth previously used, which contained 0.0015 per cent of the dye, with the new fuchsin broth in the examination of a series of waters, the percentage of false positives indicated by the confirmatory method was reduced from 15.3 to 2.5; the corresponding excess of positives with brilliant-green bile was 1.9, but Ritter favored the modified fuchsin broth because it appeared to be more inhibitive than the bile to spore-forming organisms.

Smith (1940) found brilliant-green bile, 2 per cent, to be satisfactory as a confirmatory medium, as did Kelly (1940) in his examination of sea water and shellfish. Howard, Lochhead, and McCrady (1941) reported results of a study of the brilliant-green bile confirmatory method by 5 Canadian laboratories which suggested that this method might be advantageously employed also in the examination of Canadian waters. Wattie (1943) investigated the relative accuracy of the indications furnished by confirmatory brilliant-green bile after 24

hours and after 48 hours of incubation. Raw lake water and the same water after varying degrees of chlorination were examined. The results obtained showed that, in general, B.G.B. confirmation indicated coliform densities slightly higher than those determined by the completed test; and the heavier or longer the chlorine treatment, the greater was the proportion of bile positives between the twenty-fourth and forty-eighth hours of incubation. These results attest to the necessity, likewise demonstrated by many of the reports from laboratories that have collaborated in the group studies described in this chapter, of incubating liquid media confirmatory tubes for 48 hours; otherwise, the number of positive results obtained will often be too low.

TABLE 63

Enrichment Medium	Secondary Medium	Per Cent of Sample Portions Positive	
		By Completed Test	By Gas in B.G.B.
Lactose broth	Lactose broth	31.2	....
Lactose broth	Lauryl broth	30.4	....
Lauryl broth	Lactose broth	33.8	....
Lauryl broth	Lauryl broth	33.2	....
Lactose broth	.....	....	33.4
Lauryl broth	.....	....	34.7

In the investigation, mentioned in the preceding chapter, by a number of laboratories to determine the utility of lauryl broth as a substitute for lactose broth in standard procedures, one group of observers employed brilliant-green bile for confirmation of lactose broth and lauryl broth enrichment gas-positives. The proportions of the water sample portions examined (1,732) which were indicated by the various methods of confirmation to contain coliforms are shown in Table 63, which was taken from the report by McCrady (1943). More detailed results are given in Table 64. Evidently, here again the results from the use of either of the enrichment broths followed by confirmatory brilliant-green bile compared very favorably with those obtained from the completed test regardless of whether lactose broth or lauryl broth was used, or how each was used, in the ordinary standard procedure.

Because of the generally favorable reports of its usefulness as a confirmatory method, the Committee on Standard Methods (1944) extended the application of the confirmed test, employing either the solid media, E.M.B. or Endo agar, or the liquid medium B.G.B., 2 per cent, to the routine examination of all waters, including finished

TABLE 64. RESULTS OF COMPARATIVE TESTS OF LAURYL SULPHATE BROTH AND LACTOSE BROTH

(McCrady, 1943)

	Primary Medium	No. of Sample Portions Planted	Portions Gas-positive Per Cent	Complete Confirmations Using Secondary		Gas in B.G. Bile upon Transfer from Primary Per Cent
				L.B. Per Cent	L.S.T. Per Cent	
Raw						
12 laboratories	LB	750	66.9	46.5	45.7	....
139 samples	LST	750	64.1	48.8	48.8	....
12 laboratories	LB	695	67.2	46.8	45.8	46.8
128 samples	LST	695	64.0	48.1	48.1	....
9 laboratories	LB	515	71.3	47.2	45.6	48.4
98 samples	LST	515	65.2	48.2	46.8	47.6
Unfinished						
10 laboratories	LB	535	50.7	15.3	15.3	....
99 samples	LST	535	21.1	15.9	16.3	....
10 laboratories	LB	517	50.1	15.5	15.5	17.4
95 samples	LST	517	21.7	16.2	16.6	....
8 laboratories	LB	481	53.4	16.6	16.6	18.7
92 samples	LST	481	21.8	17.5	17.7	18.9
Finished						
9 laboratories	LB	434	37.6	10.8	11.1	....
77 samples	LST	434	16.1	11.8	11.5	....
8 laboratories	LB	410	39.3	11.5	11.7	12.0
73 samples	LST	410	16.3	11.7	11.5	....
6 laboratories	LB	332	41.3	10.2	10.8	11.1
60 samples	LST	332	16.9	12.3	11.4	13.9
Pools						
5 laboratories	LB	310	35.8	17.7	16.8	....
58 samples	LST	310	24.5	19.4	20.0	....
3 laboratories	LB	90	38.9	31.9	31.9	30.0
15 samples	LST	90	37.8	34.4	34.4	....
3 laboratories	LB	78	43.6	34.6	34.6	33.3
13 samples	LST	78	41.0	38.5	38.5	38.5
Wells, Springs						
7 laboratories	LB	1,195	61.0	44.5	45.8	....
200 samples	LST	1,195	66.0	50.7	52.3	....
6 laboratories	LB	1,099	60.9	44.9	46.2	48.0
184 samples	LST	1,099	66.3	51.3	52.9	....
3 laboratories	LB	308	69.1	48.7	46.8	55.8
52 samples	LST	308	76.6	56.8	56.2	58.8
Miscellaneous (Type Not Reported)						
6 laboratories	LB	191	50.8	27.2	36.6	....
32 samples	LST	191	51.8	28.3	37.2	....
5 laboratories	LB	185	51.9	28.1	37.8	37.8
31 samples	LST	185	53.5	29.2	38.4	....
2 laboratories	LB	18	44.4	33.3	27.8	27.8
3 samples	LST	18	55.6	44.4	44.4	44.4
Sea Water						
1 laboratory	LB	40	45.0	40.0	42.5	45.0
10 samples	LST	40	55.0	50.0	50.0	....

waters and chlorinated sewage effluents, but stipulated that "laboratories responsible for the quality of the raw or finished waters supplied to large communities shall apply the Completed Test, if not exclusively to these raw or finished waters, at least to such a proportion of samples as to establish beyond reasonable doubt the applicability to them of the Confirmed Test."

Standard Routine Test for Bacteria of the Coliform Group. From the multiplicity of media and methods that have been proposed for use in the coliform test the Committee on Standard Methods (1944) has chosen those which appear to be best adapted to the generality of waters of this continent. Although the use of other procedures may in certain very special circumstances be justified, it is most important to ensure the greatest possible measure of uniformity in order that results obtained by different observers be reasonably comparable. Such uniformity is best attained by close adherence to the procedures of the present Standard Methods, with such modifications as may be incorporated therein in the future.

The Standard Methods Committee prescribes that graduated quantities of the water sample be planted in lactose or lauryl broth and the culture incubated at 35° to 37° C. for 48 ± 3 hours. It is essential that the quantities and concentrations of the enrichment broth be such that the concentrations of the ingredients in the mixture of broth and water sample are practically identical with those of the basic formula of the broth; ordinarily 1 ml. or less of sample may be added to 10 ml. of broth of normal (*N*) concentration; 10 ml. of sample to 10 ml. of 2 *N*, or 20 ml. of 1½ *N* broth; and 100 ml. of sample to 50 ml. of 3 *N* or 100 ml. of 2 *N*, or 200 ml. of 1½ *N* broth; as a rule better results are obtained with the less concentrated broths. The formation of any amount of gas in the fermentation tube within 24 ± 2 hours at 35° to 37° C. constitutes a positive presumptive test; the formation of no gas within 48 ± 3 hours constitutes a negative test; and any other finding constitutes a doubtful test which must be investigated further.

The confirmed test is to be performed in either of two ways. (*a*) An E.M.B. or Endo plate is inoculated with a trace of the gas-positive enrichment culture as soon as possible after gas has appeared, and incubated at 35° to 37° C. for 24 ± 2 hours. If typical *E. coli* colonies appear on the plate, the confirmed test is positive; if not, at least two of the colonies most closely resembling those of coliforms must be submitted to the completed test. If no colonies whatever appear upon the plate, the confirmed test is negative. (*b*) A 3-mm. loopful of the gas-positive enrichment culture is transferred, as soon

as possible after gas has appeared, to a fermentation tube containing B.G.B., 2 per cent, which is then incubated at 35° to 37° C. for 48 ± 3 hours. If any gas appears in the tube the confirmed test is positive; if no gas appears, it is negative.[1] Because of its simplicity and convenience, this method of performing the confirmed test is ordinarily the one to be preferred.

The completed test is to be applied either to the original gas-positive enrichment culture or to the gas-positive B.G.B. confirmatory culture. An E.M.B. or Endo plate is inoculated with a trace of either of these cultures as soon as possible after gas has appeared, and incubated at 35° to 37° C. for 24 ± 2 hours. If no colonies appear upon the plate the completed test is negative. If colonies do appear, one or more colonies typical of *E. coli* or, if none of these is present, two or more colonies most closely resembling those of coliforms, are each transferred both to an agar slant and a lactose or lauryl broth fermentation tube which are then incubated at 35° to 37° C. until gas formation is observed in the latter, although the time of incubation must not exceed 48 ± 3 hours. If gas formation is observed, a gram-stained preparation of the corresponding agar slant culture is examined microscopically. The formation of gas in the fermentation tube and the demonstration of gram-negative, non-spore-forming bacilli in the agar culture constitutes a positive completed test, considered to be proof of the presence of coliform organisms. The absence of gas formation in the tube, or the failure to find gram-negative, non-spore-forming bacilli constitutes a negative test. If spore-forming organisms are found in the agar slant culture, a trace of it is transferred to a fermentation tube containing formate ricinoleate broth which is incubated at 35° to 37° C. for 48 ± 3 hours; and if gas appears, a trace of the latter culture is transferred to lactose or lauryl broth and to an agar slant which are incubated at 35° to 37° C. for 48 ± 3 hours and examined. Gas formation in the tube and absence of spore formers in the agar slant culture are considered to constitute a positive completed test; if spore formers are found in the agar culture, however, the test is recorded as negative.

It is to be noted that the Standard Methods Committee recommends that *all* enrichment gas-positives be submitted to the confirmed test or the completed test when either of these tests is necessary, but states

[1] In accordance with the suggestion of Dr. F. E. Hale, brilliant-green bile cultures that are found to be decolorized should be *immediately* transferred to some inhibitive broth (formate ricinoleate may be used) to eliminate spore formers (*C. welchii*) which are often responsible for such change in color. If gas appears in this broth within 48 hours, the indication should be confirmed by the completed test.

that it is permissible, in routine work, to submit to the confirmed test or the completed test, whichever is necessary, all the enrichment gas-positives of the two highest dilutions (containing the smallest portions of sample) of sample that have produced gas. Thus, if each of three dilutions is planted in 5 enrichment tubes and gas appears in 2 of the highest dilution, 3 of the middle dilution, and 4 of the lowest dilution, only the 2 of the highest and the 3 of the middle dilution need be confirmed or completely confirmed. All other enrichment gas-positives, however, such as the 4 of the lowest dilution in the example just given, must be recorded as containing coliform organisms even though all the confirmed or completed tests made prove to be negative. Consequently, in order to avoid debiting a water with an unnecessarily excessive coliform content, it is usually advisable to confirm or completely confirm *all* enrichment gas-positives.

The Standard Methods Committee indicates the types of water to which the presumptive, confirmed, and completed tests are applicable as follows:

PRESUMPTIVE TEST: sewage, sewage effluent (except when chlorinated), water known to be heavily polluted, water not to be used for drinking water; also raw water in a purification plant if this test appears to give reasonably precise results.

CONFIRMED TEST: waters to which the presumptive test is apparently not applicable; routine samples of drinking water, unfinished and finished waters; chlorinated sewage effluents.

COMPLETED TEST: waters to which the confirmed test does not appear to be applicable; raw or finished waters supplied to large communities, or to such a proportion of these waters " as to establish beyond reasonable doubt the applicability to them of the Confirmed Test."

Technical Practices and Devices. Before leaving this subject of the technique of the coliform test attention may be directed to certain technical practices and devices that should be of particular value to laboratories in which a large volume of work is done. Archambault and McCrady (1942) showed that liquid media in fermentation tubes when held at a low temperature may dissolve so much air that a bubble of gas may appear in the inverted vial or closed arm of the tube when the latter is incubated at 37° C. Since such an air bubble might mistakenly be recorded as an indication of gas formation by coliform organisms, all fermentation tubes should be stored at a higher temperature, preferably about 25° C., in order to eliminate this source of error; but as evaporation, with resultant alteration in the concentrations of the broth ingredients, may be excessive at this temperature, the tubes should be held before use no longer than one

week. If for any reason fermentation tubes must be stored at a low temperature, they should be incubated overnight before use and the tubes containing air bubbles discarded.

To facilitate the preparation of multiple fermentation tests, McCrady (1915) described a shallow zinc box holding two rows of 5 tubes each of flat-bottomed specimen tubes, with inverted vials, which constitutes a battery of fermentation tubes; a shallow zinc cover is fitted with a strip of eiderdown which, when the cover is in place and fastened with a rubber band, serves as a cotton plug for all the tubes; the open space between the two box sections permits observation of gas formation in the tubes. Various modifications of this device have been described, notably those by Wells (1918*a*) and by Ziegler and Halvorson (1935) for use with large culture tubes.

Cowles (1939) has shown that a culture tube or a bottle of sterile liquid medium may be converted into a fermentation tube ready for use by dropping into it a sterilized glass tube bent in a special manner, the air in the tube being immediately displaced by the medium. Such a device may be of particular utility in handling water samples planted in enrichment broth in the field, for the equivalent of the inverted vial can be added at any subsequent time as, for example, when the mixture of sample and broth has been received at the laboratory.

McCrady (1920) described a means of controlling the operation of purification plants that are some distance from a laboratory; it comprises the examination of four 5-ml. samples of the water supply sent by mail each day to the central laboratory. A special mailing case (such as is used for shipping sputum specimens) holds four cork-stoppered specimen tubes, each containing 2 ml. of concentrated brilliant-green bile, 2 per cent, to which is added 5 ml. of water sample from a tap on the distribution system by filling to a 7-ml. mark on the tube. Sterile inverted vials are inserted when the samples reach the laboratory, the tubes are inclined to permit the liquid contents to fill these vials, and after 48 hours of incubation the formation of 10 per cent of gas is recorded as a positive test. This presumptive method (with confirmation if necessary) has been used by one of us (M.H.M.) as a means of state control of plant operation, supplementary to that of routine supervision, for over twenty-five years — during 1944 these daily samples were received from approximately 130 supplies — and has proved of inestimable value for discovering lapses in operation and for directing the efforts of control officers to those plants that require immediate attention. A small but important detail has been added to the original specifications for the preparation of the outfit

employed: since even protracted autoclaving occasionally fails to destroy mold spores contained in the interstices of the cork stoppers, these openings are plugged by dipping the lower part of the stoppers in hot melted paraffin after the tubes (with corks in place) have been autoclaved; upon the adoption of this procedure practically no further difficulty from mold growth was experienced.

Possibilities for the Future. In the foregoing pages an account has been given of the development during the past sixty years of the coliform test used on this continent, from the plating procedure, with or without preliminary enrichment in glucose broth, and identification of coliform organisms which was employed some time before the turn of the century, to the present procedure of enrichment in the more selective lactose media with a variety of methods of confirmation. We have seen that in the course of this development presumptive methods, including the use of glucose broth, lactose bile, and numerous other media, were tried and found wanting; that the reaction to this experience was a reversion to the complicated procedure involving enrichment, isolation, and identification, known as the completed test, which is bacteriologically sound but impracticable for use in routine control work where numerous samples have to be examined; and that an intensive effort on the part of laboratory groups as well as of individual observers to relieve the situation finally resulted in the far more flexible and practical procedures of the present Standard Methods recommendations.

Although adequate laboratory control of the quality of some water supplies may still require the use of the arduous completed test, the frequency with which it must be performed can usually be markedly reduced by employing either the selectivity of lauryl broth or the screening effect of interposed brilliant-green bile confirmation, or both. The coliform density of the great majority of waters, however, may be satisfactorily determined by means of the confirmed test using brilliant-green bile which, when combined with lauryl broth enrichment, constitutes a comparatively simple and foolproof method that is reasonably rapid in producing final results. Instead of the 3 to 5 days or more required by the use of the completed test with enrichment gas-positives, 2 to 4 days now usually suffice for an examination, and the labor of confirmation is so reduced by the use of this procedure that the increasing demand for greater frequency of laboratory examination of water supplies may be reasonably satisfied.

Despite the improvements that have been effected in the coliform test in the past few years, however, it is still far from perfect.

Peculiar and erratic results are occasionally encountered that cannot be satisfactorily explained; duplicate examinations of the same sample at times yield discrepant results which are certainly not due solely to irregular distribution of coliform organisms in the sample; and the demand for a rapid, generally applicable *presumptive* test that will furnish fairly accurate results remains unsatisfied. Improvements in the coliform test are therefore still greatly needed. To fill this need old fields of research must be reëxamined and newer fields, such as those of accessory growth factors and antibiotics, must be explored. It is hardly conceivable that from the volume of current research on bacterial selection and inhibition some useful suggestions for the improvement of the coliform test may not be forthcoming.

A word regarding the experimental procedure to be employed when evaluating the utility of a new medium or a new procedure for coliform detection may not be amiss. Too great reliance should not be placed upon the results obtained with pure cultures, for time and again past experience has shown such results to be entirely misleading. The behavior of coliforms when associated with other organisms is often very different from that of pure cultures. Natural, unfinished, and finished waters should therefore be employed when trying out new media or methods; well and spring waters and chlorinated supplies containing few coliform organisms are particularly useful for this purpose because they often very severely tax the sensitivity and selectivity of our present procedures. Furthermore, experience has amply demonstrated that the coliform and associated bacterial floras of one locality may differ markedly from those of another in their response to a given cultural environment; consequently, if possible, the collaboration of several widely separated observers should be sought when attempting to determine whether a procedure promises to be of general utility. Probably no method fully satisfactory for the examination of all waters will ever be found, but a *standard* method should be applicable to the very great majority of them.

The precision of coliform procedures employing the dilution method of examination should not be overstressed. Inherent in this method, which appears at present to be the only practicable means of examining large volumes such as 50 and 100 ml. of sample, there is a rather surprising facility of error due to irregular distribution of organisms in the well-mixed sample. It will be shown shortly that, because of this error of simple sampling, the odds are only about 32 to 1 that the coliform estimate of a sample, obtained from the use of even 40 tubes with each of 3 dilutions, lies within the range of about 60 to 150 per cent of the true coliform density; only repeated examination will

permit a reduction of this sampling error to a reasonable magnitude. Furthermore, we have found that the completed test in one extensive series of examinations failed to recover coliforms from an average of about 14 per cent of enrichment tubes containing them, and some laboratories have reported a similar failure of 20 to 30 per cent. Consequently, since present coliform procedures yield only very rough estimates of the number of coliform organisms actually present in a sample, too great precision should not be demanded of methods that promise substantially to reduce the time and effort required by the present standard procedures.

Some years ago, at a time when the completed test was required for the examination of all drinking water, one of us (Winslow, 1934) stated his conception of the attitude that should be adopted toward the coliform test in the following words.

> We have used the colon [coliform] test in its standardized form so long that it has become something sacred and untouchable; and that is always a dangerous frame of mind smacking of religion rather than of science. Let us remember that this is a test devised by plain human beings like ourselves and that it was designed to differentiate between safe and unsafe water supplies. The colon bacillus in itself has no inherent significance except as an index of sanitary quality. The isolation of all the colon bacilli present is of no importance except as an index of sanitary quality. What we want is a test which shows the greatest possible difference between good waters and bad waters, and that is all we want.
>
> The use of lactose-broth tubes followed by isolation and identification has, after all, no Divine sanction. It may be that the newer procedures correspond more closely than the old ones to actual sanitary conditions.
>
> My plea is for a fundamental reëxamination of this whole problem with an open mind, and with a clear view of the practical results to be obtained, and of the margin of error associated with their interpretation. The pressing need for economy and efficiency makes such a reëxamination peculiarly imperative at the present time. If we can cut down the cost of an examination we can make more examinations; and a fuller measure of control should vastly overweigh any 5 or 10 per cent loss in accuracy.

We believe that, in view not only of the broad range of practical interpretation of coliform results but also of the inaccuracy of current methods, the choice of standard coliform procedures should be determined by their practicability as well as by their accuracy; it avails little that a method yield precise results if it is so complex and laborious that it cannot be generally applied. The Committee on Standard Methods has already indicated its concurrence in this belief by waiving its former general requirement of isolation and identification of coliform organisms and by considerably simplifying its standard

procedure; and now that this breach has been effected it is probable that other methods, less orthodox, but more practicable than the completed test, will be regarded with more favor in the future than they have been in the past. Eventually, an ideal coliform test may be achieved, the essentials of which may be postulated as follows.

1. It should yield reasonably uniform results with the great majority of waters; otherwise, the significance of the results may vary with different water supplies.

2. It should require a minimum of judgment of the technician lest speculation influence the character of the results obtained.

3. It should require a minimum of time, skill, labor, and material in order that results may be secured rapidly and at the least possible cost.

4. Its accuracy should be such as to satisfy the purpose for which the procedure was designed, viz., to distinguish between safe and unsafe waters; greater accuracy, if occasionally required, can be secured by multiple examination or by the use of special procedures.

Quantitative Statement of the Results of the Coliform Test. The plate method yields results which may be expressed, with greater or less accuracy, in direct quantitative terms. The results of the coliform test, on the other hand, give us merely the information that coliforms are absent from a certain amount of water or are present in a given proportion of samples of a certain volume. In 1907 Phelps suggested that the reciprocal of the highest dilution in which a positive result is obtained should be used as the expression of the number of coliforms present in the sample under examination. If a negative result were obtained in one dilution and a positive result in a higher dilution, the two results should be transposed. Thus, if positive results were obtained in 1.0 ml. and 0.1 ml. with a negative result in 0.01 ml. we should say there were present 10 coliforms per milliliter. If the 1.0-ml. and 0.01-ml. tests were positive and the 0.1-ml. test negative we should reverse the two latter figures and again say there were 10 coliform organisms per milliliter. This procedure was recommended in the 1917 Standard Methods and remained the only official method for expression of coliform results until the publication of the 1936 Standard Methods.

Since more than one gas-producing organism may have been present in the water portion planted in an enrichment tube in which gas is produced, it is clear that this method of calculating the coliform density is incorrect. McCrady (1915) pointed this out and showed that the frequency of appearance of gas-producing organisms in the portion drawn from a sample is an exponential function of the number

of such organisms in the sample. For example, if 5 tubes are planted each with 1 ml. of sample and 4 of them are found to contain coliforms, the most probable density of these organisms per 100 ml. of sample is not 80, as might be inferred, but 160. It was demonstrated that for any combination of results obtained by testing one or more portions of one or several dilutions of sample, a single Most Probable Number (M.P.N.) per 100 ml. of sample is the density of organisms most likely responsible for that combination of results. This density may not correspond to the density actually present in a given sample, but in the long run the most probable numbers will represent more closely than will any other series of numbers the densities of gas-producing organisms in the samples examined. With the aid of Professor W. D. Cairns of Oberlin University, Ohio, a method of arriving at a conception of the accuracy of the M.P.N. was also developed.

Wolman and Weaver (1917) showed how, by a few approximations, McCrady's basic equation could be more readily solved; and McCrady (1918), making use of these suggestions, presented a series of tables of computed most probable numbers corresponding to various fermentation tube results that might be expected in practice, and also a general table of factors by means of which any result obtained from the usual geometric series of dilutions may readily be computed. Some of these tables were later incorporated in the British equivalent of Standard Methods (Committee, Ministry of Health, 1934 and 1939).

In the meantime, Greenwood and Yule (1917) and Stein (1919 and 1921), both apparently unaware of McCrady's work, were studying the same problem. Greenwood and Yule employed essentially the same reasoning that McCrady used, starting with a basic equation which the latter had already presented. In their development, however, they introduced a simplification, which Stein also employed, which has been generally adopted in later investigations of the problem. McCrady had estimated the M.P.N. of organisms in a volume of 100 ml.; Greenwood and Yule estimated the M.P.N. of organisms per 100 ml. in a volume of practically infinite size which might be considered the water supply itself from which the sample of, say, 100 ml. was taken. Wolman and Weaver's modification of McCrady's equation had the effect of introducing the same idea. Stein approached the problem from the viewpoint of what corresponds to the method of " maximum likelihood " estimates of Fisher (1921); he derived equations and presented graphs for interpreting single-dilution results and estimating their accuracy. The most probable numbers computed from the equations offered by Greenwood and

Yule, and by Stein, are practically identical with those computed from McCrady's equation; an occasional difference of unity in the last digit arises from the difference in the method of approach or the mathematical treatment employed.

Halvorson and Ziegler (1933*a*) derived still another equation for "most probable values," which is similar to that of Greenwood and Yule, and provided tables of factors which facilitate solution of the equation. Since the interpretation of dilution results finds an application in many biological problems, a considerable literature on this subject, which is reviewed by Eisenhart and Wilson (1943), has accumulated during the past twenty-five years.

The solution of the equations proposed for determining the M.P.N. is rather laborious unless tables of factors to facilitate the calculation are available. Consequently tables of computed M.P.N.'s corresponding to various fermentation tube results have been prepared by McCrady (1918), Reed (1925), Halvorson and Ziegler (1933*a*), Hoskins (1933 and 1934), and Swaroop (1938). Hoskins' series provides for the widest variety of combinations of tubes and dilutions, and certain of his tables are included, together with the requirement that the M.P.N. method of computing coliform densities be used for multiple-tube results, in the 1936 edition and in the latest revision of Standard Methods. For single-tube results, the dilution-reciprocal method of Phelps is retained, since it appears to serve as well as, if not better than, the M.P.N. method when an average figure for a long series of such results is desired. The fact that the number of organisms in a sample is a logarithmic function of the frequency of their appearance in the portion drawn for examination causes the average of M.P.N.'s to be somewhat inaccurate, especially those of single-tube results. When short series of single-tube results are averaged, however, the dilution-reciprocal method may yield very inaccurate results; hence the advantage of employing with each dilution multiple tubes which yield a result from which a more accurate M.P.N. may be computed from the result of each individual examination.

Several investigators have demonstrated by experiment that the most probable number expresses most satisfactorily the results of fermentation tube tests. Thus, Butterfield (1933*a*) and Ziegler and Halvorson (1935) compared the use of agar plates and that of fermentation tubes for estimating bacterial densities in suspensions of pure cultures. In both of these studies the estimates obtained by means of the most probable number compared very closely with those derived from plate counts.

In the Appendix will be found a table of most probable numbers

calculated by Hoskins (1934); it interprets fermentation tube results obtained from the use of 5 tubes with each of 3 dilutions in the usual geometric series. This table is similar to the one in Standard Methods. On page 252 is reproduced Hoskins' table, taken from the report of the Advisory Committees on Official Drinking Water Standards (1943), of M.P.N.'s for results obtained from five 10-ml. portions of sample and for those obtained from five 100-ml. portions. Since the loss, through breakage or other cause, of one or more tubes occasionally leaves the analyst with a combination of tube results not to be found in published tables, a table of factors prepared by one of us (McCrady, 1918), which permits rapid arithmetical calculation of the M.P.N. corresponding to any fermentation tube result obtained from dilutions in the usual geometric series, is also included in the Appendix.

Accuracy of the Most Probable Number. The accuracy of the most probable number has been the subject of numerous investigations, mathematical as well as experimental. Halvorson and Ziegler (1933*b*, and 1933*c*) and Swaroop (1938, 1940, 1941), are among those who have given it special attention. Halvorson and Ziegler showed that the accuracy of the M.P.N., when derived from single-dilution results, is dependent both on the number of tubes used and on the bacterial density, and that the accuracy cannot be closely estimated unless this density is known beforehand. They found, however, that when 3 or more dilutions are employed, a fair estimate of the accuracy of the M.P.N. is possible. They (Halvorson and Ziegler, 1933*c*) calculated the frequencies with which different possible combinations of tube results would be obtained if 10 tubes were used with each of 3 dilutions in the usual geometric series in the repeated examination of suspensions of certain bacterial densities varying from 0.15 to 1.5 per ml. From these and the M.P.N.'s corresponding to the various expected combinations of results, frequency distribution curves were constructed to show the frequency of occurrence of percentage deviations of the M.P.N. from the mode (the mode, of course, corresponding to the assumed bacterial density). The curves were found to be very similar (and only moderately skewed), and the calculated coefficients of variation practically constant; this indicated that when 3 dilutions are employed the accuracy of an observed M.P.N., for the range considered, is practically independent of the bacterial density.

Eisenhart and Wilson (1943) pointed out that the standard deviation (from the mode) of the M.P.N., computed by Halvorson and Ziegler in their study, hovers very closely around 45 per cent of the assumed density and that the standard deviation of the logarithm of

the M.P.N. is nearly a constant. They showed further that, since the distribution of the *logarithmic* values of the M.P.N. is more nearly symmetrical than that of the values of the M.P.N., a closer estimate of the accuracy to be attributed to the M.P.N. may be obtained by assuming the logarithm of the M.P.N. to be normally distributed, with a median limiting standard deviation of 0.166 (for 10 tubes), about the logarithm of the assumed density than by considering the standard deviation of the M.P.N. itself. Eisenhart and Wilson propose the use of this method for estimating the confidence with which an observed M.P.N. may be regarded. They suggest, for example, that 10 tubes be run with each of a series of dilutions (say, 10 ml., 1 ml., and 0.1 ml.) and that the M.P.N. per milliliter corresponding to the result be taken from one of the various published tables. If to the logarithm of this M.P.N. there is added and subtracted 1.96×0.166, the corresponding antilogarithms should indicate the confidence interval within which, with a probability of about 0.95 (odds of 19 to 1), the actual coliform density lies. (It will be recalled that ± 1.96 times the standard deviation gives the range which includes about 95 per cent of a normal distribution.) From a table prepared by Eisenhart and Wilson certain figures are reproduced in Table 65 which show some results of applying this method of calculation.

TABLE 65

Tube Result	M.P.N. per ml.	0.95 Confidence Intervals (By use of logarithm of M.P.N.)
10/10 — 7/10 — 3/10	1.53	0.72 to 3.24
8/10 — 5/10 — 1/10	0.267	0.126 to 0.563
4/10 — 2/10 — 1/10	0.086	0.041 to 0.182

Swaroop (1938) derived the following expression for obtaining the standard deviation of the estimate of the bacterial density when several dilutions are used. As Eisenhart and Wilson (1943) have pointed out, it gives accurate results only when the number of tubes employed is large.

$$\frac{1}{\sigma_n^2} = S\left(\frac{s_x E_x}{a^{2x}}\right)$$

where σ_n is the standard deviation of n (corresponding to the M.P.N.); s is the number of tubes planted with each dilution; x is the stage of dilution such as 1, 2, etc.; a is the dilution factor (usually 10); E_x is $\frac{1}{e^{n/ax} - 1}$ (where e is the Naperian logarithm base); and S indicates a

summation of the various terms corresponding to the various dilutions. Employing this equation, Swaroop (1940) found the coefficient of variation or percentage standard deviation $\left(\frac{100n}{\sigma_n}\right)$ to remain fairly constant over wide ranges of n except when the latter is very small. In Table 66 are shown some of Swaroop's calculations of the coefficient of variation when different dilution systems are employed. For the sake of brevity, only integral values are given.

TABLE 66. THREE DILUTIONS (as 0.1, 0.01, 0.001 Milliliter)

Tubes with Each Dilution	Coefficient of Variation for Various Values of n (the M.P.N.)					
	$n = 1$	$n = 2$	$n = 3$	$n = 5$	Approximate Range with Greater Values of n	Approximate Average of This Range
1	307	222	186	151	110–150	130 or less
3	177	128	107	87	64–87	75 or less
5	137	99	83	67	49–67	60 or less
20	...	..	..	..	.....	30 or less
200	...	..	..	..	.....	less than 10

Some conception of the sampling error involved when 5 tubes are used with each of 3 dilutions may also be had from the calculations, by Halvorson and Ziegler (1933c), of the frequencies with which various combinations of results are given by this system. Assuming a bacterial density of 1.5 per ml., these authors found that 97 per cent of the data (odds of about 32 to 1) were included in the range of approximately 30 to 360 per cent of this density. In other words, about once in 33 trials a suspension of this density may be expected to yield an M.P.N. above or below this range. The corresponding range, if 10 tubes were used with each dilution, was found to be 42 to 230 per cent of the assumed density; if 20 tubes were used, 50 to 174 per cent; and if 40 tubes were used, 62 to 147 per cent. Since, according to Halvorson and Ziegler, the accuracy of an observed M.P.N. is not much affected by the bacterial density when 3 dilutions are employed, they propose the use of these ranges for determining the accuracy of M.P.N.'s obtained from suspensions of unknown density. An example follows.

Employing 40 tubes with each of 3 dilutions (0.01 to 0.0001 ml.) of a suspension, there is obtained the result: $\frac{40}{40}$, $\frac{27}{40}$, $\frac{2}{40}$. The M.P.N. corresponding to this result is found, from M.P.N. tables, to be 999 bacteria per milliliter. As the range for odds of 32 to 1 is, as indicated above, 62 to 147 per cent of the true value, the M.P.N. may be too low by 38 per cent of this value

and too high by 47 per cent of this value. Solution of the following two equations determines the limits of this range.

$$x_1 + .47x_1 = 999 \qquad x_1 = 680$$
$$x_2 - .38x_2 = 999 \qquad x_2 = 1{,}611$$

We may conclude, therefore, that the odds are about 32 to 1, or the probability is 97 per cent, that the true bacterial density is within the range 680 to 1,611 per ml.

For approximate estimation of the accuracy of most probable numbers, where 3 dilutions are employed and when the dilutions have been properly chosen, this method should yield satisfactory results.

From the above brief discussion of the accuracy of the most probable number, certain conclusions may be drawn:

1. A result obtained from the use of 1 tube with 1 dilution is of practically no significance. If the result is positive, it means simply that one or some coliforms are present in an *unknown* volume. On the other hand, even if a water contains 160 coliforms per 100 ml., a negative result from testing 1 ml. may be expected 20 per cent of the time.

2. A result obtained from the use of 1 tube with each of 3 dilutions is not much more informative. It should be employed only as an explorative procedure. If the indicated coliform density is very low for the dilutions employed, the coefficient of variation is, as shown in Table 66, of a very high order.

3. A result secured by using several tubes with a single dilution is fairly satisfactory provided the proper dilution is employed and provided a sufficient number of tubes is used. When only a few tubes are employed the probability of securing a satisfactory result is greatly reduced unless the choice of dilution for the test has been a very fortunate one. The accuracy of M.P.N.'s depends on the bacterial density. When a very favorable proportion of tubes are positive (around 70 per cent) the accuracy may approach that when 3 dilutions are employed.

4. The use, in practical work, of 2 dilutions serves two purposes: it favors the chance of hitting on the proper dilution, and it usually yields more information than the single dilution regarding the density of the organisms sought. The accuracy of an M.P.N. obtained by this method may, when the proportion of positives is more or less favorable, approach that when 3 dilutions are employed. Preferably at least 5 tubes should be used with each dilution.

5. There is considerable practical advantage in employing several tubes with each of 3 or more dilutions. This procedure not only pro-

vides greater assurance of securing results from which M.P.N.'s may be computed, but the accuracy of the M.P.N.'s obtained by means of it can also be fairly satisfactorily estimated. For ordinary routine control of water supplies a minimum of 5 tubes may be used with each dilution, but for special investigations 10 or 20 tubes or more may be required with each dilution. An effort should always be made to choose such dilutions that both positives and negatives will occur in the middle dilution, because a result of this type furnishes the most accurate M.P.N. For M.P.N.'s corresponding to results obtained by using large numbers of tubes, published tables may be consulted or the corresponding M.P.N. may be calculated from the Table of Factors which will be found in the Appendix.

6. Because of the marked inaccuracy of the dilution method when 5 or fewer tubes are employed with each dilution, any tendency toward fictitious accuracy in expressing the result should be discouraged. Most probable numbers corresponding to results obtained with such small numbers of tubes may be expressed with more than sufficient accuracy by one or, at most, two significant figures with the second figure to the nearest 5.

It is of some interest to compare the accuracy of bacterial density estimates obtained from plate counts and those calculated from fermentation-tube results. It has been seen in Chapter III that the percentage standard deviation, or coefficient of variation, of individual plate counts may range under fairly favorable conditions from ± 10 to ± 20 per cent. On the other hand, Table 66 shows that, by using 3 dilutions and 5 tubes with each dilution, the coefficient of variation of the M.P.N. under favorable conditions averages close to 60 per cent. According to this table apparently some 50 to 100 tubes with each of 3 dilutions would be required to furnish an estimate equal in accuracy to a single satisfactory plate count.

Wells (1918*b*, 1919*a*, 1919*b*) and Wells and Wells (1922) proposed the use of the geometrical mean for interpreting fermentation-tube results but Cairns (1918) and others do not consider this method advantageous.

Expression of Results Obtained over a Period of Time. Whether the bacteriological data relative to the quality of a water are obtained in terms of plate counts or of most probable numbers of coliforms, some means of expressing the totality of the data accumulated over a period of time, such as a year, is desirable. Particularly is it important to indicate in some readily comprehensive manner the performance, from a bacteriological standpoint, of a water purification plant. The average, the geometrical mean, and the median are

of no value for this purpose because the sanitarian is interested particularly in the frequency of occurrence of the *higher* numbers of bacteria and coliforms. Wolman (1918, 1920), approaching this subject from the standpoint of the practical operator of a treatment plant, suggested the construction of graphs showing the relation between the weekly averages of the number of bacteria or of coliforms in the raw water and in the effluent. The resulting curves, if the purification plant performance is consistent, are practically straight lines when the densities are plotted on a logarithmic basis. The general equation of these curves is $y = x^c$, where y and x are, respectively, the raw water and final effluent 20° counts, and c is a constant for the particular plant, which Wolman found, from a survey of 19 rapid sand filtration plants, to average over 2.5 for the year.

Streeter (1922), in his investigation of the efficiency of Ohio River plants, used this method and found that, upon grouping the 20° counts, the 37° counts, or the coliform densities of the raw water, and plotting against these groups the coincident effluent estimates on a logarithmic basis, the correlated values followed closely a straight line, the equation for which assumed the general form $E = cR^n$ in which E represents the effluent content, R the raw water content, and c and n constants defining in a general way the average efficiency of purification and the relative constancy of the effluent content under different loadings respectively. A high value of c indicates a low average efficiency, and a high value of n low uniformity of the effluent under varied loadings. It will be noticed that this equation is similar to Wolman's; but the constant c, which Wolman found to approach very closely unity in his Maryland experience and therefore disregarded, is included in Streeter's equation because it attained the significant values of 4.41 for the 20° count, 0.23 for the 37° count, and 0.29 for the coliform estimate. The corresponding values of n were 0.27, 0.55, and 0.30.

An International Joint Commission adopted, as a guiding principle for the regulation of the pollution of international boundary waters between the United States and Canada, a standard providing that the average yearly loading, with respect to the coliform content of the raw water delivered to filtration plants on the Great Lakes, should not exceed 500 coliforms per 100 ml., if the plants were to produce effluents satisfying the Treasury Department standard (2 coliforms per 100 ml.) for water supplied to common carriers. Streeter calculated, from his general formula for the performance of Ohio River plants, that these could produce an effluent satisfying the Treasury Department requirements if the coliform content did not exceed 650 per 100 ml.

The close correspondence of these two standards is, as Streeter observes, most interesting because they were derived differently and were based on data obtained from very different waters.

Pursuing still further this method of determining limiting coliform standards for treatment plant loadings, Streeter (1933), after a series of experimental studies of water purification and a review of the performance of certain Ohio River, other middle western, eastern, and Great Lakes water purification plants, selected the raw-water coliform maxima shown in Table 67 to represent the limiting loadings which would permit plants of the more efficient types to produce effluents meeting the primary requirement of the *revised* Treasury Department standard (1 coliform organism per 100 ml.).

TABLE 67. LIMITING RAW-WATER COLIFORM INDEX PER 100 MILLILITERS (Streeter, 1933)

Treatment	Limiting Index	
	Ohio River	Great Lakes
1. Chlorination alone	80	50
2. Coagulation, sedimentation, and rapid sand filtration (without chlorination)	80	60
3. Same as 2 with pre-chlorination	3,500	*
4. Same as 2 with post-chlorination	6,000	4,500
5. Same as 2 with both pre-chlorination and post-chlorination	20,000	*
6. Same as 4 with double-stage sedimentation (relatively long sedimentation period)	60,000	*

* No observations.

Later observations of plant performance with highly polluted waters, however, led Streeter (1939) to conclude that pollution indicated by 5,000 coliforms per 100 ml. represents the approximate deadline between raw-water pollution from which *average* Ohio River and Great Lakes plants can produce filtered-treated effluents conforming to the Treasury Standard of an average not exceeding 1 coliform per 100 ml., and raw-water pollution from which they cannot produce such an effluent. Similarly, he considered that a pollution indicated by 50 coliforms per 100 ml. represents the corresponding limit when chlorination alone is employed with Great Lakes waters. Referring to proposals that accepted standards for treated water be made more rigid, Streeter stated that lowering the finished-water coliform standard from 1 to 0.5 per 100 ml. would probably reduce the permissible raw-water pollution limit from 5,000 to about 1,500 per 100

ml.; and lowering it still further to 0.2 per 100 ml. might reduce the raw-water limit to a level of approximately 200. As Streeter pointed out, " zero coli " effluents, under present circumstances at least, are impracticable except when the character of the raw water is exceptionally and consistently favorable. These working limits suggested by the results of Streeter's extensive studies of the subject have won wide acceptance as the maxima of raw-water pollution that can be handled successfully by average water purification plants.

CHAPTER VIII

DIFFERENTIATION OF ORGANISMS OF THE COLIFORM GROUP

Isolation of Organisms for Differentiation. Since the purpose of differentiation is to determine whether coliform types considered to be of particular sanitary significance are present in the water examined, care must be observed to choose a method of isolation which will facilitate the recovery of such types. The uncertainty of isolation of all the coliform types that may be present in enrichment tubes precludes their use for this purpose. Consequently, direct plating of water samples must be employed, for plates inoculated directly with measured portions of sample may be expected not only to yield colonies of all the coliform types present in such portions but also to permit an estimate of the frequency with which each type occurs. If the type or types of particular interest can be recognized on the plates, this method furnishes in one operation both a quantitative and a qualitative result of no uncertain value.

Litmus lactose agar, MacConkey agar, and Endo medium were used for direct plating in the early years of water bacteriology; but these have been largely superseded by the eosin methylene blue plate which yields fairly accurate results with highly polluted waters and sewage. With less polluted waters which may contain weak gas-producing coliforms, the colonies of which cannot be distinguished from those of some non-coliform bacteria, the results are not so satisfactory. Since deep colonies in E.M.B. agar are not very readily differentiated by their appearance, a method of surface planting such as that described by Gehm and Heukelekian (1935) should be employed: 1 ml. of the sample is spread by means of the pipet over the surface of a plate poured to a depth of about 3 mm. with E.M.B. agar (containing 2 per cent agar instead of the usual 1.5 per cent), the plate with cover removed is placed in the 37° C. incubator until the surface is dry (about 1 hour), and the plate with cover replaced is then incubated for 24 hours at 37° C. Gehm and Heukelekian found that the total coliform counts obtained by this method from sewage, sewage effluents, and highly polluted waters were generally slightly higher than those resulting from the use of the dilution method

employing brilliant-green bile tubes. This direct plating procedure should be of considerable aid in estimating the numbers of *Escherichia* and *Aerobacter* organisms occurring in such waters. For more complete and more accurate differentiation, a suitable proportion of the colonies on the plate may be fished and submitted to differential tests. A modification of this method for use with waters of better quality was proposed by Schulhoff and Heukelekian (1936). To 50 ml. of sample is added 1 ml. of sterile aqueous 20 per cent kaolin suspension. After being centrifuged for 20 minutes at 2,500 rpm. the supernatant liquid is decanted until 5 ml. remains. This is mixed and spread in 1-ml. portions over the surface of 5 solidified E.M.B. agar plates (E.M.B. containing 2 per cent agar and 1:120,000 crystal violet). Excellent results were obtained upon comparison of this procedure with that of Standard Methods.

A synthetic agar medium specially designed for direct plating was described by Ayers and Rupp (1918*a*). It provided nitrogen in the form of sodium ammonium phosphate and carbon in the form of lactose, and acid formation was indicated by a fuchsin-sulphite mixture similar to that employed in Endo agar. It is employed as a pour-plate medium with incubation at 37° C. for 24 hours; consequently, most of the growth is in the form of deep colonies. One of us (M.H.M.) experimented exhaustively with this medium and several modifications of it but found that although they yielded fairly accurate total counts when employed with highly polluted waters, those obtained from moderately or slightly polluted waters were frequently very unsatisfactory because of the difficulty in distinguishing between colonies of non-coliforms and those of weakly fermenting coliforms which are often present in such waters. A medium of this character is, however, suitable for isolation of coliform colonies to be differentiated since representatives of all the types of colonies present may be fished and differentiated.

A modification of Ayers and Rupp's medium, known as ferrocyanide-citrate agar was introduced by Noble (1928), and Tonney and Noble (1931*a*). It is a pour-plate medium, requiring an incubation period of 42 hours, which was proposed not only to furnish total coliform counts but also to differentiate *E. coli* from other coliforms by the appearance of the colonies alone. The originators of the method obtained very satisfactory total coliform counts and fairly accurate identification of *E. coli*, but more difficulty was experienced in recognizing the other coliform colonies. Ruchhoft, Coulter, Adams, and Sotier (1933) employed this method for the examination of sewage, sewage effluent, polluted rivers, and Lake Michigan water, and

found the direct counts of coliforms to be usually considerably below the estimates obtained by lactose broth enrichment followed by streaking E.M.B. plates and by inoculating brilliant-green bile tubes from lactose broth gas-positives, growth on E.M.B. and gas in B.G.B. being considered an indication of the presence of coliforms. Upon differentiation of a proportion of the colonies appearing on the direct plating medium, only 34 to 60 per cent of the colonies identified as *E. coli* proved to be of this species, and 51 to 82 per cent of those considered to be *Aerobacter* colonies were of this type. Colonies of intermediates were usually identified as *E. coli* but some resembled *Aerobacter* colonies.

Another pour-plate medium, brilliant-green lactose bile agar, which requires only 17 hours of incubation, was proposed by Noble and Tonney (1935) for direct plating. It is essentially a solid form of the well-known liquid brilliant-green lactose bile with various indicators added to permit differentiation of coliform colonies from those of other bacteria. Noble and Tonney reported that the new medium yielded somewhat better results than those obtained through the use of the liquid brilliant-green bile presumptive procedure, although the productivity of neither was more than about 65 per cent of that of lactose broth.

Littman and Stark (1938) developed a citrate ricinoleate agar, utilizing the inhibitive properties toward many non-coliform bacteria which is characteristic of sodium ricinoleate, and the ability of *Aerobacter* organisms to use citric acid with the formation of alkali. Peptone, milk powder, and lactose are included in the medium; neutral red and bromothymol blue are present as indicators. Littman and Stark state that with this pour-plate medium it is possible to differentiate *Escherichia* from *Aerobacter* organisms and, by flooding the plate with a protein precipitant, to distinguish between the latter and proteolytic bacteria such as *Proteus, Serratia,* and *Pseudomonas* forms. Inoculated plates are incubated for 24 hours at 37° C.

Two other pour-plate media intended for direct plating should be mentioned: violet-red bile agar (Difco Laboratories) containing bile salts and crystal violet to inhibit undesired organisms; and desoxycholate lactose agar (Baltimore Biological Laboratory) containing sodium desoxycholate, another bile salt. As all pour-plate media depend upon the appearance of deep colonies for differentiation of coliform from other colonies, a cover of a few milliliters of the agar should be superimposed upon the solidified poured plate so that any bacteria at the surface of the latter will be covered by a layer of the medium.

The use of direct plating to determine the total coliform density of water has never been widely used, although a plate count of at least a few colonies is far more accurate than an estimate derived by means of the dilution method as ordinarily employed. There are two main reasons for this neglect:

1. The plate method is inconvenient and costly to use for the examination of large portions of water sample. Not more than 10 ml. of sample can conveniently be mixed with a like quantity of double-strength agar in the ordinary 100-mm. plate; and for the examination of 50 to 100 ml. of sample 5 to 10 such plates must be poured, whereas the dilution method usually requires only a few tubes of enrichment medium and the expenditure of a comparatively insignificant amount of time and effort. The substitution of larger plates facilitates the plating procedure but the high first cost of these plates and the risk of excessive breakage militate against their general use.

2. Since no plating medium, to our knowledge, indicates whether the organisms growing in it are capable of producing gas from lactose, which is the characteristic primarily employed for detection of coliforms, results from plates cannot be expected to correlate perfectly with those obtained from methods which indicate such gas production. It is true that satisfactory coliform counts may be obtained from many waters by means of plates; but in our experience of a number of plating media, samples are encountered from time to time which yield excessively high counts of non-coliform colonies that cannot be distinguished from those of coliform organisms. Bacteria producing such colonies, whether slow lactose fermenters or atypical in other respects, cannot be accepted as members of the coliform group unless our present definition of the group is changed to include them; and for such action there appears to be very little justification. For total coliform counts of sewage and waters known to be heavily polluted, however, direct plating methods may prove convenient and useful; in such waters coliforms are nearly always so numerous that other bacteria are not likely to interfere seriously. Pending crystallization of opinion relative to the merits of the different plating media and methods proposed, however, Standard Methods has not provided for the use of direct plating methods to determine the total coliform density of waters.

On the other hand, for isolation of coliforms from water preliminary to their differentiation, direct plating in practically any of the media described above should be suitable. The modified E.M.B. medium with surface inoculation is perhaps the most convenient for the examination of highly polluted waters; one of the other media, in

poured plates, should be employed with potable or moderately polluted waters, since relatively large portions of such waters must be examined. In either event many colonies, including all types on the plate, should be subjected to the differential tests chosen until experience has proved that certain types of colony may be ignored.

Purification of Cultures Prior to Differentiation. Despite the greatest of care in preparing plates for isolation of coliforms and in fishing colonies from such plates, a proportion of the cultures obtained may not be pure but, instead, contain mixtures of organisms. Particularly is this true when deep colonies are fished. Ruchhoft *et al.* (1931) estimated that about 18 per cent of their large group of *E. coli* cultures and about 45 per cent of their regular *Aerobacter* cultures taken from E.M.B. surface colonies were mixtures of bacteria when first examined. These observers recommended the inoculation of each colony fished from the E.M.B. plate into tryptophan (tryptone) broth and, after 2 to 3 hours of incubation, streaking this broth culture on E.M.B. plates which are incubated for 20 to 24 hours; this process is repeated three times, subcultures from all types of colonies on the plate being fished each time; finally all subcultures are transferred to differential media, lactose broth, and agar slants.

Stuart, Griffin, and Baker (1938), observing "shifts" in type, or changes in the biochemical activity of certain coliform strains after repeated transfer of the pure cultures, suggested the term stabilization instead of purification to designate the procedure employed so to adapt an organism to its new environment that a culture of it is composed almost entirely of one type of cells which present constant and uniform reactions. They agree with Ruchhoft that for accurate work particular care must be exercised in order to obtain, if possible, pure and stable cultures. Occasionally, however, an unstable variant such as *Escherichia coli-mutabile* may be encountered: growing out of a colony of organisms which show no evidence of ability to ferment lactose there appear papillae, subcultures of which ferment lactose in the normal manner. Or, an organism may exhibit a change such as the citrate mutation described by Parr (1939) which, although usually of rare occurrence, was presented by 54 of 60 colonies of *E. coli* isolated from a single sample of feces: small numbers of citrate-positive, hydrogen sulfide-negative variants were given off by each parent strain. Indole variants of a similar nature were observed by Griffin and Stuart (1940). Another type of variation is that reported by Koser (1924*a*): eight M.R.+, V.P.— soil strains reversed their reactions after a few weeks of cultivation; Stuart *et al.* (1938) encountered a similar variation in some of their atypical

coliforms. A number of other examples of change in biochemical activity, particularly in the fermentation of sugars, might be cited. As Parr and Robbins (1942) pointed out, however, despite the possibility that no bacterial culture may prove perfectly stable if a sufficiently intensive examination of repeated transplants is made, most bacterial strains present little difficulty, and after a few manipulations become for all practical purposes constant in their biochemical behavior.

Early Methods of Differentiation. In addition to the *Bacterium coli-commune,* Escherich (1885) isolated from human feces another closely related organism, *Bacterium lactis-aerogenes,* which differed from the former in several respects; it fermented not only glucose and lactose, but also sucrose. Since both of these organisms had been isolated from intestinal discharges, early water bacteriologists assumed the natural habitat of both to be excreta. Before long, however, it was found that the number of *B. lactis-aerogenes* present in human and animal dejecta was small compared with that of *B. coli-commune;* and the latter, which came to be known as *Bacillus coli-communis* or *B. coli,* was recognized as the principal indicator of fecal pollution of water. The *B. lactis-aerogenes,* or *B. aerogenes,* and other allied forms were designated as atypical *B. coli* or paracolon bacilli.

The first edition of Standard Methods (1905) required *B. coli* to possess the following characteristics: motility, non-liquefaction of gelatin, coagulation of milk, production of indole in peptone solution, reduction of nitrates, and fermentation of glucose broth with the formation of about 50 per cent of gas, of which about one third is absorbed by a 2 per cent solution of sodium hydroxide. The last requirement stemmed from the observations of Theobald Smith (1895) who, with rare discernment, perceived the importance of difference in gas production by organisms of the coliform group; unfortunately some twenty years were to elapse before the significance of this carbon dioxide : hydrogen ratio, accurately determined, was to be fully appreciated. The *B. coli* of the earlier water bacteriologists, therefore, corresponds approximately to those organisms of our present genus *Escherichia* which exhibit motility and produce indole from peptone broth.

Because of the great variety of characteristics presented by different strains of *B. coli* and atypical *B. coli,* comprehensive classification of the whole coliform group was next attempted. MacConkey (1909), the first to elaborate such a classification, started with differentiation by fermentation of sucrose (as Smith had suggested in 1895) and dulcitol; he employed several of the usual characteristics, including

indole production, gelatin liquefaction, production of acetyl-methyl-carbinol (the Voges-Proskauer reaction), as well as other carbohydrate reactions to establish eventually a system of differentiation which provided for 128 coliform types. Bergey and Deehan (1908) constructed a system which increased the number of different types to 256. The 1913 Standard Methods included the Jackson classification based upon the fermentation of dulcitol, sucrose, mannitol, and raffinose, with subvarieties determined by motility, indole production, and other characteristics, which constituted another unwieldy system. Unlike the MacConkey and the Bergey and Deehan systems, it did not employ the Voges-Proskauer reaction. The *B. coli* group as then defined in Standard Methods was much more inclusive than that of the first edition; excepting the gelatin-liquefying species, *cloacae,* it corresponds very closely to our present coliform group.

It soon became apparent that these complex systems were too involved for practical coliform differentiation and served no very useful purpose. Finally, reverting to a consideration of the correlations, suggested by experience, of coliform characteristics with apparent natural habitats, water bacteriologists faced with the problem of actual control of water-supply quality came to depend largely upon indole production, gelatin liquefaction, sucrose fermentation, and the Voges-Proskauer reaction for separating the more significant coliforms from the less significant as indicators of water pollution; for indole-positive, and gelatin, sucrose, and Voges-Proskauer-negative reactions appeared to be characteristic of the majority of coliform organisms found in human and animal feces and in polluted waters. Sucrose fermentation and the Voges-Proskauer reaction were more frequently employed by English than by American laboratories, but indole production and gelatin liquefaction were very generally accepted as means of more or less useful differentiation. In the meantime Clemesha (1912*a*), in India, had departed so far from the usual systems of classification as to suggest a grouping based upon the resistance of certain individual types of coliforms to storage and other environmental hazards. A satisfactory classification of the coliform group was still wanting, however, when a series of rapid developments provided methods of differentiation which appeared to be not only scientifically sound but also of considerable practical value. The first of these developments was the demonstration of a correlation between the accurately determined carbon dioxide : hydrogen ratios of the gases produced from glucose by different coliforms and certain sources of these organisms; the second was the discovery of a simple procedure (methyl-red test) the results of which

correspond very closely with those of the complicated gas-ratio determination; the third was the recognition of the almost perfect inverse correlation between the results of the Voges-Proskauer test and those of the other two tests; and the fourth was the discovery of another simple method (citrate test) of differentiating fecal from non-fecal coliforms which promised to be of even more practical value than the other procedures.

The Carbon Dioxide : Hydrogen Ratio of Gas from Glucose. Harden and Walpole (1905–06) estimated the carbon dioxide : hydrogen ratio of gas produced from glucose by *B. coli* to be approximately 1 : 1, and that of gas produced by *B. lactose-aerogenes* (*A. aerogenes*) to be about 2.5 : 1. These ratios are almost the reverse of those observed by Theobald Smith (1895); but Keyes (1909) pointed out the inaccuracies of the fermentation-tube method employed by Smith, and Keyes and Gillespie (1913) showed that when the organisms are grown in a vacuum and the accurate method of gas measurement developed by Keyes is used the gas ratio is of prime importance in coliform differentiation.

Rogers, Clark, and Davis (1914), applying a modification of Keyes' method to coliform organisms isolated from milk, found them to be about evenly divided into two groups: a high ratio group producing gas consisting of carbon dioxide and hydrogen in the ratio of about 1.90 : 1 to 3.00 : 1, and a low ratio group producing gas with a carbon dioxide : hydrogen ratio of 1.06 : 1. The high ratio group usually fermented sucrose but not dulcitol. Rogers, Clark, and Evans (1914, 1915), using the same medium (consisting of 1 per cent Witte peptone, 1 per cent glucose, and 0.5 per cent K_2HPO_4) and the same gas-ratio method, found that of 166 organisms producing gas from glucose which were isolated from grains, 8 were of the low ratio type, 7 produced only carbon dioxide, and the remaining 151 were of the high ratio type; and of 150 strains isolated from bovine feces, 149 were of the low ratio and only 1 of the high ratio type. Naturally these results aroused great interest, for here appeared to be a test which sharply differentiated fecal from non-fecal coliform organisms.

The Methyl-red Test. The complicated method and elaborate apparatus required for the determination of the gas ratio were soon shown to be unnecessary, for Clark and Lubs (1915*a*) demonstrated that the high ratio and low ratio groups (*Escherichia* and *Aerobacter* respectively) could also be differentiated by the hydrogen-ion concentrations produced by them in a medium consisting of 0.5 per cent Witte peptone, 0.5 per cent glucose, and 0.5 per cent K_2HPO_4 after 3 to 4 days of incubation at 30° C.; and that the difference in hydro-

gen-ion concentration could be readily observed by testing the cultures with methyl-red indicator, the low ratio cultures giving an acid reaction (red color) and the high ratio an alkaline reaction (yellow color). Apparently, in the medium employed, low ratio cultures, before exhausting the sugar present, reach a limiting hydrogen-ion concentration that stops further activity of the organisms; but high ratio cultures reach hydrogen-ion concentrations that are considerably lower and which do not prevent continued activity. In fact, the latter become progressively more alkaline after attaining their maximum hydrogen-ion concentrations. The most satisfactory explanation of this reversion of reaction appears to be that offered by Ayers and Rupp (1918*b*) who suggested that both *Escherichia* and *Aerobacter* organisms ferment glucose in a similar manner, the organic acids formed combining with the phosphate of the medium to produce salts of organic acids and acid phosphates; the fermentation of the sugar and that of the organic acid salts then proceed simultaneously with the eventual formation of carbonates and bicarbonates from the latter; and the difference in end products is due simply to a difference in the rate at which the glucose and the organic salts are fermented by the two groups of coliforms. Since the color changes of methyl red occur in the interval *p*H 5.0 to 6.0, which includes the limiting hydrogen-ion concentration produced by low ratio coliform organisms and which is too low to include that of the high ratio coliforms, this indicator serves admirably to detect the type of organism responsible for the *p*H produced.

The temperature and period of incubation of the culture employed in the methyl-red test are extremely important. Although some series of cultures may yield practically identical results whether the temperature of incubation is 30° or 37° C., and the period of incubation 3 days or 5 days, other series may yield markedly different results. In the examination of 221 coliform strains presumably intermediate in character between *E. coli* and *Aerobacter*, Vaughn, Mitchell, and Levine (1939) obtained the results shown in Table 68 with incubation temperatures of 30° and 37° C. and with various incubation periods. It is evident that the incubation temperature of 30° C. favors the completion of the fermentation and associated reactions with an increase in the number of cultures becoming alkaline to methyl red, and that the use of the higher temperature is conducive to erroneous results.

Since the composition of the medium employed for the methyl-red test is of particular importance, the Standard Methods Committee specifies that only Witte, Difco, or Proteose peptone be used; and in

order to ensure a proper buffer concentration, the phosphate employed should be of such purity that a dilute solution of it gives a distinct pink color with phenolphthalein. Bacto M.R.-V.P. Medium (dehydrated) may be employed, as it yields excellent results. The broth as described above is tubed in 10-ml. quantities and sterilized by the intermittent method for 20 minutes on each of 3 successive days. After inoculation, a tube is incubated for 5 days at 30° C., when 5 ml.

TABLE 68. EFFECT OF TEMPERATURE AND PERIOD OF INCUBATION ON METHYL-RED REACTION OF 221 INTERMEDIATE STRAINS OF *Coli-Aerogenes* GROUP

(Vaughn, Mitchell, and Levine, 1939)

Temperature of Incubation ° C.	Incubation Period (Days)			
	2	3	4	5
	Number of Strains Alkaline to Methyl Red			
30	35	37	37	37
37	5	7	10	11

are transferred to a clean tube and 5 drops of methyl-red solution (prepared by dissolving 0.1 gram in 300 ml. of 95 per cent alcohol and diluting to 500 ml. with distilled water) are added. A distinct red color is characteristic of low ratio (*Escherichia*) coliforms and is recorded as positive; a distinct yellow color, characteristic of high ratio (*Aerobacter*) organisms, is recorded as negative; and an intermediate color is recorded as a doubtful result.

The Voges-Proskauer Reaction. Voges and Proskauer (1898) reported that, upon adding a 10 per cent solution of potassium hydroxide to glucose broth cultures of certain bacteria, an eosin-like coloration was produced. According to Harden (1905) and Harden and Norris (1911) this effect is the result of the action of diacetyl, formed from acetyl-methyl-carbinol produced by bacterial fermentation of glucose, on peptone in the presence of potassium hydroxide, and may be represented as follows:

$$\underset{\text{glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{acetyl-methyl-carbinol}}{CH_3COCHOHCH_3} + KOH \rightarrow$$

$$\underset{\text{diacetyl}}{CH_3COCOCH_3} + \text{peptone} \rightarrow \text{pink coloration}$$

This reaction, commonly known as the V.P. reaction, was extensively used by MacConkey and his followers in England and by Bergey and Deehan (1908) in America who found it to be character-

istic of glucose broth cultures of *Aerobacter*. West (1909) confirmed this finding; but since the V.P. reaction was not included in either the 1905 or the 1913 edition of Standard Methods, it was evidently then not very generally regarded in this country as an important means of coliform differentiation. The work of Levine (1916*a*), however, showed a marked negative correlation between the results of the V.P. test and those of the methyl-red test, and interest in the V.P. reaction developed rapidly. As the test was then performed, considerable time was required for the appearance of the pink color in positive cultures and frequently the color was so indistinct that reading of results was difficult. Numerous modifications to improve the test were proposed. Many of these included the addition of oxidizing reagents to hasten the reaction: West (1909) advised shaking the culture with air or blowing air through it as recommended by Rivas; Levine, Weldin, and Johnson (1917) proposed the use of hydrogen peroxide; and Bedford (1929) that of sodium peroxide. Werkman (1930) suggested the addition of ferric chloride; this results in the production of a persistent deep copper coloration in positive cultures; apparently the ferric chloride catalyzes the oxidation of the acetyl-methyl-carbinol to diacetyl, for the reaction does not occur in the absence of a hydrogen acceptor, and the diacetyl reacts with the peptone to give the copper coloration. O'Meara (1931) used creatine to speed the reaction; and Levine, Epstein, and Vaughn (1934) recommended a modification of O'Meara's technique which appeared to reduce the time required for a positive reaction from the 2 to 5 days ordinarily required to only 7 to 11 hours. One of the best procedures suggested for the V.P. test is that of Barrit (1936) which consists in adding 0.6 ml. of 5 per cent α-naphthol in absolute ethyl alcohol, and 0.2 ml. of 40 per cent potassium hydroxide to 1 ml. of the culture to be tested. A crimson-to-ruby color develops in a positive culture within 2 to 4 hours; as this color may fade upon standing, results should be read not later than 4 hours after addition of the reagents.

Since acetyl-methyl-carbinol, according to several observers, including Paine (1927) and Tittsler (1938), may eventually be destroyed by some coliform strains, the time and temperature of incubation of the cultures employed in the V.P. test are very important. Vaughn, Mitchell, and Levine (1939) compared the use of different temperatures and periods of incubation and of various reagents for testing 221 presumably intermediate coliform strains; the results are shown in Table 69. Obviously the use of α-naphthol with cultures of these strains yielded the largest number of positive results.

In a study of the time required for the color to develop after addition of the reagents, it was found that 2 hours after adding the α-naphthol all the V.P.+ cultures were showing the color, whereas only 45.5 per cent were positive when 10 per cent potassium hydroxide was employed as the reagent, and 75.3 per cent when creatine-potassium hydroxide was used. Table 69 shows also that incubation at 30° C. gives the better results and that only 24 to 48 hours at this temperature is necessary. A further series of 138 *Aerobacter* strains giving positive V.P. reactions upon incubation for 1 day as well as for

TABLE 69. EFFECT OF TEMPERATURE, PERIOD OF INCUBATION, AND TEST REAGENT ON V.P. REACTION OF 221 INTERMEDIATE STRAINS

(Vaughn, Mitchell, and Levine, 1939)

Test Reagent	Days of Incubation				
	1	2	3	4	5
	Per Cent Positive at 30° C.				
10% KOH	20.4	21.7	21.7	21.7	21.3
Creatine-KOH	23.1	23.1	23.1	22.6	22.6
α-Naphthol	23.1	23.1	23.1	23.1	23.1
	Per Cent Positive at 37° C.				
10% KOH	9.5	11.8	15.4	15.4	16.3
Creatine-KOH	17.2	19.0	19.5	19.5	19.9
α-Naphthol	19.9	19.9	19.9	19.9	20.4

5 days at 30° C. were tested by Vaughn *et al.* after incubation at 41° to 43° C.; only 72.5 per cent were positive after 1 day and 63.8 per cent after 5 days. Admitting that the "presumably intermediate" group of organisms studied by Vaughn *et al.* was particularly adapted to expose the variation in reaction resulting from the use of different conditions of incubation and different test reagents, there appears to be no doubt that incubation at 30° C. for 24 to 48 hours and the use of Barrit's α-naphthol reagent give the best results, and the Standard Methods Committee specifies these conditions for the Voges-Proskauer test.

In practice, a tube containing 10 ml. of the glucose broth described for use with the methyl-red test is seeded with the coliform strain to be examined and incubated at 30° C. for 24 to 48 hours, when 5 ml. of the culture is withdrawn aseptically for the V.P. test. The original glucose broth tube is then replaced in the incubator and after a period

of 5 days (including the previous incubation) the contents of the tube are subjected to the methyl-red test.

Correlation of Methyl-red and Voges-Proskauer Results. Since the time and temperature of incubation recommended for the methyl-red and V.P. tests by Standard Methods varied from 5 days at 30° C. in the 1917 and 1923 editions to 3 to 4 days at 37° C. in the 1936 edition, and since the use of 10 per cent potassium hydroxide was recommended for the V.P. test, it is obvious that some allowance for error must be made when considering the significance of the correlation between the results of these two tests which was reported during the greater part of the past thirty years. Nevertheless, the correlation noticed by Levine between the M.R. and the V.P. results was soon confirmed by a number of investigators including Greenfield (1916*b*), Burton and Rettger (1917), Chen and Rettger (1920), Koser (1926*a*), and Wood (1920) in England. Although this correlation was found to be not always perfect (McCrady, 1916; Burton and Rettger, 1917; Perry and Montfort, 1921) it was so marked that there appeared little doubt that the differentiation effected by these two tests was of fundamental importance. Classification of the coliform group of organisms based upon the results of these procedures assumed the following form:

	Methyl Red	Voges-Proskauer
E. coli section	+	−
A. aerogenes section	−	+

The Citrate Test. Upon investigating the utilization of organic acids by coliform bacteria, Koser (1923) found that both his M.R.+, V.P.— strains and *Aerobacter* strains isolated from soil were able to utilize sodium or potassium citrate as the sole source of carbon, whereas his *Escherichia* strains isolated from feces did not possess this ability. The possibility of thus dividing *Escherichia* organisms into a citrate-positive and a citrate-negative group was soon confirmed by numerous other observers both here and abroad. Raghavachari (1926) in India, Bardsley (1926) in England, and Ruchhoft *et al.* (1931) in this country contributed particularly interesting data attesting to the value of this means of differentiation.

The M.R.-positive, V.P.-negative, citrate-positive coliform organisms were designated as intermediate by the earlier observers. Braak (1928), finding that trimethylene glycol was produced from glycerol by intermediate coliforms but not by *E. coli* or *Aerobacter* controls, gave them the name *E. freundii* because they appear more closely related to *Escherichia* than to *Aerobacter* organisms and because A. Freund observed, in 1881, the production of trimethylene glycol as

a result of the fermentation of glycerol. Werkman and Gillen (1932) studied in detail 15 cultures of intermediates and suggested the creation of the genus *Citrobacter* to include these organisms; but Bergey *et al.* (1939) have retained the designation *E. freundii* as proposed by Braak. Vaughn and Levine (1942) suggested a classification of the intermediate group based principally upon hydrogen sulphide production in proteose-peptone ferric-citrate agar, and further division of the hydrogen sulphide-negative section into two groups according to the motility of the organisms and their ability to attack starch and inositol. These observers give a detailed description of the appearance of the hydrogen sulphide-negative group colonies on eosin methylene blue agar.

The sodium citrate test, as described in Standard Methods, consists in lightly inoculating with the pure agar culture to be tested a medium containing 1.5 g. of sodium ammonium phosphate (microcosmic salt), 1 g. of potassium dihydrogen phosphate, 0.2 g. of magnesium sulphate, and 2.5 to 3.0 g. of sodium citrate crystals per liter, distributed in tubes in 5-ml. quantities and sterilized at 15 pounds pressure for 15 minutes, and incubating at 35° to 37° C. for 72 to 96 hours. A turbidity in the medium is considered a positive result. Extremely light inoculation should be practiced, for the introduction of even a small amount of nutrient material with the transfer may permit *E. coli* to multiply and give a positive result. Vaughn, Mitchell, and Levine (1939) showed that either 30° or 37° C. may be employed as the incubation temperature.

Classification on the basis of the citrate, methyl-red, and Voges-Proskauer reactions takes the following form; it has been very generally employed in the past.

	M.R.	V.P.	Citrate
E. coli	+	−	−
E. freundii	+	−	+
A. aerogenes	−	+	+

An interesting modification of the citrate test was proposed by Simmons (1926) who added agar and bromothymol blue to Koser's medium. When streaked on a plate or slant of this solid agar, *E. coli* cultures are markedly or completely inhibited and do not change the color of the medium, whereas *Aerobacter* and *E. freundii* form greenish blue colonies and produce sufficient alkali to color the medium blue. Lewis and Pittman (1928) proposed another agar medium, containing ferric ammonium citrate, on which a positive reaction is indicated by the deposition of iron hydroxide.

The Eijkman Test. Eijkman (1904) utilized an incubation temperature of 46° C. to differentiate coliforms in feces of warm-blooded animals from those in cold-blooded animals. He contended that only the former could produce gas in a broth containing 10 per cent glucose, 10 per cent peptone, and 5 per cent sodium chloride to which had been added one eighth of its volume of the water to be tested. Leiter (1929) reviewed the favorable results obtained by a number of German observers and reported that his own work on isolated strains showed a close correlation between gas production in Eijkman's medium at 46° C. and indole production, and failure to utilize sodium citrate and uric acid by coliform organisms isolated from feces of warm-blooded animals; coliforms from the intestines of cold-blooded animals gave negative results. Brown and Skinner (1930), however, from their work with feces and polluted water, reported that only a small percentage of the *Escherichia* from human feces gave positive Eijkman results, that many "typical *B. coli*" from the water gave negative results, and that the test failed to eliminate some of the *Aerobacter* and intermediates present. Employing lactose broth at 46° C for 48 hours in the study of 161 coliform organisms, Wagner (1931) found so many *Aerobacter* strains giving positive results and so many *E. coli* reacting negatively that he considered the Eijkman test untrustworthy.

Perry and Hajna (1933), observing that *E. coli* could seldom be isolated from positive tubes after 24 hours of incubation at 46° C., modified the original Eijkman medium by adding a buffer and reducing the glucose content to 0.3 per cent in order to create an environment more favorable to the growth and viability of this organism. With this modified medium *E. coli* strains were found to produce gas in 24 hours at 46° C.; 4 of 10 *Aerobacter* strains produced gas, but only after 48 hours; and attempted isolation was successful after 96 hours or even longer periods of incubation. Wilson *et al.* (1935) reported that by using MacConkey broth with water-bath incubation at 44° C. satisfactory differentiation of *E. coli* from other coliforms could be effected. In view of these and other conflicting reports on the value of the Eijkman test, Hajna and Perry (1939) made a careful study to determine the conditions of incubation and culture most favorable for this procedure. They found that their modified Eijkman medium, with either water-jacketed air incubation or water-bath incubation to maintain the tube cultures at a temperature of 44° C. as recommended by Wilson, gave unsatisfactory differentiation of coliforms although the results were much better than those obtained with MacConkey broth. Comparing tube temper-

atures of 42°, 44°, and 46°, and glucose, lactose, and mannitol as the fermentable substance in the medium, they concluded that the use of a water-jacketed incubator providing an air temperature of 45.5° to 46.0° C. (maintaining the tubes at a temperature of 45.2° to 45.7°) furnished the most favorable environment, and that either glucose or lactose might be employed in the medium.

Stuart *et al.* (1942) used incubation in a water bath maintained at 45.5° C. with a variation of less than 0.1°, and the Perry-Hajna modified Eijkman lactose broth (Difco dehydrated) containing bromothymol blue indicator, to test a large number of coliform and related organisms. None of 14 *Serratia* cultures produced even any acid within 48 hours (glucose was substituted for lactose in the medium because lactose-fermenting *Serratia* strains are rarely encountered), and only 3 of 24 *Erwinia* cultures produced acid even with heavy inoculations. Light inoculation from agar slant cultures of coliform organisms gave the following frequency of *gas* production in 48 hours: *E. coli,* 1,269 of 1,283 strains, or 98.9 per cent; *E. freundii,* 4 of 882 strains, or 0.5 per cent; *Aerobacter* (citrate-positive), 17 of 863 strains, or 2.0 per cent; and a small group of *Aerobacter* (citrate-negative, cellobiose-positive or -negative), none of 31 strains, or 0.0 per cent. Evidently these results constitute a striking confirmation of Perry and Hajna's contention of the differential value of the Eijkman test when properly performed.

The Standard Methods Committee specifies the use of the modified Eijkman medium containing 15.0 grams of tryptose, 3.0 grams of lactose, 4.0 grams of K_2HPO_4, 1.5 grams of KH_2PO_4, and 5.0 grams of sodium chloride per liter. A tube of the medium is inoculated with the culture to be tested and placed immediately in a carefully controlled water-jacketed incubator maintained within 0.2° of 45.5° C. Any gas production within 48 hours constitutes a positive test.

The Indole Test. At the beginning of the century the identification of *B. coli-communis* depended principally upon three characteristics: gas production from glucose (and in later years, lactose), production of indole from peptone water, and non-liquefaction of gelatin. The test for indole described in the first edition of Standard Methods (1905) consisted of the addition of 2 drops of concentrated sulphuric acid and 1 ml. of 0.01 per cent solution of sodium nitrite to a peptone-water culture which had been incubated 4 days at 37° C.; the appearance of a pink color indicated the presence of indole. A ring test using similar reagents and known as the Salkowsky test was an improvement, but Kligler (1914*a*) found the Ehrlich test, employing *p*-dimethyl-amino-benzaldehyde and hydrochloric acid, to be most

reliable. Since indole is liberated from the amino acid, tryptophan, Kligler suggested the use of tryptophan broth in order to provide an ample quantity of this ingredient. In accordance with his suggestion tryptone, a peptone particularly rich in tryptophan, is now very generally used for the indole test.

Perhaps the best method for detection of indole is the modification of Ehrlich's test proposed by Kovācs (1928). In a study of the sensitivity of this procedure Ruchhoft, Kallas, Chinn, and Coulter (1931) found that it gave a positive reaction with all of 38 strains of *E. coli* after 18 hours of incubation, and after 24 hours of incubation positive results were obtained with only 0.1 ml. of the cultures. To perform this test, a 5-ml. tube of sterile 1.0 per cent tryptone broth is seeded with the culture to be tested and incubated at 35° to 37° C. about 24 hours. To it is then added 0.2 to 0.3 ml. of amyl alcohol indole reagent and the tube is shaken well and set aside for about 10 minutes; a dark red color in the amyl alcohol surface layer indicates the presence of indole. The test reagent is prepared by dissolving 5 grams of C.P. *p*-dimethyl-amino-benzaldehyde in 75 ml. of amyl alcohol (reagent grade) and adding 25 ml. of concentrated hydrochloric acid; the color of the finished reagent should be yellow. The above procedure is that specified at present by Standard Methods.

There was a time when the indole reaction was considered by many observers to be one of the least reliable of differential tests, but the results obtained with improved methods of testing have won for it a place in several of the more recent systems of differentiation. It should be noted that, as Ruchhoft *et al.* (1931) have pointed out, a particularly valuable feature of the indole test is its freedom from interference by other organisms. These observers found that indole formers, even when mixed with a variety of different bacteria, persisted in their production of indole; and that once formed, the indole appeared to be not readily broken down by other organisms.

The Gelatin-liquefaction Test. The classic means of differentiating *A. aerogenes* from *A. cloacae* is the gelatin-liquefaction test. A tube of ordinary nutrient gelatin is seeded with the culture to be tested by a stab into the solid medium and then incubated for at least 20 days at 20° C., when it is examined to determine whether any liquefaction has occurred. An improvement of this procedure is incubation at 37° C. for 20 to 30 days followed by cooling in ice water. Extended periods of incubation are advisable; Johnson and Levine (1917) found 106 of 202 coliforms isolated from soil to liquefy gelatin after 34 days whereas only 38 were indicated as liquefiers after 20 days at 20° C.

Kligler (1914*b*) proposed the fermentation of glycerol to differentiate these two closely related organisms, and the production of acid and gas from this alcohol has been generally accepted as a differential test as valuable as, if not more valuable than, gelatin liquefaction because it appears to be the more stable characteristic. *A. aerogenes* ferments glycerol, but *A. cloacae* does not. An incubation period of at least 21 days was found necessary by Griffin and Stuart (1940).

Hajna and Damon (1934) proposed a more rapid procedure to take the place of the gelatin-liquefaction test. A loop of a tryptone broth culture is planted in 5 ml. of a medium containing 5.0 grams of NaCl, 0.2 gram of $MgSO_4 \cdot 7H_2O$, 1.0 gram of $(NH_4)H_2PO_4$, 1.0 gram of K_2HPO_4, and 3.0 grams of sodium hippurate. The appearance of turbidity in the medium after 3 days is considered a positive result. Hydrolysis of the hippurate may be detected by the usual ferric chloride procedure but inspection is reported to furnish sufficiently accurate results. Positive reactions were obtained by Hajna and Damon with 57 *A. aerogenes* cultures, and negative reactions with 30 *A. cloacae,* 49 *E.* coli, and 21 intermediate coliforms.

Other Differential Tests. Koser (1918), in a study of the utilization of simple nitrogenous compounds by coliform organisms, observed that *A. aerogenes* grows readily in a medium containing uric acid as the sole source of nitrogen. Apparently *Aerobacter* cultures are able to attack the purin ring of the uric acid and utilize its nitrogen, but *Escherichia* cultures lack this ability. Chen and Rettger (1920) obtained perfect correlation between results of the V.P. test and the uric acid test with 447 V.P.-positive strains from soil and 173 V.P.-negative strains from feces, but half of the 20 V.P.-negative coliforms from soil were able to grow in the uric acid medium.

The fermentation of cellobiose, a disaccharide, was found by Jones (1924) and Jones and Wise (1926) to be characteristic of *Aerobacter* but not of *E. coli* cultures. Confirming this observation, Koser (1926*a*), Lewis and Pittman (1928), Tittsler and Sandholzer (1935), and others reported that intermediate coliforms (M.R.-positive, V.P.-negative, citrate-positive) also usually fermented the sugar with production of acid, or acid and gas. Stuart *et al.* (1942) recently reported fermentation (acid or acid and gas) of cellobiose by 96 of 1,283 M.R.+, V.P.—, citrate—, 746 of 882 M.R.+, V.P.—, citrate+, 850 of 863 M.R.—, V.P.+, citrate+, and 23 of 31 M.R.—, V.P.+, citrate— cultures.

Levine (1921*c*) suggested that the inhibitory action of boric acid might be utilized for differentiation of coliform bacteria, and Levine, Epstein, and Vaughn (1934) reported that in a special proteose-

peptone medium containing 3.25 grams of boric acid per liter, 97.3 per cent of 150 *E. coli,* 6.1 per cent of 181 *Aerobacter,* and none of 57 intermediate cultures produced gas in 48 hours.

Vaughn and Levine (1936), following up a previous suggestion by Levine and his associates that the production of hydrogen sulphide from proteose peptone might serve to differentiate intermediates from other coliform organisms, found that satisfactory results were obtained with a medium containing 2.0 per cent proteose peptone, 1.5 per cent agar, 0.1 per cent K_2HPO_4, and 0.05 per cent ferric citrate. The proportion of agar was discovered to be important. Inoculation was made by a stab into the middle of the tubed medium and another along the wall of the tube. Upon incubation at 37° C. for 48 hours, 74.0 per cent of 169 intermediate, 1.8 per cent of 229 *E. coli,* and none of 194 *A. aerogenes* and 105 *A. cloacae* strains gave positive reactions (blackening of the medium). The classification by Vaughn and Levine of the intermediate group of coliforms on the basis of hydrogen sulphide production has been mentioned.

Other means of differentiation, including fermentation tests with various sugars and alcohols, have been proposed but none is very generally employed at the present time, at least in this country.

Correlation between the Results of Differential Tests. Numerous examples have already been given to show the relationship between some of the results obtained from certain of the numerous differential tests mentioned in this chapter. No attempt will be made to review thoroughly the extensive literature on this subject, but a few additional examples of results obtained with various tests may be cited in order to indicate the bases of some of the more recent coliform classifications.

Koser (1918), in a study of 74 cultures of *E. coli* isolated from feces and 50 of *A. aerogenes* from soil, obtained the results shown in Table 70 with the uric acid, the M.R., and V.P. tests (cultures incubated 4

TABLE 70

	Uric Acid		Methyl Red		Voges-Proskauer	
Cultures	+	−	+	−	+	−
74 *E. coli*	0	74	72	2	0	74
50 *A. aerogenes*	50	0	0	50	50	0

days at 37° C.). Evidently, in this series of examinations, the results of the uric acid test were perfectly correlated with those of the V.P. test; and the M.R. results were almost perfectly inversely correlated with those of the other two tests.

Table 71 shows the differential reactions obtained in the study, by Levine *et al.* (1934), of a number of coliforms representing each of the four principal coliform species. In this table the term *Escherichia* corresponds to *E. coli* and the term *Citrobacter* to *E. freundii*.

TABLE 71. DIFFERENTIAL REACTIONS IN THE COLIFORM GROUP
(Levine, Epstein, and Vaughn, 1934)

Genus:	*Escherichia*	*Aerobacter*		*Citrobacter*
		Cloacae	*Aerogenes*	
No. of Strains:	155	80	123	43
Character	Per Cent Positive Reactions			
Growth in citric acid	0	100	100	100
V.P.	0	100	100	0
M.R.	100	0	0	100
H_2S	1	0	0	100
Indole	97	0	60	0
Glycerol	47	0	100	100
Aesculin	73	34	100	0
Salicin	71	74	100	0
Starch	0	1	99	0

The results shown in Table 72 are taken from a more extensive table by Stuart *et al.* (1942) who employed several tests in the examination of a large number of coliforms of various types. They divided their strains into 3 groups primarily on the basis of indole and Voges-Proskauer reactions, and into 19 types.

From the results obtained through the use of the various differential tests, numerous classifications of the coliform group of organisms have been proposed. Some of the earlier systems have been mentioned in a previous section; later groupings include those of Winslow *et al.* (1919), Levine (1921*a*), and those intended principally for the use of the bacteriologist engaged in practical sanitary control of water quality by Ruchhoft, Kallas, Chinn, and Coulter (1931), Stuart *et al.* (1942), and the Committee of the British Ministry of Health (1939). The last, which is somewhat similar to the classification of Ruchhoft *et al.*, is reproduced in Table 73.

Stuart *et al.* (1942), considering the inverse relationship between the results of the M.R. and V.P. tests, would dispense with the former; they also favor the use of the Eijkman test. Their simplified classi-

TABLE 72. EIJKMAN REACTIONS OF COLIFORM BACTERIA FROM AGAR SLANT INOCULATIONS

(From Stuart *et al.*, 1942)

Group	Type	Indole	Methyl Red	Voges-Proskauer	Citrate	Cellobiose	Eijkman Test 48 hr., 45.5° Difco Eijkman Medium Per Cent*	Cultures
A	1	+	−	+	+	+	0	183
	2	−	−	+	+	+	2.6	652
	3	−	−	+	+	A†	0	15
	4	−	−	+	+	−	0	13
	5	−	−	+	−	+	0	7
	6	−	−	+	−	A	0	16
	7	−	−	+	−	−	0	8
B	8	−	+	−	+	+	0.4	394
	9	−	+	−	+	A	0	220
	10	−	+	−	+	−	0	109
	11	−	+	−	−	+	0	9
	12	−	+	−	−	A	0	11
	13	−	+	−	−	−	100.0	114
C	14	+	+	−	+	+	0	81
	15	+	+	−	+	A	0	51
	16	+	+	−	+	−	7.4	27
	17	+	+	−	−	+	91.2	57
	18	+	+	−	−	A	100.0	19
	19	+	+	−	−	−	99.2	1,073

* Per cent of cultures (in last column) giving positive results. † A = acid.

TABLE 73. DIFFERENTIATION OF THE COLIFORM GROUP

(Committee, Ministry of Health, 1939)

	M.R.	V.P.	Growth in Citrate	Indole	Gas in Mac-Conkey at 44° C.	Gelatin Lique-faction 7 days	Probable Habitat
Bact. coli, type I, fecal	+	−	−	+	+	−	Human and animal intestine
Bact. coli, type II	+	−	−	−	−	−	Doubtful; probably not primarily intestinal
Intermediate, type I	+	−	+	−	−	−	Mainly soil
Intermediate, type II	+	−	+	+	−	−	Mainly soil
Bact. aerogenes, type I	−	+	+	−	−	−	Mainly vegetation
Bact. aerogenes, type II	−	+	+	+	−	−	Mainly vegetation
Bact. cloacae	−	+	+	−	−	+	Mainly vegetation
Irregular, type I	+	−	−	+	−	−	Human and animal intestine
Irregular, type II	+	−	−	−	+	−	Doubtful
Irregular, other types	Reactions variable						Doubtful

fication is shown in Table 74. They suggest the use of the Eijkman test in routine differentiation of coliform strains, because it appears readily to separate the so-called fecal from the other types. This

TABLE 74. SIMPLIFIED GROUPING OF THE COLIFORM BACTERIA

(Stuart *et al.*, 1942)

	Indole	Voges-Proskauer	Growth on Citrate	Eijkman Reaction 48 hr., 45.5° C.	Probable Origin
E. coli					
Variety I	+	−	−	+	Fecal
Variety II	−	−	−	+	Fecal
Intermediate					
Variety I	−	−	+	−	Non-fecal
Variety II	+	−	+	−	Non-fecal
A. aerogenes					
Variety I	−	+	±	−	Non-fecal
Variety II	+	+	+	−	Non-fecal

simple means of separating *E. coli* from other coliforms merits exhaustive experimentation to determine whether it is generally applicable to coliform organisms throughout the continent.

TABLE 75. CLASSIFICATION OF *Escherichia* AND *Aerobacter* COLIFORM ORGANISMS OF TRIBE I, *Escherichieae*

(Bergey *et al.*, 1939)

(Rearranged in Tabular Form)

	M.R.	V.P.	Citrate	H_2S	Glycerol (Acid and Gas)	Gelatin
Genus I. *Escherichia*	+	−	±			
Species I. *E. coli*	+	−	−	−		
Species II. *E. freundii*	+	−	+	+		
Genus II. *Aerobacter*	−	+	+			
Species I. *A. aerogenes*	−	+	+		+	−
Species II. *A. cloacae*	−	+	+		−	+

The key to the principal features of the taxonomic classification of coliform organisms (included in genera *Escherichia* and *Aerobacter*) by Bergey *et al.* (1939) is shown in Table 75. This classification is perhaps the one most generally employed at the present time.

Finally, we bring together in Table 76 the reactions usually obtained with these four species of coliforms when they are submitted to the various differential tests indicated. It should be remembered

that the correlations shown are never absolutely constant: aberrant combinations of reactions are not infrequently encountered. Organisms that produce such combinations of reactions are said to be irregular in type, and it is seldom that the study of a considerable number of coliform organisms does not show one or more to be irregular. The first four tests of the table are those most commonly

TABLE 76. CORRELATION OF REACTIONS OF COLIFORM ORGANISMS

	Indole	M.R.	V.P.	Citrate	H_2S	Glycerol	Gelatin	Sodium Hippurate	Eijkman	Uric Acid	Cellobiose
E. coli	±	+	−	−	−	±	−	−	+	−	−
E. freundii	±	+	−	+	±	+	−	−	−	−	+
A. aerogenes	±	−	+	+	−	+	−	+	−	+	+
A. cloacae	−	−	+	+	−	−	+	−	−	+	+

employed, and these in the order given have been designated by Parr (1936) as the "Imvic" group of tests, the mnemonic indicating the order of the tests: (I) indole; (M) methyl red; (V) Voges-Proskauer; and (C) citrate test. Thus the usual "Imvic" result given by *E. coli* is + + − −.

It will be observed that each of the three tests, the citrate, the Eijkman, and the cellobiose, serves to separate *E. coli* from the other species of coliforms. The cellobiose test is not only more costly but it also takes more time than the others. The citrate test (often supported by the V.P. test) is the simplest of the three but requires 3 to 4 days for completion. If an accurately controlled water bath is available, perhaps the Eijkman test with incubation for 48 hours at 45.5° C. ± 0.2° may prove the simplest and most informative single test yet devised for distinguishing between *E. coli* and the other organisms of the coliform group.

Antigenic Relationships of Coliform Organisms. From the turn of the century to the present time attempts have been made to discover some degree of serological homogeneity in the coliform group, but with little success. Recently, more intensive efforts, taking into consideration all the various species of coliforms as well as the paracolon organisms ("aberrant" coliforms from man), have proved somewhat more fruitful. Stuart, Baker, Zimmerman, Brown, and Stone (1940) found a measure of antigenic specialization in the coliform group. Marked diversification, with few antigenic fractions, was observed in the *Aerobacter* group of strains studied; less diversification, with many antigenic fractions, in the *Escherichia* group; whereas the intermediate group appeared, in this respect, to

occupy a position between the other two. The paracolon group investigated seemed not so closely related to typical *Escherichia* as were the other three groups to one another. Stuart, Wheeler, Rustigian, and Zimmerman (1943) reported much more marked serological relationships among paracolon bacteria than among normal coliforms. The results of tests of normal *Aerobacter*, intermediate, and *Escherichia* coliforms with their respective normal antiserums indicated that 0.0, 8.2, and 33.0 per cent respectively were identical or closely related; upon testing corresponding groups of paracolon cultures with paracolon antiserums 47.8, 52.5, and 67.2 per cent showed such relationship. Paracolon strains from one geographical section were usually identical, but strains from different localities were seldom identical.

Wallick and Stuart (1943), in a study of 650 *E. coli* strains isolated from one person, found 85.3 per cent of them to be serologically identical when tested with 10 antiserums against strains from this individual. Upon similar testing of 75 cultures from 3 members of this person's family, however, only 16 per cent proved to be identical; and of 100 cultures from different persons only 1 per cent was found to be identical when tested with these antiserums. Subsequent tests of *E. coli* from this individual showed a continuous succession of different antigenic types and, after a few months, an apparently complete disappearance of the antigenic types first isolated; later examinations, however, revealed a reappearance of many of these types, to be again followed by their almost complete absence a short time afterward. Wallick and Stuart suggested the presence of a bacteriophage to account for these disappearances of types, although an attempt to isolate one was unsuccessful. Antigens common to coliform and *Salmonella*, or to coliform and *Shigella* organisms have also been reported by several observers, notably Peluffo, Edwards, and Bruner (1942), Edwards, Cherry, and Bruner (1943), Wheeler (1944), and Stuart *et al.* (1943). This last group of investigators concluded: "As more information is collected, it seems likely that a continuous series of antigenic relationships can be shown for the bacteria included in the coliform, paracolon, *Salmonella* and *Shigella* groups."

On the whole, however, because of the lack of marked serological homogeneity in either the coliform or the paracolon group, and the lack of any evidence of a significant correlation between the source of a strain and its antigenic type, it appears at the present time rather improbable that the water bacteriologist will derive much practical advantage from attempts to differentiate coliform and related organisms by serological means.

CHAPTER IX

SIGNIFICANCE OF THE PRESENCE OF COLIFORM ORGANISMS IN WATER

Coliform Organisms in the Intestines of the Lower Animals. Coliform bacteria are by no means confined to the human intestine. Dyar and Keith (1893) found *Escherichia* organisms to be the prevailing forms in the cat, dog, hog, and cow. Smith (1895) recorded the presence of the same bacteria, in almost pure cultures, in the intestines of dogs, cats, swine, and cattle; and he also found them in the organs of fowls and turkeys after death. Numerous other reports of the isolation of *Escherichia* organisms from animal intestines soon followed these early observations; only a few need be cited. Eyre (1904) found them in 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. Ferreira, Horta, and Paredes (1908*b*) isolated 81 lactose-fermenting bacteria from 38 species of mammals and 8 species of birds, including monkeys, bears, wolves, foxes, hyenas, lions, panthers, tapirs, a camel, deer, and ostriches from the zoölogical gardens; these cultures were studied by an elaborate series of tests and 93 per cent of them proved to be typical *Escherichia* organisms. Konrich (1910) examined 170 samples of feces from a large number of different animals, including man, moles, earthworms, an adder, and a trout; *Escherichia* bacteria were isolated from every species of animal examined and from all but 14 of the samples of feces. These and similar results reported by many other observers proved conclusively that coliform organisms, principally of the *Escherichia* type, are generally present in the feces of all warm-blooded animals. Foote (1937) has indicated a possible exception to the general rule; upon examining 37 specimens of feces from 13 animal species, he rarely succeeded in isolating coliform-like bacteria, even after enrichment of portions of the samples in lactose broth, in the feces of *Herbivora* of cleanly habits such as the deer, elk, and moose, when in their native state and habitat; the feces of animals of the scavenger type, however, such as the bear and coyote, and those in captivity or otherwise exposed to contamination from domes-

tic stock or human habitations, were usually found to contain these organisms.

In cold-blooded animals the occurrence of coliform bacteria is less constant. Amyot (1902) failed to find *Escherichia* organisms in the intestines of 23 fish representing 14 species; Johnson (1904), however, isolated them from 47 of 67 fish caught in the polluted Illinois and Mississippi rivers. Bettencourt and Borges (1908*b*) isolated 29 cultures of coliform-like bacteria from the intestines of 17 types of fish, reptiles, and amphibia; only 8 formed gas in lactose broth and only 2 (from an eel and an adder) proved to be typical *Escherichia* organisms. Browne (1917) in a later study found that, of 93 scup taken from generally unpolluted waters near Woods Hole, 27 contained *Escherichia* but not bacteria of the *C. welchii* group; 18, members of the *C. welchii* but not of the *Escherichia* group; 10, members of both groups. More recently Kline and Fuller (1932) reported finding coliforms in the intestines of 66 per cent of salamanders frequenting springs in New York State during the months of May and June, but in those of only 11 per cent during the month of February. The evidence, therefore, appears to support the conclusion of Fromme (1910) that whereas coliform bacteria are regularly present in warm-blooded animals, they are present often, but not regularly, in cold-blooded animals.

Types of Coliform Organisms in Human and Animal Excreta. Early observers were generally agreed that indole-forming organisms of the *Escherichia* group comprise the great majority of the coliforms present in human and animal feces. By means of the more modern differential methods it was soon established that the M.R.+, V.P.—, citrate— coliform, or *E. coli,* is the predominant fecal type and that it is usually an indole former. Levine (1921*a*) summarized the results of 13 studies of coliforms found in human dejecta by various observers; exclusive of a high percentage obtained in one investigation in which selective methods of isolation were employed, the average percentage of M.R.+, V.P.— coliforms isolated was 96.6. Ruchhoft, Kallas, Chinn, and Coulter (1931) presented another summary of results obtained in various countries from six studies of human and animal feces undertaken during the preceding decade from which it may be calculated that the average reported percentage of citrate— organisms (presumably *E. coli*) was 92.9. These observers, very carefully purifying, or stabilizing, 486 cultures of coliforms which they isolated from 32 samples of human and animal feces, found 83.2 per cent to be indole+ *E. coli* and 11.5 per cent to be indole— *E. coli*; only 5.3 per cent were of other types (3.3 per cent *Aerobacter*

and 2.0 per cent intermediate) despite the fact that not only direct plating but also a method favoring selection of citrate+ forms was employed for isolating the organisms. Gray (1932) reported all of 200 coliform cultures obtained from feces by direct plating on MacConkey agar to be of the M.R.+, V.P.— (*Escherichia*) type; direct plating on citrate agar, however, showed that all but 3 samples (taken from the same person) of the 40 examined contained some M.R.—, V.P.+ (*Aerobacter*) organisms; apparently a few intermediates were found. Burke-Gaffney (1933) studied 500 coliform strains from human feces and, on the basis of their methyl-red and citrate results, found that 87 per cent were *E. coli,* 8 per cent *Aerobacter,* and 4 per cent intermediate forms. Employing selective methods, Bardsley (1938) succeeded in isolating citrate+ coliforms from 61 of 100 human feces specimens. Reedy and Puncochar (1940), also by selective methods, demonstrated the presence of *A. aerogenes* in 87 per cent of 250 samples of feces from normal adult women.

Parr (1938) made a detailed study of 235 specimens of feces from 100 persons, 93 of whom were found to be discharging *E. coli,* although occasional specimens did not yield this type of coliform. The 7 subjects not yielding this organism were babies under 2 weeks of age. *Aerobacter* types were found in the feces of 33 persons, *A. cloacae* in those of 14, and intermediates (including M.R.+ or indole+ aerogenes types) in those of 31. Of the total of 235 specimens, no coliforms were isolated from 11 even with enrichment methods. Seven specimens yielded only citrate utilizers, 1 only intermediates, and 1 only *A. aerogenes* types. *E. coli* was found in 218 specimens, *Aerobacter* forms in 66 (*A. cloacae* in 21), and intermediates in 51. Nineteen specimens contained *E. coli, Aerobacter,* and intermediate types. The results of this study are particularly significant because of the large number of organisms examined: 1,987 strains were isolated, purified, and studied in detail and 4,837 additional strains were subjected to citrate tests.

Very few of the reports cited above included results of a quantitative character. The usual first step in the procedure of isolation was to place an unweighed particle of the feces sample or a loop of a suspension of unknown feces content into an enrichment tube, or to smear it over the surface of an agar plate. Tonney and Noble (1930), employing direct plating in cyanide-citrate agar, recorded an average of 92.8 million coliforms per gram in 132 samples of human feces, of which only 0.37 per cent were judged by the appearance of their colonies on the plate to be *Aerobacter* types; in 324 samples of animal feces the average coliform density was 689 million, of which 0.58 per

cent were considered *Aerobacter* forms. According to figures obtained after biochemical differentiation of isolated organisms the samples of human feces contained an average of at least 200,000 *Aerobacter* or intermediate coliforms per gram, and the animal feces at least 640,000 *Aerobacter* or intermediates per gram. Reedy and Puncochar (1940), by direct plating in E.M.B. agar and in citrate agar, found the ratio of citrate utilizer to *Escherichia* counts of 253 feces samples from normal adult women to vary from an average of 1 : 110, represented by 90 ratios within the range of 1 : 1 to 1 : 500, to an average of 1 : 28,249, represented by 16 ratios ranging from 1 : 10,000 upward; 4 ratios, averaging 1.5 : 1, indicated that citrate utilizers outnumbered the other coliforms in these 4 samples; the over-all average ratio (calculated from the data presented) is 1 : 3,696, but this does not include 82 samples in which citrate utilizers were not found in the 1 : 10,000 dilution (although from 54 samples they were recovered by enrichment in citrate), nor 4 samples in which no *Escherichia* coliforms were found in that dilution. In 166 samples the citrate utilizers numbered at least 10,000 per gram of feces; they were detected in 220 samples.

Although the normal kidney and bladder are free from bacteria, the urine from individuals with genito-urinary infection may contain coliform organisms of various types. The proportion of *Aerobacter* to *Escherichia* strains in such urine appears to be usually greater than that generally found in feces. Hill, Seidman, Stadnichenko, and Ellis (1929), in a study of 179 coliforms isolated from persons with genito-urinary infection, reported results which permit grouping these organisms as follows: *E. coli,* 30 per cent; intermediates, 26 per cent; *A. aerogenes,* 32 per cent; and *A. cloacae,* 12 per cent. Burke-Gaffney (1933) found that of 1,000 coliforms isolated from such urines in the tropics only 33 per cent were *E. coli,* 10 per cent intermediates, and 52 per cent *Aerobacter* types. Sandholzer (1936), on the other hand, reported 83 per cent of 530 coliforms isolated from 283 individuals with urinary infection to be *Escherichia* and 13 per cent to be *Aerobacter* strains.

The effect of storage upon the coliform flora of excreta is of special interest to the water bacteriologist because the sporadic pollution to which natural waters of fairly good quality are often subjected usually derives from human or animal excreta that have been " stored " for varying periods, either in a privy vault or manure pit, or on or in the ground. Jordan (1926) studied the changes in the coliform flora of feces held at various temperatures and found that although multiplication of some of the contained bacteria followed evacuation

of the feces, *E. coli* did not multiply but gradually died off until after 2 to 3 months at —11° C. it could not be recovered. The period of survival was shortened by storage at higher temperatures. Parr (1937, 1938) reported the results of extensive studies of feces that were suspended in saline and stored at 37° C. and in the cold room. At the higher temperature the coliforms usually persisted only about 3 months, but in the cold room those of some samples were viable after nearly 3 years. *E. coli,* when the only coliform type contained in the feces, survived longer than a year in one specimen and remained true to type throughout this period; when accompanied by other coliform species, however, *E. coli* was usually supplanted by them. This longevity of coliforms in stored feces is the more interesting in view of the fact that, as Parr points out, they must compete with a great variety of other bacteria such as anaerobes, cocci, spirochetes, and the "Bacillus P" of Clemesha (1912*a*) — a resistant, non-sporing aerobe, not fermenting lactose but usually fermenting glucose, which is generally present only in small numbers in fresh feces but which becomes very abundant in long-stored feces.

It may be concluded from these and other data that fresh feces usually contain from 5 to 500 million or more coliform organisms per gram; that well over 95 per cent of these are *E. coli,* the great majority of which are indole formers; that only a very small proportion, probably much less than 5 per cent, are *A. aerogenes;* and that even a smaller proportion are *E. freundii* (intermediates). *A. cloacae* may also be present in small numbers. It must be remembered, however, that the proportions of types vary in different samples: practically all the coliforms in a sample may be *A. aerogenes,* and at least a large proportion may be *E. freundii* or *A. cloacae;* the coliform flora of a single person may also vary markedly from time to time. Furthermore, although the percentage of coliforms other than *E. coli* is usually extremely small, their numbers per gram may be large; for example, *A. aerogenes* probably occurs in most feces samples in numbers ranging from 10,000 to 500,000 or more per gram. Intermediates likewise occur frequently and in considerable numbers in feces, although less frequently and in fewer numbers than *A. aerogenes.* A considerable proportion of both of these types may be indole formers. In urine from genito-urinary infections, on the contrary, *E. coli* appears to be often outnumbered by *Aerobacter* coliforms, and the proportion of *E. freundii* strains approaches that of *E. coli.* The numbers and proportions of the different coliform types in urine, as in feces, vary greatly. Storage of feces results in the ultimate extinction of all species of contained coliforms, but they may persist

for several months at body temperature and for 2 to 3 years or more at low temperatures. The citrate ultilizers usually remain viable much longer than *E. coli*.

Coliform Organisms in Dust and Soil. Winslow and Kligler (1912) showed that coliform bacteria may be very abundant in the dust of city streets and houses, as might naturally be expected. They examined 24 samples of street dust and 72 samples of house dust (all in New York City). All of the street dusts and 63 of the 72 house dusts contained coliforms in at least 1 of 3 duplicate 0.01-gram portions. In 2 street samples the numbers rose to 330,000 and 660,000 per gram respectively, but the largest indoor result was 60,000. The average for the indoor dusts was between 1,000 and 2,000 per gram and for the street dusts over 50,000 per gram. This dust was dust deposited on surfaces and would be carried up into the air only by currents of some force. It is well known that coliform organisms are, as a matter of fact, rarely present in street or house air. Konrich (1910) exposed open Petri dishes of glucose broth to the air of Jena streets for 24-hour periods, daily, for 3 months and found coliform bacteria only 11 times. The coliforms in street dust may, however, account for the anomalous positive results sometimes obtained in reservoirs bordered by roadways.

The occurrence of *E. coli* in soils was the subject of several early investigations by Houston, Savage, and others, the results of which indicated that this organism was rarely to be found in virgin soils, but in soils known to be polluted with human or animal excreta it was usually present, sometimes in large numbers (1,000 per gram). A particularly interesting contribution to the study of coliform organisms in soil was made by Konrich (1910). Out of 547 samples of soil, 65 per cent showed *E. coli* in portions of between 0.1 and 0.5 gram. The farther removed from cultivation a sample was, the less were the chances of positive results. He concluded that *E. coli* is widely distributed in the outer world, that it is almost always found in soil from cultivated fields or from traveled places, and that the farther a source is removed from travel and from cultivation the more rarely is the organism found; but it is never altogether absent. On plants or parts of plants it is frequently found when they come from cultivated land; on plants from waste places it is rarely found. It seems probable that coliforms may be even more widely distributed in a thickly settled and intensively cultivated country like Germany than in the great rural areas of some parts of the United States and Canada.

These early observations and conclusions were amply confirmed by

later investigators. Johnson and Levine (1917), employing direct plating and enrichment methods, examined 42 samples of soil from various sources, including fallow and cropped experimental plots. Coliform organisms were found to be abundant in the soils of all the 11 cropped plots, but in those of only 4 of the 13 fallow plots; they were rare in the soils of 2, and absent in those of the other 7 fallow plots. Of 177 strains isolated from these soils, 80.4 per cent were *Aerobacter*, the majority of which were *A. cloacae*, and 19.6 per cent were *Escherichia* organisms. Burton and Rettger (1917), using glucose broth enrichment at 37° C. for isolation, obtained very similar results from 1,000 presumably uncontaminated samples of soil, leaves, berries, sand, twigs, etc. Chen and Rettger (1920) employed direct plating, usually on plain agar, with incubation at 30° C. for 48 hours, in the examination of 317 samples of soil, most of which were presumably uncontaminated, and found only 4.3 per cent of the 467 strains isolated to be of the *Escherichia* type and only 3.6 per cent to be *A. cloacae*. It is interesting to note that no coliform-like organisms were isolated from 60 per cent of the soils although colonies fished from the agar plates were transferred to lactose broth and incubated for 5 days at 30° C.

Similar results were reported by Koser (1924*a*). Using lactose broth enrichment at 30° C. he isolated, from 70 relatively uncontaminated soils, 72 coliforms, 14 of which required cultivation for several days before they produced gas in lactose even at 30° C. From 10- to 15-gram quantities of 17 of these soils no coliforms were recovered. Only 3 per cent of the strains proved to be *E. coli*; 32 per cent were intermediates, and 65 per cent were *Aerobacter* types. Koser (1926*a*) reported another series of results employing lactose broth enrichment and direct plating for isolation of coliforms from soils of cultivated fields and grazing pastures. Of 41 samples from the comparatively uncontaminated fields 7 failed to yield any coliforms although quantities of soil up to 10 grams were examined. All the 11 pasture samples yielded coliforms, some from a dilution of 1 : 1,000. Of 104 strains isolated from the cultivated fields 67 per cent were *Aerobacter* in type, 8 per cent were intermediates, and 23 per cent *E. coli;* of 33 strains from the pastures 33 per cent were *Aerobacter* forms, 3 per cent were intermediate, and 64 per cent *E. coli.* Koser remarks upon the apparent irregularity of the distribution of coliforms in soil; 0.1-gram quantities of one sample might yield these organisms whereas none could be recovered from 10-, 1-, or 0.1-gram quantities of another sample from the same field.

An examination by Frank and Skinner (1941) of 356 strains of

coliforms isolated by means of brilliant-green bile enrichment from virgin soils, or field soils not manured for 10 years, showed about 21 per cent to be *E. coli*, 34 per cent intermediate, and 45 per cent *Aerobacter* types.

The preponderance of *Aerobacter* types and the frequent presence of intermediates among the few coliforms found in relatively unpolluted soil has been reported also from other parts of the world. Raghavachari (1926) found 93.5 per cent of 518 soil organisms to be *Aerobacter* strains; 5.0 per cent were *E. coli* and 1.5 per cent were intermediates. Hicks (1927) reported 80 per cent of his Shanghai soil cultures to be citrate utilizers. Bardsley (1934) studied 86 samples of English soil, three fourths of which yielded no coliforms although 15 grams of each were examined by enrichment of various dilutions in MacConkey broth. Of 152 cultures of coliforms recovered, 31 per cent were *E. coli*, 66.4 per cent intermediate, and only 2.6 per cent *Aerobacter* strains. Burke-Gaffney (1932) found only *Aerobacter* types of coliforms in Dar-es-Salaam soils that were considered to be unpolluted, whereas 29 per cent of the coliforms isolated from remotely polluted soil were intermediates and 67 per cent were *Aerobacter* types.

The fate of coliforms when deposited in or on the soil has also been studied extensively. This subject is of particular interest to the water bacteriologist because of the possibility that coliform types with different survival periods might serve as indicators to permit distinction between recent and remote pollution, and because of the possibility that certain coliform types might change to other types with a consequent effect upon the interpretation of the results. Much effort has been expended in the attempt to induce variation or mutation of one coliform type to another, but with the exception of the loss or gain of a minor characteristic such as the ability to form indole or to ferment a carbohydrate — changes that may frequently be induced by means of physical, chemical, or serological treatment of cultures — reports of success in effecting fundamental changes in type, such as the transformation of *E. coli* to *A. aerogenes*, are extremely rare. As already indicated, an occasional strain of *E. coli* may give off citrate+ variants and the M.R. and V.P. results of a citrate+ strain may be reversed upon long cultivation; but even these variations which, after all, are limited in extent, occur very seldom. It may be concluded, therefore, that, although instability on the part of an occasional coliform organism may result in a limited departure from its original type, the probability of *radical* change in coliform type may be safely ignored by the water bacteriologist.

As remarked earlier, the survival periods of the various coliform types in soil are of much practical interest. Horrocks (1903), after adding *Escherichia* suspensions to various soils, was successful in recovering the organism from sandy soil 60 days later. Clemesha (1912*a*) reported *A. cloacae* to be the only coliform found in earth from trenching grounds 1½ to 2 years after it had been polluted. Skinner and Murray (1926) found the number of coliforms in soil inoculated with cow feces to be steadily reduced from 600,000 per gram at the beginning of the experiment to none after 122 days. *E. coli* and *A. aerogenes* broth cultures were added to separate portions of soil and a mixture of the two cultures to another portion; the separately inoculated coliforms were not recovered after 176 to 218 days, and the survival period of the mixed cultures was of about the same duration. No multiplication of the coliforms in the soil was observed. Kulp (1932) seeded separate sterilized soils, occasionally wetted with sterile tap water, with 15 *E. coli* and 9 *A. aerogenes* cultures, and after 3 years and 7 months recovered organisms of 6 of the former and of 2 of the latter cultures. Kline (1935) inoculated sterilized soil contained in a 3-inch stove pipe 20 feet long with a purified culture of *E. coli* and kept the soil wet with sterile water. After 410 days, when the experiment was discontinued, the identical organism was recovered. At intervals after about 45 days, however, an occasional organism atypical with respect to the methyl-red, citrate, or Eijkman reaction was recovered. Kline also soaked small stones in similar cultures and then transferred them every 24 hours to flasks of sterile water; in some experiments the organisms died out within a few days; in others, they persisted for long periods — at least 544 days in one instance. No change in the type of coliform recovered was observed until after 185 days, when several atypical forms were isolated.

It appears, therefore, that coliform organisms are not abundant in relatively uncontaminated soils and that, of the few bacteria of this group which may be present, *Aerobacter* types, with *A. cloacae* well represented, are usually the most numerous. Intermediates also frequently constitute a considerable proportion of the coliforms found in such soils, but *E. coli* is usually present, if at all, in comparatively small numbers. The distribution of coliforms in soil is evidently very irregular, samples of considerable volume often failing to yield them; and the more remote an area is from human and animal pollution the less likely are coliforms to be found in the soil. Once a soil is contaminated with these bacteria, however, months or years may pass before they die out either from lack of food or moisture or because of antagonism of other organisms. *E. coli* apparently suffers the most

rapid reduction in number, whereas *A. aerogenes,* and especially *A. cloacae,* are much more resistant. Finally, there is little evidence to indicate that a long sojourn in the soil will effect any marked change in coliform type.

Coliform Organisms on Plants and Plant Products. The possibility was long ago suggested by various observers that coliform bacteria may live in a semi-parasitic fashion on plants as well as on animals. Of a series of 47 cultures of lactic-acid-producing organisms isolated chiefly from cereals, flour, bran, cornmeal, oats, barley, etc., by one of us (Prescott, 1902*a*, 1903, 1906), 25 were found to give the reactions of typical " colon bacilli," as then defined, even to pathogenicity upon inoculation into guinea pigs. From Germany identical results were reported by Papasotiriu (1901). Klein and Houston (1900), on the other hand, found typical coliforms in only 3 of 24 samples of wheat and oats obtained from a wholesale house; rice, flour, and oatmeal bought at one retail shop were found to contain *Escherichia* organisms but none was found in those from another shop. Clark and Gage (Massachusetts State Board of Health, 1903) were unable to isolate *Escherichia* bacteria from standing grains. Winslow and Walker (1907) examined 178 samples of grain and 40 of grasses for these organisms without success. Konrich (1910) obtained positive results from 46 per cent of 0.1- to 0.5-gram samples of cultivated plants, 6 per cent of samples of leaves, grasses, and herbs in waste places, 91 per cent of hay, and 55 per cent of grain samples. The work of Rogers and his associates, to which reference has been made in the preceding chapter, indicated that the coliform-like organisms found on grains are generally of the *A. aerogenes* and not of the *Escherichia* type. Of 152 cultures of such organisms isolated by means of enrichment in glucose broth at 30° C. from dried grains (most of which were taken from cars on the track) and 14 cultures isolated from 2 of 3 samples of green oats, 7 usually failed to ferment lactose, 8 appeared to be of the *Escherichia* type, and 151 (91 per cent) of the *Aerobacter* type. Forty of this last group liquefied gelatin and produced a yellow pigment. Even the 8 *Escherichia*-type organisms formed a somewhat similar pigment, and could be distinguished from the ordinary *Escherichia* bacteria by this characteristic. It may be noted that Gililland and Vaughn (1943) also described a number of pigmented (yellow to orange) coliform cultures.

McCrady (1916), employing enrichment in lactose broth at 37° C. for isolation, studied 179 coliforms obtained from 12 samples of dried grains collected in the Montreal Harbor elevators; 76 per cent of them gave M.R.—, V.P.+ results. Stokes (1919) found 74 per cent

of 77 cultures obtained from various prepared breakfast foods and grains to yield cultures of the *Aerobacter* type. Particularly interesting results in this connection were reported by Allen and Harrison (1936). Employing bile salt broth in the dilution method with incubation at both 30° and 37° C., these observers found 1,000 to 1,000,000 organisms producing gas at 30° C. per gram of grass from grazed pastures and those not recently grazed, but only 1 of 6 samples yielded coliforms in the 1 : 100 dilution (the lowest used) upon incubation at 37° C. Experiments with grass silage showed a rapid development of lactose fermenters producing gas at 30° C. to as many as 10,000,000 per gram, but those capable of forming gas at 37° C. seldom numbered more than 100 per gram. The predominating type of organism fermenting lactose at 30° C. was a gelatin-liquefying citrate utilizer which did not produce gas in lactose at 37° C. Wilson *et al.* (1935) examined samples of grass, hay, straw, decaying leaves, swedes, water, various foodstuffs, grains, and grain products, employing enrichment in MacConkey broth with incubation at 37° C. Only 36 of 65 samples of grain and grain products yielded coliforms, and of 57 strains isolated, 23 per cent were *E. coli* or related irregular types, 5 per cent intermediates, 46 per cent *A. aerogenes,* and 23 per cent *A. cloacae.* Of the other 25 samples (grass, hay, etc.) coliforms were found in only 11; and a study of the 23 strains isolated showed 56 per cent to be *E. coli* or related irregulars; 22 per cent, intermediates; 9 per cent, *A. aerogenes;* and 13 per cent, *A. cloacae.*

Stuart *et al.* (1938), upon streaking E.M.B. plates with decayed parts of fruits and vegetables, and employing incubation at room temperature for 48 hours, found that of 42 cultures only 13 produced acid and gas in lactose broth at 37° C. (and at room temperature), 24 produced gas only at room temperature, and 5 produced acid alone at either temperature. All the gas formers utilized citrate; 10 of these were intermediate in type and 27 were of the *Aerobacter* group.

Unfortunately many of the coliform organisms reported to have been found on plants were isolated by means of glucose broth or other special media, or by using lower temperatures than that employed in water work, because it was realized that these conditions favor the growth of certain " grain types " and " soil types " of these bacteria. It is probable that a proportion of these organisms, had they been contained in water samples, would not have been included in coliform estimates of those waters. Nevertheless sufficient data obtained by the use of lactose broth and incubation at 37° C. are available to establish beyond doubt that coliform organisms, as defined by the water bacteriologist, do occur on presumably uncontaminated plants,

leaves, grasses, etc. As in relatively unpolluted soil, however, their numbers are generally small; a considerable proportion of samples of such materials may yield no coliforms.

All types of coliform organisms are found on plants, grains, etc., but *A. aerogenes* strains are usually in the majority, with *A. cloacae* well represented. Intermediates are also frequently found. *E. coli* organisms, however, generally constitute a modest minority.

The Natural Habitat of Coliform Organisms. The appellations "grain type," "soil type," and "fecal type" have been frequently employed to designate coliforms exhibiting certain characteristics; and just as the animal body has been designated as the natural habitat of *E. coli,* plants and even soil have at times been designated as the natural habitat of other coliform species. We have found, however, that *E. freundii* and *Aerobacter* organisms constitute a small but rather persistent proportion of the coliforms in human and animal feces, and that the number of *Aerobacter* per gram varies roughly from a few to 500,000 or more — not an insignificant figure. In some feces specimens *A. aerogenes* or intermediates may be the only coliforms found, and in urine from genito-urinary infections and in feces from intestinal disturbances *A. aerogenes* may be very numerous. The animal body, therefore, serves as a constant reservoir of all types of coliform organisms. Man and other animals, including birds, fish, etc., that have been in direct or indirect contact with man or unclean animals, nearly all apparently disseminate coliforms of various types. Birds, especially, may be expected to contaminate soils, grasses, leaves, etc., even in places far from man and other animal coliform carriers. Houston (1928) estimated the average discharge of coliforms from a gull to be 50 to 100 millions per day.

When coliforms leave the animal body, a struggle for existence ensues. Parr (1938) showed that coliforms usually multiply in fresh feces at first, but that later a reduction occurs, *E. coli* dying off more rapidly than the other coliforms. It should be noted that coliform organisms are not particularly fastidious in their food requirements. Houston (1916) was the first to direct attention to the ability of certain coliforms (producing indole and not liquefying gelatin) to grow on leather washers of water taps; and several observers, including Greer (1928), Spaulding (1929), and Caldwell and Parr (1933*b*), have reported similar growths on leather washers or leather packing. Caldwell and Parr found *A. aerogenes,* both indole+ and indole— types, and also intermediate coliforms, to be involved in such "pump infection." Spaulding (1931) reported the multiplication of coliforms on jute packing used in the joints of water mains. Leahy (1932)

discovered an indole— *Aerobacter* strain multiplying on a cotton guard-rope partly immersed in a swimming pool, and Parr (1937) showed that a pure culture of *E. coli* derived from feces could be grown on bits of string in tap water (the whole previously autoclaved) even at ice-box temperature. Tonney and Noble (1930) examined 51 samples of decaying wood and found an average of 380 coliforms per gram by their direct plating method; biochemical differentiation indicated that approximately 2.5 per cent of these organisms were citrate-negative. Tonney and Noble (1931*b*) reported also that *E. coli* and *A. aerogenes* suspensions, prepared from feces and from cultures, survived for 9 to 228 days when planted in decayed stumps of trees.

Beckwith (1931), in a study of wood-pulp slime, concluded that the causative organism was a bacterium apparently *A. aerogenes* in type, which he usually recovered from the water-soaked portion, but not from the sound interior, of a sectioned pulp log. Sanborn (1944), reviewing the literature relative to slime-producing organisms of the coliform type, pointed out that varieties of *A. aerogenes* have been implicated in the production of ropiness in dairy products, in maple syrup, and even in tea. He reported that examination of slime growths from 340 pulp and paper mills has shown that coliform bacteria were prevalent or the principal cause of slime in 52.6 per cent of them. Of 175 samples of slime examined, *A. aerogenes* was the principal slime producer in 35 per cent, and organisms more closely related to *Escherichia* varieties in 6 per cent; *A. cloacae* predominated in 59 per cent of the samples. The Imvic reactions (Parr, 1936) of 82 *Aerobacter* organisms were − − + + (75%), − − − + (19%), and − − + − (6%); the reactions of 12 *E. coli* were + + − − (7 times), + − − − (4 times), and − + − − (once). All of Parr's 16 possible Imvic combinations were represented. A few of these mucoid variants caused partial rotting of carrots and softening of potato, resembling in this respect *Erwinia carotovora,* but most of them did not show this characteristic. On the other hand, many resembled *Klebsiella* species in certain important details. In any event, bacteria of this kind that produce gas from lactose in 48 hours at 37° C. must be included in the coliform group of organisms.

It is evident from the above examples that coliform organisms may derive nourishment from a variety of vegetable, as well as of animal, substances. Consequently, when these bacteria are deposited on or in the soil, on leaves, grasses, twigs, or decaying wood, they apparently often encounter conditions that favor the survival of some

strains for days, months, or longer. Although *Aerobacter* and intermediate strains usually outlast those of *E. coli*, it is probable that occasionally conditions are such as to permit the latter to outlive its associates; a temperature of 45° to 46° C., for example, might well favor *E. coli* by inhibiting the growth of other coliforms.

Since available data indicate, however, that very few coliforms are usually found in virgin soils, on plants, leaves, etc., it appears that any multiplication taking place there must ordinarily be fortuitous and very restricted; and the environment where such limited multiplication occurs hardly deserves to be designated a *habitat* of the coliform organisms present. Taylor (1942), reviewing the literature on the ecology of coliforms found in water, and remarking upon the paucity of reported *quantitative* data regarding the occurrence of coliform bacteria on grains, plants, etc., and upon the lack of any proof that coliforms multiply in the soil or on fresh grasses or grains, concludes, " there is insufficient evidence to justify the definite statement often made that *Bacterium aerogenes* and intermediates are normal inhabitants of soils, grasses and grains." It seems, nevertheless, that vegetation may constitute a natural habitat for certain slime producers, and probably a few members of the tribes *Erwineae* and *Serrateae* which conform to the definition of coliforms. Fortunately, these bacteria apparently very seldom occur in water in sufficient numbers seriously to affect the results of the coliform test. With this possible exception, there seems to be little ground for belief that the natural habitat of the coliform organisms found in water is other than the animal body. They may be widely distributed in nature, they may survive for varying periods outside the animal body, and in exceptional circumstances they may even multiply extensively; but the available evidence indicates that when outside the animal body they practically all eventually succumb to the unfavorable influences of an unnatural environment.

The Viability of Coliform Organisms in Water. Observers are agreed that some coliform organisms may persist for long periods in water. MacConkey (1905) reported the survival of *E. coli* for about a year in a filter candle immersed in tap water. Rogers (1917) found *Escherichia* and *Aerobacter* organisms still surviving 9 months after inoculation of water with feces, the latter type predominating by a wide margin at the end of the experiment. In sewage held in running water the proportion of *A. aerogenes* forms changed in 7 days from about one third to more than one half of the coliforms present. Winslow and Cohen (1918*a*) recovered both *E. coli* and *A. aerogenes* organisms from water 60 days after inoculation. These

types were isolated by Platt (1935) from some of his inoculated water samples 80 days after seeding. Frost and Streeter (1924) estimated that in the stretch of the Ohio River extending from Cincinnati to Louisville the percentage of coliforms remaining (during the seasonal period April–November) was about 64 after 10 hours of flow, 41 after 20 hours, 10.9 after 50 hours, 1.4 after 100 hours, and 0.0921 after 300 hours, the maximum time of flow. A continuation of the survival curve indicated that 0.00186 per cent would remain after 1,000 hours. Thus, although the great majority of the coliforms which enter water die off at a logarithmic rate in proportion to the time of storage, as shown by these figures and also by the work of other investigators, including Winslow and Cohen (1918*a*) and Cohen (1922), it is clear that a few survive for long periods.

Several investigators have reported *Aerobacter* to outlive *E. coli* strains in water, but Ruchhoft *et al.* (1933) found only an insignificant change in the proportion of *E. coli* among the coliforms present in polluted water after self-purification of the latter which resulted in a coliform reduction of over 99 per cent.

It may be concluded, therefore, that coliforms of various types may survive in fresh water for weeks or months unless conditions are very unfavorable. Whether some types outlast the others appears to depend upon existing circumstances, for the reported experience of observers with respect to the relative survival periods of the different species of coliforms varies considerably.

In sea water, on the other hand, it is generally agreed that coliform organisms die off very rapidly. For example, an experiment reported by the Massachusetts Department of Health (1936) showed that a coliform count of 500 per ml. in a mixture of sea water and 1 per cent sewage was reduced to 100 after 24 hours, to 50 after 48 hours, and after 96 hours coliforms were present only in 10-ml. portions of the mixture.

Significance of the Various Coliform Types in Water. The sanitary significance attributed by early bacteriologists to the presence in water of coliform organisms which formed indole and failed to liquefy gelatin was not without some basis in fact; for, as we have seen, such bacteria are abundant in feces. It was soon realized, however, that many *Aerobacter* strains derived from soil also possessed these characteristics, and that alternative methods of differentiation such as fermentation of sucrose and other carbohydrates likewise failed to furnish adequate information relative to the source of the various water-borne coliforms. The advent of the methyl-red test, therefore, aroused considerable interest, for it appeared to furnish

a very simple method of distinguishing fecal from non-fecal contamination of water. Recognition of the inverse relationship between methyl-red and Voges-Proskauer results, which followed a little later, provided still another means of effecting the same differentiation. It seemed reasonable to expect a significant proportion of the coliforms found in unsafe waters to be M.R.+, V.P.— (*Escherichia*) in type, the great majority of those found in safe waters to be M.R.—, V.P.+ (*Aerobacter*) in type, and the ratio of *Escherichia* to *Aerobacter* coliforms present in a sample to vary more or less directly with the fecal contamination to which the water had been subjected.

That the problem of distinguishing safe from unsafe waters might not be so simply solved was soon demonstrated by several investigators. Rogers (1918), employing direct plating on asparagine agar, examined samples taken from a small stream flowing through a town of 4,000 inhabitants. At the lower municipal limits, the untreated city sewage entered the stream which then flowed through partly wooded farming country for 15 miles without further sewage pollution. Above the town where there was no sewage pollution 7 cultures all proved to be *A. aerogenes*. At a point 2 miles below the city sewer 10 of 11 strains were *Escherichia* organisms, and 2 miles lower down only 2 of 20 strains were of this type. All of 10 strains from a point 2 miles still farther downstream were *A. aerogenes*. Although these results conform in a general way to expectations, a grievous error might be committed were the water from the last sampling point adjudged safe simply because all of 10 coliforms isolated from it proved to be *A. aerogenes*. Rogers examined several other waters from some of which coliforms were isolated with characteristics that correlated very well with the sanitary quality of the supplies, but the types found in other waters did not correspond so well. For example, one spring that was carefully protected yielded 5 coliforms, all of the *A. aerogenes type;* but another carefully protected spring, distant at least a mile from any house and with no apparent source of contamination in the vicinity, yielded 5 coliforms of which 3 were *Escherichia* organisms and 2 were *A. aerogenes*. Two small brooks flowing through woods, contamination of which was only remotely probable, gave similar confusing results: 1 of 4 coliforms from one, and 2 of 4 from the other brook, were identified as *Escherichia* types. Rogers suggests several possibilities that might explain these results, such as pollution by wild animals, by fish, or by contamination from some other source not detected by the sanitary survey of these waters. But the fact remains that there were several instances where the

ratio of coliform types found in water samples was of very little sanitary significance.

Another discouraging report was that of Winslow and Cohen (1918*b*), who examined cultures isolated from polluted and unpolluted sources and from stored raw waters. Lactose broth enrichment was employed in this study; and the fact that 94 per cent of the cultures from the unpolluted sources were isolated from 100-ml. or 10-ml. sample portions testifies, as the authors point out, to the validity of their judgment regarding the sanitary quality of these waters — apparently the sanitary surveys of the sources were made with particular care. Differentiation of the 225 coliform strains isolated yielded the results shown in Table 77. It is evident that the percent-

TABLE 77. PERCENTAGE OF CULTURES FROM WATERS OF DIFFERENT QUALITIES EXHIBITING VARIOUS CHARACTERISTICS

(Winslow and Cohen, 1918*b*)

Type of Water	Methyl-red Negative	V.P. Positive	Indole Not Formed	Adonitol Not Fermented*	Gelatin Liquefied
Polluted	23	24	29	41	5
Unpolluted	24	24	49	10	3
Stored	15	15	38	42	5

* This test applied only to 53 methyl-red negative strains.

age of *Aerobacter* strains among the coliforms found furnished no indication of the relative sanitary quality of these waters. The indole results were more helpful in this respect, but even they could not be considered particularly informative. Rogers and his associates had remarked that all their *A. aerogenes* strains from feces fermented adonitol whereas few of their "grain type" organisms were adonitol fermenters; the results in the above table show that the fermentation of adonitol by M.R.— strains was just the opposite of what might have been expected. The authors frankly state, "Our study of a limited number of cultures . . . does not therefore seem to show any connection between the type of organism and corresponding source."

Greenfield (1916*b*) likewise found a certain uniformity in the proportion of V.P.+ coliforms in waters of different sanitary quality; and Lewis (1926) reported surprisingly high percentages of *Escherichia* organisms in samples from deep fissure springs of large volume in Texas at the immediate source of which no pollution was possible, although pollution of sheep-grazing lands on the catchment area might explain the unexpected results.

When Koser (1923) showed that the *Escherichia* group of coliforms

could be differentiated by the citrate test, and that the majority of soil coliforms of the *Escherichia* type are citrate+, it appeared probable that the citrate— type (*E. coli*) of the group might serve as a reliable indicator of fecal pollution. Koser (1924*a*) found the sanitary quality of water to correlate considerably better with citrate-test results than with those of the uric acid, or the M.R. and V.P. tests. The percentages of coliforms from his polluted and unpolluted water samples which failed to utilize citrate were 64.5 and 16.7, respectively, whereas the percentages that gave M.R.+, V.P.— reactions were 80.4 and 73.3. Lewis and Pittman (1928) employed the citrate test in an examination of 320 coliform cultures from 53 sources. Primary isolation was effected by means of various enrichment broths and the incubation temperature used at all stages of the work was 30° C. Of 83 strains from polluted waters, 86.7 per cent were of the M.R.+, V.P.— type; and of 237 strains from waters of high sanitary quality, 78 per cent, or nearly the same proportion, were of this type. The results with the citrate test were more interesting: 73.5 per cent of the polluted water strains and 34.1 per cent of the strains from waters of high quality were citrate—. Even this difference, however, is not very striking and probably would not be of much practical use. Table 78 shows the results of the various tests employed.

TABLE 78. RESULTS OF DIFFERENTIAL TESTS

(From Lewis and Pittman, 1928)

	Polluted Water		High Sanitary Quality Water	
	Number	Per Cent	Number	Per Cent
M.R.+, V.P.−	72	86.7	185	78.0
M.R.−, V.P.+	10	12.0	43	17.7
M.R.−, V.P.−	1	1.2	9	4.1
Citrate−	61	73.5	81	34.1
Cellobiose acid−, gas−	56	67.4	72	30.4
Indole+	56	67.4	101	42.0
Cellobiose acid+, gas−	0	0.0	21	8.9

Lewis and Pittman also grouped their coliform strains in accordance with a classification suggested by Levine (1921*a*), but found almost identical distributions of the coliforms obtained from the two grades of waters. Consequently this grouping, too, proved of little practical value in distinguishing the polluted from the unpolluted waters.

Employing the cyanide-citrate direct plating method followed by confirmatory biochemical tests of cultures from representative colonies, Tonney and Noble (1930) reported that whereas the ratio of M.R.+, V.P.— strains to M.R.—, V.P.+ strains in 92 sewage samples was about 1 : 1, the ratio of citrate— to citrate+ strains was about 1 : 2; the corresponding ratios of coliforms isolated from 45 surface runoff waters were 1 : 3.7 and 1 : 13.5. By means of a similar method of examination Ruchhoft, Coulter, Adams, and Sotier (1933) determined the percentage of *E. coli* among coliforms in Chicago raw sewage to be 35.7, in sewage plant effluent it was 33.0, and in Lake Michigan water at a pumping station intake it was 22.4. The percentages of intermediates in these waters were 10.6, 12.5, and 1.3, respectively. Here, the proportion of *E. coli* in the sewage, which contained a total of 13,815 coliforms per milliliter, was not much different from that in the lake at the water intake which contained 0.219 coliform per milliliter.

Another example of failure of the coliform ratio to assist in distinguishing between waters of different sanitary quality is furnished by the results obtained from a study of certain English lakes by Taylor (1941). A total of 288 coliform-like strains were isolated by using enrichment in MacConkey broth followed by plating on brilliant-green bile agar. The percentage of *E. coli* among the coliforms found in the samples from two polluted lakes and their inflows varied from 37.1 to 58.3, but the proportions found in the pure waters of a lake and its inflows, from which the city of Manchester draws an average of 40 million gallons per day, and around which special care had been taken to eliminate sources of pollution, were 87.5 and 98.2 per cent respectively. Very few (2.5 and 0.0 per cent) intermediates were found in the pure waters whereas they constituted from 9.0 to 29.6 per cent of the coliforms isolated from the polluted waters. Taylor states further that tests with uric acid and cellobiose furnished no additional information.

It appears, therefore, that the ratio of *E. coli* to other coliforms cannot be relied upon to furnish, consistently, helpful information relative to the sanitary quality of the water in which these organisms are found. The fact that even in raw sewage *E. coli* usually constitutes only about 50 per cent or less of the coliform population suggests that the proportion of this species present in a water of fair quality should be extremely small. Experience proves, however, that only too frequently, in such water, it approaches or even exceeds the percentage found in sewage. In unsafe waters, on the other hand, a large proportion of *Aerobacter* organisms usually accompanies any

E. coli that may be present. Consequently, although the ratio of *E. coli* to other coliforms is often well correlated with the results of the sanitary survey of a supply, too many instances of lack of such correlation are encountered to render this ratio of much practical value, except in cases of " coliform infection " of materials in contact with water, which will be discussed in a succeeding section.

Although type ratios may not prove particularly useful in the interpretation of coliform results, it does not necessarily follow that the sanitary significance of the presence in water of one coliform type should be considered exactly the same as that of every other type. The fact that usually nearly all the coliforms in feces are *E. coli* and most of those in soil are *Aerobacter* or intermediate types cannot be ignored. It appears reasonable, therefore, generally to regard the presence of the *E. coli* type in a water with more concern than that of other coliforms. Moreover, since members of the genus *Aerobacter,* particularly, are noted for their persistence in unfavorable surroundings, it seems reasonable generally to consider their presence in water an indication of pollution more remote than that indicated by the presence of *E. coli* organisms. Difficulties are very often encountered, however, when an attempt is made to apply these general considerations to a specific problem of interpretation of coliform results. In view of the fact that *Aerobacter* organisms are usually present in fresh feces in considerable numbers, that the coliforms of ordinary sewage are about equally divided between *Escherichia* and *Aerobacter* types, that the presence of *Aerobacter* organisms often affords one of the earliest indications of imminent danger, and that even soil contamination or surface wash must be regarded with grave suspicion because such contamination may include not only human fecal pollution but also animal *Salmonella* types pathogenic for man (discussed in Chapter X), it would obviously be extremely hazardous to disregard generally, as of no significance, the presence of intermediate or *Aerobacter* organisms in a water sample. The question confronting the interpreter of a result of coliform differentiation may be simply stated thus: how much less significance may be attached to the presence in water of *Aerobacter* or intermediates than to the presence of *E. coli?* To one who is concerned with the safety of the public health there can be but one answer: the margin of probable difference in significance is too doubtful to be given very serious consideration, unless the results of the sanitary survey of the water supply in question are completely in accord with definite indications furnished by the coliform differentiation. Such support will often be forthcoming in cases of " coliform infection," coliform multiplication

in mains and reservoirs, and occasionally in supplies apparently free from possible human or animal contamination; but often the results of the sanitary survey will not prove so helpful and, to be on the safe side, the interpreter of coliform results will of necessity attribute almost, if not the same, significance to the presence in water of *Aerobacter* or intermediate coliforms as to that of *E. coli.*

Because the practical advantage derived from differentiation of the coliform organisms found in a water is somewhat questionable except in certain circumstances, and because differentiation is ordinarily an arduous procedure involving isolation, purification, and biochemical examination of a number of organisms, it is not very generally employed, at least on this continent. If a very simple method of numerical determination of the various types present were available, it might prove worth the effort involved. The Committee, British Ministry of Health (1939), proposed a rapid method of examination based on the use of a modified Eijkman test and the citrate test. MacConkey broth gas-positives are transferred to MacConkey tubes for incubation at 44° C. for 24 to 48 hours, gas formation being considered a presumptive indication of the presence of *E. coli;* if none or a small proportion of such tubes show gas formation, the original 37° C. tubes remaining negative on subculture at 44° C. are transferred also to Koser's citrate broth and incubated at 37° C. for 2 to 3 days, growth being taken as a presumptive indication of the presence of I.A.C. (intermediate, *A. aerogenes,* or *A. cloacae*) organisms. The practice of testing a mixed culture of water organisms for citrate utilizers is open to criticism because of the variety of non-coliform bacteria that utilize citrate; and one is inclined to question the result obtained from testing a mixed culture after several days of incubation. Reports on the utility of this and proposed alternative procedures differ. The Committee points out that the citrate test is not strictly specific under these conditions. Sahib, Raghavachari, and Iyer (1939) found that 60 to 70 per cent of "areogenes-like" organisms isolated from Indian waters produced gas at 44° C., and considered their results to indicate the procedure to be inapplicable to tropical waters. Ferramola (1940) reported incubation at 44° C. to give excellent results with Argentine waters, and 92 per cent of citrate positives from 24-hour MacConkey gas-positives were confirmed as due to citrate utilizers; but only 61 per cent of the 48-hour MacConkey gas-positives were so confirmed, the organisms in the remaining 39 per cent consisting of non-lactose-fermenting, gram-negative bacilli. Incidentally, Ferramola found MacConkey broth to be superior to lactose broth for determination

of coliform organisms in the waters examined. For many years the differentiation of coliform organisms on the basis of their resistance to adverse conditions, as proposed by Clemesha (1912*a*), was employed by his successors at King Institute, Calcutta, in the examination of Indian waters; recently, however, Raghavachari and Iyer (1939) of the same Institute reported that their experience has indicated the *total coliform count* to be the only reliable measure of potability. Borman, Robinton, and Stuart (1941), in a study of Connecticut surface waters, likewise found no advantage to derive from differentiation.

The available evidence, therefore, appears to warrant the following conclusions relative to the sanitary significance of the presence in water of the different types of coliform organisms:

1. The ratio of the number of *E. coli* to that of other coliforms in a water cannot be relied upon as an indication of its sanitary quality.

2. Although the presence of *E. coli* in water may be considered, in general, more indicative of recent and dangerous pollution than is the presence of other coliforms, this generality is so subject to exception that its usefulness in the practical interpretation of coliform results is, save in a few particular instances, problematical.

3. The limited information, relative to water quality, furnished by coliform differentiation does not appear to warrant the expenditure of any but a minimum of effort to effect such differentiation. Whether the application of a very simple test, somewhat along the lines of that proposed by the British Ministry of Health, may prove worth while in the routine examination of waters on this continent remains to be determined.

The Number of Coliform Group Organisms as an Index of Pollution. From the data which have been cited, it is clear that bacteria of the coliform group are usually present in very large numbers in human and animal feces and also may be present, but ordinarily in very small numbers, in some soils and on some plants, leaves, etc. That they are not typical water organisms is shown by the rapidity with which the great majority of them die off in water. A few, however, as we have observed, may survive for long periods. When, therefore, a single coliform organism is found in a sample of water, it may have come directly from human feces or it may have fallen into the reservoir on a leaf, weeks before. The real question is whether coliforms from non-fecal sources do, or do not, appear in unpolluted waters in large numbers. This is a question which can be settled only on the basis of practical experience in the examination of a great many representative waters from various sources. Such experience

is at hand, in volume ample to demonstrate the significance of coliform bacteria when they are present in considerable number; but the interpretation of their significance must always be made in the light of the following considerations:

1. Bacteria conforming to the definition of coliform organisms are by no means confined to the animal body but are widely distributed elsewhere in nature.
2. The finding of a few coliforms in large samples of water, or their occasional discovery in small samples, does not necessarily have any special significance.
3. The detection of coliforms in a considerable proportion of 1-ml. samples is imperatively required as an indication of *recent* sewage pollution.
4. The *number* of coliform organisms in water, rather than their mere *presence* or their *type,* should be used as the principal criterion of recent pollution by sewage.

With these qualifications the value of the coliform test was never more firmly established than it is today. Organisms of the coliform group find in the intestine of the higher vertebrates an environment better suited to their growth and multiplication than any other which occurs in nature. We have found the number of *E. coli* per gram of human feces to vary, as a rule, from 5,000,000 to 500,000,000 or more, and the number of *Aerobacter* organisms to range from a few to 500,000. 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, coliform organisms will be found in comparative abundance. If pollution has been remote the number of coliforms 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 perhaps 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 coliforms in water we owe to Theobald Smith (1893*a*) who in 1892 outlined a plan for a study to be made by the New York Board of Health on the Mohawk and Hudson rivers. 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. von Freudenreich (1895) at the same time indicated the necessity for taking into account the number of coliforms present. He recorded the isolation of the

organisms from unpolluted wells when as large a quantity of water as 100 milliliters was used, and concluded that it was entirely absent only from waters of great purity, and present in large numbers only in cases of heavy pollution. He also quoted Miquel as having found coliforms in almost every sample of drinking water, if only a sufficient portion were taken for analysis.

The practical results of the application of the coliform test from this standpoint have proved of the greatest value. As originally outlined by Smith, it comprised the inoculation of a series of glucose tubes with small portions of water, tenths or hundredths of a milliliter. It was first used by Brown (1893) in 1892 for the New York State Board of Health, and showed from 22 to 92 fecal bacteria per milliliter 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 coliform organisms in the Mississippi River were found to vary from 3 to 7 per ml.

Hammerl (1897) used the presence of *E. coli* as a criterion of self-purification in the river Mur. He considered that when a water contained large numbers of coliforms, 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 direct plate cultures. Hammerl generally failed to find coliforms in the river except immediately below the various towns situated upon it; at these points of pollution he discovered a few coliform colonies upon his plates, not more than 4 to 6 per ml. 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." 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 *E. coli* at all, but that water from other sources need not be condemned unless the organism was found in 20 ml. or less. When coliforms were found only in greater quantities than 100 ml. the water might be considered as probably safe. 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 ml. of the water. . . . I would say that a water which contained *B. coli* so sparingly that 200 ml. required to be tested in

order to find it had probably been polluted with sewage, but the contamination was not of recent date."

Coliform Organisms in Surface Waters. One of the first elaborate applications of the coliform test was made by Jordan (1901) in the examination of the fate of the Chicago sewage in the Desplaines and Illinois rivers. The results were very significant. In fresh sewage a positive result was obtained about one third of the time in 0.00001 ml. and almost constantly in 0.0001 ml. The Illinois and Michigan canal proved almost as bad, giving positive results on 7 days out of 28 in dilutions of 1 : 100,000 and on 28 days out of 32 in a dilution of 1 : 10,000. At Morris, 27 miles below Lockport, where the canal enters the bed of the Desplaines River, and 9 miles below the entrance of the Kankakee, the principal diluting factor, the numbers were so reduced that positive results were obtained only on 11 days out of 20 in 0.001 ml., on 20 days out of 30 in 0.01 ml., and on 20 days out of 23 in 0.1 ml. At Averyville, 159 miles below Chicago, coliforms were isolated on only 4 days out of 27 in 0.1 ml. and on 13 days out of 31 in 1 ml. Chick (1901) reported 6,100 coliforms per milliliter 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, no coliforms were found in 31 of 38 unpolluted streams and rivulets, and fewer than 1 per ml. in the other 7. Liverpool tap water, snow, rain, and hail contained no coliform bacteria. In a study of the Potomac River, Cumming (1916) reported illuminating data relative to the influence of self-purification upon the coliforms in water. Just below the city of Washington these organisms ranged from 24 to 617 per ml. (monthly averages), according to season; at Marshall Hall, 14 miles below, the averages ranged from 24 to 137; at Maryland Point, 42 miles below, from 0.04 to 6.9; and in the salt water stretch, 64 to 102 miles below, from 0.01 to 0.04 per ml. Frost and Streeter (1924), in their study of the Ohio River, found the 1916 average monthly estimates of coliform organisms just above Cincinnati to be 3 to 61 per ml.; immediately below this city they increased to 55 to 5,689 per ml.; and 123 miles down river, above Louisville, they dropped to 6 to 128 per ml. The average monthly discharge of the river just below Cincinnati ranged from 18,200 to 304,000 second-feet; at this point, as might be expected, the higher coliform counts were usually obtained when the river stage was low, viz., when the sewage from Cincinnati was not greatly diluted.

Clark and Gage (Massachusetts State Board of Health, 1903) published the results of certain studies of Massachusetts ponds which indicated clearly the coincidence of the distribution of *E. coli* in

single milliliters of surface waters with actual sanitary conditions. They indicated also the slight significance of the test for this organism in larger volumes of water. Almost every source gave positive tests in 100 ml., whereas with 1-ml. samples only those lakes appeared suspicious which were, in fact, exposed to dangerous pollution. (See Table 79.) These results are somewhat similar to those, shown in

TABLE 79. DISTRIBUTION OF TOTAL BACTERIA AND *E. coli* IN SURFACE WATERS

(Clark and Gage, 1903)

Lake	Population of Watershed per Square Mile	Bacteria per ml.	*E. Coli* Per Cent Positive Tests	
			1 ml.	100 ml.
1*	1,400	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.

Table 9, page 9, obtained from 20 lakes in Eastern Canada. There the coliforms ranged from 0 to only 27 per 100 ml., with not more than 3 per 100 ml. in 11 of the lakes despite the fact that a few summer homes were located on their shores.

It will be recalled that Winslow and Cohen (page 182) found the ratio of *E. coli* to other coliform species isolated from various surface and ground waters to correlate very poorly with the results of the sanitary surveys of these supplies. In marked contrast with this lack of correspondence is the correlation between the relative densities of coliforms, indicated by the frequency of isolation of these organisms from different dilutions of sample, and the sanitary survey results, as shown in Table 80.

In sea water the number of coliforms is usually very low except when exposed to heavy pollution. Houston (1904*a*) reported that none of 168 samples of sea water near the English coast showed the presence of *E. coli* in 1 ml.; 97 samples gave negative results in 10 ml., 45 in 100 ml., and 4 in 1,000 ml.

TABLE 80. FREQUENCY OF ISOLATION OF COLIFORMS FROM DIFFERENT DILUTIONS OF SAMPLE

(Winslow and Cohen, 1918*b*)

	100 ml.	10 ml.	1 ml.	0.1 ml.	0.01 ml.	0.001 ml.	0.0001 ml.
Polluted, per cent	2	19	38	27	8	5	1
Unpolluted, per cent	64	30	5	1			

Coliform Organisms in Ground Waters. Ground waters, as a rule, should contain fewer organisms of the coliform group than good surface waters, because during their passage through the soil they have not only been more or less thoroughly filtered but have also been completely protected from occasional contamination by birds and other animals which continually adds to the coliform population of all surface waters. Even in sources of excellent quality, however, we should expect to find, and we do sometimes find, these organisms in large volumes of the water. Abba, Orlandi, and Rondelli (1899) showed by experiments with *Erythrobacillus 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 permit coliform bacilli to appear only in large samples of water is infinitesimal.

An interesting contribution to the bacteriology of ground waters was made by the 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 bottles as delivered for sale to the public. In the water of one spring *E. coli* was found (presumably in 100 ml.) 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 instances *E. 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 *E. 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 (Massachusetts State Board of Health, 1903), in

the examination of 170 samples of water from tubular and curb wells of good quality used as sources of water supply, found *E. coli* only 5 times, once in 1 ml. and 4 times in 100 ml.

Houston (1903*b*) made an instructive comparison of some more or less polluted shallow wells at Chichester with deep ground waters of high quality at Tunbridge Wells. Table 81 shows the value of the 1-ml. sample in discriminating between good and bad waters.

TABLE 81. DISTRIBUTION OF *E. coli* IN GOOD AND BAD WELL WATERS
Percentage of Positive Tests
(Houston, 1903*b*)

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

Fromme (1910) brought out the relation between *E. coli* and total numbers of bacteria in 120 samples of well waters near Hamburg as shown in Table 82. Similar data relative to the number of *E. coli*

TABLE 82. RELATION BETWEEN TOTAL NUMBERS OF BACTERIA AND *E. coli*
(Fromme, 1910)

Colony Count	Number of Samples	Per Cent Positive *E. coli* tests in 10 ml.
Over 200	35	40.0
50–200	19	15.8
Under 50	66	3.0

or coliforms found in some wells in the United States and Canada have been cited in Chapter I (pages 20–21) and Chapter V (pages 63–64).

Mackenzie (1938) directed attention to the fact that nearly a million consumers are supplied with well water by the London Metropolitan Water Board. Certain results obtained from the untreated waters of a few of the Kent wells, shown by chemical analysis to be of high organic purity, are given in Table 83.

It is evident that deep well waters usually contain very few coliforms. Shallow well waters, on the other hand, even when not exposed to pollution, may contain considerable numbers of these bacteria. Very probably the principal cause of the greater coliform density frequently found in satisfactorily constructed shallow wells is multiplication of the organisms. Stagnation of the water in such wells

due to low velocity of the underground stream, with resulting rise in water temperature, especially during warm weather; the presence of nutrient material in the form of accumulated vegetable matter in solution and deposited on the walls; pump washer infections; any or all of these factors may favor multiplication of the few coliforms that enter the well in the ground water. Consequently, a coliform density that might be considered alarming if found in a deep well water may be regarded with a measure of tolerance when found in the water of a shallow well if the latter is apparently unexposed to contamination. Particularly is such tolerance indicated if the great majority of the coliforms present are slow lactose fermenters. The Committee of the British Ministry of Health (1939), in its discussion of private supplies in Britain, states that "By relatively simple measures, such as

TABLE 83. RESULTS OF BACTERIOLOGICAL EXAMINATION OF KENT WELLS
(From Mackenzie, 1938)

Wells	Number of Samples	Average Colony Count Agar, 37° C. per ml.	Per Cent Samples Yielding *E. coli* by Minimal Volumes				Coliform Types Isolated	
			Neg. 100 ml.	Pos. 100 ml.	Pos. 10 ml.	Pos. 1 ml.	*E. coli*	I.A.C.*
Crayford No. 2†	252	0.3	96.8	3.2	...	...	8	2
Crayford No. 3	149	0.2	98.0	1.3	0.0	0.7	5	6
Darenth†	250	0.1	99.6	0.4	...	...	1	.
Orpington No. 1	120	0.2	95.8	4.2	...	...	5	5
Wansunt†	266	0.4	99.6	0.4	...	...	1	2
West Wickham	137	0.1	98.5	1.5	...	...	2	0
Wilmington No. 1†	245	0.3	99.2	0.8	...	...	2	5
Wilmington No. 2†	232	0.5	97.8	1.7	0.4	...	5	9

* Intermediate, *A. aerogenes*, and *A. cloacae* types. † Supplied without treatment.

the removal of obvious sources of contamination from the catchment area, and by attention to the coping, brick lining, and covering of the well, it should usually be possible to reduce the coliform count for a shallow well water to a level between 10 and 25 per 100 ml. Persistent failure to do this, especially if the water frequently gives a presumptive coliform count of 50 or over per 100 ml., should lead as a rule to condemnation of the supply, though there are circumstances, such as where the pollution appears to be mainly of non-human origin, when an exception may be made to this rule." It should be pointed out that a thorough cleaning of a well followed by disinfection with chloride of lime to provide an available chlorine concentration of

at least 50 ppm. in the well water for a period of 12 to 24 hours, or even the disinfection alone, will often permit a shallow well with an initial high coliform content to yield a water beyond suspicion. Such disinfection is frequently necessary immediately after construction or repair of any type of well.

Ground waters, like other supplies, should be subjected to regular bacteriological examination. That they are not necessarily exempt from accidental pollution has been proved by many unfortunate experiences. Defects in construction of wells, catchment basins, or reservoirs, the development of leaks, or breaks in seals offer means of entry to contamination which sooner or later may lead to disaster. A number of instructive examples of such accidental pollution of wells and reservoirs have been described by Ferguson and Klassen (1929). It should be recalled that the work of Stiles *et al.* (1927) and of Caldwell and Parr (1937) showed that bacterial pollution underground travels in the same direction as the ground water; that wet weather, increasing the ground-water velocity, tends to extend the travel of the pollution; that a horizontal band of pollution, instead of expanding widely as it leaves its source, contracts (after perhaps a slight expansion) and eventually narrows to its apex; and, finally, that if the direction of the ground-water flow is not known, distance constitutes the best measure of safety of the supply.

Interesting data relative to the time required for recovery of deep wells from contamination caused by reworking have been reported by Sauls and Harvill (1933). One of the deep wells, sunk in unconsolidated sands, which supply the city of Houston, Texas, was repaired in the month of April. Coliforms were present in 85.0 per cent of the 10-ml. portions of water from this well during April, in 30.7 per cent during May, and 0.0 per cent during June. Another well, repaired in February, showed the presence of coliforms in 21.8 per cent of the 10-ml. portions during that month, in 37.3 per cent during March, in 50.0 per cent during April, and in 1.2 per cent during May. Thus, despite every precaution to prevent contamination, it was not until 2 or 3 months after reworking that the quality of the well water, as indicated by its coliform content, returned to the normal. In this connection, it is interesting to note that Stiles *et al.* (1927) recovered *Escherichia* organisms from ground water more than 2 years and 8 months after it had been experimentally infected with excrement.

Coliform Bacteria in Filtered and Treated Waters. One of the most important applications of the coliform test is in the control of the operation of municipal water purification plants. It has been used for this purpose for 45 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 recorded the presence of *E. coli* in 60 per cent of the 1-ml. samples from the Ohio River, whereas the effluent from either slow sand or mechanical filters gave positive results only half the time in samples of 50 ml. The results

TABLE 84. *E. coli* IN MERRIMAC RIVER AND LAWRENCE FILTER EFFLUENT

	Merrimac River, Per Cent of 1-ml. Samples Containing *E. coli*	Merrimac River, Number of *E. coli* per ml.	Filtered Water, Per Cent of 1-ml. Samples Containing *E. 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.

of the examinations carried out at Lawrence for 6 years are brought together in Table 84 from the Annual Reports of the Massachusetts State Board of Health.

At Harrisburg, Pa., mechanical filtration combined with chlorine disinfection yielded the results shown in Table 85.

TABLE 85. *E. coli* IN RAW AND TREATED WATER AT HARRISBURG, PA. (Harrisburg, 1913)

	Per Cent Positive Tests in 1 ml.	
Year	Raw Water	Treated Water
1906	71.9	2.7
1907	64.0	1.0
1908	65.7	1.1
1909	63.1	1.0
1910	55.4	0.2
1911	77.3	0.6
1912	46.9	0.8

The effectiveness of modern methods of water treatment in eliminating large numbers of coliforms is well illustrated by Table 86, which shows the coliform content of one of the St. Louis water supplies at various stages in its purification.

The results of the daily tests made at water purification plants are usually expressed in monthly or yearly averages as in the above tables. 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 contain coliforms in 2 or 4 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

TABLE 86. COLIFORM RESULTS OBTAINED AT HOWARD BEND (ST. LOUIS) FILTRATION PLANT*

Month	Number of Coliform Bacteria per 100 ml.						
	River Water	Coagulated Water	Settled Water	Influent Water	Effluent Water	Clear Water Basin	Water to Mains
1942							
April	5,800	600	250	4.0	0.04	0.00	0.00
May	9,300	700	300	0.2	0.00	0.00	0.00
June	11,500	850	550	0.6	0.01	0.00	0.00
July	5,200	600	190	0.2	0.04	0.00	0.00
August	5,000	700	275	1.5	0.00	0.00	0.00
September	6,800	750	375	0.0	0.04	0.00	0.00
October	6,000	800	500	1.5	0.01	0.00	0.00
November	5,900	850	425	0.7	0.00	0.00	0.00
December	5,000	750	400	0.7	0.30	0.09	0.00
1943							
January	3,100	600	250	2.5	0.04	0.00	0.00
February	3,300	475	180	3.0	0.02	0.01	0.00
March	3,400	500	180	0.2	0.00	0.00	0.00
Average	5,860	680	320	1.3	0.04	0.01	0.00

* Annual Report of Water Commissioner, 1943.

given month 3 per cent of the 1-ml. samples tested contain coliforms, the effluent may or may not be safe. If on each of 20 days 3 coliforms or thereabouts were present in 100 ml. of the water it is probably a safe one. If on 19 days no coliforms were present, and on the twentieth day 100 ml. contained 60 coliforms, the average result would be the same, but the water on one day was of a dangerous character. With properly managed 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 coliform tests may include and cover up dangerous and significant high numbers at particular periods.

Borman *et al.* (1941) emphasized the importance of examining samples from the distribution system. They found, in a detailed

study of various treated waters, that the presence of coliforms in tap samples frequently reflected lapses in adequate treatment occurring as long as 3 weeks before sampling. Such coliforms appeared to persist longest in laterals and feeders of small diameter and at points most distant from the distribution source. These investigators concluded: "This suggests that examination of samples from the distribution system may produce more useful results than inspection of plant records or examination of samples of plant effluent when water supplies are suspected of having caused gastrointestinal disturbances." They point out that inadequate treatment can be detected in plant effluent samples only if those samples are taken from the body of insufficiently treated water as it passes the sampling point.

Data obtained by Levine, Heller, and Bender (1942) in their examination of treated waters suggest that the cause of the higher incidence of coliforms sometimes found in tap samples, compared with that in finished water samples at the plant, is not recovery of coliform bacteria from injury by chlorination, but rather ordinary after-growth in the distribution system. These observers found 36 *E. coli*, 33 intermediate, and 108 *Aerobacter* organisms in the final chlorinated effluents of the four plants included in their study. Coliforms were very seldom found in 10-ml. portions when the residual chlorine was 0.4 ppm. or more, but non-coliforms producing gas in lactose broth were occasionally present even when the residual was 0.6 to 0.8 ppm., after 48 to 72 hours of contact. No special relation was observed between the incidence of these gas formers and that of coliforms in the samples examined. These results tend to confirm the general opinion that complete elimination of non-coliform gas formers, although desirable, is, as a rule, neither absolutely necessary from a public health standpoint nor always economically practicable. Nevertheless, in order to provide an additional factor of safety, a number of operators reduce the gas-former density of their supplies to a minimum through the use of superchlorination or of break-point chlorination.

Coliform Organisms in Waters of Good Quality. When it is considered that the droppings from a bird, or a squirrel, or other animal, on a bit of vegetation, a twig, decaying wood, or soil, may result in the survival for a considerable time, or even in a temporary multiplication of the various organisms contained in the feces, it is not surprising that coliforms of any type (and especially *E. coli*, the predominant type in feces) are occasionally recovered from streams, springs, or lakes, contamination of which appears to be impossible. It must be remembered that such sources are the natural watering

places of birds and other animals. Kline and Fuller (1932), for example, found certain New York springs to be contaminated by salamanders which were often located in crevices far back in the rock from the point of emergence of the springs.

When coliforms from such accidental pollution, or from soil which likewise has been polluted, gain access to water, some may persist there for a long period. Because of this survival, the coliform organisms entering a body of water in small numbers from time to time may accumulate until they become numerous. Furthermore, coliforms occasionally may multiply in water, especially during the warm summer months and when organic matter sufficient for their needs is available. Bardsley (1934) and Taylor (1941) both observed an increase of coliforms in English surface waters during the summer, for which the available data offered no explanation. Taylor stated that the coliform density of all the lakes he studied increased in the same month, June, and that a temperature rise was one of the few common factors involved. He suggested prolonged viability, and perhaps multiplication of the coliforms washed into the lakes, as the most reasonable explanation of the phenomenon. A similar increase in the coliforms during certain months of the year has been observed in apparently uncontaminated reservoirs. Ewing and Hopkins (1930) reported a seasonal rise in the coliform content of the surface water of certain reservoirs from May to September each year, when the water attained a temperature of about 68° F. and nutrient material from the lining of the reservoirs or from algae and plants was plentiful. Incidentally, they point out that tap water drawn from the colder bottom water of such reservoirs may remain comparatively free from coliforms if the stratification of the water is not disturbed by fluctuations in level or other cause. Sauls and Harvill (1933) found an average increase of 300 per cent in the coliform content of Houston, Texas, reservoir waters taken from deep wells. The normal temperature in these reservoirs was 80° F.

It is a frequent experience of those entrusted with the control of water supplies to find a few coliform organisms in tap waters that are normally coliform-free whenever the contents of a section of the distribution system are subjected to violent agitation, such as may occur at the time of a large fire, a break in a main, etc. This sudden appearance of coliforms is usually caused by disturbance of the sediment in the water pipes which often contains, together with organic and inorganic matter, a residuum of resistant coliform organisms which not only survive there but very probably also multiply occasionally, although at a very slow rate. The coliforms in such sedi-

ment may be of any variety, but those of the genus *Aerobacter* are generally the most numerous.

Coliform Infections. Another source of coliforms in distribution systems, particularly after the laying of new mains, is the calking material employed in the joints. Numerous observers have reported the presence of coliforms in hemp, jute, cotton, etc., and their multiplication when these materials are placed in water. Adams and Kingsbury (1937) found coliforms in all of 11 samples of jute; and 3 days or more after placing the samples in water enormous increases in the number of these organisms were observed. Autoclaved jute, immersed in sterile water, inoculated with typhoid organisms and held at 70° F., yielded from 7 *Eberthella typhosa* per milligram at the beginning of the experiment to a maximum of 23,000 in 9 days; after 14 days the number dropped to 700 per mg. Calvert (1939) found jute to support bacterial multiplication better than hemp; even good quality cotton yarn caused heavy aftergrowth in tap water. This observer records that a new main, laid with untreated calking material, flushed, and treated with chlorine to give a residual of 90 ppm. after 43 hours, yielded no coliforms and bacterial counts of 5 per ml. or less, 20 minutes after the disappearance of the last trace of chlorine; but 21 hours later, 18 of 20 water portions of 1 ml. contained coliforms and the bacterial counts averaged 500 per ml. Even after 56 days, 5 per cent of the 1-ml. water portions showed the presence of coliforms and bacterial counts above normal. Other observers have reported the sudden appearance of coliforms, caused by infected jute or hemp, long after the mains were laid. Fortunately several substitutes such as rubber, asbestos rope, and cement, which apparently do not support bacterial growth, have been reported satisfactory as calking materials.

Reference was made in a previous section to similar multiplication of coliform organisms on leather washers of pumps. The incidence of such " pump infection " is perhaps far greater than is yet realized. Caldwell and Parr (1933*b*), who have devoted considerable attention to the subject, report numerous instances of washer infection. A piece (5 × 7 cm.) of one washer, which was found to be contaminating the water passing it, was tranferred after immersion for 3 to 4 weeks to a jar containing 1400 ml. of sterile water. Upon shaking twenty-five times, 10,000 *A. aerogenes* per milliliter were recovered. Obviously such a source of contamination in a pump might very seriously affect the results of coliform tests of the water discharged from it. Caldwell and Parr found 11, or 7.3 per cent, of 150 wells frequently tested to show pump infection, and 5 of these were from wells yielding good

water. *A. aerogenes* (both indole-positive and indole-negative), intermediate coliform, and *Pseudomonas* strains were implicated in the infections. New as well as old pumps became infected. The water discharged from certain pumps in which infection was well established contained the infecting organism in from 10-ml. to 0.1-ml. portions. Upon attempting to infect pumps artificially by pouring 1 to 2 liters of broth cultures of organisms through them, it was found that both *A. aerogenes* and human fecal *E. coli* infections, persisting for months, could be established in this manner. It is interesting to note that replacement of washers and chlorinating did not eliminate the difficulty because a few organisms often remained in the pump and infected the new washers, whereupon multiplication again proceeded. Heat (boiling water in contact for 10 to 30 minutes) appeared to be the most practicable method of ridding the pumps of these infections.

One of us (M. H. M.) encountered a similar infection of several taps in a public building; all the coliforms found proved to be identical *A. aerogenes*. Replacement of the old with new washers eliminated the infection. Another instance of coliform infection was discovered at a small spring, located in an area far removed from human habitation and domestic animals, and well protected by a tight, clean, wooden box. Samples of the water yielded coliforms, all *A. aerogenes*, in 10-ml. and 1-ml. portions. After substitution of a new box no coliforms were found in 10-ml. portions of the water: evidently the organisms were growing on the wood of the box. Tonney and Noble (1932) mentioned several instances of coliform infection of various materials, including wood.

The detection of coliform infection is one problem the solution of which is often greatly facilitated by differentiation of the organisms implicated, because usually only a single type of coliform is present. *A. aerogenes* is the most frequent offender in such infections, but *E. freundii* or one of the other species may be involved. *E. coli* infections, however, appear to be very rare. Whenever a coliform infection is suspected, the water should be examined very thoroughly by direct plating methods and all coliform-like colonies carefully purified. The resulting cultures are then submitted to various differential tests such as the M.R., V.P., and citrate tests. If all, or practically all the cultures prove to be of a single type the presumption is very strong that they derive from an infection of some material with which the water has been in contact, and bacteriological examination of such materials may reveal the source of the infection.

Bacteria Closely Related to the Coliform Group. The procedures specified by Standard Methods for the detection of coliform organ-

isms in water have the effect of limiting the group to aerobic and facultative anaerobic, gram-negative, non-sporing bacilli that produce gas from lactose *within 48 ± 3 hours upon incubation at 35° to 37° C.* Naturally, this arbitrary limitation excludes many bacteria found in water which are apparently very closely related to the coliforms. Some of these organisms are found among the slow lactose fermenters which Stuart, Mickle, and Borman (1940) prefer to term "aberrant coliforms," and which they describe as indicated below. It is to be noted that they consider aberrant coliforms to be those which produce less than 20 per cent gas within 48 hours at 37° C. In accordance with the suggestion of Stuart, Wheeler, Rustigian, and Zimmerman (1943) we include "non-lactose-fermenting coliforms" in the aberrant coliform classification.

1. MICRO-AEROGENIC COLIFORMS. These are the slow lactose-fermenting coliforms, producing from a bubble to 10 per cent of gas in 48 hours at 37° C. They grow more slowly at lower temperatures. On E.M.B. agar they produce white, or black, or black and white colonies with the black portion appearing as a triangular sector. They may be *E. coli*, intermediate, or *Aerobacter* organisms. All three sections of these slow fermenters have been found in public water supplies and in human feces, although in small numbers. The great majority of those that have been isolated from soil, water, and milk proved to be of the genus *Aerobacter*. They were usually associated with typical, rapid gas-producing coliforms.

2. PSEUDOMICRO-AEROGENIC COLIFORMS. These bacteria are coliform-like organisms for cultivation of which the optimum temperature seems to be 20° to 30° C. Some produce gas in 48 hours at 37° C. (in which event they must be included in the Standard Methods coliform group), but others do not. On E.M.B. agar they form colonies varying from typical intermediate and *Aerobacter* growths to small white colonies. Some of these bacteria can be "trained" by serial transplants in lactose broth every 48 hours eventually to produce gas at 37° C. Their biochemical reactions are usually those of the intermediate or *Aerobacter* coliforms. Since organisms of this kind were isolated from water, soil, and milk, but not from any of 600 fecal specimens, Stuart *et al.* considered them to be of little sanitary significance; and since initiation of growth at room temperature may induce some of them to produce gas at 37° C., these observers proposed warming water samples and preheating the enrichment broth, in which large portions of samples are to be planted, to 37° C. so as to prevent ultimate production of gas by these organisms.

3. PAPILLAE-FORMING COLIFORMS. These bacteria present the char-

acteristics, relative to lactose fermentation, of *Bacterium coli-mutabile.* On E.M.B. agar streaked from a gas-positive lactose broth tube, both black and white colonies are produced; the black colonies appear to be typical coliforms, fermenting lactose with the formation of 20 per cent or more gas in 24 hours at 37° C., and breeding true; but white colonies produce gas from lactose very slowly. A white colony on E.M.B. agar, after 2 to 5 days, is found to have several small, black, daughter colonies developing out of the parent colony, or on it; and subcultures from the black papillae produce gas just as typical coliform cultures do. These peculiar "unstable variants" were isolated from the feces of 52 per cent of 100 patients and food handlers in the course of a study of an outbreak of gastro-enteritis; they appear to be frequently associated with such outbreaks although conclusive evidence of any causal relation is lacking. Organisms of this sort usually present the biochemical reactions of *E. coli,* but intermediate and *Aerobacter* types are also encountered. As Stuart *et al.* pointed out, papillae-forming coliforms should perhaps be considered of more sanitary significance than any of the other "aberrant coliforms." Since many do not produce gas from lactose within 48 hours at 37° C., however, Standard Methods procedures frequently fail to detect them.

4. ANAEROGENIC COLIFORMS. Organisms conforming to our definition of coliforms, but which produce acid without gas from lactose, belong to this group. Occasionally a strain may give rise to a gas-forming variant. On the other hand, it is well known that occasionally a typical coliform strain is encountered which loses its property of gas production. No suggestion relative to the sanitary significance of the anaerogenic coliforms is offered.

5. NON-LACTOSE-FERMENTING COLIFORMS. These bacteria present many of the characteristics of coliform organisms, but produce neither acid nor gas from lactose. They may be *E. coli, E. freundii,* or *Aerobacter* in type. They have been isolated frequently from normal and pathogenic feces. Some, which react like *Escherichia* but show no serological relationship to *Salmonella,* are designated as "paracoli" by Parr (1939). Stuart *et al.* (1943), however, reserve the term "paracolon" for "aberrant coliforms" isolated from man.

It will be observed that all those organisms of the first of the above groups, and some of the second and third, are at present included in the coliform group as defined by Standard Methods. Whether an effort should be made to detect the presence in water samples of a larger proportion of certain of the "aberrant coliforms," or whether practically all such organisms should be disregarded as of

no significance, is a question of considerable importance. Some sanitarians maintain that even the present definition of the coliform group is too inclusive; that an excessive incidence of coliforms is frequently encountered in waters known to be of good quality; that coliforms producing only a bubble, or less than 10 per cent of gas in the enrichment or confirmation tube should be disregarded because they are attenuated or devitalized forms representing, at worst, remote pollution no longer of sanitary significance. On the other hand, many sanitarians argue that slow lactose fermenters are often found in considerable numbers in feces; that they are usually the last representatives of the coliform group to be recovered after excremental pollution of a water; that factors other than attenuation, such as scarcity of coliforms, overgrowth, or antagonism of associated bacteria, may diminish gas production by typical coliforms, as indicated by the results of Borman *et al.* (1941); that certain of the slow-lactose-fermenting and other "aberrant coliforms" are associated with, if not the cause of, some gastroenteric disturbances; and it is often intimated that the coliform group should be so defined as to include more of these bacteria. The discussions by Hale (1926), Goudey (1938), Ziegler (1939), Dulaney and Smith (1939), Borman *et al.* (1941), and Parr and Friedlander (1942) well illustrate the differences of opinion on this subject.

In order to obtain some conception of American opinion regarding the significance in water of coliforms confirmed from enrichment tubes in which less than 10 per cent of gas has been produced within 48 hours at 37° C., a questionnaire was submitted to about 30 bacteriologists by McCrady (1939*b*). Of 25 replies received, 15 unqualifiedly favored inclusion of such organisms in the coliform group regardless of the amount of gas; 6 indicated a practice of ignoring enrichment tubes containing little gas because of experience of failure to recover coliforms from such tubes, but the implication is clear that, had the experience of confirmation been more favorable, small amounts of gas would not be disregarded; 1 reply favored ignoring small quantities of gas in tubes planted with natural waters; and 3 frankly expressed doubt of the sanitary significance of the slow lactose fermenters. It is evident, therefore, that the great majority of the bacteriologists questioned considered the production of gas, whatever the amount, in 48 hours at 37° C. to be of sanitary significance.

Levine (1941) made a special study of the incidence of "aberrant coliforms" in chlorinated supplies. He found 83 (42 per cent) of the coliforms isolated from such waters to produce less than 10 per cent of gas in 48 hours at 37° C. Of these, 15 were tentatively

classified as micro-aerogenic and 68 as pseudomicro-aerogenic coliforms. Among the latter 11 gave the reactions of *E. coli*, 27 those of intermediate, and 30 those of *Aerobacter* organisms. Since 67 of the 68 were isolated from water samples submitted during the summer, Levine suggests that they may have been soil bacteria washed into the water with spring rains.

We believe there is some justification for considering the presence of slow lactose fermenters in *natural* waters to be of somewhat less significance than that of typical coliforms, just as we consider the finding of coliforms other than *E. coli* to be of somewhat less significance than that of *E. coli*. We recognize the fact that slow lactose fermenters frequently constitute a large proportion of the few coliforms found in unpolluted or slightly polluted waters; and if the results of the sanitary survey are very favorable, we are inclined to regard these organisms with a measure of tolerance. As when attempting to judge the relative significance of the different coliform types found in a sample, however, it must be remembered that the presence of the less typical coliforms conforming to Standard Methods' definition of the group is often as significant of danger as is that of any other type. In no sense may they be generally ignored.

On the other hand, we question very much the advisability of admitting other atypical coliform-like bacteria into the group. Although it is likely that our present definition of coliforms excludes some types that may be of sanitary significance, we should anticipate much more serious difficulty from the inclusion of organisms such as those, for example, which grow only at temperatures other than 37° C. or which require more than 48 hours at this temperature to produce gas. Aside from the inconvenience attending an attempt to detect the presence of such bacteria in water, there is the danger that the few additional organisms of sanitary importance which might thus be included in the coliform estimate would be accompanied by a much larger number of other bacteria of little or no significance. We feel, therefore, that the present Standard Methods' definition of the coliform group is, at least for use in water work, sufficiently inclusive.

In filtered, treated, or filtered-treated waters, the presence of slow fermenters should be considered from a different viewpoint. Since water-borne disease organisms are assumed to be no more resistant to filtration or treatment than are coliform bacteria, whether slow fermenters or other varieties, the coliform estimate of such waters serves, not primarily as a measure of pollution, but rather as a measure of the elimination of any associated disease organisms that may have been present in the raw water. Here there is no question

of tolerance. To be reasonably certain that all typhoid, dysentery, and similar bacteria have been removed or destroyed, *all* coliforms must likewise be eliminated; if not, a thoroughly satisfactory explanation of their presence should be forthcoming.

The coliform density of such waters may occasionally, of course, serve also its usual purpose of indicating dangerous accidental pollution, such as may result from a cross-connection, toilet back-siphonage, etc. Obviously, the lower the normal coliform density in the finished water, the more readily will such occasional contamination be detected. Plant operators — and they are many — who are able to hold the coliform content of their finished waters to a density far below that required by accepted standards are in a very advantageous position to detect accidental pollution, if and when it occurs.

The Proper Use of the Coliform Result. The presence of a moderate number of coliform organisms in a water should not be considered a sure sign of dangerous pollution, but rather an indication of *possible* pollution. The analyst's immediate reaction upon encountering such an indication should be, not to condemn the water out of hand, but to institute a search for its cause. As we have observed, this may be a coliform infection, growth in a reservoir or dead end, disturbance of sediment in a main, or other circumstance associated in no way with dangerous pollution. Only when no cause devoid of sanitary significance can be found by an exhaustive sanitary survey of the surroundings or by other means should a moderate coliform density *per se* be regarded as a possible reason for considering the supply to be of doubtful or unsatisfactory quality. Since considerable time may be required to complete an investigation of the kind described, however, experience has proved the wisdom of immediately warning the consumers, particularly when the consumer population is large, at the first definite indication of possible danger.

Altogether the evidence is conclusive that the coliform test is the most valuable and the most generally applicable single test which we possess for demonstrating the sanitary quality of a water. That when coliform organisms are present their number, with few exceptions, constitutes a reasonably accurate index of the amount of pollution in a water, appears to be proved beyond cavil.

CHAPTER X

OTHER INTESTINAL BACTERIA WHICH HAVE BEEN USED AS INDICES OF POLLUTION

The Sewage Streptococci. Bergey *et al.* (1939) classify the aerobic streptococci as shown in the first 2 columns of Table 87. Certain of the characteristics of the various species are also indicated in the table, including those especially pertinent to the discussion of the "sewage streptococci" which follows.

TABLE 87. THE GENUS STREPTOCOCCI (AEROBIC)

(From Bergey *et al.*, 1939)

Group	Species	Lancefield Group	Hemolysis	Growth at 45° C.	Survival at 60° C., 30 min.	Acid from		
						Lactose	Mannitol	Raffinose
Pyogenes	*S. pyogenes*	A	+	−	−	+	−	−
	Streptococcus sp. (animal pyogenes)	C	+	−	−	+	−	−
	S. equi	C	+	−	−	−	−	−
	S. agalactiae	B	±	−	−	+	−	−
Viridans	*S. salivarius*		−	±	−	+	−	±
	S. bovis		−	+	+	+	±	+
	S. thermophilus		−	+	+	+	−	±
	S. equinus		−	+	±	−	−	±
Lactic	*S. lactis*		−	−	±	+	±	−
	S. cremoris		−	−	±	+	−	−
Enterococcus	*S. faecalis*		−	+	+	+	+	±
	S. durans	D	+	+	+	+	−	−
	S. liquefaciens		±	+	+	+	+	±

Note: Occasional variations not indicated.

Sherman (1937) stressed our lack of knowledge of the streptococci in the following words: "For the most part, the known species of streptococci are those which have brought themselves clearly to our attention as the agents of disease or as more or less dominant organisms in familiar habitats . . . It should be recognized that the species which are now clearly defined represent only a portion, perhaps a

very small fraction, of those which actually exist." The classification in Table 87, therefore, probably includes by no means all the streptococci that may be encountered in water.

The principal source of every species indicated in the classification, except *Streptococcus thermophilus, Streptococcus lactis,* and *Streptococcus cremoris,* which derive chiefly from milk or milk products, appears to be man or other animals: *Streptococcus pyogenes,* the human pathogen, may be found not only in the human throat but also in the feces from a person with a throat infection; *Streptococcus agalactiae* is the usual cause of bovine mastitis; *Streptococcus salivarius* is the predominant streptococcus of the human throat; *Streptococcus equinus* is the principal representative of the group in the feces of the horse, as is *Streptococcus bovis* in the feces (and mouth) of the cow; and the Enterococci are the real fecal streptococci of man, although some of them are found at times also in animal feces. It will be observed in Table 87 that all the principal species just mentioned, except *S. equinus,* produce acid from lactose, and that nearly all of them grow at 45° C. and survive heating at 60° C. for 30 minutes.

The term sewage streptococci, as generally used, very probably covers not only the majority of the species of the Bergey classification but also numerous other forms not yet defined. Many of these cocci do not occur in well-marked chains. Those most commonly found grow feebly on the surface of ordinary nutrient agar, producing faint, transparent, rounded colonies, but under semi-anaerobic 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 sugars and they ferment glucose 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 indole 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. 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 coliform group, these bacteria have been isolated so frequently from polluted sources and so rarely from normal waters that it seems reasonable to regard their presence as indicative of pollution. Originally reported by Laws and Andrewes (1894), their importance was not emphasized until 1899 and 1900, when 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 6 rivers extensively sewage-polluted, he found streptococci in 0.1 to 0.0001 ml. of the water examined, although in some instances the chemical analysis would not have indicated dangerous pollution. On the other hand, 8 rivers, not extensively polluted, showed no streptococci in 0.1 ml., although the chemical and the ordinary bacteriological tests gave results which would condemn the waters. Horrocks (1901) found these organisms in great abundance in sewage and in waters which were known to be sewage-polluted, but which contained no trace of *E. coli*. He found by experiment that *E. coli* gradually disappeared from specimens of sewage kept in the dark at the temperature of an outside veranda, whereas the commonest forms which persisted were varieties of streptococci and staphylococci.

In America attention was first called to these organisms by 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. Prescott (1902*b*) showed that they are abundant in fecal matter and often overgrow *E. coli* in a few hours when inoculations are made from such material into glucose broth. 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, Winslow and Nibecker (1903) found streptococci in only one sample. Clemesha (1912*a*) reported that streptococci, in India, were present in 0.0001 or 0.00001 g. of feces, but were rare in waters that were not very grossly polluted. In a series of bottle experiments in the laboratory and in the study of an artificially polluted tank outdoors he found that they disappeared very rapidly in water, within 2 or 3 days at the most. 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.

Enumeration of Sewage Streptococci. The number of streptococci in a water sample may be estimated by direct plating or by means of liquid cultures. On a litmus lactose agar plate made directly from

polluted water, the colonies of the streptococci are generally 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. Representative colonies should be fished and examined to determine their morphology.

In the fermentation tube, streptococci will generally appear in abundance after a suitable period of incubation. Prescott and Baker (1904) found that with mixtures of *E. coli* and streptococci in glucose broth, the initial ratios of the latter to the former varied from 1 : 94 to 208 : 1; the coliforms developed rapidly during the early part of the experiment, reaching a maximum after about 14 hours, and then diminished as rapidly. The streptococci first became apparent after 10 to 15 hours and reached their maximum after 20 to 60 hours, depending upon the number originally present. When the same method was applied to polluted waters, similar periodic changes were observed; nearly pure cultures of *E. 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.

The successive growth of these two intestinal groups in the same glucose broth tube suggests the following method for detection of the presence both of coliforms and of streptococci. A glucose broth enrichment tube containing a portion of the sample is incubated at 37° C., and if gas appears an attempt is made to isolate coliform organisms. The tube is returned to the incubator, and after a total period of incubation of 36 to 48 hours, a plating from the tube usually results in an almost pure culture of streptococci, if these bacteria were originally present in the water.

Since a similar succession of growth occurs in lactose broth, the ordinary lactose broth enrichment tube may be employed in a like manner. Mallmann and Gelpi (1930) suggested, in connection with its use, a modification of the procedure described above. After gas-positives are confirmed in accordance with the usual coliform procedure, the incubation of the lactose broth tubes is continued until a total period of 48 hours has elapsed. The tube is then centrifuged to concentrate the suspended organisms, the liquid is decanted, the sediment smeared on a glass slide, stained, and examined for cocci. Or, after the 48-hour incubation period, the tube may be allowed to stand at room temperature for 1 to 3 days to permit sedimentation of the bacteria. A heavy sediment in the bottom of

the tube similar to that of the deposit in a macroscopic agglutination test is an indication of the presence of streptococci, but it should be confirmed by microscopic examination. Mallmann and Cary (1933) recommended holding the incubated tube to the light and looking for a granular precipitate, flocs of which may adhere to the wall of the outer tube or to the outer surfaces of the inverted vial. They stated that this indication is not always confirmed microscopically but that confirmation is not necessary if the granular precipitate is absent.

As other organisms may interfere with the isolation of streptococci from the enrichment tube, Houston (1930) studied methods of taking advantage of the greater resistance to drying and to high temperatures displayed by these organisms in order to facilitate their isolation. One method which, he states, was suggested to him by Dr. Okell, is still recommended by the Committee of the British Ministry of Health (1939) for this purpose. After the contents of the enrichment tube is mixed (it will be recalled that MacConkey broth is generally used in Britain) 1 ml. is added to 9 ml. of sterile water in a tube which has been standing for some time in a water bath held at 60° C. The greatest of care must be taken to avoid side-smearing. This tube is then immersed in the 60° C. water bath so that the level of the liquid in the tube is considerably below that of the water in the bath. After about 15 minutes, when the great majority of coliforms and other interfering bacteria are probably destroyed, the tube is removed, shaken, and 1 drop is spread over the surface of a solidified agar plate which is then incubated at 37° C. for 24 hours and examined. Colonies resembling those of streptococci are fished and transferred to lactose broth (with Andrade indicator), to the condensation water of a nitrate agar slope (nutrient agar plus 0.1 per cent KNO_3; *p*H 7.5), and to the slope itself. Acid production, without gas, from lactose, with a characteristic floury precipitate near the bottom of the tube, usually signifies the presence of streptococci. The finding of short chains of cocci in the microscopically examined condensation water of the nitrate agar slope, and the absence of any indication of nitrate reduction (no brown coloration) upon the addition to the latter of a few drops of nitrite test reagent (5 per cent metaphenylenediamine hydrochloride solution containing 1 to 2 drops of dilute sulphuric acid), confirm the presence of these organisms. In the classification of the streptococci given earlier in this chapter it will be observed that nearly all the species except those of the *pyogenic group* survive heating at 60° C. for 30 minutes.

Since many individual streptococci may be killed by the combina-

tion of acid and the temperature of 60° C., even when exposed to it for only 15 minutes, another method for isolation of these bacteria was proposed by Harold (1936). One or two drops of the enrichment-tube culture is spread, without heating, on the surface of a solidified tellurite agar plate which is incubated for 24 hours at 37° C. The agar medium contains 1.0 per cent peptone, 0.5 per cent lactose, 0.2 per cent K_2HPO_4, 0.5 per cent NaCl, 2.0 per cent agar; since reduction of potassium tellurite occurs upon heating, enough of this substance to give a final concentration in the medium of 1 : 15,000 should be dissolved in part of the water of the medium at a temperature below 40° C., and added to the sterilized medium (0.5 ml. of 1 : 1,000 potassium tellurite may be added to 7 ml. of sterilized medium). Small bluish black colonies about 1 mm. in diameter, with a peripheral opalescence, are fished and examined as described above. The ability of the organisms to develop characteristic colonies on MacConkey agar plates is considered an important requirement.

Mallmann (1940) suggested the use of a broth (Darby and Mallmann, 1939) containing 2 grams of Bacto-tryptose, 0.5 gram of lactose, 0.4 gram of K_2HPO_4, 0.15 gram of KH_2PO_4, 0.5 gram of NaCl, and 100 ml. of distilled water, to which is added sodium azide to give a concentration of 1 : 5,000; final *p*H, 6.8. This medium was found to grow streptococci but to inhibit coliform organisms, and it proved useful in estimating the number of the former group of bacteria in sewage. Hajna and Perry (1943) proposed another selective medium for fecal streptococci, containing 1 : 2,000 of sodium azide, to be used with incubation at 45° C. The ingredients of this " SF medium " are Bacto-tryptone, 20 grams; NaCl, 5 grams; glucose, 5 grams; K_2HPO_4, 4 grams; KH_2PO_4, 1.5 grams; sodium azide (NaN_3), 0.5 gram; 1.6 per cent alc. sol. of bromocresol-purple, 2 ml.; and distilled water, 1,000 ml. Growth and the production of acid in this medium, after incubation for 24 to 48 hours at 45.5° C., are stated to be almost complete evidence of the presence of *S. faecalis*. A study of the inhibition of gram-negative bacteria by sodium azide has been reported by Snyder and Lichstein (1940).

Streptococci as Indicators of Recent Pollution. Houston (1932) reported the examination of 14 mixtures of 10 human stools each, sampled at a sewage works, and found one mixture to contain 10,000 streptococci per gram, another mixture 10,000,000 per gram, and the other 12 mixtures 100,000 to 1,000,000 per gram. As a rule, feces contain somewhat fewer streptococci than coliforms, but the numbers

of both are very high. Raw sewage, however, contains far fewer streptococci than coliforms. Houston found a series of sewage samples to yield from 10 of the former per milliliter to 1,000,000 per ml., the median density being 1,000 per ml. In the raw water of the polluted Thames River coliforms were usually from 10 to 100 times as numerous as streptococci.

Horrocks (1901) maintained, largely on the strength of certain experiments with stored sewage, that the streptococci persist after the coliforms have disappeared, and indicate contamination with old sewage which is not necessarily dangerous. It seems likely that in sewage where there is a large amount of organic food material present the streptococci may kill out the coliform organisms as they do in the fermentation tube, and as we know they frequently do in shellfish. This would explain Horrocks' results. There is good evidence, however, that streptococci are less resistant than coliforms to the unfavorable conditions which exist in water of ordinary organic purity. Savage and Wood (1918), in an experimental study of the viability of streptococci in water, found that they died out in parallel with the coliforms, although a trifle more rapidly. In waters of potable character the latter are frequently present without the streptococcus; and a negative test for streptococci has little significance. A positive result, 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 coliform test 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; but in this country the 1913 Standard Methods concluded that "the information afforded by the occurrence of these organisms seems to be of less value than in the case of *B. coli* and it is believed that for the present at least, the streptococcus test is of subordinate importance." Later editions do not mention this method of examination.

Savage and Read (1917) examined an extensive series of waters for coliforms and streptococci. To detect the latter, they added the water sample to glucose neutral-red broth and made a direct microscopic examination for chains after 40 to 48 hours at 37°. They found a general correspondence between the results of the two tests, indicated in Table 88, for a group of 974 surface waters. It will be observed, however, that a number of samples containing many coliform organisms gave negative results with the tests for streptococci.

In one respect the streptococci appear to possess a decided advantage over the coliforms as indicators of pollution in ordinary waters: available evidence indicates that they rarely multiply in water as do, occasionally, the coliforms. In general, however, the streptococcus

TABLE 88

Subgroup	*B. coli*	Number of Samples	Percentage of Each Subgroup Containing Streptococci in			
			0.1 or 1 ml.	10 ml.	30 ml.	Not in 40 ml.
A	In 0.1 or 1 ml.	443	53	32	9	6
B	In 10 ml.	249	19	35	24	22
C	Not in 10 ml.	282	3	18	22	56

test is less delicate than the coliform test and, except when corroborative data are required or when the coliform results are suspected, it adds little to the information furnished by the test for coliform organisms.

Use of the Streptococci to Distinguish between Human and Animal Pollution. It has been frequently suggested that the streptococci might prove of assistance in the important task of differentiating between human and animal pollution, a task in which all other tests have so far failed. Unlike the coliform bacteria, streptococci from equine, bovine, and human feces appear to belong to more or less distinct types. Early English observations of specific differences in the streptococci from these sources were confirmed by Winslow and Palmer (1910); their most important results are shown in Table 89.

TABLE 89. COMPARATIVE FERMENTATIVE POWER OF STREPTOCOCCI FROM THE HORSE, THE COW, AND MAN

(Winslow and Palmer, 1910)

Streptococci	Percentage of Positive Results (300 Strains)		
	Lactose	Raffinose	Mannitol
Human	62	6	28
Equine	8	4	2
Bovine	52	28	6

The rarity of lactose-fermenting streptococci in the horse makes it probable that this group can be used for distinguishing pollution by road washings from that due to domestic sewage; and the fact that a

larger proportion of human strains attack mannitol and a larger proportion of bovine strains ferment raffinose might make it possible to use the ratio between results with these sugars to distinguish between the wash from pastures and cultivated land, and sewage. Clemesha (1912*a*), in India, found that both human and bovine fecal streptococci fermented raffinose, sucrose, and salicin, but not mannitol. Fuller and Armstrong (1913), however, confirmed the results obtained by Winslow and Palmer; they found that lactose-fermenting streptococci of any kind are rare in horse dung, that the streptococci of bovine feces characteristically ferment lactose and raffinose, whereas the fermentation of lactose and mannitol is usual among human fecal strains. The results of these workers were more consistent than those shown in Table 89, 65 per cent of the human strains being of the *S. faecalis* type (mannitol-positive), and 64 per cent of the bovine strains of the *S. salivarius* (raffinose-positive) type. Rogers and Dahlberg (1914) also found that streptococci from bovine feces typically ferment raffinose and not mannitol, and that those from the mouth of the cow are frequently of the *S. salivarius* type. Houston (1930) plated mixtures of 20 stools each, sampled at a sewage works, on various days, and found that 168 of the streptococci isolated fermented lactose but not raffinose, 115 attacked both of these carbohydrates, 1 fermented raffinose but not lactose, and 36 fermented neither. The results obtained on different days were very irregular; on one day 20 colonies fermenting raffinose and none failing to ferment it were isolated, whereas on another day the results obtained were exactly the opposite, despite the fact that in each instance a mixture of 20 stools was examined. Thus, although the mannitol-positive streptococci do appear to be more common in human feces, and the raffinose-positive more common in bovine feces, the evidence available does not indicate the ratio of the two types to be of great practical assistance in evaluating the sanitary quality of water.

A special use was made of the streptococcus test by Pettibone, Bogart, and Clark (1916), who employed it for the detection of mouth pollution in an examination of bubble fountains at the University of Wisconsin.

The Spore-forming, Lactose-fermenting Anaerobes. English bacteriologists have ascribed a certain importance as indicators of sewage pollution to another group of organisms, the lactose-fermenting, spore-forming anaerobes, of which the form first described by Welch and Nuttall (1892) and now called *Clostridium welchii*, or *Clostridium perfringens* (Bergey *et al.*, 1939), is that most commonly sought in water.

A method long employed for detection of the presence of *C. welchii* depends upon the production of "stormy fermentation" in milk. A portion of the water sample to be examined is added to a tube of sterile milk which is first heated to 80° C. for 10 minutes to destroy vegetative cells, then cooled and incubated at 37° C. under anaerobic conditions. If the *C. welchii* type of organism is present the appearance of the tube after 18 to 72 hours will be somewhat like that described by Klein (1898, 1899) who, in the course of an epidemic of diarrhea, isolated an anaerobe which he named *Bacterium enteritidis sporogenes:* "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."

Early reports on the sporing anaerobes isolated from sewage and water usually referred to them as *B. sporogenes,* but it seems likely that these organisms were either *C. welchii,* or a mixture of *C. welchii* and *Clostridium sporogenes,* a form found in feces, sewage, and manured soil, which tends to grow in mixed cultures. The stormy fermentation of milk, described above, is particularly characteristic of *C. welchii,* whereas the organism now known as *C. sporogenes* does not attack lactose.

Since *C. welchii* 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 2,000 ml. of water by filtration through a Pasteur filter was recommended by Horrocks (1901) as a necessary prelude. The 1913 Standard Methods recommended the following enrichment procedure for the isolation of *B. sporogenes.* Various dilutions of the water to be tested are incubated in fermentation tubes containing glucose liver broth for 24 hours at 37° C. If the organism is present gas will be evolved and a characteristic vile odor will be produced. If this reaction is obtained, the contents of each positive tube are transferred to an Erlenmeyer flask or large test tube and heated at 80° C. for 10 minutes to destroy vegetative cells. One milliliter of broth containing sediment is withdrawn from the bottom of each flask and enriched once more in a fresh liver broth tube. A gelatin stab culture made from these 24-hour liver broth tubes will show, after 48 hours' incubation at 20° C., a distinct liquefying anaerobic

growth beginning about 2 cm. below the surface, with gas bubbles at the top of the liquefied area. In order to obtain absolutely pure cultures it is necessary to fish from liver broth tubes only 3 to 5 hours old as only young vegetative cells will grow on plates. Transplants from the closed arm of such tubes will grow on glucose liver agar plates incubated under anaerobic conditions.

Another method for isolating this organism suggested by Wells is simpler than the foregoing. He recommended the use of freshly boiled lactose broth tubes (heated in the Arnold sterilizer) brought to a temperature of 70° C., at which temperature the inoculation is made. The inoculated tubes are then kept at 70° C. for 10 minutes and subsequently incubated at 37° C. By this means the spore forms develop into vegetative cells in the course of 24 hours and give rise to a rapid evolution of gas. Aerobic plates made from these tubes will generally give no growth. On the other hand, if coliforms were present, gas would ordinarily be produced in 18 hours and aerobic plates, either of E.M.B. or Endo agar, would show an abundance of colonies.

A plating method for estimating the number of *C. welchii* in water or sewage, suggested by Wilson and Blair (1925) and by Wilson (1931), is offered as an alternative to the stormy fermentation test by the Committee of the British Ministry of Health (1939). The procedure recommended by this committee follows. Two plates containing the portions of water to be tested are prepared as described below. After 24 to 36 hours of aerobic incubation at 37° C. the black colonies in the depth of the agar are counted. Large colonies, 5 mm. or more in diameter, are usually of *C. welchii* but occasionally contain coliforms; medium colonies, 3 to 4 mm. in diameter, also may be either *C. welchii* or coliforms; smaller colonies are of less significance and, though absent from pure water, probably consist of saprophytic water bacteria that may be found in potable water during the warmer months. More than one large black colony per 40 ml. of sample is considered by Wilson to show the presence of *C. welchii* and to justify suspicion of fecal pollution. In order to reduce interference by other bacteria further Wilson (1938) suggested incubation of the plates at 44° C.

The special Wilson and Blair medium consists of 100 ml. of ordinary nutrient agar (3 per cent agar), 10 ml. of 20 per cent solution of anhydrous sodium sulphite, 5 ml. of 20 per cent solution of glucose, and 1 ml. of 8 per cent solution of ferrous sulphate crystals. "In practice a stock sodium sulphite glucose solution may be prepared by dissolving 200 gm. of anhydrous sodium sulphite in 1 litre of boiling

water, and mixing when cool with a solution containing 100 gm. of glucose in 500 ml. of water. When the medium is required for use 100 ml. of nutrient agar are melted, and 15 ml. of the stock sodium sulphite glucose solution are added together with 1 ml. of a stock 8 per cent solution of ferrous sulphate. The medium is distributed into wide tubes in 20 or 40 ml. quantities, cooled to 55° C., mixed with an equal volume of the water to be tested, and poured into Petri dishes of suitable size. Some workers prefer to heat the water beforehand to 80° C. for 10 minutes to destroy non-sporing organisms."

C. welchii is a short, stout, gram-positive, non-motile rod, sometimes occurring in chains. In old cultures some elements may be gram-negative. Spores, occasionally present in ordinary media, are oval, central to excentric, and do not markedly swell the rods. *C. welchii* liquefies gelatin vigorously and on agar produces a circular, moist, slightly raised colony with an opaque center. Glucose, lactose, and sucrose are fermented rapidly with production of acid and gas. Numerous other types of lactose-fermenting, spore-forming anaerobes are found in water and soil, however. Some are motile; some are long, thin rods; and in some the rods are swollen by their spores, which may be central, excentric, or terminal.

Much work was done during and since the World War I relative to the systematic relationships of this group of anaerobic spore formers which proved to be of great importance in connection with wound infections. Space forbids an analysis of this material which is well reviewed, from the standpoint of the water bacteriologist, by Levine (1921*a*).

Significance of the Anaerobes in Water Analysis. The researches of Klein and Houston (1898, 1899) showed these organisms to occur in English sewage in numbers varying from 30 to 2,200 per ml., and to be frequently absent from considerable volumes of ordinary surface waters. In Boston sewage they were usually found in 0.01 to 0.001 ml. by Winslow and Belcher (1904).

Since the spores of an anaerobe may persist for an indefinite period in polluted waters, their presence need not necessarily indicate recent or dangerous pollution. Levine (1921*a*) expressed the opinion that the general use of the anaerobic spore formers as indices of pollution in water is undesirable because of their extreme resistance, their abundance in animal manure, in decomposing organic matter, and in soil, and their frequent failure to occur in numbers that correlate with the results of the sanitary survey. He cites with approval Cumming's statement that "unlike *B. coli* which varies many thousand per cent,

from several hundred per cc. to less than 1 in 10 cc., according to the intensity of pollution, these spores were found often in the best river water in 10 cc. and seldom showed an average much above 4 to 5 per cc. . . . Their number furnishes no clue to the degree of pollution and purification as does the number of *B. coli.*"

Wilson (1931), however, suggests that since *C. welchii* is essentially a fecal organism and since its spores may persist long after other indicators of contamination such as coliform bacteria have disappeared, this anaerobe may well serve as an indicator of *intermittent* pollution. Occasional contamination of shallow wells, for example, might be detected by tests for the presence of this organism. Harold (1935) reported that the density of *C. welchii* in various London supplies did not correlate as well as the streptococcus density with the coliform index. Incidentally, he found only 78.6 per cent of large and medium colonies on Wilson and Blair's plates to produce stormy fermentation in litmus milk. Some of the results obtained from Lee Valley wells are shown in Table 90.

TABLE 90. RESULTS OF EXAMINATION OF LEE VALLEY WELLS

(From Harold, 1935)

Sample	Coliforms (lactose+, indole+) Present in	Streptococci Present in	Milk Test		Plate Method
			10 ml.	1 ml.	Colonies per 10 ml.
Chadwell Spring	0.1 ml.	0.1 ml.	+		8 large
Amwell Marsh	0.01 ml.	0.01 ml.	+		10 large 9 small
Hoddesdon	0.1 ml.	0.1 ml.	+	+	80 large
Rye Common	1.0 ml.	neg. in 100 ml.	+		3 large

By employing the plating method, heating the sample for 10 minutes at 80° C. before plating, and incubating the plates at 44° C. for 24 hours, the Metropolitan Water Board (Mackenzie, 1938) examined various polluted English waters during the years 1936, 1937, and 1938, and found the percentages of large and medium black colonies on plates to be 91.6, 94.2, and 91.0 respectively. Upon fishing a considerable number of large and medium colonies to litmus milk and incubating for 24 hours at 37° C., stormy fermentation was produced by 78.6, 85.6, 78.4, and 71.0 per cent respectively, during the four years 1935–38. In raw river water 90 to 95 per cent of the black colonies were of large or medium size, and 70 to 80 per cent of these were *C. welchii.* It was concluded from these studies that "the

TABLE 91. MEAN RESULTS OF EXAMINATION OF THE RIVER THAMES
May, 1934 — December, 1938

(From Mackenzie, 1938)

	Bacteria Colonies per ml.		*B. coli* (lactose+, indole+) Per Cent Samples Positive		Streptococci (Heating Method) Per Cent Samples Positive			*C. welchii* Milk at 37° C. Per Cent Samples Positive			Plates at 45° C. Large and Medium Colonies in 10 ml.
	Agar 20–22° C. 3 days	Agar 37° C. 20–24 hr.	0.1 ml.	0.01 ml.	100 ml.	10 ml.	1 ml.	10 ml.	1 ml.	0.1 ml.	
Keel's Boathouse, Tilehurst	3,650	277	37.8	5.4	40.5	18.9	8.1	43.2	16.2	2.7	7.1
Sonning Bridge	3,442	357	54.1	5.4	43.2	18.9	5.4	78.4	16.2	...	9.3
Hambledon Lock	2,850	307	37.8	13.5	37.8	21.6	2.7	70.2	10.8	2.7	7.3
Townsend's Boathouse, Bourne End	2,508	283	29.7	5.4	40.5	10.8	...	43.2	5.4	...	5.3
Ruddle's Pool, Clewer	4,420	940	61.1	16.5	55.5	25.0	2.8	66.6	16.5	...	6.6
Bells of Ouseley	3,635	645	75.0	13.8	47.2	22.2	2.8	55.5	8.3	2.8	8.2
Littleton Intake	3,671	501	62.4	10.8	45.9	13.5	5.4	83.7	16.2	...	10.3
Walton Intake	4,683	542	61.1	16.4	47.2	13.8	5.5	86.1	22.2	2.8	10.1

appearance of black colonies 3 mm. or more in diameter in a plate containing a volume of water is, therefore, highly significant of *Clostridium welchii* and justifies the presumption that fecal pollution of recent or remote origin has taken place." It was pointed out, however, that the test is not sufficiently delicate for use with waters of good quality (in 100 ml. of which coliforms are absent), because there is little likelihood of *C. welchii* being present in 10 to 20 ml. of such waters.

TABLE 92. RESULTS OF COMPARISON OF TESTS FOR THE PRESENCE OF SULPHITE-REDUCING BACTERIA AND COLIFORM ORGANISMS

(From Lewis, Green, and Hamilton, 1930)

THE NUMBER OF SULPHITE-REDUCING BACTERIA IN FILTERED WATER WHICH CONTAINED NO COLIFORM BACTERIA

Sulphite-reducers per 100 ml.	Number of Samples	Per Cent of Total Samples	Quality of Water by Wilson-Blair Test
0	50	28.8	Good
1–6	47	27.2	Good
6–10	41	23.7	Good
11 and above	35	20.3	Bad

SULPHITE-REDUCING BACTERIA ABSENT IN 40 ML. AND CITRATE-NEGATIVE COLIFORMS PRESENT IN 10 ML.

Number of 10-ml. Portions Containing Citrate-negative Coliforms	Number of Samples	Per Cent of Total	Quality of Water Based on Coliform Test
1	16	40	Questionable
2	11	27.5	Bad
3	7	17.5	Bad
4	2	5	Bad
5	4	10	Bad

NUMBER OF SULPHITE-REDUCING BACTERIA IN WATER CONTAINING CITRATE-NEGATIVE COLIFORMS IN ALL OF FIVE 10-ML. PORTIONS

Number of Sulphite-reducers per 100 ml.	Number of Samples	Per Cent of Total	Quality of Water by Wilson-Blair Test
1–6	8	24.2	Good
6–11	7	21.2	Good
11–26	4	12.1	Bad
26 and above	14	42.4	Bad

Table 91 presents certain of the results obtained from systematic bacteriological surveys of the Thames River, covering periods of drought as well as excessive rainfall, through the years 1934–38.

An interesting comparison of the numbers of sulphite-reducing and coliform organisms in American waters was made by Lewis, Green, and Hamilton (1930). Employing 40 ml. of sample in the *C. welchii* plating method described by Wilson and Blair (1925), and five 10-ml. portions in the Standard Methods procedure specified by the U. S. Treasury Department for the examination of water supplied to common carriers, these investigators obtained the results indicated in Table 92 from three groups of sand-filtered effluent from the purification plant at Austin, Texas. The last column in the first and third sections of the table shows the quality of the water according to Wilson and Blair's criterion that a potable water should contain not

TABLE 93. CORRELATION OF TEST FOR CITRATE-NEGATIVE COLIFORMS AND SULPHITE-REDUCING BACTERIA IN SPRING WATER

(From Lewis, Green, and Hamilton, 1930)

Number of Sample	Positive Tubes from 5 10-ml. Portions	Sulphite-reducers per 40 ml.	Number of Sample	Positive Tubes from 5 10-ml. Portions	Sulphite-reducers per 40 ml.
1	4	56	11	1	53
2	2	7	12	5	27
3	4	212	13	5	5
4	5	269	14	0	0
5	4	105	15	0	0
6	4	404	16	0	0
7	0	4	17	1	20
8	2	93	18	5	123
9	5	32	19	5	585
10	5	69	20	5	84

more than 4 sulphite-reducers per 40 ml. Evidently, the correlation between the results obtained by the two methods is not very satisfactory. The comparative results secured from a series of spring waters are shown in Table 93; here the correlation is better.

Matheson (1937*b*) compared the results obtained from the plain agar plate (presumably at 37° C.), the coliform test, and the *C. welchii* test employing Wilson and Blair's medium and 10 ml. of sample to the plate, in a survey of certain waters at Hamilton, Ontario. These results are shown in Table 94. The industrial shore of Burlington Bay is polluted by sewage, drainage, and industrial wastes. The other three shores are comparatively clean, except when winds

bring pollution from the industrial shore. The lake is subject to contamination from the water of the Beach Canal; and the Desjardin Canal, with little flow, is polluted by sewage from a neighboring municipality at its upper end. The correlation between the average results shown in the table is fairly satisfactory, but Matheson states that the correspondence between the coliform and sulphite-reducer estimates in individual samples was not so marked. Although the

TABLE 94. COMPARISON OF BACTERIOLOGICAL RESULTS
(From Matheson, 1937*b*)

Zone	Samples	Agar Count per 1 ml. (average)	Coliforms per 1 ml. (average)	*C. welchii* per 1 ml. (average)
Industrial shore	78	190,000	12,000	13.9
Beach shore	51	24,000	340	2.9
North shore	42	7,600	10	1.1
West end	30	3,800	80	4.9
Desjardin Canal	23	730	550	7.5
Center of Bay	27	24,000	24	1.6
Lake Ontario	72	365	1.1	.75

bay samples all had a coliform index of 1 per ml. or greater, only 41 per cent showed a *C. welchii* count of 1 per 10 ml., and most of the samples yielding no *C. welchii* had rather high coliform indices. The greater persistence of the spore formers was shown by the fact that 32 per cent of the comparatively unpolluted lake water samples showed the presence of one *C. welchii* per milliliter although only 31 per cent of these samples had a coliform index of 1 per ml. or more. The coliform counts in the lake decreased much more rapidly than did the counts of sulphite-reducers.

It is clear, from the examples which have been given, that the *C. welchii* test is not nearly so delicate a means of detecting pollution in water as is the coliform test, and that the results obtained with it do not follow very closely those given by the latter procedure. Moreover, its indications may relate to pollution so remote as to be of little or no sanitary significance. It is also an impracticable test to employ in the analysis of ordinary waters because the numbers of *C. welchii* in such supplies are so small that inconveniently large samples must be examined for their presence. Except, possibly, as a special test for intermittent pollution or as an occasional corroborative test for moderate or gross pollution, the search for lactose-fermenting, spore-forming anaerobes in water appears to yield little information that cannot be furnished by the tests for coliforms or for streptococci.

The question of whether the ingestion of these anaerobes may cause disease has been raised from time to time (Larner, 1922), but the general opinion at present seems to be that the numbers ordinarily occurring in our water supplies are devoid of danger to the consumer.

The Isolation of Typhoid and Related Pathogens from Water. In view of the fact that *E. typhosa* is the organism most to be feared in a water it might naturally be asked why we do not use this organism itself as an index of pollution. The answer is that it can be found in water so rarely as to be of little practical value for such a purpose. There are, it is true, numerous instances on record in which this organism has been isolated from polluted water, as by Kübler and Neufeld (1899), who examined a farmhouse well at Neumark in 1899, and Fischer and Flatau (1901), who discovered an organism responding to a most exhaustive series of tests for *E. typhosa* in a well at Rellingen in 1901. In these examinations the water was directly plated upon Elsner's medium or phenolated gelatin with no preliminary process of enrichment. Willson (1905) summarized the instances in which the organism had been isolated from infected drinking water, up to 1905, and included, in addition to the above-mentioned instances, 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 the use of carbol gelatin plates.
3. By Jaksch and Rau, in 1904, from the water supply of Prague, and also from the river Moldau, by caffein nutrose crystal-violet agar.
4. By Ströszner, in 1904, from a well near Budapest, by the same method.

Several other instances in which the isolated organisms gave positive agglutination tests, as well as the usual cultural reactions, are also cited by Willson.

Since 1905 a number of successful isolations of typhoid bacteria have been reported in America. An organism obtained from the water supply of Scranton, Pa., in 1907, by simple enrichment in Parietti bouillon, was identified as *E. typhosa* by Professor Fox after a very careful series of tests with immune sera (Pennsylvania, 1908). The most important results were achieved, however, by Jackson with lactose bile enrichment and subsequent plating on Hesse agar. He reported the isolation of the organism from 10-ml. samples of the Grass River at Canton, N. Y., and of a pond and stream at Hastings, N. Y. (both used as sources of water supply) and from two 1-ml. samples of the Hudson River near Hastings at the time of the typhoid

epidemic there (Jackson and Melia, 1909). Pettersson (1919) reported finding *E. typhosa* in a polluted well at Visby by the iron sulphate precipitation method. Geiger, Macmillan, and Gillespie (1917) even isolated this organism, by direct plating on litmus lactose agar, from a brook associated with a typhoid epidemic at a labor camp in California. The water of the brook in question was, however, so heavily polluted as to be more properly designated as dilute sewage.

The search for typhoid or related organisms is usually suggested when an outbreak of 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 that any of the specific bacteria once present will have disappeared. Although elaborate experiments have shown that *E. typhosa* may persist in sterilized water for upwards of 2 months and in unsterilized water from 3 days to several weeks, the number of the organisms present is always very rapidly reduced. Even in highly polluted water their number is proportionately small, as is well shown by the experiments of Houston (1928, 1929), who examined a large number of samples of raw River Thames water for these bacteria. Each sample consisted of a mixture of equal parts of hourly samples collected over a period of 24 hours. One liter of the composite sample was dosed with 50 ppm. of alum, then divided into two equal portions, to one of which a certain number of *E. typhosa,* separately determined by agar culture, was added. After centrifuging, the deposit from each water portion was spread over 16 plates of Wilson and Blair's bismuth sulphite agar and incubated, and a considerable number of the resulting suspected colonies were fished and submitted to the usual cultural and serological tests. One organism ultimately identified as *E. typhosa* was isolated from each of 2 non-infected samples of the 34 examined in 1928, and a similar result was obtained from the examination of 34 samples in 1929. As indicated in Table 95, an average of about 35 per cent of the typhoid organisms added to the purposely infected 500-ml. samples was recovered by means of the method employed. Houston's recovery of 2 typhoid organisms from approximately 2,000 colonies examined, compared with a similar recovery from the study of 20,771 colonies in other investigations made before the use of bismuth sulphite agar, testifies to the marked effectiveness of this new medium.

Wilson (1938) reported several recoveries of *E. typhosa* from water and sewage effluents in the course of epidemiological investigations. In fact, the isolation of typhoid organisms, when fairly numerous, from sewage and sewage effluents by means of bismuth sulphite agar

has become a comparatively simple procedure. Wilson (1928) examined Belfast sewage on four occasions, plating out 4, 11, 10, and 5 ml. On these sets of plates there developed 31, 67, 64, and 71 black colonies respectively, the great majority of which fermented sucrose gelatin (with an added indicator). Finally, the four groups of colonies yielded 4, 2, 7, and 8 strains of *E. typhosa,* or approximately 1 per ml. of sewage examined. Incidentally, one of these samples contained 200 *C. welchii* per milliliter. Houston (1930), following the procedure described above which he employed in his search for typhoid organisms in Thames River water, except that 0.1 ml. of the sample was diluted and spread over 16 to 20 bismuth sulphite agar

TABLE 95. RESULTS OF EXAMINATION OF THE RIVER THAMES FOR *E. typhosa*
(From Houston, 1928, 1929)

Period	Number of Samples	Non-infected Samples		Infected Samples		
		Colonies Studied	Samples Containing *E. typhosa*	*E. typhosa* Added	Colonies Studied	Average Number of *E. typhosa* Recovered
1928	34	2,107	2	68	2,370	14
1929	34	1,746	2	25	1,868	18

plates, examined 34 specimens of London sewage and on 4 occasions isolated a single *E. typhosa.* Of the average number of 47 organisms purposely added to another 0.1 ml. of sewage in each of the experiments, an average of 10 was recovered.

Ruchhoft (1935*a*), by means of a modified bismuth sulphite agar, isolated typhoid organisms from sewage sludge when for each *E. typhosa* present there were 1,000 coliforms and 25,000 other bacteria.

S. schottmülleri (*Bacterium paratyphosum B*) may also be readily isolated from water or sewage if present in any considerable number. Gray (1929) identified this organism in 7 of 20 samples of 2.5 ml. each of Edinburgh sewage, finding it in only 1 of 4 districts investigated. Although comparatively free from paratyphoid fever at the time of this investigation, the population of Edinburgh had suffered a certain incidence of the disease in the past and very probably a number of carriers were still discharging paratyphoid organisms into the city's sewers.

In January, 1931, an epidemic of paratyphoid B fever, caused by milk, occurred in Epping, a town of about 5,000 population situated some 17 miles distant from London. There were 255 cases of the disease. A part of the town's sewage after treatment by land irriga-

tion is discharged into Cobbins Brook which, in turn, flows into the River Lee, one of London's sources of raw water supply. Houston (1930), in the preceding year, had examined London sewage for *S. schottmülleri*, as he had for *E. typhosa*, and a study of 1,800 colonies from nine 0.1-ml. portions of sewage plated on bismuth sulphite agar had yielded no organisms of this type, although of an average of 114 organisms added to each of 9 other portions of the sewage an average of 5 had been recovered. Consequently, it is particularly interesting that, in the course of numerous examinations made during the year of the outbreak, Houston (1931) isolated from Epping raw sewage an average of 312 paratyphoid B organisms per milliliter and a maximum of 3,400; and from the sewage effluent an average of 57 and a maximum of 355 per ml. Since in one Epping carrier's feces some 450 million of these organisms per gram were found, it is not especially surprising that on one day, according to a calculation by Harold (1935), 143 billion paratyphoid B organisms were discharged into the brook. The very slow decline in the number of these bacteria in the sewage and effluent during succeeding years is noteworthy. Mackenzie (1938) reported the recoveries shown in Table 96.

TABLE 96

Year	Para B in Sewage per ml.	Para B in Effluent per ml.	Cases of Para B Fever
1931	312	57	255
1932	446	55	
1933	219	16	Outbreak (due to milk) 22 cases late in 1933
1934	227	7	
1935	68	3	2 cases in 1935
1936	102	3	
1937	111	1.6	

The results of numerous examinations of sewage from English communities other than Epping reported by Houston and by Floris during the years 1931–33 showed that paratyphoid organisms could only rarely be isolated from the sewage of places which had suffered very little or no paratyphoid fever and no recent paratyphoid epidemics. For example, Houston (1932), in the examination of 28 samples of sewage and sewage effluents, isolated 1 paratyphoid B organism from each of 3 samples and 1 *Salmonella typhimurium* from each of 3 other samples; and Floris (1933) found only 1 paratyphoid B organism in a total of 24 samples of 0.05 ml. each of sewage from 6 different communities.

Upon storing, at a temperature of about 10° C., an Epping sewage effluent from which 1 paratyphoid organism per milliliter had been isolated, Houston (1931) found that after 3 weeks none could be recovered from 500 ml. of the effluent.

The isolation of other *Salmonella* organisms from sewage was reported by Houston (1932) and the British Ministry of Health (1931); the organisms isolated were identified as *S. typhimurium, Salmonella sp.* (Newport type), and *Salmonella enteritidis.*

In this country, Ruchhoft (1934) succeeded in isolating *E. typhosa* from samples of activated sludge, and Hajna (1935) reported the recovery of the same organism from several samples of sewage and sewage sludge collected in the Baltimore district. A review of results of some recent attempts to isolate pathogens from sewage, and an interesting discussion of the indicated relations between the *E. typhosa* density of sewage and water, the coliform density, and the typhoid fever morbidity in the community, was presented by Kehr and Butterfield (1943).

Methods of Isolating Typhoid and Related Pathogens from Water. The methods used for the isolation of typhoid, paratyphoid, and dysentery organisms from water fall into three main groups: (*a*) direct isolation on differential solid media; (*b*) isolation as above, after preliminary cultivation in a selective enrichment medium; (*c*) isolation, with or without enrichment, preceded by physical concentration produced by agglutination, chemical precipitation, or filtration.

Among the earlier differential media proposed for direct plating may be mentioned litmus lactose agar, phenolated gelatin, Drigalski-Conradi agar (Drigalski and Conradi, 1902), Endo agar (Endo, 1904), malachite-green agar (Loeffler, 1903, 1906; Lentz and Tietz, 1903, 1905; Doebert, 1906; Nowack, 1905), and brilliant-green agar (Torrey, 1913; Krumwiede and Pratt, 1914). All these media depend on the theory that the typhoid bacillus is more resistant than *E. coli* to the dyes present in the media. The semi-solid media of Hiss and Hesse (Hiss, 1902; Jackson and Melia, 1909; Jackson, 1907; Stokes and Hachtel, 1912) depend, on the other hand, on the greater motility of *E. typhosa* and its consequent tendency to form branching colonies and turbid zones in such media.

Among the more important enrichment media, supposed to favor the growth of *E. typhosa* at the expense of *E. coli,* are phenol broth (Parietti, 1890; Hankin, 1899), media containing caffein (Roth, 1903; Hoffman and Ficker, 1904; Klaumann, 1904; Willson, 1905), lactose bile (Jackson and Melia, 1909), and brilliant-green broth. Rakieten

and Rettger (1927) advocated the use of tubes of peptone water containing brilliant green in varying amounts to give concentrations of 1 : 650,000 and less. Houston (1930) found direct plating of sewage on bismuth sulphite agar to yield much better results than enrichment in brilliant-green peptone water followed by plating.

The solid media more generally employed at the present time for isolation of typhoid and related organisms are bismuth sulphite agar (Wilson and Blair, 1926, 1931; and Wilson, 1938), which is particularly inhibitive to the coliform group of bacteria but which favors the growth of *E. typhosa* and a large proportion of the *Salmonella* organisms; S.S. (*Shigella-Salmonella*) agar, developed by Difco Laboratories, which also markedly inhibits coliforms and favors the growth not only of *E. typhosa* and *Salmonella* organisms but also that of the dysentery group of bacteria (genus *Shigella*); desoxycholate agar, with or without citrate (Leifson, 1935), another medium which restricts coliforms but favors the development of typhoid, paratyphoid, and also dysentery bacteria; eosin methylene blue agar (Holt-Harris and Teague, 1916), an old favorite, which gives good differentiation between organisms which do and those which do not ferment lactose; and MacConkey agar which, like the desoxycholate medium, depends largely upon its bile salt content to restrict the growth of undesired organisms. Of these, only bismuth sulphite agar and MacConkey agar have been used extensively for isolation of enteric disease bacteria from water, but it is probable that others would also prove useful for this purpose. Ruchhoft (1935*a*) suggested a pour-plate modification of Wilson and Blair's bismuth sulphite agar for the isolation of typhoid organisms in water, sewage, and sewage sludge. He found the latter medium to be only 1.7 per cent as productive of *E. typhosa* as nutrient agar, whereas a productivity of 92 per cent is claimed for agar of the modified formula. It will be remembered that Houston found the productivity of the Wilson and Blair medium to average about 20 to 35 per cent.

Commonly employed enrichment media are selenite broth, first proposed by Guth (1916) and developed further by Leifson (1936); and tetrathionate broth, first suggested by Müller (1923). Numerous observers have reported these to be useful in diagnostic work. Both media inhibit coliforms and permit the growth of *E. typhosa* and *Salmonella* organisms, but their effect on the multiplication of dysentery bacteria is not so favorable. Mackenzie (1938) reports that a limited comparison of a modification of Müller's medium described by Szper (1935), a sodium selenite broth, and Wilson and Blair's direct plating method, in the examination of Epping sewage

and effluent, did not permit any definite conclusion as to the relative value of the two enrichment media: each detected the presence of paratyphoid B organisms on three occasions when the other did not. The selenite method permitted the isolation of these organisms once, and the Szper method three times, when bismuth sulphite direct plates failed, but the plating procedure showed their presence four times when selenite enrichment failed and six times when the Szper enrichment failed. Apparently, as Mackenzie intimates, there is little advantage in using enrichment when examining sewage, but it might prove to be helpful in the examination of water.

A physical concentration of the organisms sought precedes enrichment or isolation in the procedure recommended by many authors. Filtration through a Berkefeld or similar type of filter, agglutination, and chemical precipitation have been the means more commonly employed.

Agglutination was proposed by Adami and Chopin (1904) as the basis of a method of isolating *E. typhosa* from water. Two-liter samples, to each of which 20 ml. of 1 per cent glucose broth was added, were incubated for 18 to 24 hours at 37° C. Ten-milliliter portions were then withdrawn and graded amounts of typhoid serum added. After 2 to 3 hours the deposit in tubes having the greatest dilution in which agglutination was apparent was washed two or three times, plated on various media, and the organisms in the resulting colonies were examined. Klotz (1904), employing this method, isolated an organism which, although agglutinated, proved to be not a typical *E. typhosa* but a form probably intermediate between that organism and *E. coli.* It may be recalled that Frost (1910) isolated a bacillus of the *Proteus* group from filtered Potomac water which agglutinated with typhoid serum in high dilutions. Schepilewski (1903) and Altschuler (1903) also used agglutination as a means of precipitating the bacteria after enrichment cultivation in broth. Schepilewski isolated *E. typhosa* from water containing 1 loopful of a broth culture in 50 liters; Altschuler was successful in isolating the organism from a suspension of 150 in 1 liter of water.

A number of methods for concentrating typhoid bacteria in water by chemical precipitation have been tested experimentally, with some degree of promise. Vallet (1901) was the first to employ this principle; he made use of sodium hyposulphite and lead acetate. The mixture was centrifuged and the precipitate dissolved in more hyposulphite. The clear solution was then plated. Schüder (1903) and Ficker (1904) proposed modifications of this procedure; the latter, employing ferric sulphate, dissolving the precipitate in potas-

sium tartrate, and plating on Drigalski-Conradi medium, claimed that 97 to 98 per cent of the typhoid bacteria were carried down with the precipitate. Müller (1905) recommended the use of ferric oxychloride because of its quicker and less destructive action. Willson (1905) added 0.5 gram of alum per liter of the water to be examined, centrifuged the mixture, suspended the precipitate in a little water and plated it. Nieter (1906) used ferric sulphate and sodium hydrate, centrifuged the mixture, and filtered it through a sterile filter, recovering small numbers of bacteria. Employing iron oxychloride as the precipitant, he confirmed the results of Müller.

By the use of a combination of enrichment and chemical precipitation, Ditthorn and Gildemeister (1906) isolated typhoid organisms 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 *E. typhosa*. This led Ditthorn and Gildemeister to use bile and bile agar as enrichment media. After precipitation by Müller's method, the whole of the precipitate was added to 100 ml. of sterile ox bile and grown at 37° for 24 hours, after which time 1-ml. 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 was also pointed out that iron oxychloride is bactericidal in 48 hours.

Filtration, as might be expected, has frequently been employed for concentrating the bacteria present in water. A neat apparatus for filtering large volumes of water, consisting of a Berkefeld filter of medium (N) porosity encased in a metal cylinder and attached to a water meter, was designed by Ford and Gunderson (1942). Burns (1941) described the concentration of bacteria by means of a small diatomaceous disc, and reported the isolation of *S. typhimurium* from a city water conforming to the requirements of the U.S.P.H.S. standards.

Drigalski (1906) and Starkey (1906) employed methods aimed at taking advantage of the greater motility of typhoid organisms in order to separate them from other bacteria. Drigalski isolated *E. typhosa* from two springs by allowing tall milk cans containing 5 to 10 liters of the water to stand at room temperature for 1 to 2 days and plating samples from the surface. Starkey employed glass tubing, bent to provide four successive connected loops, which was filled with phenol broth, inoculated at one end, and incubated anaerobically. The more actively motile bacteria were sought by plating from the fourth loop. Houston (1911) tried this procedure but obtained only negative results

even with a water artificially infected with about 14 typhoid organisms and 21 *S. enteritidis* organisms per milliliter.

The most promising method of isolating enteric disease organisms from water appears to be either filtration as described above, or precipitation by some simple method such as Houston employed in his search for *E. typhosa* in water: addition of about 50 ppm. of alum, centrifuging, resuspending the precipitate in 1 or 2 ml. of water, and plating on several plates of bismuth sulphite agar, S.S. agar, and some less inhibitive medium such as MacConkey or eosin methylene blue agar, with incubation for 24 hours at 37° C. Since some organisms will grow only on one medium, and others on different media, at least three plating media should be used. It is well to incubate the bismuth sulphite plates for another 24 hours to permit full development of some of the *Salmonella* organisms, notably *S. schottmülleri.*

If Ruchhoft's pour-plate modification of bismuth sulphite agar is employed for the enumeration of *E. typhosa,* colonies conforming to his description should be sought: ". . . small, with a typical delicate brown halo." Ruchhoft states that larger colonies with slightly darker halo are produced by some other sulphite-reducers.

Colonies of the types described below should be fished to triple sugar agar (Krumweide and Kohn, 1917) containing considerable sucrose, for interfering organisms fermenting this sugar may be numerous in sewage-polluted waters. (*a*) On ordinary bismuth sulphite agar after 24 hours, discrete black colonies surrounded by a black or brownish black halo, which is often several times the area of the colony itself and which shows a distinct metallic sheen, may consist of *E. typhosa.* If the colony is not well separated from others this characteristic appearance may not be evident; consequently, since it is likely that pathogens, if present, are very few in number, heavy inoculation as practiced in diagnostic work should be avoided and only a small portion of the precipitate spread over each plate. Colonies like those described but more moist may be growths of *S. schottmülleri;* flat or slightly raised green colonies may consist of *Salmonella* organisms; and brownish, raised colonies with depressed centers may consist of Flexner or Sonne types of dysentery bacteria. Even if colonies such as those described are not in evidence, others should be fished; for they may be pathogenic organisms which, for one reason or another, have not developed normally. After suspected colonies are fished, the plates are returned to the incubator and examined again after another 24 hours, as slowly developing paratyphoid B or other *Salmonella* colonies may then be present. (*b*) On S.S. agar after 24 hours, colorless, smooth (usually) colonies, that are

either opaque, transparent, or translucent, may consist of *E. typhosa, Salmonella,* or *Shigella* organisms. Coliform colonies usually possess some color, although certain forms of *Aerobacter* organisms produce larger, white or cream colored, dense, mucoid colonies. (*c*) On desoxycholate, eosin methylene blue, and MacConkey agars after 24 hours, small, colorless, transparent, apparently flat, or "dewdrop" colonies may be growths of *E. typhosa, Salmonella,* or *Shigella* organisms.

Since the above media may inhibit, but not destroy, undesired bacteria, the greatest of care is to be observed, when fishing the colony, to secure a pure culture. Only the center of the colony should be touched, very lightly, with the needle used for making the transfer. The triple sugar agar is inoculated by passing the needle over the surface of the slant and then stabbing the butt. Triple sugar agar contains 1 gram of beef extract, 12 grams of proteose peptone No. 3 (Difco), 10 grams of lactose, 10 grams of sucrose, 1 gram of glucose, 5 grams of sodium chloride, 15 grams of agar, 62.5 ml. of 0.04 per cent phenol red, made up to 1 liter with distilled water. Tubes of the agar are cooled in a slanted position to form a butt of about 1 inch and a slant 1 to 1.5 inches long.

Typhoid and related pathogens will not produce sufficient acid from the small amount of glucose in the triple sugar agar to color the indicator at the surface of the slant and, since they ferment rapidly neither lactose nor sucrose, the surface of the agar slant should remain neutral or alkaline in reaction after incubation for 24 (or preferably 30 to 40) hours at 37° C. if any of these organisms are present in pure culture. They all produce, however, sufficient acid from the glucose in the agar butt to color the indicator; and some of them, in addition, produce gas from that carbohydrate. Consequently, if there is no indication of acid on the surface of the slant, but an indication of acid, or of acid and also gas, in the butt of the triple sugar agar tube, the reactions should be recorded and the culture examined further. Cultures showing an acid reaction at the surface of the slant may be discarded.

A triple sugar agar slant culture showing a neutral or alkaline slant and acid *and* gas in the butt may contain *Salmonella paratyphi* (*Bact. paratyphosum A*), *S. schottmülleri* (*Bact. paratyphosum B*), *Salmonella enteritidis* (Gaertner's bacillus), *Salmonella typhimurium* (*Bact. aertrycke*), *Salmonella suipestifer, Proteus morganii, Shigella sp.* (Newcastle type), or slow-lactose-fermenting coliform-like organisms (aberrant coliforms); whereas one showing a neutral or alkaline slant, and acid *without* gas in the butt, may contain *E. typhosa,* one

of the *Salmonella* or *Shigella* organisms not mentioned above, *P. morganii* (which varies in its ability to attack sucrose), or again slow-lactose-fermenting coliform-like organisms (aberrant coliforms).

When a suspected organism has finally been isolated, its identity must of course be established by exhaustive microscopical, biochemical, and serological tests. This procedure will rarely be adopted by the water bacteriologist. If it is attempted, recourse may be had to textbooks of medical bacteriology and particularly to the volume entitled *Diagnostic Procedures and Reagents* prepared by the Committee on Standard Methods of the American Public Health Association. Because, however, of the frequent overlapping of the physiological characteristics of coliform, paracolon, *Eberthella, Salmonella,* and *Shigella* bacteria, which may render identification difficult if not impossible, cultures of suspected organisms should preferably be referred to a laboratory which is well provided with the wide variety of serums required for practical identification of such bacteria and which is thoroughly familiar with work of this kind.

Relative Resistance to Chlorine of *E. typhosa* and Coliform Organisms. Because of the almost universal use of chlorination in the treatment of water supplies the comparative effect of this type of treatment upon pathogenic organisms and indicator organisms such as coliforms is obviously of very great importance. The fact that experience of marked coliform reduction after chlorination is generally accompanied by very favorable epidemiological experience with respect to reduction in mortality from enteric disease was long accepted as practical proof that coliforms were at least as susceptible as *E. typhosa* and related pathogens to this treatment. The typhoid epidemic in Minneapolis in 1935, which persisted for two or three months although routine bacteriological examinations (approximately 80 per month) failed to reveal any excessive coliform density in the finished water, aroused considerable speculation, accompanied by a measure of anxiety, as to whether our usual standards of water quality based upon coliform density were adequate.

In addition to favorable experimental results, which earlier had been reported by numerous investigators, relative to the comparative resistance of enteric disease organisms and coliform bacteria to chlorine and related compounds, Tonney, Greer, and Danforth (1928) had published the results of their studies of the minimal " chlorine death points " of *E. typhosa,* numerous other pathogens, and *E. coli,* which indicated that *E. coli* constituted a very suitable indicator organism for control of chlorination because, according to these observers, (*a*) it appeared to be as resistant (if not more resistant)

to free chlorine as the pathogenic organisms studied; (*b*) it grows rapidly on ordinary media; (*c*) it is easily detected.

As part of the report on the Minneapolis typhoid epidemic the Minnesota Department of Health (1935) included findings of Heathman, Pierce, and Kabler (1936) on the comparative resistance to chlorine and chloramine of *E. typhosa* and various coliform strains, from which it appeared that certain recently isolated strains of the pathogen were more resistant to these disinfectants than were older laboratory strains, that at low temperature the resistance of both coliforms and *E. typhosa* is greater than at high temperatures, and that "there is a possibility" of the pathogen surviving treatment longer than coliforms.

Butterfield, Wattie, Megregian, and Chambers (1943) studied the resistance to free chlorine (with chlorine-addition products such as chloramine excluded) of *E. typhosa, S. dysenteriae, E. coli, A. aerogenes,* and *P. aeruginosa,* with special reference to the influence of temperature and hydrogen-ion concentration. Their results indicate that the time of exposure to free chlorine is of prime importance, that the bactericidal effect of the disinfectant is enhanced by a rise in temperature, and that the kill is markedly increased as the *p*H is lowered. Furthermore, these observers found the *E. coli* and *P. aeruginosa* strains tested more resistant to free chlorine than strains of *E. typhosa* at *p*H intervals of 8.5, 9.8, and 10.7; but at *p*H 6.5, 7.0, and 7.8, when the concentration of the disinfectant was 0.03 or less, the former organisms were apparently slightly less resistant than the *E. typhosa* strains. A 100 per cent kill of *E. coli* and *E. typhosa* was effected at *p*H 7.0, 8.5, 9.8, and 10.7 by 0.1 to 0.29 ppm. of chlorine in 1, 10, 20, and 60 minutes, and 1, 3, 5, and 20 minutes respectively, when the temperature ranged from 20° to 25° C. At the lower temperatures of 2° to 5° C., an exposure of 2 to 12 times that required at the higher temperatures, or a chlorine concentration 2 to 10 times greater, was necessary to produce a 100 per cent kill of *E. coli.*

It appears from the results of these investigations that, for adequate control of chlorination procedures, the coliform content of the treated water must be watched very closely. Frequent sampling and maintenance of consistently low coliform densities are necessary if reliance is to be placed on standards of treated water quality based upon coliform concentration, for there seems to be very little, if any, favorable margin between the resistance of *E. typhosa* and *E. coli* to free chlorine and chloramine under the conditions of temperature and hydrogen-ion concentration that usually obtain in practice.

Significance of the Occurrence in Animal Discharges of Organisms Pathogenic for Man. The danger to the consumer from drinking water moderately polluted by animals, such as birds, fowl, swine, horses, cows, wild or domestic, was for many years regarded as more or less negligible, if we except the possibility of infection due to ingestion of bovine tubercle bacilli. The expression, "subject to contamination only from cultivated fields," very commonly encountered in descriptions of surface waters considered of fair quality, despite the fact that such cultivated fields are usually manured, often with excrement from a variety of animals, is an evidence of this indifference toward animal pollution. The discovery of the multiplicity of *Salmonella* organisms common to man and animals, however, may change this attitude. Edwards and Bruner (1943) reported data showing that in their collection of *Salmonella* bacteria, isolated in the United States and possessions during a period of only a few years, some 30 types have been secured from both man and one or more animals such as fowl, horses, and so on. Although Mallmann, Ryff, and Matthews (1942) were unsuccessful in producing apparent disease in monkeys fed with cultures of various *Salmonella* types isolated from chickens, Edwards and Bruner stated that they possess unpublished data which "include a number of instances in which infection in man was traceable directly to animals." Brucellosis is another infection which conceivably might be transmitted by water polluted with discharges from animals. In addition to the danger from direct pollution by diseased animals there is the possibility of water being infected by fowls or other animals serving merely as vehicles of human pathogens. *E. typhosa,* for example, has been isolated from the feces of gulls which had apparently fed on material contaminated with typhoid organisms. It is quite probable that, as we learn more regarding the transmission to man of infectious diseases caused by animal discharges containing human pathogens, an increasing realization that animal pollution can be considerably more dangerous than heretofore suspected may dispel the measure of complacency with which such pollution has been regarded in the past.

Other Pathogenic Organisms Isolated from Water. From the sanitary point of view *E. typhosa,* and *Salmonella* and *Shigella* organisms are the most important of the pathogens that may infect American supplies, for these are obviously the germs of disease which here are most likely to be disseminated through water. According to the accounts of numerous investigators, however, a wide variety of pathogenic organisms has been isolated from this medium. For example, Dunbar (1896*a, b*), making practical use of the "cholera-

red" reaction of Dunham (1887), succeeded in isolating *Vibrio comma* (*Spirillum cholerae*) from the water of the Elbe in 1892 during the cholera epidemic at Hamburg; Houston (1902*c*) isolated *B. anthracis* from sewage and from the mud of a river bank; and Brown, Petroff, and Heise (1916), by examining large volumes of water, were able to isolate tubercle bacilli from the stream which receives the sewage from Saranac Lake at points as far as 3½ miles from the sewer outlet. Parker, Steinhaus, and Kohls (1943) reported the recovery of *Pasteurella tularense* from the water and mud of three streams, in northwestern states, in which animals had died of tularemia during an epizoötic of this disease among beavers and muskrats; and *S. paratyphi* (*Bact. paratyphosum A*) was isolated under the direction of one of us (M. H. M.) from ice presumably infected by the backflow of sewage through a drain onto the floor where the ice was crushed (Foley and Brandon, 1936).

CHAPTER XI

THE SIGNIFICANCE AND APPLICABILITY OF THE BACTERIOLOGICAL EXAMINATION

Sanitary Inspection and Sanitary Analysis. The first act of the expert called in to judge the value of a potable water should be to make a thorough sanitary inspection of the pond, stream, well, or spring from which it is derived. As explained in Chapter II, 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 the soil, and the liability to contamination by surface wash are of supreme importance in interpreting the analyses to be made. In many parts of the country protests arose during the early years of the use of the Treasury Department standard (*infra*, p. 248) against too literal and rigid an application of any arbitrary analytical standards (Morse and Wolman, 1918; Hinman, 1920). In the first Progress Report of the Commission appointed by the Treasury Department to Recommend Standards of Purity for Drinking Water supplied to the Public by Common Carriers Engaged in Interstate Traffic (1914) it was stated that "It is a fact so well established as to need no further discussion that the results of bacteriological and chemical examination of a sample of water ought always to be correlated with the knowledge of the source, treatment and storage of the supply in order to enable a just estimate of the sanitary quality of such supply." The importance of the field survey was further stressed in a report of the Advisory Committee (1925) appointed to review these standards and in the report of the Advisory Committee (1943) appointed to revise them again. The *Manual of Recommended Water Sanitation Practice* accompanying the last report and prepared by the U. S. Public Health Service (1944) will be found very helpful in pointing out features to which particular attention should be given when judging the safety of a supply.

The field survey and the laboratory analysis are mutually complementary. Whittaker (1917) reported that out of 344 water supplies found to be polluted in Minnesota 52 per cent were condemned on the ground of both field survey and laboratory analysis; 40 per cent were condemned from the survey although the analysis on the date of examination was satisfactory; in 8 per cent of the cases the danger

was revealed only by analysis, the first survey indicating nothing suspicious. 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 reconnaissance 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.

Sanitary Chemical Analysis. 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 gives a historical picture which may be of value in special cases. 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 must 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 event, it is a warning of possible pollution, and the determination of free ammonia, nitrites, carbonaceous matter as shown by "oxygen consumed," and dissolved oxygen may throw some light upon 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 information about the antecedents of a sample. The results of the chlorine determination are indeed perhaps clearer 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. The chief value of this test lies in the fact that, if a high chlorine content is noted in a ground water which at the moment shows no bacteriological evidence of contamination, it may be well to reëxamine the water under other weather conditions to see if bacterial as well as saline contamination has been introduced. 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.

Information Furnished by Bacteriological Examinations. The decomposition of organic matter is closely related to bacterial multiplication. Thus, the standard plate count at 20° 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. The bacteriologist can usually declare the innocence of such waters 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 a normal variety of forms is included, 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 agar at 37° C., since organisms which thrive at body temperature are characteristic of the intestinal tract and usually occur in small numbers in normal waters.

Finally, the search for coliform organisms 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 full bacteriological analysis should then consist of three parts, the 20° C. plate count, as an estimate of the amount of organic decomposition in process; the 37° C. plate count, as a measure of the organisms which thrive at body temperature; and the application of an accepted procedure for the isolation of coliform organisms.

Special Advantage of the Bacteriological Examination. 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 estimate 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 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 coliform 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, whereas chemical evidence of pollution persisted for 6 miles beyond the point of entrance, the bacteria introduced could be detected for 4 miles farther (Woodman, Winslow, and Hansen, 1902).

The statement has sometimes been made that although bacteriological methods may be more delicate for the detection of pollution in surface waters, contamination in ground waters may best be discovered by chemical analysis. That such is not so was well shown by Whipple (1903) who cited 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 *Bact. 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 *Bact. 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 *Bact. 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 called attention to the report on the Chemical and Bacteriological Examination of Chichester Well-waters by Houston (1901), in which the results of chemical and bacteriological examinations of 30 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 *E. coli*, *C. welchii*, and streptococci. On the other hand, the chlorine and the albuminoid ammonia showed no correspondence with the bacteriological results.

Vincent (1905) cited 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 milliliter before the arrival of a regiment of troops. Its subsequent history is indicated in Table 97.

TABLE 97. PROGRESSIVE POLLUTION OF A WELL
(Vincent, 1905)

	Bacteria per ml.	*E. coli* per ml.
Before arrival of troops	200	0
6 days after arrival	770	0
14 days after arrival	4,240	1
41 days after arrival	6,960	2
60 days after arrival	14,900	10

Thirdly, negative tests for coliform organisms 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, except under the most extraordinary circumstances, that a water could contain typhoid organisms without giving clear evidence of pollution in the fermentation tube or on the 37° plate.

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

teriological examination offers a more delicate and 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 coliform organisms could be readily isolated from 75 per cent of the 1-ml. 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 (ppm.) is given in Table 98. The bacteriological

TABLE 98

Color	None
Turbidity	None
Sediment	None
Odor (hot)	None
Odor (cold)	None
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 ammonia	0.0000
Nitrites	0.0000
Nitrates	0.0000

examination showed a total count of 375 bacteria per milliliter and a 37° count of 350 per ml. The presumptive tests for coliforms showed that gas-producing organisms were present in a majority of 1-ml. samples, and typical coliforms were isolated. In this water the contamination was caused by cattle gaining access to the area immediately surrounding the spring; but the same conditions might easily have led to infection from human beings.

A special case in which the bacteriological examination proves of unique value is in the study of bottled waters which are always liable to contamination in handling, a type of contamination which can generally be revealed only by bacteriological methods. Various investigators, whose work is reviewed by Koser and Skinner (1922), reported high counts and the presence of coliforms in soft drinks, and the systematic examination of such products which are so commonly exposed to various possibilities of hand contamination is now receiving the serious attention of health authorities.

Donald, Jones, and MacLean (1924) indicated that carbonation of beverages is associated with marked reduction in the bacterial count. *E. coli* appears to be more resistant to carbonation than *E. typhosa*. The germicidal effect of the CO_2 becomes apparent when not less than 4.8 volumes of the gas are introduced and it increases with increase of CO_2 pressure.

The Committee on Microbiological Examination of Foods of the Food and Nutrition Section, American Public Health Association (1942) has suggested a series of examinations of bottled beverages which includes both microbiological tests of the finished product for the presence of mesophilic bacteria, yeasts, and molds, and bacteriological tests to determine whether the product meets the Committee's specification that it conform to " the requirements for drinking water according to the A.P.H.A. *Standard Methods for the Examination of Water and Sewage.*" The Committee points out that excessive depression of the *p*H of the enrichment broth, employed in coliform tests, by acids in the beverage may be eliminated by buffering the broth with 5 grams of K_2HPO_4 per liter; and that since formation of gas in the inoculated broth tube may be due to fermentation of sugar in the beverage by yeasts instead of coliforms, gas-positive tubes must be confirmed to determine whether the latter organisms are present.

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, because of their excessive delicacy. In studying the heavy pollution of small streams, the treatment of trade 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 derives 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 relative to the persistence of separate streams of water in immediate contact with each other. Heider (1893) 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 24 miles. 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 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 never be omitted.

The Record of the Analysis and Its Interpretation. A complete record should be kept of all the observations made in the course of the examination of a water. Very frequently these details will prove helpful in the interpretation of the results. For example a note, relative to a high plate count, indicating that practically all the colonies were of one or two types, immediately suggests multiplication of bacteria of no sanitary significance. In general, the greater the variety of colony types on a plate, the more unfavorable is the indica-

tion, for polluted waters usually contain a multiplicity of bacterial species.

The ratio of the 37° to the 20° plate count should be noted. If this ratio is low — below 10 per cent — and particularly if the body temperature count is low for the type of water examined, the indication is favorable. Low counts at both temperatures are very reassuring. Not infrequently extremely low counts in the presence of somewhat doubtful coliform results, especially in the examination of ground waters, furnish conclusive confirmation of favorable sanitary-survey findings. On the other hand, an increase in the count of a water that usually contains few bacteria may afford the first indication that something is wrong.

Every detail of the coliform results should also be recorded, including the amounts of gas formed after 24 hours and after 48 hours of incubation, both in the enrichment tube and in the confirmatory tube; and if complete confirmation is practiced, the types of colonies on the E.M.B. plate, the details of gas formation in the secondary lactose tube, and the results of the microscopical examination should be noted. The first day's gas readings are particularly significant, for the production of gas in 24 hours in the enrichment tube usually means that normal coliforms are present. On the other hand, we have remarked that, more often than not, the appearance in practically all the gas-positive enrichment and confirmatory tubes of gas only after 48 hours and in only small amount — a bubble or 5 per cent — is associated with a more or less favorable inspection report, particularly when the supply in question is a well or a spring. Too great reliance upon the significance of this rate of gas production must be guarded against, however, because, as explained in Chapter IX, typical coliforms are often present in tubes in which gas is forming slowly; and confirmation of the indication should be sought in the other features of the analytical results, bacteriological and chemical, and in the findings of the sanitary survey. Of course, in a treated or filtered-treated water, practically all the previously contained coliforms should have been destroyed; consequently, the rate of gas production of coliforms found in waters of this sort is of little significance, particularly when the samples are taken at the plant.

The number of types of colonies appearing upon the E.M.B. plates streaked from the gas-positive tubes of a sample is also important. If all these tubes yield only the same type of suspected coliform colony, and if there is no apparent explanation for the presence of coliforms in the sample, the possibility of coliform infection of material in contact with the water (washer, pump, etc.) is suggested;

and it may be advisable to submit a number of the colonies to differential tests to determine whether they are really all of the same type. If, however, a considerable variety of coliform-colony types are found on the plates, pollution of the sample by sewage is indicated.

The interpretation of results is much simplified if regular and frequent bacteriological examination of a water supply is practiced. As pointed out before, a single analysis of a water reveals only its bacterial content at the moment of sampling. Repeated examination, however, furnishes a conception of the normal variations to which the bacterial population of the supply may be subject, and thereby greatly facilitates evaluation of the significance of departures from the normal that otherwise might be overlooked or misconstrued. It also permits early detection of accidental pollution of intakes, reservoirs, or distribution systems to which, apparently, few water supplies are immune.

There has been a tendency in recent years, probably the result of a too general application of the bacteriological procedure specified in the U.S.P.H.S. standards for waters served on common carriers, to depend exclusively upon the results obtained from coliform tests for information relative to the sanitary quality of water supplies. We believe this is unwise. Even for routine work we consider it very advisable to include at least one of the plate counts, for an estimate of the bacterial content of a water frequently furnishes information that a coliform result does not. In the control of supplies serving large populations, the greatest practicable variety of tests should be employed, including non-standard as well as standard methods of examinations. As we have shown in the preceding chapters there are available, besides the standard coliform test and plate counts, many tests, one or more of which, although perhaps not generally applicable, may prove useful in the examination of a particular water. Among these may be mentioned the non-standard rapid presumptive tests, the test for acid formers in a 37° lactose agar plate, the streptococcus test, the sulphite-reducer test, and the Eijkman test. Nor should certain chemical methods, such as the chlorine-demand test and the determination of nitrogen in its various forms be overlooked; one or more of these may answer a special need. A Committee on Methods of Water Treatment and Laboratory Control (1941), appointed by the Water Purification Division of the American Water Works Association, concluded, after an exhaustive study of the adequacy of current treatment and laboratory practices, that "notwithstanding the occurrence from year to year of a few obscure intestinal outbreaks, the mass of evidence supports the effectiveness of water treatment

procedures and water quality standards, *provided these procedures are properly applied and controlled.*" By implication, the Standard Methods upon which the water quality standards are based are likewise considered, subject to the same condition, effective. Nevertheless, those charged with the laboratory control of water supplies should be ever on the alert for applications of procedures, other than the Standard Methods of the moment, that may furnish helpful additional information regarding the quality of the waters under their supervision. Standard Methods should always be employed in order to secure official data that may be compared with those obtained from other waters and by other agencies; but experience with further procedures may discover supplementary means of control that will enable the laboratory to fulfill even more effectively its function of ensuring the safety of water supplied the consumer.

The Problem of Standards. It has been emphasized earlier in this chapter that sound judgment in regard to the sanitary quality of a particular water supply should be based on a consideration of the facts brought out by a careful sanitary inspection as well as on analytical data. Conclusions based on arbitrary bacteriological standards will never completely fit all circumstances. Nevertheless, for practical administrative purposes, something in the nature of a standard is often almost essential. The most important standards thus far proposed in the United States must therefore be briefly reviewed.

The first standard of this sort which exerted an important influence was that formulated by the United States Public Health Service for the control of water served by common carriers in interstate commerce, and defined in the Progress Report (1914) of the commission appointed for this purpose. The principal features of the standard imposed at that time are outlined below.

1. 37° C. plate count not to exceed 100 per ml.
2. Not more than one of five 10-ml. portions of any sample examined to show the presence of *B. coli* upon complete confirmation of lactose broth enrichment tubes containing more than 5 per cent gas after 48 hours of incubation at 37° C.
3. Evidence of the presence of *B. coli* to comprise (*a*) the formation of gas in the original enrichment tube; (*b*) the development of acid-forming colonies on litmus lactose agar or bright-red colonies on Endo agar plates; (*c*) formation of at least 10 per cent of gas, by organisms fished from the above agar plates, in secondary lactose broth.

By this it is clear that a water containing more than one typical

coliform organism in 50 ml., or yielding an average 37° plate count of more than 100 per ml., was considered unsatisfactory.

This Treasury Department Standard, as it has commonly been called, was criticized from many quarters, but on the whole it exerted a very useful influence. Although the standard was intended only for control of the quality of water on common carriers, it was natural that many municipalities should attempt to make their water supplies, even if not used by common carriers, conform to its requirements.

A revision of the standard was made in 1925 by an Advisory Committee (1925) appointed by the Surgeon-General of the U. S. Public Health Service. From this revision plate counts were omitted. The following statement of the Committee relates to this action:

> The omission of plate counts from the standard is not to be construed, however, as denying or minimizing their importance in routine examinations made in connection with the control of purification processes. On the contrary, the committee wishes to record its opinion that one or both plate counts are of definite value in such examinations, and to emphasize that it is chiefly in the interest of simplicity that they have been omitted from the standard here proposed.

The new standard specified that:

1. The index organism should be any of the *B. coli* group as defined by the 1923 edition of Standard Methods (Completed Test).
2. Not more than 10 per cent of all the 10-ml. portions of water examined should show the presence of these organisms.
3. When the number of standard samples (5 portions of 10 ml. each) is 20 or more, not more than 5 per cent should show three or more of the five 10-ml. portions of a sample to be positive; and when the number of standard samples is less than 20, not more than one sample should show such a result.

It will be observed that this standard is more exacting in several respects than its predecessor. It implies a limiting density of about 1 coliform organism per 100 ml. of sample instead of 1 per 50 ml.; the limitation of coliforms to those that have produced more than 5 per cent of gas in the primary enrichment tube is omitted, for the 1923 Standard Methods definition of the *B. coli* group requires simply the formation of *any* amount of gas in the primary tube; the requirement relative to the type of colony appearing on the confirmatory agar plate is omitted, for the 1923 Standard Methods accepts any type of coliform colony; and the limitation of coliforms to those producing at least 10 per cent of gas in the secondary lactose tube is omitted, for *any* amount of gas in this tube is accepted as a characteristic of coliforms by Standard Methods.

It will be noticed, too, that although an *average* coliform density of not more than about 1 per 100 ml. is permitted, the standard makes a generous allowance for variations from a constant density, caused by errors of simple sampling, that may be expected. This is particularly essential because, in addition to limiting the total percentage of positive portions the standard must limit also the frequency of *high* results in single samples in order to avoid the use of temporarily dangerous supplies; yet the unavoidable variations due to simple sampling must be allowed for.

Another revision of the Official Standards was made in 1942 by an Advisory Committee (1943) appointed for the purpose. Its principal features may be summarized as follows:

1. Coliform bacteria are defined as set forth in the 1936 edition of Standard Methods and include those organisms the presence of which has been demonstrated by (*a*) the usual completed test; (*b*) the confirmed test when liquid confirmatory brilliant-green bile 2 per cent is used, the formation of *any* gas in this medium after 48 hours of incubation at 37° C. to be considered a positive confirmed test; (*c*) the confirmed test, when crystal-violet lactose broth, fuchsin lactose broth, or formate-ricinoleate lactose broth, is used. It is recommended, however, that the selection of any one of these media be based upon correlation of the results thus obtained with those from a series of completed tests, and that the medium chosen be that yielding results most nearly corresponding to those of the completed test.

2. The standard sample is considered either 5 portions of 10 ml. or 5 portions of 100 ml.

3. In any disinfected supply the sample must be freed of disinfecting agent within 20 minutes of its collection.

4. The number of water samples from representative points on the distribution system to be collected each month varies from 1 for a supply serving a population of 2,500 or under, to 100 when the population served is 100,000, and to 500 when the population is 5,000,000, in accordance with the indications furnished by a graph accompanying the report.

5. The standards impose limitations on the number of positive results as set forth in the following extract from the report.

> 3.2. *Application.* Applications 3.21 and 3.22 given below shall govern when ten milliliter (10 ml.) portions are used and applications 3.23 and 3.24 shall govern when one hundred milliliter (100 ml.) portions are used.[1]
>
> 3.21. Of all the standard ten milliliter (10 ml.) portions examined per month in accordance with the specified procedure, not more than ten (10) percent shall show the presence of organisms of the coliform group.
>
> 3.22. Occasionally three (3) or more of the five (5) equal ten milliliter

[1] It is to be understood that in the examination of any water supply the series of samples for any one month must conform to both of the above requirements, either 3.21 and 3.22 or 3.23 and 3.24, respectively.

(10 ml.) portions constituting a single standard sample may show the presence of organisms of the coliform group, provided that this shall not be allowable if it occurs in consecutive samples or in more than

(*a*) Five (5) percent of the standard samples when twenty (20) or more samples have been examined per month.

(*b*) One (1) standard sample when less than twenty (20) samples have been examined per month.

Provided further that when three or more of the five equal ten milliliter (10 ml.) portions constituting a single standard sample show the presence of organisms of the coliform group, daily samples from the same sampling point shall be collected promptly and examined until the results obtained from at least two consecutive samples show the water to be of satisfactory quality.[2]

3.23. Of all standard one hundred milliliter (100 ml.) portions examined per month in accordance with the specified procedure, not more than sixty (60) percent shall show the presence of organisms of the coliform group.

3.24. Occasionally all of the five (5) equal one hundred milliliter (100 ml.) portions constituting a single standard sample may show the presence of organisms of the coliform group, provided that this shall not be allowable if it occurs in consecutive samples or in more than

(*a*) Twenty (20) percent of the standard samples when five (5) or more samples have been examined per month.

(*b*) One (1) standard sample when less than five (5) samples have been examined per month.

Provided further that when all five of the standard one hundred milliliter (100 ml.) portions constituting a single standard sample show the presence of organisms of the coliform group, daily samples from the same sampling point shall be collected promptly and examined until the results obtained from at least two consecutive samples show the water to be of satisfactory quality.[3]

3.25. The procedure given, using a standard sample composed of five standard portions, provides for an estimation of the most probable number of coliform bacteria present in the sample as set forth in the following tabulation [Table 99]:

This standard not only includes a new requirement, that of a certain frequency of sampling from the distribution system, and takes cognizance of the dependability of the confirmed test as an indication of the presence of coliforms, but also suggests the alternative use of

[2] When this occurs, and when waters of unknown quality are being examined, simultaneous tests should be made on multiple portions of a geometric series ranging from 10 ml. to 0.1 ml. or less.

[3] When this occurs, and when waters of unknown quality are being examined, simultaneous tests should be made on multiple portions of a geometric series ranging from 100 ml. to 0.1 ml. or less.

TABLE 99

Number of Portions		Most Probable Number of Coliform Bacteria per 100 ml.	
Negative	Positive	When Five 10-ml. portions are examined	When Five 100-ml. portions are examined
5	0	Less than 2.2	Less than 0.22
4	1	2.2	0.22
3	2	5.1	0.51
2	3	9.2	0.92
1	4	16.0	1.60
0	5	More than 16.0	More than 1.60

100-ml. portions of sample. These are all logical developments: the simplicity of the liquid confirmatory test permits the desired greater frequency of examination which was practically impossible when the arduous completed test was required for confirmation; and the use of 100-ml. portions furnishes results which give warning of an approach to the limiting coliform density of about 1 per 100 ml., whereas no such warning is provided by the results from 10-ml. portions of sample. The latter point is well shown in Table 99. To be sure, the use of 100-ml. portions requires considerably more enrichment media and much more incubator space than does the use of 10-ml. portions; but since the employment of the larger portions is not made a requirement, laboratories may continue to examine 10-ml. portions of sample if they so desire. There is an obvious tendency among waterworks laboratories toward the use of 50-ml. or 100-ml. portions for the control of water supplies serving large populations, and it is probable that, in time, this practice will be generally adopted for controlling all but very small supplies.

It should be observed that the requirements of the above standards as regards procedure are necessarily *minimum* requirements, and bacteriological control of water supplies should not be limited by them. In order to detect really significant departures from the normal density it is usually advisable to examine, say, 5 portions of each of *at least* two dilutions of the water. For example, when the normal coliform density of the supply is fairly accurately indicated by the results of examination of five 100-ml. portions, these quantities of sample and also five 10-ml. portions should be tested. If, for one reason or another, the water does actually become unsafe, the procedure employed in its examination must be such as to give, *as soon as possible, unmistakable* warning of the danger. The application of

this more intensive search for coliforms to *all* the samples collected from a water supply may be impracticable, but at least the water entering the distribution system should be regularly examined in this manner.

It is interesting to compare the U.S.P.H.S. standards with the classification suggested by the Committee of the Ministry of Health (1939) for waters in Britain, as shown below.

Piped Supplies (entering the distribution system):

		Presumptive Coliform Count per 100 ml. (Acid and Gas in MacConkey Broth)
Class 1	Highly satisfactory	Less than 1
Class 2	Satisfactory	1–2
Class 3	Suspicious	3–10
Class 4	Unsatisfactory	Greater than 10

Throughout the year 50 per cent of samples should fall in Class 1; 80 per cent should not fall below Class 2; and the remainder should not fall below Class 3.

In chlorinated piped supplies the water ought to come in Class 1.

Waters of Class 1 and Class 2 of the above classification evidently conform very closely to the requirements of the American standards. Although the use of the MacConkey broth *presumptive* test is specified, it must be remembered that in Great Britain the presumption afforded by a positive with this test is reported to be very high; and since the numbers of coliforms isolated from MacConkey and lactose broth gas-positives (631 and 643, respectively) by a group of American and Canadian workers (McCrady, 1939*a*) were very similar, the use of the MacConkey presumptive test with waters in Great Britain probably yields results fairly comparable with those obtained by means of our standard completed test.

It is obvious that, although these methods of examination and standardization may be employed with large public water supplies in which bacterial determinations are made regularly and frequently, they are not so easily applied to the supplies of smaller towns, institutions, dairy farms, and private residences, where the evaluation of the quality of the water is desirable but where an occasional test, or two or three examinations in the course of the year, is often the common procedure.

Furthermore, standards of the type described should not be confused with criteria for differentiating polluted from unpolluted waters. The

natural water of an *unpolluted* lake or stream may, as we have observed in Chapter IX, contain coliform organisms in numbers considerably greater than those permitted by the standards and still constitute, under ordinary conditions, a safe drinking water. Because of the possibility of accidental pollution, to which few surface waters particularly are immune, such supplies are nevertheless usually treated before distribution to larger consumer populations. Economic considerations, however, ordinarily justify neglecting the provision of insurance afforded by treatment of water of this quality when supplied to very small communities or to private residences. It should be noted that even if typhoid fever or other similar disease occurs on an individual farm, for instance, it is likely to be spread by other means than by water — the extra hazard of contamination of the private well is far less serious than is that of a communal supply. Consequently it is not surprising that a large proportion of our population, especially that of rural districts, is drinking water which by no means conforms to the U.S.P.H.S. standard with respect to coliform density, but which is nevertheless reasonably safe.

Examination of Swimming Pools and Bathing Places. Bacteriological examination has for many years been a recognized method for sanitary control of swimming pools and other public bathing places. The dangers from bathing in polluted waters, although less than from their employment for drinking purposes, are nevertheless too real to condone the use of waters showing positive evidence of intestinal pollution. Furthermore, as Horwood, Gould, and Schwachman (1933) pointed out, since sinus, eye, and ear infections, as well as respiratory diseases, are much more likely to be contracted from the waters of swimming pools than are intestinal infections, an index of sanitary quality other than the coliform density may be needed for such waters. This subject will be discussed in a succeeding paragraph. Frequent and regular examinations for total numbers of bacteria growing at 37° C. in 24 hours, as well as for organisms indicative of intestinal pollution, are also generally considered essential for swimming pool control. The examination of public bathing waters is therefore a procedure from which the whole bacteriological picture should be obtained: on the one hand, from the viewpoint of general safety of the bather, and on the other hand as a means of controlling any purification processes that may be in operation.

The degree of bacterial purity which has been demanded in public pools in different cities and in those of semi-public organizations has been variable, but it is now generally recognized that such waters should be adequately protected from pollution of any sort. The

American Public Health Association (1942) in its official report on bathing waters recommends the following bacterial standards for swimming pools:

> Not more than 15 per cent of the samples of pool waters covering any considerable time shall contain more than 200 bacteria per milliliter growing on agar at 37° C. in 24 hours, nor shall show positive confirmed coliform tests in any of five 10-ml. portions of water, at times when the pool is in use.

In well-managed pools, it is possible to keep the 37° count always below 100 per ml.; and this more rigid standard has been established in some cities.

It is evident that the recommended coliform standard is practically equivalent to that of the U.S.P.H.S. standards for drinking water served on common carriers. It must be remembered, however, that the waters with which swimming pools are originally filled are usually of drinking water quality; that any subsequent pollution found in them is derived chiefly from the bathers themselves; and that, since this pollution is fresh and may be highly infective, it must be rendered innocuous as soon as possible after its discharge into the water. A high quality of swimming pool water must therefore be maintained at all times, particularly when the bathing load is heavy.

In the control of outdoor bathing places, it is scarcely possible to apply general enforcement of the high standards maintained in indoor pools. The Official Report mentioned above recommends, for practical purposes, the use of the lactose broth presumptive test (1 tube with each dilution) for estimating the coliform density of such waters, but specifies no standards of purity for them. Classification of bathing places, as practiced in some areas, is suggested as "the most promising program for public health workers to follow." The classification recommended, which is practically that suggested by Scott (1931) for Connecticut shore bathing waters, is shown below.

	Average *E. coli* per 100 ml.
Class A	0–50
Class B	51–500
Class C	501–1,000
Class D	Over 1,000

The Official Report states that "proposed bacteriological standards by various agencies have seemed to hit mainly upon two widely divergent limits for standards of acceptability for bathing waters, one of which is 50 *E. coli* per 100 ml. and the other of which is 1,000 *E. coli* per 100 ml. . . . It is perhaps reasonable to conclude that, subject to interpretation of analytical studies from proper angles, waters better

than the lower limit (1,000 *E. coli* per 100 ml.) are fairly acceptable." It should be noticed that the density of *E. coli* is to be estimated, according to the Official Report, by the results of the Standard Methods presumptive test: consequently, as acknowledged by the Committee, the results secured really furnish merely *presumptive* evidence of a *coliform,* rather than an *E. coli,* density.

A list of several bathing water standards, in use or proposed, has been compiled by Schroepfer (1942), from which the following are taken:

California State Department of Health: 100 coliforms per 100 ml.

Winslow and Moxon (1928): Average not over 100 coliforms per 100 ml. with a maximum of not over 1,000 (based on a study of Connecticut waters).

Indiana State: Coliforms shall not exceed 100 per 100 ml. in more than 50 per cent of samples, and shall not exceed 1,000 per 100 ml. in any single sample.

New York City Health Department: 3,000 coliforms per 100 ml.

Eddy and Greeley: 1,000 coliforms per 100 ml. suggests a warning of excessive contamination at times, and an average index in excess of 3,000 per 100 ml. shows that the water may be dangerous to the public health (suggested for Boston Harbor).

Michigan Stream Control Commission (1933): 100–500 coliforms per 100 ml., interpreted as indicating a bathing water free from detrimental pollution, might be attributed to land wash; 1,000 per 100 ml., suspicious (but *dangerous* in proximity to fresh sewage pollution); 10,000 per 100 ml., menace to health.

The original bacterial classification proposed by Scott for Connecticut waters was found by him to correlate very well with the results of sanitary surveys of the bathing places studied. Defining good conditions as Class A and very poor conditions as Class D areas, with intermediate conditions designated as Class B and Class C, 62.1 per cent of the shore area studied showed an exact correlation between the sanitary survey and the bacterial classification.

As Schroepfer has noted, the extremes of the limits just described represent a ratio of 1 to 60 (50 to 3,000 coliforms per 100 ml.). It is unfortunate that such a divergence of opinion should exist; but pending the accumulation of sound epidemiological evidence upon which reasonable standards can be firmly based, existing conditions have probably weighed heavily in the formulation of the more lenient standards.

Streptococci as Indices of Buccal Pollution. In the control of the water of natural outdoor swimming places, the test for coliform organisms is usually of primary importance. In artificial pools, however,

the water introduced is generally of high quality with respect to fecal pollution and the major problem is oral and skin pollution from the bathers themselves. Under these conditions, the acid-forming streptococci are of far greater significance than the coliform organisms. Mallmann (1928) and Mallmann and Sypien (1934) found that the streptococcus density of bathing waters corresponded much more closely with the bathing load than did the density of coliforms or of bacteria growing at 37° C.; and since coliforms apparently multiplied at times overnight, whereas streptococci did not, the numbers of the latter organisms proved the better indices of pollution. Mallmann and Sypien directed attention to the fact that, whereas the numbers of coliforms and bacteria growing at 37° C. may considerably exceed the streptococci estimate in an unchlorinated water, especially in a beach water where conditions are often favorable for bacterial multiplication because of the presence of organic matter and high temperatures near the shore, the opposite prevails in a chlorinated water where the coliforms and other bacteria are particularly affected by the chlorine. Mallmann and Gelpi (1930), in a study of the relative resistance of *E. coli* and streptococcus strains, noted that the former developed a tolerance to chlorine, but the latter did not: another advantage of the streptococcus over the coliform estimate.

In a study of several swimming pool waters in the Boston district, Horwood, Gould, and Schwachman (1933) employed the 37° count, the completely confirmed coliform test, the streptococcus test of Mallmann and Gelpi, and a blood agar plate test for hemolytic streptococci. In each of these tests 1 ml. of sample was used. The results, together with the residual chlorine figures, are shown in Table 100.

TABLE 100. RESULTS OF VARIOUS TESTS OF SWIMMING POOL WATERS

(Horwood, Gould, and Schwachman, 1933)

Pool No.	Presence of Streptococci	Presence of Hemolytic Streptococci	Bacteria on Agar 37° C., 24 hr.	Coliforms (Completed Test)	Residual Chlorine ppm.
1	0	0	2	0	0.5
2	+	+	100	0	0.2
3	+ +	+	350	0	0.25
4	+ +	+	250	0	0.2
5	+ + + +	+	400	0	0.2
6	+ + +	+	1,500	0	0.0
7	+	0	50	0	0.3
8	+ +	+	150	0	0.25
9	0	0	50	0	0.3
10	0	0	40	0	0.25

An excellent correspondence is shown, in this table, between the results of the two streptococcus tests and those of the 37° count. Moreover, it was found, after a sanitary survey of each of these pools, that " where the administration, sanitary maintenance, and sanitary control of bathers in the operation of swimming pools are excellent, the bacterial results are especially satisfactory." The coliform results, on the other hand, furnished little information relative to the sanitary condition of the pools examined.

Standard Methods (1936) recommended a study of the sediment of 48-hour enrichment broth cultures, as described in the preceding chapter, for estimating the number of streptococci in water. In the control of the swimming pools at Yale University it has been found that such determinations of the presence of lactose-fermenting cocci in 10-ml., 1-ml., and 0.1-ml. lactose broth tubes or, better yet, a plate count on differential media of this group of organisms, are the best index which the laboratory can provide.

As the Official Report mentioned above points out, however, although the numerous streptococci derived from the bathers, especially from their nasal and buccal discharges, are undesirable, complete elimination of these organisms from the water of pools cannot be effected without marked increases in chlorine residuals and sharp reductions in bathing loads, either of which may limit the usefulness of the pool. Acid-forming cocci are often present even with chlorine residuals above 1.0 ppm., which swimmers could not tolerate. Reliable standards might be developed by operating pools in accord with proper sanitary regulations, with the free chlorine residual (without using ammonia) at, say, 0.4 to 0.6 ppm., with the water *p*H 7.1 to 7.3, and the water temperature 74° to 76° F., and with bathing loads averaging, say, one bather per 50 square feet of pool surface, and by comparing under these conditions counts of cocci with total plate counts and coliform estimates.

In connection with total plate counts, it is interesting to note the results obtained by Riddick (1941) upon comparing the relative value of agar counts at 37° C. with 24- and 48-hour periods of incubation for the control of several New York pools. He found that 15 per cent of undechlorinated samples gave 24-hour counts greater than 8 per ml.; a like percentage of dechlorinated samples gave 24-hour counts greater than 52 per ml. (6.5 times the former count); and the same proportion of dechlorinated samples yielded 48-hour counts greater than 340 per ml. (6.5 times the second count). All the samples were iced and examined within 4 hours of collection. A fairly satisfactory correlation was shown between the 48-hour 37° count, the

coliform density, the residual chlorine, and the bathing load, whereas little correlation was evident between the 24-hour 37° count and these other factors. Riddick, therefore, favors the use of the longer incubation period.

Collection of Samples from Swimming Pools and Other Bathing Places. If the water is treated with chlorine, any residual chlorine present must be destroyed within a few minutes of collection of the sample. Bottles for sampling such bathing waters should therefore contain sodium thiosulphate, as described in Chapter II.

The sample should be collected in the manner generally indicated for sampling a surface water: by plunging the open bottle in an arc down into, through, and out of the water with one continuous, sweeping motion. Care must be observed to avoid rinsing the thiosulphate out of the bottle. Samples should be taken only when the pool is being used; and periods when the bathing loads are heaviest should be chosen for collecting the samples. Mallmann and Sypien (1934) have emphasized the necessity of repeated sampling and the unreliability of occasional catch samples.

Standard Methods requires that a residual chlorine test be made at the pool side when the sample is collected, and also at the laboratory in order to make sure that the thiosulphate was not removed when the sample was taken.

If samples must be shipped, they should be well iced. Ritter (1937) reported the results obtained from 145 samples of chlorinated swimming pool water, collected in duplicate in thiosulphate-treated and untreated bottles and shipped uniced; they were in transit for 12 to 93 hours. The percentages of counts under 200 per ml. in the treated and untreated samples were 22.8 and 56.5 respectively. The results from a short series of iced samples examined within 4 hours of collection showed no such marked bacterial increase in the thiosulphate-treated samples.

Of course, some difference between the counts of dechlorinated and undechlorinated *iced* samples must usually be expected because of the effect of the residual chlorine left in the latter. A series of results, shown in Table 101, obtained from iced samples received at Montreal after 18 to 24 hours in transit, is typical. Since multiplication of coliforms in iced samples is almost inconceivable (in fact, their numbers practically always decrease in iced samples), the figures in the last column of the table may be taken to represent a conservative estimate of the coliforms originally present in these waters; and if these coliforms were all eliminated by the residual chlorine in the untreated sample, as they appear to have been, there is little doubt

that the divergence between the 37° counts in the treated and untreated samples is also largely due, not to multiplication of bacteria in the former, but to reduction caused by the residual chlorine left in the latter. When high pool residuals are employed, the difference in counts is not so great, because the bacteria in the original water

TABLE 101. RESULTS FROM SWIMMING POOL SAMPLES; ICED, 18 TO 24 HOURS IN TRANSIT

Water Number	Number of Bathers in Pool	Residual Cl in Pool, ppm.	Without Thiosulphate		With Thiosulphate	
			Count 24 hr., 37° C.	Coliforms per 100 ml.	Count 24 hr., 37° C.	Coliforms per 100 ml.
1	15	0.3	7	0	27	54
2	19	0.3	7	0	100	4.5
3	12	0.3	9	0	24	0
4	15	0.25	130	0	240	0
5	16	0.25	18	0	25	0
6	30	0.2	13	0	28	0
7	35	0.35	8	0	65	0
8	35	0.3	36	0	110	7.9
9	6	0.45	17	0	32	0
10	14	0.15	75	0	800	13

have already been reduced to a small number of rather resistant forms. In order to avoid confusion in the interpretation of bacteriological results from chlorinated swimming pool samples, therefore, such samples should always be treated with thiosulphate, and either examined practically immediately or, if the examination is to be delayed, packed in ice.

CHAPTER XII

BACTERIOLOGY OF SEWAGE AND SEWAGE EFFLUENTS

Bacteriological and Chemical Examination of Sewage. The first object of modern sewage disposal is the oxidation of putrescible organic matter. Chemical, rather than bacterial, purification is usually 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 2-foot rule — the saw will do the cutting, but the rule will measure the work cut." (W. J. Dibdin.)

In certain instances, however, bacterial as well as chemical purity must be effected. 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 should be so treated as to protect those using the water. In seaboard cities, where sewage effluents are likely to contaminate oyster beds and other layings of edible shellfish, the problem assumes great importance. When bacterially impure 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, in any event, from the pollution necessarily incident to surface waters. Unless the bacterial condition of a stream or lake is made 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. The whole question is one of relative costs. Under certain circumstances sanitary authorities may rightly demand that bacteria, as well as putrescible organic matter, shall be removed by sewage treatment. Under such conditions the bacterial control of sewage purification plants is as essential as is that of water plants.

Methods of Bacteriological Examination of Sewage and Effluents. 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, suggested various tests involving the use of litmus milk, peptone solution, gelatin tubes, and neutral-red broth, as

well as the inoculation of animals. He considered the determination of the numbers of *E. coli* and *B. sporogenes* (*C. welchii*) particularly important, the identification of streptococci of value in certain instances, and the enumeration of liquefying bacteria, spore-forming aerobes, thermophilic bacteria, and hydrogen sulphide-producing bacteria of subsidiary importance. Rideal (1906) subsequently recommended a somewhat less extensive series of tests, including aerobic and anaerobic counts, both at 20° and 37°, with 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, anaerobic 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 the *C. welchii* and sewage streptococcus densities tells the observer little more than he can learn from the routine use of the coliform test. In the United States the practice of sewage bacteriologists has crystallized around the total count and the estimation of coliform organisms. In the absence of evidence regarding the specific value of other data, the routine control of purification plants may well be limited to these two determinations.

Practice in the use of the bacterial counts varies. Some bacteriologists employ the 20° count, some the 37° count, and a few use both; and occasionally a count of acid formers is obtained by means of lactose agar as a check on the coliform estimate. The ratio of the 37° to the 20° count varies with different sewages. At Boston the body temperature count has been reported as 70 to 80 per cent of the total count; at Lawrence, Gage (1906) found it to be proportionately much lower.

It should be borne in mind, as Lederer and Bachmann (1911) pointed out, that the sampling error is a very serious one with sewage. Duplicate tests made at 1-minute intervals for a period of 10 minutes in their experiments gave extreme values of 190,000 and 550,000 per ml. Samples of sewage and effluent should, of course, be examined as soon as possible after collection. If not examined immediately, they should be well iced; and no more than a few hours should intervene between sampling and examination. Separate samples, not composite samples, should be used for bacteriological work.

The determination of the number of coliform organisms should constitute an integral part of bacteriological sewage examination, for it is important to know whether the reduction in the number of intestinal bacteria is proportional to that of total bacteria. Standard

Methods permits the use of the presumptive test for estimating the coliform density of sewage and effluent, except chlorinated effluent in which a proportionately considerable number of spore-forming lactose-fermenters may be present. Either the confirmed test or the completed test is specified for the examination of chlorinated effluent.

An interesting series of results obtained from a comparison of various methods employed for estimating the number of coliforms in the raw sewage and the chlorinated-settled sewage at Buffalo has been reported by Symons and Simpson (1942). The samples tested represented the 10:00 A.M. and 1:00 P.M. sewage before and after chlorination, the chlorinated samples being treated with thiosulphate immediately and all samples being examined within 10 to 15 minutes after their collection. Difco dehydrated media were used throughout the investigation. Table 102 shows the results obtained both from confirmation of lactose broth gas-positives by transfer to brilliant-green bile 2 per cent and from plating on E.M.B. agar. Since previous work had shown that more than 99.9 per cent of lactose broth tubes inoculated with raw sewage and showing gas within 24 hours were positively confirmed by B.G.B., it was not considered necessary, in this study, to confirm such gas-positives; those showing gas in the second 24-hour period, and both the 24-hour and the 48-hour gas-positives from chlorinated sewage, however, were confirmed as indicated in the table. The results show that a large proportion

TABLE 102. COMPARISON OF B.G.B. 2 PER CENT BROTH WITH E.M.B. AGAR AS A CONFIRMATORY MEDIUM

M.P.N. Coliform Bacteria per Milliliter (39 Observations)

Sample	M.P.N. Presumptive Coliform Bacteria	M.P.N. by Confirmation		Ratio of M.P.N. by Confirmation to Positive Presumptive M.P.N.	
	Lactose Positive	B.G.B. Broth	E.M.B. Agar	B.G.B Broth	E.M.B. Agar
Raw sewage	60,900	50,100	37,500	86%	72%
Chlorinated-settled sewage	76.2	40.5	19.9	53%	26%

(86 per cent) of the lactose broth presumptives from raw sewage was positively confirmed by B.G.B., and almost as large a proportion (72 per cent) by E.M.B. plating. Only 53 per cent of the presumptives from chlorinated sewage were positively confirmed by the liquid medium, however, and only 26 per cent by plating.

A comparison was also made of the confirmed test employing B.G.B. and direct planting into the same medium, the latter procedure constituting a presumptive test with B.G.B. Evidently this method did not indicate as high coliform densities as did the confirmed test, especially when employed with chlorinated sewage. The results are

TABLE 103. COMPARISION OF DIRECT PLANTING IN B.G.B. 2 PER CENT BROTH WITH LACTOSE PRESUMPTIVE GAS FORMERS AND CONFIRMATION IN B.G.B. BROTH

M.P.N. Coliform Bacteria per Milliliter

Sample	Number of Observations	Positive Presumptive Gas Formers	Confirmed B.G.B. Broth	Direct Planting B.G.B. Broth	Ratio of M.P.N. to Presumptive M.P.N.		Ratio M.P.N. by Direct Planting to M.P.N. by Confirmation
					Confirmation	Direct Planting	
Raw sewage	621	73,000	68,500	60,100	93.8%	82.3%	88%
Chlorinated-settled sewage	478	84.0	42.3	31.8	50.4%	37.8%	75%

given in Table 103. Direct planting of samples into lauryl broth also yielded considerably lower coliform figures, as shown in Table 104.

In order to determine whether the temperature of the sewage might influence the results, a comparison of formation of gas in lactose broth,

TABLE 104. COMPARISION OF DIRECT PLANTING IN L.S.T. BROTH AND IN B.G.B. 2 PER CENT BROTH WITH CONFIRMED RESULTS

M.P.N. Coliform Bacteria per Milliliter

Sample	Number of Observations	Confirmed in B.G.B. Broth	Direct Planting	
			B.G.B. Broth	L.S.T. Broth
Raw sewage	229	38,000	38,600	29,000
Chlorinated-settled sewage	153	81.3	68.8	58.7

the confirmed test using B.G.B., and direct planting into this medium was carried through a period of nine months, July to March inclusive. Rather low proportions of lactose broth gas-positives from the chlorinated sewage were positively confirmed by B.G.B., and similarly low coliform densities were indicated by direct planting into this medium during four months, August to November, but no significant variation

occurred during the other months. The averages for the whole period were very similar to the figures shown in Table 103. Another series of samples was examined by the confirmed test, direct planting in B.G.B., and direct plating on violet-red bile agar. The results, presented in Table 105, show that the confirmed test, using the liquid

TABLE 105. COMPARISON OF DIRECT PLANTING IN VIOLET-RED BILE AGAR WITH RESULTS IN LIQUID MEDIA

M.P.N. Coliform Organisms per Milliliter

Sample	Number of Observations	Positive Lactose Gas Formers	Confirmed B.G.B. Broth	Direct Planting B.G.B. Broth	Direct Planting Violet-red Bile Agar
Raw sewage	21	93,000	76,700	63,600	8,200
Chlorinated-settled sewage	20	34.7	18.9	2.9	1.3

confirmatory medium, gave a much higher number of positives with chlorinated sewage than did the other two procedures; and the coliform density of the raw sewage indicated by direct plating on violet-red bile agar was very much lower than the densities shown by the confirmed test and the B.G.B. presumptive test.

The figures given in Tables 102 through 105 show very clearly the necessity of confirming enrichment gas-positives when examining chlorinated effluents. With raw sewage, however, the presumptive test alone usually suffices for practical work.

It will be recalled that Gehm and Heukelekian (1935) obtained results from direct plating of sewage on E.M.B. agar that compared very favorably with those secured from the B.G.B. presumptive test.

Numbers of Bacteria in Sewage. The total number of bacteria and the number of coliform organisms 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 20° counts from 1,000,000 to 5,000,000 in the sewages of Essen, Berlin, Charlottenburg, Leeds, Exeter, Chorley, and Oxford; 2,000,000 to 10,000,000 in the sewages of London, Walton, and W. Derby; and over 10,000,000 in the sewages of Paris, Ballater, and Belfast (Winslow, 1905). The number of coliforms 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 milliliter. At Columbus, Johnson (1905) reported an average of 3,600,000 bacteria per milli-

liter; the individual numbers varied from 320,000 to 27,000,000. The number of coliforms varied from 50,000 to 1,000,000 and averaged 500,000. At the Easterly plant of Cleveland, Ellms (1943) reported an average 20° count of 2,700,000, a 37° count of 1,960,000, and a presumptive coliform estimate of 293,000 per ml. 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 milliliter. 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, 11,488,000 in September, 3,693,000 in October, 587,100 in November and 712,000 in December (Winslow, 1905). In Buffalo sewage the coliform density, determined by means of the confirmed test, using B.G.B. 2 per cent, was 27,300 per ml. in December, 1940, when the temperature was 49.2° F., and 117,000 per ml. in August, when the temperature was 72.5 (Symons and Simpson, 1941). In fact, the coliform density curve closely paralleled that of the temperature throughout a period of about three years.

There is also a marked diurnal variation in the bacterial content of sewage, since the flow contains a smaller proportion of excreta at night than at other times. For example, a series of hourly samples, collected August 13–14 in 1903 at the Sewage Experiment Station of the Massachusetts Institute of Technology, showed 20° counts of bacteria varying from 400,000 per ml. between 3:30 and 7:30 A.M. to 4,600,000 between 3:30 and 7:30 P.M. Symons and Simpson (1941) found a marked diurnal variation in the coliform density of Buffalo sewage sampled at five different hours of the day; the monthly average, July–August, 1939–40, ranged from 88,000 per ml. at 8:00 A.M. to 195,800 per ml. at 1:00 P.M. In the other three seasons of the year, the 3:00 A.M. and the 8:00 A.M. samples contained about the same minimum number of coliforms, but the 1:00 P.M. coliform densities were always the highest. It is evident that many published results of bacterial examinations of sewage are in excess of the average values, because they often refer to day samples only.

When certain industrial wastes are present, abnormally low bacterial counts may be found in municipal sewage. Thus at New Haven (Winslow and Mohlman, 1918) a test carried out for one week at intervals during the daytime gave the results shown in Table 106. At 8 A.M. and on Sundays the bacterial content was reasonably normal; but during the working day the 20° count was kept down to less than 200,000 and the gas formers to less than 20,000 per ml. by copper salts introduced from a munition plant.

TABLE 106. BACTERIAL CONTENT OF NEW HAVEN SEWAGE

	Agar 20° Count per ml.				Gas Formers per ml.			
	8 A.M.	10 A.M.	1 P.M.	4 P.M.	8 A.M.	10 A.M.	1 P.M.	4 P.M.
Average, 6 week days	1,295,000	115,400	14,700	159,000	100,000	19,000	385	17,000
Sunday	.....	3,355,000	2,275,000	2,535,000	.....	100,000	100,000	100,000

Bacterial Content of Sewage Effluents. The bacterial content of sewage effluents varies widely according to the process of purification adopted and the efficiency of the particular plant. Of the ordinary treatment processes adopted, activated sludge treatment and intermittent sand filtration are the only ones which produce a notably purified effluent from a bacteriological standpoint.

The activated sludge process may be made to yield almost any bacteriological results desired by adjusting the aeration and sedimentation periods, the amount of air, and the proportion of activated sludge. This is well illustrated by the results from the Milwaukee experiments, presented in Table 107.

TABLE 107. RELATION BETWEEN AERATION TIME AND BACTERIAL PURIFICATION

(Milwaukee, 1915)

Aeration period, hours	0	1	2	3	4	5
Per cent removal of bacteria	0	52	81	92	95	98

In actual practice very high degrees of purification are not usually attained. Thus, at the permanent Milwaukee plant the monthly average 20° count varied in one period from 714,000 to 1,761,000, and the activated sludge effluent from 35,000 to 190,000.

According to Heukelekian and Rudolfs (1929), aeration, as employed in the activated sludge and trickling filter processes, performs the useful function of maintaining a numerous flora and fauna, including large numbers of ciliates, which feed on the bacteria in the sewage and reduce their densities to low levels.

Intermittent sand filters, when operated with care, may give a bacterial purification well over 99 per cent, as shown by bacteriological examinations at the Brockton (Mass.) filters, reported by Kinnicutt, Winslow, and Pratt (1910). (See Table 108.) Such high efficiencies as this table indicates are often not realized under the actual working conditions of a municipal plant. At Vineland, N. J., for example, the intermittent filters showed a reduction of 99 to 95 per cent

TABLE 108. BACTERIA IN SEWAGE AND EFFLUENTS AT BROCKTON, AVERAGE OF FOUR EXAMINATIONS, AUTUMN OF 1908

	Bacteria per ml. Gelatin 20°	Coliforms per ml. Lactose Bile
Sewage	3,150,000	150,000
Effluent A	1,900	400
" B	6,300	15
" D	125	0
" E	1,400	5
" F	2,000	1

in total bacteria and a somewhat higher reduction of coliforms. The results of three examinations made in 1906 are given in Table 109.

Because of their frequent use, it is interesting to note that small septic tanks, about 2 feet wide and 40 inches deep with a capacity of about 185 gallons, operated singly and in pairs in series, were found

TABLE 109. BACTERIA IN SEWAGE AND SAND FILTER EFFLUENT AT VINELAND, N. J.

(N. J. State Sewerage Commission, 1907)

Date	Bacteria per ml.		Coliforms in	
	Sewage	Effluent	Sewage	Effluent
March 2	480,000	20,000	0.0001 ml.	0.01 ml.
July 26	496,000	61,000	0.0001 ml.	0.001 ml.
July 26	511,000	38,000	0.00001 ml.	0.001 ml.

at the Lawrence (Mass.) Experiment Station to effect a reduction of only 20 to 42 per cent in the number of bacteria developing in 4 days at 20° C. (Wright, 1944).

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 1,000,000 to 2,000,000 bacteria in the effluents of contact beds and from 750,000 to 1,900,000 in effluents from trickling filters. The average data, shown in Table 110, for two of the earlier constructed large trickling filter plants in the United States are cited by Kinnicutt, Winslow, and Pratt (1910).

Streeter (1930) suggested the rough limiting efficiencies to be expected from various sewage purification processes presented in Table 111.

It is obvious that effluents from these processes cannot be considered satisfactory from the standpoint of bacterial purification. Houston (1904*b*) found that *Pseudomonas pyocyanea* appeared in the effluent

TABLE 110. BACTERIAL CONTENT OF SEWAGE AND EFFLUENTS FROM TRICKLING FILTERS

Place	Period	Screened Sewage	Bacteria per ml.	
			Septic Effluent	Filter Effluent
Reading, Pa.	1908–09	3,100,000	1,800,000	600,000
Columbus, Ohio	1909	2,370,000	1,050,000	560,000

of a trickling bed 10 minutes after application to the top and continued to be discharged for 10 days. In septic tanks and contact beds, the same germ persisted for 10 days. Rideal (1906) quoted experiments by Pickard at Exeter, which show that typhoid bacteria may persist for 2 weeks in a septic tank and that contact-bed treatment effects only a 90 per cent removal of these organisms. Mom and Schaeffer (1940) reported that during the passage of sewage through

TABLE 111. LIMITING EFFICIENCIES OF SEWAGE PURIFICATION PROCESSES

Process	Per Cent Reduction	Process	Per Cent Reduction
Fine screening	15–20	Activated sludge	90+
Plain sedimentation	50–60	Intermittent sand filters	98–99
Chemical precipitation	80–90		
Trickling filters	90+	Final settling tanks	50–70

an Imhoff tank installation in Netherlands India only about 80 per cent of the typhoid bacteria present settled with the sludge; and some could still be isolated from the digested sludge, although after the sludge was dried none could be recovered. Ruchhoft (1934) recovered *E. typhosa* from artificially infected activated sludge 13 to 14 days after storage at 68° to 72° F., and 83 days after storage at 50° to 60° F.

Disinfection of Sewage Effluents. When greater bacterial purity than that effected by the ordinary methods of sewage purification is required, some special process of disinfection must be combined with them. For this purpose treatment with chloride of lime or liquid chlorine is generally used; and in connection with this process bacteriological control is 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; 50 parts would reduce their number to 20 per ml. Coliform organisms were reduced from

1,000,000 per ml. to less than 1 per ml. by 30 parts of chlorine. In septic effluent 25 to 44 ppm. reduced the coliforms from 2,500,000–4,500,000 to less than 1 per ml. With contact effluents smaller amounts of chlorine proved efficient. The primary effluent required 20 ppm., the secondary effluent 10.6 ppm., and the tertiary effluent 2.5 ppm., to reduce the number of coliforms so that they could not be isolated from 5 ml.

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 Table 112 smaller amounts of chlorine than were used by Rideal give good results with more dilute American sewages.

TABLE 112. 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 ml.		Coliforms, Jackson Bile Test	
	Before	After	Before 0.000001 ml.	After 1.0 ml.
1906				
August 11	270,000	69	+ 0	+ 0
" 13	630,000	41	0 0	+ 0
" 14	135,000	406	+ +	+ 0
" 15	230,000	21	0 0	0 0
" 16	250,000	37	+ 0	0 0
" 18	110,000	40	0 0	+ 0
" 20	90,000	54	+ 0	0 0
" 21	220,000	22	0 0	0 0
" 23	.....	...	+ 0	0 0
Average	240,000	86	33%+	22%+
Average removal	99.96%		99.993%	

The success of chemical disinfection varies with the qualities of the sewage or effluent treated, since the organic matter present consumes a certain amount of the disinfectant and renders it inoperative. Early methods of operation of chlorination plants were based largely upon minimum fixed dosages of disinfectant, but Tiedeman (1927) showed the advantages of employing residual chlorine minima for this purpose. He made an intensive investigation of the operation of a sewage treatment plant at Huntington, Long Island, which consisted of shallow Imhoff tanks converted to settling tanks. The effluent

from a series of these tanks, high in suspended solids and organic matter, was treated with liquid chlorine. Tiedeman reported an extensive series of bacteriological results which demonstrated that this effluent could be effectively disinfected when chlorine sufficient to leave a residual of 0.2 ppm. was applied with a contact period of 5 minutes. Under these conditions the fine suspended solids were penetrated by the chlorine; and, furthermore, a permanent reduction in the biochemical oxygen demand of the effluent was obtained. After this experience, operation control of chlorination on a residual chlorine basis soon became generally established.

The greater the concentration of residual chlorine left in a sewage or effluent, of course, the greater is the bacterial reduction obtained. Thus Scott and Pool (1927) obtained the results shown in Table 113 upon chlorinating sewage of medium strength at Bridgeport (Conn.) after its passage through Reinsch-Wurl screens with $\frac{3}{64}$-inch slots. The contact period in this particular instance was 10 minutes.

TABLE 113. EFFECT OF RESIDUAL CHLORINE ON DISINFECTION

Residual Cl after 10 min. Contact	37° Count per ml.	Coliforms per ml.
1.0	390	3
1.0	280	0
0.6	2,400	0
0.3	150	10
0.25	2,200	100
0.2	1,320	170
0.1	5,300	375

Rudolfs, Ziemba, and Gehm (1934) reported some interesting observations on the effect of chlorine dosage upon the reduction of coliform organisms in sewage. Partially chlorinated sewages were examined after various contact periods. It was found that the chlorine consumption rate and also the coliform kill were almost instantaneous in both fresh and stale sewage. A 90 per cent coliform kill was obtained in 15 seconds or less, with thorough mixing, upon satisfying only 50 per cent of the chlorine demand; the extent of coliform reduction depended especially upon the chlorine dosage, not upon the proportion of chlorine demand satisfied, although the kill increased as more of the chlorine demand was satisfied. These observers point out that in a strong sewage, containing more substances likely to absorb chlorine, the coliform reduction after satisfaction of a given proportion of the demand is greater than in a dilute sewage; and since some of this absorbed chlorine may be slowly active for a period,

contact time is necessary if it is to be utilized. The presence in sewage of nitrogenous trade wastes may increase the chlorine demand, but in this event germicidal " chloro " products are not formed in significant amounts and no secondary reduction of coliforms is effected.

Since raw sewage contains large particles of suspended matter which cannot readily be penetrated by chlorine, some form of treatment of the sewage to remove the coarser solids usually precedes any attempt to disinfect it. Screening of sewage, although it may not take out a large proportion of the suspended matter, leaves only finer particles that may be successfully treated with moderate doses of chlorine or hypochlorite. Thus, at Daytona (Fla.), Simons (1919) reported that the application to the raw sewage of 20 pounds of chlorine per million gallons reduced the bacteria and coliforms 75 per cent and 90 per cent respectively; but after passing the sewage through $\frac{5}{64}$-inch screens, which removed only 7 per cent of the suspended solids, a dosage of only 5 pounds destroyed 80 per cent of the bacteria. The purpose of this installation was to protect oyster beds from excessive pollution by the sewage.

TABLE 114. CHLORINATION OF SEWAGE AND EFFLUENT

	Place	Year	Chlorine, ppm.		Per Cent Removal	
			Dosage	Residual	Bacteria 37° C.	Coliforms
Prechlorination on Imhoff tank	Portsmouth, O.	1932–3	10.1	0.13	99.79	99.77
Chlorination of settled sewage	Huntington, N.Y.	1926	6.5–13.0	0.2–0.6	99.9	99.9
Chlorination of trickling filter effluent	Allentown, Pa.	1932	2.8–5.23	....	99.9+	99.9+
Chlorination of activated sludge	Pasadena, Cal.	1928–9	2.5	0.22	....	30 per ml. remaining

The Committee on Sewage Disposal of the Public Health Engineering Section of the American Public Health Association (1934), in its comprehensive report on chlorination in sewage treatment, compiled numerous data relative to the results obtained from chlorination of sewage and effluent. A few of these data are shown in Table 114.

The bacterial results that may be attained under conditions where an extremely high degree of purification is required are well illustrated

by the plant at Mt. Kisco, N. Y.; the sewage from this village was treated successively by septic tanks, contact beds, sedimentation, sand filters and chlorination before it entered a tributary of the New York water supply. (See Table 115.) The final chlorinated effluent

TABLE 115. BACTERIAL PURIFICATION, MT. KISCO

(Coffin and Hale, 1916)

	Sewage	Septic Effluent	First Contact Effluent	Second Contact Effluent	Settled Effluent	Sand Effluent	Chlorinated Effluent
Bacteria per ml. (37°, agar)	1,480,000	400,000	560,000	470,000	460,000	38,000	38
Coliforms, per cent 0.001 ml. samples positive	37	22	20	16	14	0	0

showed coliforms in only 39 per cent of a series of 10-ml. samples and in only 8 per cent of a series of 1-ml. samples.

Rudolfs and Gehm (1935) found that the bacteria and coliform organisms remaining in chlorinated sewage multiplied rapidly, after a short lag period, upon incubation at 20° C. Aeration after chlorination reduced the lag period. When 100 per cent of the chlorine demand of the sewage was satisfied the increase in rate of growth was delayed, but when less was satisfied the rate increased within 6 hours after chlorination. The original numbers of bacteria (20° count), however, were never reached until after 24 hours of incubation. Although increasing the dosage of chlorine retarded the multiplication, it increased the ultimate bacterial or coliform density attained. Incidentally, protozoa appeared to be eliminated when 50 to 75 per cent of the chlorine demand was satisfied. Reduction of the fauna that feed on bacteria, and the possibility that the chlorine-resistant organisms remaining after chlorination may have high normal population levels, are suggested explanations for the marked aftergrowths observed.

Standards for Sewage Effluents. The science of sewage bacteriology is still in an unadvanced state. We know that bacterial counts will vary with the degree and type of purification but it is difficult to give any general rules for the interpretation of bacteriological exam-

inations designed to indicate whether disposal plants are operating successfully or not. Houston stated provisionally in 1902 that the 20° count should be under 100,000 and the 37° count under 10,000, and that coliforms should be absent from 0.001 ml. and *C. welchii* from 0.1 ml. This standard now seems to us far too lenient, especially in view of the great improvements which have been made in methods of treatment. It seems wisest at the present time to avoid fixing any definite 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 materially to increase the bacterial content of the body of water into which it is discharged.

Bacteriology of the Sewage Plants Themselves. 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. Notwithstanding the great advances in the development of the art of sewage disposal, knowledge of the microörganisms concerned and of the exact conditions which favor their activity is greatly needed; such knowledge is woefully deficient. In a general way we know of the nitrifying organisms long ago discovered by Winogradsky. Later work, like that of Schultz-Schultzenstein (1903), Boullanger and Massol (1903), and Calmette (1905), slightly increased our knowledge concerning these forms. In regard to the specific bacteria functioning in the septic tank and contact bed we are almost wholly in the dark. Present opinion seems to indicate that certain specific types of bacteria play a predominant part. Septic tanks work well with some sewages and badly with others; and presence or absence of the necessary bacteria is probably largely responsible for the different results. In some plants, as at Plainfield, N. J., the seeding of a tank with cesspool contents produced a material improvement in septic action. The process of inoculation or seeding is now not uncommon, e.g., in Imhoff tanks. The fact that a filter works best when "ripe" suggests the presence of specific organisms with rather definite growth and reacting conditions.

Gage (1905) made a suggestive study of the bacteria which carry on the reducing changes in sewage. His method consisted in planting sewages and effluents and 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 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 produce certain changes. The results of further studies made by Clark and Gage (1905), on sewages and on sand, contact, and trickling effluents, showed that there may be important differences between various sewages in this respect which must more or less facilitate their purification. They indicated that the effluents obtained from intermittent sand filters in cold weather contain larger numbers of ammonifying and denitrifying bacteria than appear at other seasons, a fact which may help to explain the poorly nitrified effluents obtained in the winter season.

Birge (1915) studied the effects of certain common aerobic bacteria grown alone and together in sterilized sewage and demonstrated the reducing power of *E. coli* and *A. cloacae* and the ammonifying power of *B. subtilis* (under aerobic conditions) and of *Proteus* forms (under anaerobic conditions). Buswell and Long (1923) presented interesting results in regard to the biological agents which function in activated sludge, with particular reference to the higher fungi and protozoa. Butterfield (1935) found that certain zoögleal formations are the predominant bacteria in activated sludge, and Ruchhoft, Butterfield, McNamee, and Wattie (1939) showed that these are the active agents in activated sludge: in pure culture they can produce activated sludge and can effect the oxidation and purification accomplished by natural activated sludge. In a later study of the growths found in trickling filters, Butterfield (1941) isolated similar zoögleal organisms from the surface of the stones, where they occurred in numbers of at least 300 million per milliliter of growth. Pure cultures of these bacteria produced adherent growths on filter stones, and a filter so prepared appeared to possess purifying power similar to that of normal trickling filters. Furthermore, a mixture of 9 pure cultures of these organisms (which seemed to be more effective than single pure cultures), when applied to trickling filter and activated sludge model plants, effected the same degree of purification as that produced when all fauna and flora of natural sewage were functioning. It appears, therefore, that zoögleal bacteria of this general type are the organisms chiefly responsible for the purification accomplished by biological sewage treatment plants. Research work along such lines as these promises to be highly fruitful of results.

During recent years this subject has been attacked with new vigor

in the laboratories of the Chicago Sanitary District, in the sewage substation of the New Jersey Agricultural Experiment Station at New Brunswick, N. J., and in the Stream Pollution Investigations Station of the United States Public Health Service at Cincinnati, Ohio. Since the present volume is concerned primarily with water bacteriology, these researches cannot be reviewed in detail; but the student of the bacteriology of sewage treatment will find them invaluable for reference.

CHAPTER XIII

BACTERIOLOGICAL EXAMINATION OF SHELLFISH

Shellfish and Disease. The pollution of areas devoted to the growing of shellfish and the consequent pollution of the shellfish themselves is a matter of much sanitary importance. Oysters, clams, and mussels are the shellfish commonly used as food, and, since they are likely to be eaten in an uncooked or partially cooked condition, it is important to be assured of their bacteriological characteristics. In their normal habitats, in clean sea water, or in river estuaries free from pollution, shellfish are unquestionably free from dangerous bacteria, although their feeding habits make it probable that the types of bacteria indigenous to the waters in which they are found might be present in considerable numbers. With the pollution of streams by unpurified sewage the areas in which oysters and clams develop may readily become infected by organisms of intestinal types and, as a consequence, typhoid bacteria and other pathogens may pass from the sewage into the intestinal tract of the consumer of raw oysters or clams.

The early history of this subject was well summarized by Newlands and Ham (1910), from whose excellent report the following paragraphs are adapted:

Attention was first drawn to the danger from shellfish by the remarkable outbreak of typhoid fever which occurred in Middletown (Conn.) in 1894, as a result of serving raw oysters at college fraternity banquets. The oysters used were all derived from a certain portion of Long Island Sound, where they had been put down, or planted, in order to fatten. Investigation showed that a stream entering the Sound at this point was highly polluted and, furthermore, that at a nearby house there were two severe cases of typhoid fever from which intestinal discharges were turned into a drain and thence into the stream without disinfection. The course of the passage of the bacteria from the diseased patient to the oysters, and so on to the young men at the banquets was, therefore, traced in a most complete and thorough way. The results of this investigation, which was conducted by Professor H. W. Conn of Wesleyan University, caused the immediate initiation of further studies relative to shellfish in this country.

In 1893 Thorne-Thorne, in a report to the Local Government Board, wrote that in his opinion certain cases of cholera which had occurred that year at various inland towns in England were due to contaminated oysters from beds at Grimsby, where there had been a small cholera epidemic. Following the suggestions embodied in this report the English Government began a series of investigations which made many important additions to our present knowledge of the subject.

In 1902 the famous oyster epidemics at Winchester and Southampton, England, were proved beyond reasonable doubt to have been caused by contaminated oysters taken from grounds at Emsworth. Here again we have to deal with banquets given in different cities where the only common source of infection appears to have been contaminated oysters. Of the 267 guests at these banquets 118 were attacked with intestinal disorders and 21 cases of typhoid fever developed, of which 5 were fatal.

Although a great many sensational attacks, based on insufficient or no proof, have been made against oysters as carriers of disease germs, the above-mentioned investigations and others, among which might be mentioned those of Thresh, Marvel, and Soper, brought out ample trustworthy evidence to show that contaminated oysters must be considered a real factor in the dissemination of typhoid fever and other water-borne diseases. To estimate the exact extent to which such illness is due to oysters would be impossible. The careful supervision exercised by health authorities over oyster culture during recent years has certainly greatly minimized the danger. The last major epidemic in the United States due to shellfish was in the winter of 1924–25 when some 700 cases of typhoid fever in New York and several hundred cases in Chicago, Washington, and other cities were all traced to oysters from a single lot. In most of the American outbreaks which have been clearly traced to shellfish it was demonstrated that the oysters which caused the outbreak had been "floated" or "fattened" in brackish water near the mouths of polluted streams; this practice is no longer permitted by sanitary authorities.

Valuable earlier studies of the relation between shellfish and disease were those of Bulstrode (1911), Wilhelmi (1911), and Stiles (1912). An indication of the frequency with which shellfish have been implicated in the transmission of infection is furnished by a compilation entitled "A Tabulation of Some Instances in Which the Occurrence of Disease Has Been Ascribed to Contaminated Shellfish," prepared by the Committee on Shellfish of the American Public Health Association (1937).

Effect of Cookery upon Polluted Shellfish. It should be noted that, unfortunately, not only raw shellfish are responsible for the spread of disease. Most of the processes of cookery to which these foods are subjected are insufficient to destroy pathogenic germs. Clark (1906*b*) found that clams and oysters in stews and fried and scalloped in the usual manner were generally free from coliforms and streptococci. In steamed clams, however, the bacteria present could not be destroyed except by a temperature high enough and prolonged enough to ruin the clams for eating. Rickards (1907) confirmed these indications of the danger from steamed clams, but found fried clams and clams in chowder and scalloped oysters to be practically sterilized. Oyster stew, however, is not exposed to long-continued heat as is clam chowder, and fried oysters are less thoroughly heated than fried clams in the ordinary processes in use. Oysters in both of these forms and fancy roast oysters may still contain coliforms and streptococci. Buchan (1910) reported that the ordinary methods of cooking mussels do not remove the risk of typhoid infection. It was noted by Perry (1929), moreover, that not only shellfish but also the utensils used in handling infected shellfish may be sources of contamination.

Bacteriological Examination of Shellfish. Without further discussing the general sanitary aspects of the subject it is important to consider just how one may determine whether the oysters from a given region are polluted. The methods which have been developed for this work are essentially modifications of the methods used in water examination. Although total counts of bacteria at different temperatures have sometimes been sought, special emphasis has been placed on the application of the various tests for the determination of coliform organisms. Here, as in the examination of water, these bacteria may be taken as an index of pollution, and their occurrence in considerable numbers must be looked upon as indicating that the shellfish themselves may contain bacteria of pathogenic importance, such as *E. typhosa, S. dysenteriae,* and *Salmonella* organisms. Determinations of the pollution of the water above the beds are frequently made, because these may bear indirectly and inferentially on the possibility of the pollution of the shellfish contained therein. Results of the two determinations are not always in close agreement, however, owing to the rapidly changing local conditions due to tide, etc. The general relations and the individual variations between water and shellfish determinations are well illustrated in Table 116, taken from the report by Newlands and Ham (1910) on conditions in New Haven Harbor.

Fisher and Acker (1935) reported similar discrepancies in their

TABLE 116. BACTERIA IN WATER AND SHELLFISH, NEW HAVEN HARBOR

Station	Water				Oysters		
	Samples Taken	Average Number of Bacteria per ml.		Average Number of Coliforms* per ml.	Average Number of Coliforms* per ml.	Number of Oyster Samples	Quality of Bottom
		37° C.	20° C.				
Ferry St. Bridge	12	210	1,260	43	...	..	Soft
Tomlinson Bridge	15	910	2,650	34	...	..	"
No. 1	15	510	1,680	51	...	..	"
No. 2	15	375	910	73	...	..	"
No. 3	16	255	835	9	72	16	"
Buoy 10	15	155	450	10	...	..	"
No. 4	15	160	1,720	9	...	..	"
No. 5	17	615	1,340	74	308	13	"
Buoy 5	23	315	715	15	...	..	"
Buoy 8	15	205	410	8	...	..	"
No. 6	16	145	485	8	37	6	Seaweed
No. 7	21	215	740	29	425	11	Hard
No. 9B	11	220	260	7	64	10	"
No. 9A	13	100	185	9	46	10	"
No. 9	12	195	200	17	37	10	"
No. 7A	11	120	240	10	255	11	"
No. 8	16	180	270	7	370	6	
No. 10	23	300	615	9	100	1	Soft
No. 11	11	405	510	8	10	4	"
Buoy 6	21	815	1,690	9	...	..	
Buoy 3	17	175	590	6	291	8	Hard
No. 12	14	620	1,190	4	6	5	"
No. 13	7	240	120	10	10	8	"
No. 14	12	285	1,100	1–†	7	8	"
Buoy 4	7	375	1,400	4			
No. 15	7	455	1,680	1	45	15	Hard
No. 16	14	280	1,025	1–	1–	3	Soft
No. 17	14	300	1,260	1–	...	..	Hard
No. 19	1	800	300				
No. 20	10	135	860	1–	10	8	Hard
Buoy 2	11	375	905	2			
No. 22	8	305	560	1–	7.3	3	Hard
Buoy 1	6	115	995	1–	4	12	"
No. 18	10	255	675	1–			
No. 24	4	710	1,340	4	9	10	Hard
No. 23	6	450	240	1–			"
No. 25	2	130	1,000	1–			
No. 26	5	130	465	1–			
No. 27	10	630	695	1–	...	..	Soft
No. 28	4	415	1,400	1–	4	3	Mud and sand
No. 29	5	370	1,700	1–	...	..	Soft
No. 30	5	185	440	1–	1–	15	Hard
No. 31	7	320	130	1–	1–	15	"
No. 32	5	70	1,050	1–	3	15	"
No. 33	4	485	405	1–	1–	10	"
No. 34	4	535	495	1–	1	10	"
No. 35	4	120	270	1–	1	15	Sticky

* Jackson's lactose bile presumptive test used.

† Minus sign after figure 1 indicates that the average was less than 1.

results of the bacteriological examination of oysters and overlying waters in Narragansett Bay (R.I.). As a rule the oyster coliform "score" (see below) increased with the water score, but a high oyster score was often observed when the water score was low. The median scores of the water and oyster samples for the six-month season were 5 and 3 respectively. Temperature appeared to influence the relationship of the two series of samples in that the oyster scores were usually less than the water scores when the temperature was below 2° C.

It should be noted that the feeding habits of the oyster may explain many of the variations in numbers of bacteria in oysters from the same source.

Standard methods for the bacteriological examination of shellfish, as first developed, were based principally upon the results of studies and investigations conducted at the Lawrence (Mass.) Experiment Station by Gage, at the Sanitary Research Laboratory of the Massachusetts Institute of Technology by Phelps, at Brown University by Gorham, and in New York by Pease. Recognizing the importance of the problems involved, the Laboratory Section of the American Public Health Association appointed a Committee on Standard Methods for the Examination of Shellfish which made its first report in 1910. The Standard Methods prepared by the Committee and published in 1922 were, with a few changes later reported by the Referee on Oyster Analysis (1933) for the Committee on Standard Methods, in very general use in America throughout the period 1922–43. Briefly, these methods (as revised in 1933) specified the examination of five 1-ml., five 0.1-ml., and five 0.01-ml. quantities of the mixed shell liquor of 12 oysters, or of a mixture of equal parts of shucked oyster meats and sterile 2 per cent salt solution, employing lactose broth enrichment and the presumptive, confirmed, and completed tests for coliforms as required. Dilutions were prepared with sterile sea water or 2 per cent salt solution. Contrary to previous reports, no bacterial counts were recommended. The dilution-reciprocal method was specified for determining the numerical values indicated by the results, and these values were summed to give a "score." Anomalous results were balanced in the usual manner as shown in Table 117.

Throughout the years 1922–43 the above methods of examination were almost universally employed in America for the control of shellfish and overlying waters, and of the operation of cleansing and conditioning plants. A vast amount of bacteriological work was accomplished during this period by health departments and shellfish

commissions of the different states, the U. S. Public Health Service, and the shellfish industry. Criticism of these methods, however, finally led to a very thorough revision by the Committee on Standard Methods (1943), which promises to meet more satisfactorily than did the 1922 Standard Methods the demands of both the control agencies and the industry itself. Since very few data based on this revision have yet accumulated, we shall defer for the moment a review of the new methods in order to mention briefly some of the results obtained through the application of the older procedures.

TABLE 117. RESULTS OF COLIFORM TESTS IN DILUTIONS INDICATED

Tubes	1.0 ml.	0.1 ml.	0.01 ml.	Value
1	*	*	0	10
2	*	*	0	10
3	*	*	0	10
4	*	0	0	10
5	*	*	*	10
				Score: 50

Tubes	1.0 ml.	0.1 ml.	0.01 ml.	Value
1	*	*	0	10
2	*	*	0	10
3	*	*	*	100
4	*	*	*	10
5	*	0	0	10
				Score: 140

* Positive, confirmed coliforms in fermentation tube. 0 = Negative, coliforms absent.

Tonney and White (1926) determined the coliform scores on successive days in four series of samples of fresh unchlorinated oysters kept at 5.8° C. The percentage increase in the coliform score of one lot at 5.8° C. was 458 in 12 days and, of another lot, 1,490 in 11 days. Shucked oysters gathered in the early part of the season and stored at 5.8° C. increased more rapidly in coliform content than those gathered in midwinter and stored under similar conditions. Living shell oysters did not increase in coliform content under dry storage conditions at 5.8° C. from the eleventh to the eighty-third day after shipment. After the twenty-eighth day a consistent decrease was noted and after the sixtieth day the coliform tests were usually negative. A study of 856 routine samples of shucked oysters received in the Chicago market during the season of 1924–25 showed that 71 per cent would have passed a coliform standard score of 50, and all (100 per cent) of 164 samples of shell clams would have passed the

same score. Tentative maximum coliform scores of 140 for shucked oysters and 50 for shell oysters and hard-shell clams were recommended and later adopted as official standards for the Illinois market.

Seasonal Variation of Bacteria in Oysters. It was observed by Gorham (1912) and others that the examination of oysters from certain regions made in the summer failed to agree with similar analyses from the same beds made in the winter. With the advent of cold weather there seems to be a great improvement in the sanitary quality, so that oysters taken from beds in close proximity to the outfalls of large sewers show in the colder months entire absence of any evidence of contamination (if judged solely by the bacteriological data). Thus, Gorham found in the summer of 1910 that all oysters on the beds in the Providence and Warren rivers and the upper part of Narragansett Bay were so badly polluted by sewage as to be unfit for food. Coliform organisms were detected in the "shell water" of every oyster in amounts as small as 0.01 of a milliliter or less. Chemical and bacteriological examination of the waters over these beds showed them to be heavily polluted with sewage. In December of the same year the analyses of the oysters were strikingly different, although the condition of the water was apparently unchanged. In the examination five oysters were selected for each test, the average total number of bacteria per milliliter was determined and the presence of coliforms was detected by bile enrichment and subsequent isolation and identification of the organisms. Table 118 shows the numbers of bacteria found, and the proportion of the five oyster samples in which coliforms were present in milliliter amounts and also in 0.1 and 0.01 ml.

The conclusions arrived at by Gorham were that during the cold weather the oysters assume a condition of rest or hibernation, during which time ciliary movement ceases and the process of feeding is suspended. No organisms are therefore taken in from the outside water and those inside the oyster are gradually eliminated, so that the total number of organisms is reduced very considerably and the oyster becomes practically free from coliform organisms.

Regarding shellfish-producing waters, Miller (1936) indicated, in the extracts quoted below, the general policy adopted by administrative agencies for their sanitary control.

> In recent years there has been considerable discussion among those engaged in sanitation activities related to shellfish as to what shall constitute a clean area for growing purposes. The Report of the Committee on the Sanitary Control of Shellfish in the United States in its supplementary report of 1927 treats this subject as follows:

TABLE 118. SEASONAL VARIATION IN THE BACTERIAL CONTENT OF OYSTERS
(Gorham, 1912)

Date	Average Total Bacterial Count of Shell Water of 5 Oysters	Proportion of 5 Oysters Showing Coliforms in			Score	Coliforms Present in Water in	Temperature of Water ° C.
		1 ml.	0.1 ml.	0.01 ml.			
BED NO. 8, PROVIDENCE RIVER							
Dec. 20, 1910	1,000	3	1	0	4	0.01 ml.	−1°
Jan. 14, 1911	750	5	3	1	41		
Jan. 25	80	4	3	0	23	0.01 ml.	1°
Jan. 27	23	5	3	0	32		
Feb. 10	130	2	2	0	4	1.0 ml.	0.1°
Feb. 28	140	0	0	0	0	0.0001 ml.	1°
March 11	200	5	4	0	41	0.01 ml.	1.75°
April 14	275	5	2	0	23	0.01 ml.	8.5°
April 28	700	5	5	4	410	0.0001 ml.	12.5°
May 12	1,700	5	5	5	500	0.0001 ml.	15°
BED NO. 44, PROVIDENCE RIVER							
Jan. 7, 1911	425	5	5	1	140	.....	0.25°
Feb. 10	250	4	0	0	4	.....	0°
Feb. 28	240	5	1	0	14	.....	0.5°
March 11	100	5	2	0	23	.....	2°
April 14	210	2	0	0	2	.....	8.5°
April 28	1,000	5	5	4	410	.....	11.75°
May 12	1,100	5	5	4	410	.....	14.75°
BED NO. 204, WARREN RIVER							
Jan. 25, 1911	600	5	4	1	50	.....	0°
Feb. 10	140	0	0	0	0	1.0 ml.	0°
Feb. 28	400	0	0	0	0	0.01 ml.	0.75°
March 4	750	3*	3*	0*	*	.....	0.75°
March 11	60	1	0	0	1	0.01 ml.	3°
March 14	3,400	0	0	0	0	0.01 ml.	8.75°
April 28	1,050	5	5	4	410	0.01 ml.	13°
BED NO. 205, WARREN RIVER							
Dec. 22, 1910	250	3	0	0	3	.....	−1°
Feb. 10, 1911	325	0	0	0	0	1.0 ml.	0°
Feb. 28	450	4	2	0	14	0.01 ml.	1°
March 4	600	2	2	1	5	.....	0.75°
March 11	85	2	1	0	3	0.01 ml.	2°
April 14	325	1	1	0	2	0.01 ml.	8.25°
April 28	4,000	5	5	5	500	0.01 ml.	11.5°

* Only 3 oysters used.

As to what constitutes satisfactory evidence that an area is fully protected against contamination with disease-producing microörganisms, judgment in any given case should be based upon all the facts available, considering different observations not separately but in their relation to each other. Thus, the correct interpretation of bacteriological examinations depends to a considerable extent upon what is shown by sanitary inspection and vice versa. Hence it would be unwise to attempt to set up any specific and inflexible standards of acceptability in terms of the findings on sanitary survey or bacteriological examination or both. It is our opinion, however, that on the basis of such examinations as have been outlined, areas may be classified generally as follows with respect to their fitness for the taking of shellfish for market:

(1) Areas from which the taking of shellfish should not be permitted.

(2) Areas which may be approved for the taking of shellfish without serious question. This class includes the areas which are so protected against human fecal contamination by distance from sources of such pollution, by dilution, and by the time afforded for natural purification, that there is no discoverable likelihood of dangerous contamination.

(3) Areas which are intermediate between the first and second class as regards exposure to and protection against fecal pollution.

The Public Health Service after having done considerable work of this character has arrived at the conclusion that generally not more than 50 per cent of the 1 cc. tubes in an area should show the presence of *B. coli* if that area is to be used for the taking of shellfish for the market. This is not an inflexible figure and can be used only as a guide when weighing all the evidence collected. This figure is in general use today for our field work and was applied in this specific survey.

Cleansing of Shellfish. A shellfish, in the course of its breathing and feeding, filters daily a surprisingly large volume of water. Bacteria that may be present in this water are either ejected at once or are caught, with the other microscopical particles which constitute the food of the shellfish, and passed along to the mouth. Those that survive passage through the stomach and gut are discharged about 5 hours after their entrance. Because of the rapidity with which these processes of filtration, digestion, and elimination are performed, cleansing of contaminated shellfish by placing them in clean water or even in chlorinated water is not only feasible but is practiced commercially on a large scale. In the control of such cleansing, bacteriological examinations are of the greatest importance.

Phelps (1911) showed that oysters which develop in sewage-polluted waters may be entirely freed from coliform organisms by removal to purer waters, and that after a time they will have so cleansed themselves that they may be considered safe even for con-

sumption in the raw state. The length of time necessary for this self-purification to take place is obviously of considerable importance. From the commercial standpoint it is desirable to make it as short as possible, but from the sanitary standpoint it must be long enough to ensure a thorough and satisfactory removal of all traces of polluted matter. Oyster beds which are free from pollution or which are sufficiently good for relaying polluted oysters are difficult to find and limited in area because of their proximity to sources of pollution. The investigations in question were conducted by Phelps in the Providence River and the upper part of Narragansett Bay. The oysters were removed from heavily polluted regions and carried to waters which were practically free from pollution, where they were planted. Examinations were made from day to day in order to determine the length of time that these particular oysters showed pollution and it was found that within 4 days the organisms of the coliform type were practically all eliminated.

The purification of oysters was extensively developed on a practical scale by Wells (1916), the essential principle of the process being the storage of the oysters in a special basin containing chlorinated water. A later contribution by the same author (Wells, 1923) described the first purification plant of this type to be formally recognized by a certificate from the Conservation Commission of the State of New York. It included two concrete basins with a capacity of 6,000 gallons each and each capable of handling 200 bushels of oysters. The chlorine for disinfecting was obtained by electrolysis of sea water. The oysters were exposed for two successive periods of, respectively, 6 and 12 hours to the action of the chlorinated water, with the result that initial scores of 50 to 500 were reduced by the first " drinking interval " to 5 to 50 and by the second treatment to scores of 0 to 5.

Krumwiede, Park, *et al.* (1926) showed that in oysters artificially contaminated with large numbers of *E. typhosa* and stored at refrigerator temperature, these organisms survived for a period of 14 to 40 days in small numbers, whereas oysters subjected to repeated changes of sea water showed less than 1 per cent remaining in or on the oysters after the third change.

Wells stated, in 1929, that a commercial plant for the cleansing of mussels had been functioning successfully in England since 1916, that seven plants for cleansing or for sanitary conditioning of oysters, treating about 250,000 bushels a year, were constructed in New York State during the period 1922–29, and that a plant for cleansing soft clams was being built as a result of studies by the Massachusetts State Department of Health. Table 119 shows a summary of the

scores obtained during a marketing season at the largest of the New York plants. This installation, which used chlorine, had a capacity for treating over 1,000 bushels of oysters per day, and the over-all reduction effected in the median score after 24 hours of treatment was 93 per cent.

TABLE 119. RELATION BETWEEN OYSTER SCORES AND TIME TREATED
September, 1926, to May, 1927

(From Wells, 1929)

Hours Treated	Number of Samples	Per Cent of Scores Above		
		0.5	5	50
0	68	89.7	63.3	42.7
0 to 6	123	76.5	44.7	17.9
6 to 12	97	69.1	29.9	6.2
12 to 24	96	59.4	17.7	2.1
24 to 120	96	56.2	19.8	1.0

Some results reported by E. Wright to the Shellfish Committee (1940) of the Engineering Section of the American Public Health Association on the operation of two Massachusetts clam treatment plants are shown in Table 120.

TABLE 120. MEDIAN SCORES OF RAW AND TREATED SHELLFISH, 1938

Month	Raw		Treated		Average Treatment Period (Hours)	
	Newbury-port	Scituate	Newbury-port	Scituate	Newbury-port	Scituate
January	50	41	23	4	29	25
February	50	32	18	3	34	27
March	50	32	23	3	34	25
April	140	32	23	3	39	26
May	50	23	14	3	37	24
June	50	23	14	3	30	24
July	50	23	14	3	32	24
August	50	..	14	..	30	..
September	41	23	14	3	27	26
October	41	41	14	3	30	27
November	41	32	14	3	31	27
December	32	50	14	4	32	26
Yearly median	50	32	14*	3†	..	..
Average (hours)					32	26

* Maximum for year, 50. † Maximum for year, 32.

Criticisms of the 1922 Shellfish Methods. It was stated in a previous section that the 1922 Standard Methods, although very generally used for a number of years, were not exempt from criticism. One feature of these methods which was frequently attacked was the limitation of the examination to the shell liquor. Clark (1906*b*) had reported the results from clams (shown in Table 121) which indicated that there was considerable danger from overgrowth by streptococci when the body of the clam was planted in glucose broth, the enrichment medium employed at that time. The Referee's report of 1933 stated that "practically nothing is gained by attempting to get at the stomach and intestinal contents in addition to the shell liquor." Because, however, of the uneven distribution of both *E. coli* and other coliforms in the shell liquor, body, and intestine of oysters, the Committee on Standard Methods (1943) recommended, as we shall see in the next section, the use of the entire body for the examination.

TABLE 121. *E. coli* AND STREPTOCOCCI IN DIFFERENT PORTIONS OF THIRTY CLAMS

	Per Cent of Samples Showing			
	Fermentation in Glucose Broth	*E. coli*	Streptococci	*E. coli* and Streptococci
Shell water	90	83	47	40
Gills	77	53	25	15
Stomach (intestine)	55	35	22	12
Rectum (intestine)	82	45	43	13
Liver	37	18	15	3
Visceral tissue	18	8	7	2

Further representative criticism of the 1922 Standard Methods was expressed by Wachter (1925) and Perry (1928). Perry (1929), using the Standard Methods technique, found coliform scores in oysters higher than would be expected from a consideration of their habitat. He explained this by the fact that many of the coliforms in oysters are non-fecal, and suggested that a test for *E. coli* be substituted for the coliform test in the examination of these shellfish. Such a routine test would include the use of the Eijkman method with incubation at 46° C., plating after 18 and 48 hours on E.M.B. agar, and transferring from suspected colonies to cellobiose, citrate, indole, and lactose broths with incubation for 24 hours at 37° C. Cellobiose-negative, citrate-negative, indole-positive and lactose-positive coliforms would be considered *E. coli*. Further arguments and data sup-

porting the use of *E. coli* as the indicator for pollution of oysters and oyster waters were presented by Perry and Bayliss (1936). They maintained that a close correlation was found between the concentration of *E. coli* in oysters, as well as in overlying Maryland waters, and the extent to which the latter were known to be polluted. Although a fair correlation was observed between the coliform estimate and pollution of the waters, no such correspondence was apparent between the coliform concentration in the oysters and the amount of pollution. During the summer months the coliforms increased extensively in both water and oysters; and " enormous " increases were observed in oysters when the water was warm, whether the areas were grossly polluted or not. In this work the modified Eijkman medium of Perry and Hajna (1933) was employed, because this medium was found to yield greater numbers of *E. coli* than did lactose broth with complete confirmation. In another report by Perry and Hajna (1935), the same medium was recommended for the examination of crabmeat as well as of oysters, shucked oysters, and waters.

Dodgson (1937) and Payne (1938), however, both encountered difficulties when attempting to estimate *E. coli* densities by means of the Eijkman test at 46° C. Dodgson concluded that this temperature is too high for culture of *E. coli;* and Payne, working with oysters, clams, and shellfish-growing waters, reported that a larger number of coliforms and a larger number of *E. coli* were indicated by the use of lactose broth with complete confirmation than by the use of the modified Eijkman medium. The latter medium failed to eliminate many of the coliforms other than *E. coli,* and inhibited the growth of some strains of this organism. Minkevich *et al.* (1936) considered 46° C. the maximum temperature for *E. coli,* and found that heavy inoculations were necessary to ensure growth.

More recently Hajna and Perry (1943) suggested the use of their " EC medium " (modified Eijkman medium with 0.15 per cent Bacto Bile Salts No. 3 and with tryptose peptone substituted for Bacto peptone) at a temperature of 45.5° C. for the estimation of *E. coli;* and Perry and Hajna (1944) have reported further experience of this medium which indicates that it may prove a rival of their earlier modified Eijkman medium. The recently recommended Standard Methods, however, propose the use of the latter with incubation at 45.5° $\pm$ 0.2° C. as an alternative to isolation and identification of *E. coli.*

The use of the " score " to express the coliform results of examination of shellfish and overlying waters has also been frequently

attacked as an arbitrary form of expression, misleading, and meaningless except to the well-initiated. The new methods require the coliform or *E. coli* results to be expressed in the more logical form of most probable numbers per 100 ml. of sample.

Another suggestion advanced by Perry (1928) for the examination of shellfish was a return to the use of the bacterial count as a measure of the cleanliness with which shucked oysters, especially, are handled. It has been realized for some time that a bacterial index of general sanitation (which includes, of course, refrigeration when necessary) is needed, and the revised Standard Methods specify the use of the 37° count on tryptone glucose extract agar, with *48 hours* of incubation, for this purpose.

The Revised Standard Methods for the Examination of Shellfish and Shellfish Waters. This revision of the shellfish methods, prepared by the Standard Methods Committee for the Examination of Shellfish and reported by the Committee on Standard Methods (1943) of the American Public Health Association, can be only briefly outlined here. The complete report, published separately by the Association, should be consulted for details. The following statement from the report indicates the principal departures from the 1922 Standard Methods.

> Since the publication of the first standard method, dissatisfaction with its inadequacies in the practical sanitary control of the shellfish industry has been growing. It has become apparent from biological studies of shellfish and the shellfish-growing waters that special problems are involved. Under certain conditions it is desirable to make not only a quantitative estimate of the coliform bacteria but of the relative proportion of *Escherichia coli* present.
>
> The examination of large numbers of water and shellfish samples makes it impractical to determine the presence of coliform bacteria as required in the latest edition of Standard Methods of Water Analysis. More rapid and economical procedures are desirable and, since there is no direct plating medium of practical value in estimating the numbers of coliform bacteria, the committee has recommended the estimation of the number of these organisms in the product from the results obtained by inoculating several tubes of medium with varying dilutions of the product on test. The Most Probable Number (M.P.N.) is considered mathematically more accurate and less likely to give erroneous interpretations than the "Score," and therefore tables for determining the M.P.N. have been included as the best method of expressing the results.
>
> Two rapid methods of confirming the presence of *Escherichia coli* are recommended. The first requires plating out on eosin-methylene-blue agar (E.M.B.) and confirming on Koser's citrate medium. The second

provides a transfer from the original positive lactose broth tubes to modified Eijkman lactose medium. Dodgson in England and Perry in this country have found the latter test of particular value in the practical control of the shellfish industry. Either method is sufficiently accurate to warrant practical use.

Both *Escherichia coli* and other coliform bacteria have been found unevenly distributed in the shell liquor, body and intestine of oysters. It has seemed advisable, therefore, to use the entire oyster. A new method of cleansing and disinfecting the shells has also been introduced. Of particular importance is the examination of both shellfish and their growing waters in a comparable manner. Other changes will be noted.

Shellfish Defined — The term " shellfish " as here used refers to oysters, soft-shell clams, quahaugs, and mussels.

Coliform Group — For the purpose of examination of shellfish and shellfish-growing waters, the *coliform group* shall be considered as including all organisms which, upon transfer from a positive presumptive test (gas positive in lactose broth), show fermentation with gas formation in lactose medium containing 0.00133 per cent of brilliant green and 2.0 per cent of bile (brilliant green lactose bile broth).

For the purpose of this report *Escherichia coli* shall be considered to be any member of this group which in pure culture fails to grow in Koser's citrate medium within 24 hours, or which shows definite gas formation in modified Eijkman medium incubated at 45.5° C.

Procedure for Shellfish-growing Waters. " Samples of water from shellfish-growing areas should be collected at various stages of the tide and should also be collected at different depths if there is any indication of variations in salinity or pollution due to stratification. The samples should be collected in sterile bottles and should be fully protected against contamination both during sampling and after collection. They should be kept at a temperature at or below 10° C. (50° F.) and should be examined as soon as possible after collection. Every sample should *preferably* be tested within 12 hours from the time of collection and in no case should samples which have been held for more than 30 hours be tested."

Not less than 3 tubes of lactose broth are inoculated with each of three dilutions (usually 10 ml., 1 ml., and 0.1 ml.) of the water sample and incubated for 48 hours at 37° C. Gas-positives are confirmed by transfer to brilliant-green bile (2 per cent), as in the confirmed test of the Standard Methods for water analysis. The production of any gas in B.G.B. within 48 hours at 37° C. constitutes a positive test for the presence of coliform organisms. Absence of gas in either the lactose broth primary or in the confirmatory B.G.B. after 48 hours of incubation constitutes a negative test for the presence of coliforms.

Three methods are given for the estimation of *E. coli.*

(*a*) The usual confirmed test with transfer to eosin methylene blue plates, and inoculation from several colonies developing on each plate to Koser's citrate. Absence of growth in this medium after 24 hours' incubation at 37° C. constitutes a positive test for *E. coli.*

(*b*) Transfer from gas-positive lactose broth tubes to modified Eijkman medium with incubation in a water-jacketed incubator at 45.5° ± 0.2° C. The production of any gas in the latter medium within 48 hours is considered a positive test for *E. coli.*

(*c*) The usual completed test, with identification of *E. coli* by the use of the citrate, methyl-red, and Voges-Proskauer tests as indicated in Standard Methods of Water Analysis.

Procedure for Shellfish. Ten or more representative oysters, clams, or mussels of the average size of the lot, with deep bowls and shells tightly closed, are selected. If shucked shellfish are to be sampled, not less than one-half pint is collected. Samples must be handled aseptically and kept at or below 10° C. until examined, but the shellfish should not be permitted to come in direct contact with ice. Shell oysters should preferably be examined within 12 hours of collection and no samples held for more than 30 hours may be tested. Shucked oysters should, if possible, be examined within 4 hours after collection.

Detailed directions for opening aseptically the shells of unopened shellfish are given in the report. After the shells are opened the shell liquor is drained off into a heavy sterile bottle, graduated at 200 and 400 ml., and the body of the shellfish is very carefully removed, cut into about 10 pieces with sterile scissors or knife, and added to the shell liquor. Not less than 200 ml. of the mixture and not less than 6 shellfish may constitute a sample. After the addition of a tablespoonful of 5-mm. imperforate glass beads, the bottle is shaken 50 times within 30 seconds, each shake having an excursion of about 1 foot. To 200 ml. of the shaken mixture 200 ml. of sterile distilled water is added and the whole is well mixed by shaking. The sample is then permitted to settle for 2 minutes.

A sample of shucked shellfish is prepared for examination in an analogous manner, and 200 ml. of the mixed shellfish and shell liquor is added to 200 ml. of sterile distilled water after the bodies of the shellfish have been cut into pieces as described in the preceding paragraph.

Five tubes of lactose broth are each inoculated with 2 ml. of the water and composite shellfish mixture, five tubes each with 1 ml. of a dilution made by adding 2 ml. of the latter mixture to 8 ml. of

sterile distilled water, and five tubes each with 1 ml. of another dilution made by adding 1 ml. of the preceding dilution to 9 ml. of sterile distilled water. By proceeding in this manner, 5 tubes will each contain 1 ml. of the shellfish and liquor composite sample, 5 tubes will contain 0.1 ml., and 5 tubes will contain 0.01 ml. The lactose broth tubes are then incubated for 48 hours at 37° C. and gas-positives are confirmed for evidence of the presence of coliforms or *E. coli* as described in the previous section. It is considered advisable, according to the report, to complete all tests for *E. coli* according to the differential procedure recommended in the Standard Methods of Water Analysis, unless the shellfish samples are known to have been freshly collected from growing areas, for such *E. coli* results " may become the basis of legal action and shortcuts are not recommended." Coliform and *E. coli* results are to be expressed as most probable numbers per 100 ml.

A standard colony count, made by plating various amounts of the prepared sample of shellfish into tryptone glucose extract agar and incubating at 37° C. for 48 hours, is required, to serve as an index of general sanitation and refrigeration. The procedure to be employed is that specified for counts of bacteria in milk by *Standard Methods for the Examination of Dairy Products*, published by the American Public Health Association, except that the agar employed does *not* contain added milk. The procedure is, in fact, essentially that which we have described in Chapter V for obtaining 37° counts of water except that the incubation period is extended to 48 hours and the use of only tryptone glucose extract agar is permitted.

The report also requires the preparation of field records of environmental conditions at the time samples of overlying water or shellfish are collected. Forms for recording field data, and methods for preparing recommended culture media are included in an appendix to the report.

APPENDIX

TABLES FOR OBTAINING THE MOST PROBABLE NUMBER FROM DILUTION DATA

(Dilutions in Geometric Progression, Ratio of 10)

The Significant Part of the Result. A general observation that applies to any result secured from the use of more than 3 dilutions is that it may be put in the form of a 3-dilution result by selecting the three dilutions which are significant. In this way the M.P.N. from the results of several dilutions may be obtained, with practically no loss of accuracy, from a 3-dilution table. To select these three significant dilutions, take the highest which has all the tubes positive but below which no dilution has any negative tubes, and the next two higher dilutions. The following examples illustrate this procedure. Each dilution result is given in the form of a fraction, the denominator indicating the number of tubes employed, and the numerator the number of positive tubes. The three significant dilution results are underlined.

	10 ml.	1 ml.	0.1 ml.	0.01 ml.	0.001 ml.
(*a*)	5/5	<u>5/5</u>	<u>3/5</u>	<u>0/5</u>	0/5
(*b*)	<u>5/5</u>	<u>0/5</u>	<u>1/5</u>	0/5	
(*c*)	<u>5/5</u>	<u>4/5</u>	<u>5/5</u>	0/5	
(*d*)	<u>5/5</u>	<u>0/5</u>	<u>1/5</u>	1/5	
(*e*)	<u>5/5</u>	<u>0/5</u>	<u>2/5</u>	0/5	

When one or more positives occur with a dilution higher than the 3 significant dilutions, as in example (*d*), they should be thrown back into the highest significant dilution, making the result read as in example (*e*). Or, the M.P.N. may be calculated from the table of factors which provides for data from four dilutions.

Another important feature of the M.P.N. is that the results obtained from dilutions higher or lower than those given in M.P.N. tables may be thrown backward or forward and the M.P.N. corresponding to this new result multiplied or divided by 10, 100, etc., according to the number of dilutions the result has been shifted. Thus, a result such as that given in example (*f*) below may be thrown backward 1 dilution to make it read as in example (*g*); the corresponding M.P.N., taken from the Table of Most Probable Numbers on page 296, is 14; since the result was shifted back-

ward only 1 dilution, this M.P.N. must be multiplied by 10; consequently the M.P.N. corresponding to (*f*) is 140.

	100 ml.	10 ml.	1 ml.	0.1 ml.	0.01 ml.	M.P.N. per 100 ml.
(*f*)			2/5	3/5	1/5	
(*g*)		2/5	3/5	1/5		14
(*h*)	3/5	1/5	2/5			
(*j*)		3/5	1/5	2/5		17

Similarly, the result in example (*h*) is shifted forward 1 dilution to give the result in example (*j*), the M.P.N. for which, taken from the table, is 17. Since the result was shifted forward 1 dilution, it is divided by 10; and the M.P.N. corresponding to (*h*) is therefore 1.7. This procedure can be applied to any number of dilutions or tubes.

Use of the Table of Factors for any Result from Dilutions in Geometric Series (Ratio of 10). This table, which assumes sampling from any original volume of 1,000 liters in order to facilitate calculation, is based on the general formula

$$10\,(p+q) + 1(r+s) + \cdots = p\frac{10}{1-.99999^{10{,}000x}} + r\frac{1}{1-.999999^{10{,}000x}} + \cdots$$

where $(p+q)$ = number of tubes inoculated with 10 ml. of sample
$(r+s)$ = number of tubes inoculated with 1 ml. of sample
p = number of tubes positive with 10 ml. of sample
r = number of tubes positive with 1 ml. of sample

as determined either by appearance of gas alone or by more rigid definition depending on isolation of some particular type of organism.

In the table, under each value of x (coliforms per 100 ml. of sample) are given the values of $p\frac{10}{1-.99999^{10{,}000x}}$ and like terms, when p, r, etc. (the numbers of positive tubes), are 1, 2, 3, 4, or 5. Therefore to solve the equation:

1. Calculate the left side of the equation by adding the products obtained by multiplying the number of tubes inoculated each with any portion of the sample, by the number of milliliters in that portion. Thus, if 5 tubes are inoculated with 10 ml., and 3 tubes with 1 ml., the left side of the equation becomes 53.

$$\begin{aligned} 5 \times 10 &= 50 \\ 3 \times\ 1 &= \underline{\ 3} \\ &\quad\ 53 \end{aligned}$$

2. Calculate the right side of the equation by assuming a value of x (number of coliforms per 100 ml.) which may correspond to the result in question;

TABLE OF MOST PROBABLE NUMBERS

(After Hoskins, 1934)

Most probable numbers per 100 ml. of sample, planting 5 portions in each of 3 dilutions in geometric series

Positives with 10 ml.	1 ml.	0.1 ml.	M.P.N.	Positives with 10 ml.	1 ml.	0.1 ml.	M.P.N.	Positives with 10 ml.	1 ml.	0.1 ml.	M.P.N.	Positives with 10 ml.	1 ml.	0.1 ml.	M.P.N.	Positives with 10 ml.	1 ml.	0.1 ml.	M.P.N.
0	0	0	...	1	0	0	2.0	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4.0	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6.0	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8.0	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9.0	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4.0	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1,600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81				

then find under this value of x the factors corresponding to the number of positives and the quantity of sample inoculated into each tube, for each dilution, and add them.

3. This sum is compared with the other (53 in the example above), and that value of x which gives a sum nearest to this other sum is the number of coliforms per 100 ml. of sample.

Examples

Given the result

1 ml.	0.1 ml.
2/5	1/3

Left Side	*Right Side*	
$5 \times 1 = 5.0$	Try 70. In table, under 70, find	
$3 \times .1 = 0.3$	1 ml., 2 positives	3.973
5.3	0.1 ml., 1 positive.....	1.479
		5.452
	Try 80. In table, under 80, find	
	1 ml., 2 positives	3.632
	0.1 ml., 1 positive.....	1.301
		4.933

But 5.452 is nearer the left-hand value of 5.3 than is 4.933. Therefore the probable number is 70 coliforms per 100 ml. of sample.

Given the result

10 ml.	1 ml.	0.1 ml.	0.01 ml.
3/3	3/3	0/3	0/3

$3 \times 10 = 30.0$	Try 200:	
$3 \times 1 = 3.0$	10 ml., 3+	30.0
$3 \times .1 = 0.3$	1 ml., 3+	3.47
$3 \times .01 = 0.03$	0.1 ml., 0+	0
33.33	0.01 ml., 0+	0
		33.47
	Try 300:	
	10 ml., 3+	30.000
	1 ml., 3+	3.157
		33.157

But 33.47 is the nearer to the left-hand value of 33.33 so the above result means 200 coliforms per 100 ml. of sample. (By interpolation, since 33.33 is about midway between the two other values, the probable number of organisms is more nearly 250 per 100 ml.).

Here again, as when using the other table, if the result to be interpreted includes dilutions outside the range of the table, the result should be thrown backward or forward to bring it within the range of the table, and the corre-

TABLE OF FACTORS FOR CALCULATION OF MOST PROBABLE NUMBERS PER 100 ML. OF SAMPLE FROM RESULTS OBTAINED FROM DILUTIONS IN GEOMETRIC PROGRESSION (RATIO OF 10) (McCrady, 1918)

Dilution	Number of Positives	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10 ml.	1	105.08	55.167	38.583	30.332	25.415	22.164	19.864	18.160	16.851	15.820	14.990	14.311	13.746	13.273	12.872
	2	210.17	110.33	77.166	60.665	50.830	44.327	39.729	36.319	33.702	31.640	29.979	28.621	27.493	26.546	25.744
	3	315.25	165.50	115.75	90.997	76.245	66.491	59.593	54.479	50.254	47.459	44.969	42.932	41.239	39.819	38.617
	4	420.34	220.67	154.33	121.33	101.66	88.655	79.457	72.639	67.405	63.279	59.958	57.242	54.985	53.092	51.489
	5	525.42	275.83	192.91	151.66	127.08	110.82	99.322	90.799	84.256	79.099	74.948	71.553	68.732	66.366	64.361
1 ml.	1	100.50	50.500	33.836	25.503	20.504	17.172	14.792	13.007	11.619	10.508	9.600	8.843	8.203	7.655	7.179
	2	201.01	101.00	67.673	51.006	41.009	34.343	29.583	26.013	23.237	21.017	19.200	17.687	16.406	15.309	14.359
	3	301.51	151.50	101.51	76.509	61.513	51.515	44.375	39.020	34.856	31.525	28.800	26.530	24.610	22.964	21.538
	4	402.01	202.00	135.35	102.01	82.018	68.686	59.166	52.026	46.474	42.034	38.400	35.373	32.813	30.618	28.717
	5	502.52	252.50	169.18	127.52	102.52	85.858	73.958	65.033	58.093	52.542	48.000	44.217	41.016	38.273	35.896
$\frac{1}{10}$ ml.	1	100.	50.050	33.378	25.050	20.052	16.717	14.335	12.550	11.161	10.050	9.141	8 384	7.742	7.193	6.717
	2	200.	100.10	66.756	50.100	40.104	33.434	28.670	25.100	22.321	20.101	18.282	16.767	15.485	14.385	13.434
	3	300.	150.15	100.13	75.150	60.156	50.150	43.005	37.649	33.482	30.151	27.422	25.151	23.227	21.578	20.150
	4	400.	200.20	133.51	100.20	80.208	66.867	57.340	50.199	44.643	40.201	36.563	33.535	30.969	28.771	26.867
	5	500.	250.25	166.89	125.25	100.26	83.584	71.675	62.749	55.804	50.252	45.704	41.918	38.712	35.963	33.584
$\frac{1}{100}$ ml.	1	100.	50.	33.333	25.	20.	16.667	14.286	12.5	11.111	10.	9.091	8.333	7.692	7.143	6.671
	2	200.	100.	66.666	50.	40.	33.333	28.571	25.0	22.222	20.	I8.182	16.667	15.385	14.286	13.342
	3	300.	150.	99.999	75.	60.	50.000	42.857	37.5	33.333	30.	27.273	25.000	23.077	21.429	20.013
	4	400.	200.	133.33	100.	80.	66.667	57.143	50.0	44.444	40.	36.364	33.333	30.769	28.571	26.684
	5	500.	250.	166.67	125.	100.	83.333	71.429	62.5	55.556	50.	45.455	41.667	38.462	35.714	33.356

Dilution	Number of Positives	16	17	18	19	20	30	40	50	60	70	80	90	100	110	120
10 ml.	1	12.530	12.235	11.980	11.759	11.565	10.524	10.187	10.068	10.025	10.009	10.003	10.001	10.000	10.	10.
	2	25.059	24.470	23.961	23.517	23.130	21.048	20.373	20.136	20.050	20.018	20.007	20.002	20.001	20.	20.
	3	37.589	36.706	35.941	35.276	34.696	31.572	30.560	30.203	30.075	30.027	50.010	30.004	30.001	30.001	30.
	4	50.119	48.941	47.921	47.035	46.261	42.096	40.746	40.271	40.100	40.036	40.013	40.005	40.002	40.001	40.
	5	62.649	61.176	59.902	58.794	57.826	52.620	50.933	50.339	50.125	50.046	50.017	50.006	50.002	50.001	50.
1 ml.	1	6.763	6.397	6.071	5.779	5.517	3.858	3.033	2.542	2.216	1.986	1.816	1.685	1.582	1.499	1.431
	2	13.527	12.793	12.141	11.558	11.033	7.717	6.066	5.083	4.433	3.973	3.632	3.370	3.164	2.998	2.862
	3	20.290	19.190	18.212	17.337	16.550	11.575	9.100	7.625	6.649	5.959	5.448	5.025	4.746	4.497	4.293
	4	27.053	25.586	24.282	23.116	22.067	15.433	12.133	10.166	8.865	7.946	7.264	6.710	6.328	5.996	5.724
	5	33.817	31.983	30.353	28.895	27.583	19.291	15.166	12.708	11.082	9.932	9.080	8.426	7.910	7.495	7.155
$\frac{1}{10}$ ml.	1	6.3	5.933	5.606	5.313	5.05	3.384	2.550	2.050	1.717	1.479	1.301	1.162	1.051	0.960	0.884
	2	12.6	11.865	11.211	10.626	10.10	6.767	5.101	4.101	3.434	2.958	2.601	2.324	2.102	1.920	1.769
	3	18.9	17.798	16.817	15.940	15.15	10.151	7.651	6.151	5.151	4.437	3.902	3.486	3.153	2.880	2.653
	4	25.2	23.730	22.423	21.253	20.20	13.535	10.201	8.202	6.869	5.917	5.203	4.647	4.203	3.840	3.537
	5	31.5	29.663	28.028	26.566	25.25	16.918	12.752	10.252	8.586	7.396	6.503	5.809	5.254	4.800	4.422
$\frac{1}{100}$ ml.	1	6.254	5.889	5.562	5.269	5.005	3.338	2.505	2.005	1.672	1.433	1.255	1.116	1.005	0.914	0.838
	2	12.508	11.779	11.123	10.538	10.010	6.676	5.010	4.010	3.343	2.867	2.510	2.232	2.010	1.828	1.677
	3	18.762	17.668	16.685	15.806	15.015	10.013	7.515	6.016	5.015	4.300	3.765	3.348	3.015	2.742	2.515
	4	25.016	23.557	22.247	21.075	20.020	13.351	10.020	8.021	6.687	5.734	5.020	4.464	4.020	3.656	3.353
	5	31.270	29.446	27.809	26.344	25.025	16.689	12.525	10.026	8.358	7.167	6.275	5.580	5.025	4.570	4.192

Dilution	Number of Positives	130	140	150	160	170	180	190	200	300	400	500	600	700	800	900
10 ml.	1	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.
	2	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.
	3	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.
	4	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.
	5	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.
1 ml.	1	1.375	1.327	1.287	1.253	1.224	1.198	1.176	1.157	1.052	1.019	1.007	1.002	1.001	1.000	1.000
	2	2.749	2.655	2.574	2.506	2.447	2.396	2.352	2.313	2.105	2.037	2.014	2.005	2.002	2.001	2.000
	3	4.124	3.982	3.862	3.759	3.671	3.594	3.528	3.470	3.157	3.056	3.020	3.007	3.003	3.001	3.000
	4	5.499	5.309	5.149	5.012	4.894	4.792	4.703	4.626	4.210	4.075	4.027	4.010	4.004	4.001	4.000
	5	6.873	6.637	6.436	6.265	6.118	5.990	5.879	5.783	5.262	5.093	5.034	5.012	5.005	5.002	5.001
$\frac{1}{10}$ ml.	1	0.820	0.765	0.718	0.676	0.640	0.607	0.578	0.552	0.386	0.303	0.254	0.224	.199	.182	.169
	2	1.641	1.531	1.436	1.353	1.279	1.214	1.156	1.103	0.772	0.607	0.508	0.449	.397	.363	.337
	3	2.461	2.296	2.154	2.029	1.919	1.821	1.734	1.655	1.157	0.910	0.762	0.673	.596	.545	.503
	4	3.281	3.062	2.872	2.705	2.559	2.428	2.312	2.207	1.543	1.213	1.017	0.898	.795	.726	.671
	5	4.102	3.827	3.590	3.382	3.198	3.035	2.889	2.758	1.929	1.517	1.271	1.122	.993	.908	.843
$\frac{1}{100}$ ml.	1	0.774	0.719	0.672	0.630	0.593	0.561	0.531	0.505	0.338	0.255	0.205	0.175	.148	.130	.116
	2	1.548	1.439	1.343	1.260	1.187	1.121	1.063	1.010	0.677	0.510	0.410	0.349	.296	.260	.232
	3	2.323	2.158	2.015	1.890	1.780	1.682	1.594	1.515	1.015	0.765	0.615	0.524	.444	.390	.349
	4	3.097	2.877	2.687	2.520	2.373	2.242	2.125	2.020	1.353	1.020	0.820	0.698	.592	.520	.465
	5	3.871	3.596	3.358	3.150	2.966	2.803	2.657	2.525	1.692	1.275	1.025	0.873	.740	.650	.581

Dilution	Number of Positives	1,000	1,100	1,200	1,300	1,400	1,500	1,600	1,700	1,800	1,900	2,000	3,000	4,000	5,000	6,000
10 ml.	1	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.	10.
	2	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.	20.
	3	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.	30.
	4	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.	40.
	5	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.	50.
1 ml.	1	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	1	1.	1.	1.
	2	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.
	3	3.	3.	3.	3.	3.	3.	3.	3.	3.	3.	3.	3.	3.	3.	3.
	4	4.	4.	4.	4.	4.	4.	4.	4.	4.	4.	4.	4.	4.	4.	4.
	5	5.	5.	5.	5.	5.	5.	5.	5.	5.	5.	5.	5.	5.	5.	5.
$\frac{1}{10}$ ml.	1	.158	.150	.143	.137	.133	.128	.125	.122	.120	.118	.116	.105	.102	.101	.100
	2	.316	.300	.286	.275	.265	.257	.251	.245	.240	.235	.231	.210	.204	.201	.201
	3	.475	.450	.429	.412	.398	.386	.376	.367	.359	.353	.347	.316	.306	.302	.301
	4	.633	.600	.572	.550	.531	.515	.501	.489	.479	.470	.463	.421	.407	.403	.401
	5	.791	.749	.716	.687	.664	.644	.626	.612	.599	.588	.578	.526	.509	.503	.501
$\frac{1}{100}$ ml.	1	.105	.096	.088	.082	.077	.072	.067	.064	.061	.058	.055	.039	.030	.025	.022
	2	.210	.192	.177	.164	.153	.144	.135	.128	.121	.116	.110	.077	.061	.051	.044
	3	.315	.288	.265	.246	.230	.215	.203	.192	.182	.173	.166	.116	.091	.076	.066
	4	.420	.384	.354	.328	.306	.287	.271	.256	.243	.231	.221	.154	.121	.102	.089
	5	.525	.480	.442	.410	.383	.359	.338	.320	.304	.289	.276	.193	.152	.127	.111

sponding number calculated as above, multiplied or divided by 10, 100, etc., according to the number of dilutions the result has been shifted.

Here again, also, if the result contains more than four dilutions, the most significant three or four should be chosen, and the table entered with these.

If, because of the use of more than 5 tubes with any dilution, the number of positives is greater than 5, the corresponding factor is obtained by adding together factors corresponding to such numbers of positives as will equal the number of positives obtained in the tube result. Thus, if 9 positives occur, the factors for 5 positives and 4 positives may be added together to obtain the factor for 9 positives. Similarly, the factor for 17 positives is obtained by adding together the factors corresponding to 5, 5, 5, and 2 positives.

The accuracy of the M.P.N.'s calculated from this table is ample for all practical purposes.

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