

A SYSTEM FOR THE BACTERIOLOGICAL EXAMINATION OF WATER

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INTRODUCTION

A tremendous literature has accumulated during the last several years on methods for the bacteriological examination of water. The subject has attracted considerable interest chiefly from the standpoint of the interpretation of results and, also, with a view to the elimination of false or spurious positive presumptive tests, with the aid of various dyes incorporated into the media.

Organisms which were at one time classed as unsafe are now no longer held, by the majority of workers, to have the same sanitary significance. In other words, the colon group is now divided into the coli and aerogenes sections of the colon group.

The elimination of false or spurious presumptive tests with the aid of various dyes incorporated into the media has occupied the attention of many investigators. This is a very important forward step as it not only makes the results more clear cut, but effects a great saving in time and materials. The need at the present time is to obtain results as quickly as possible and the addition of dyes to the media is a step in this direction.

Considering all of the newer methods being advocated, the majority of workers have failed to appreciate the fact that the borderline or atypical strains are not eliminated. We are no nearer to a solution of these atypical strains now than we have ever been. It is relatively easy to identify a typical *B. coli* but a very difficult matter to classify an atypical strain. It is not

believed that a medium will ever be devised to entirely eliminate these borderline organisms but if many or most of them are classed as colon or not colon a great step forward will have been made.

Unfortunately many of the procedures have tended to complicate rather than simplify the subject. The need at present is to simplify the methods as much as possible and at the same time produce the desired results.

A complete system for the bacteriological examination of water is here presented. It is believed that the method will not only prove simple and effect a great saving in time and materials, but that the atypical or borderline strains will be reduced to a minimum. The method has already been shown to possess great possibilities, proving far more satisfactory to the writer than any now in use.

A NEW MEDIUM FOR THE PRESUMPTIVE TEST

Churchman (1912) as early as 1912 showed that gentian violet in a concentration of 1:100,000 exhibited a marked inhibitory action on the Gram positive organisms but that it had very little, if any, effect on those organisms which stained Gram negatively. This applies not only to vegetative cells but also to bacterial spores, although Churchman (1912), Burk and Skinner (1925), Wiesner (1926), and others have shown that a higher concentration of dye is necessary. Churchman stated that the above effect is better described as bacteriostatic rather than bactericidal because all organisms are not necessarily killed but merely prevented from multiplying. These results have been further verified in subsequent papers by the same author (Churchman, 1921, 1922 and 1923) by Gay and Beckwith, (1922), and others. This same principle has been made use of by Petroff (1915) in the isolation of the tubercle bacillus from sputum and feces; by Krumwiede, Fielder and Watson (1918) for the purification of vaccine virus; by Bernstein and Loewe (1919) for the isolation of the influenza bacillus, and by many others for various special investigations.

Various dyes are being used more and more for the elimination

of positive presumptive tests that do not confirm for the members of the colon group. These false tests are attributed to three causes: (1) bacterial symbiosis, (2) aerobic spore formers, and (3) anaerobic spore producers. Elimination of the above three causes means a saving of a considerable amount of time in obtaining results.

Bacterial symbiosis

By bacterial symbiosis is meant the production of acid and gas from carbohydrate media by two organisms living together, neither of which acting alone is capable of producing gas. This phenomenon which was first investigated by Sears and Putnam (1923) has actually been found to be the cause of many false presumptive tests. Others who have brought forth convincing evidence as to the existence of bacterial synergism are Leitch (1925), Castellani (1926), and Holman and Meekison (1926). Dyes have been very useful in the elimination of the majority of spurious tests due to bacterial symbiosis.

Spore formers

Aerobic spore bearing bacilli have been responsible for many positive presumptive tests which have failed to confirm for the presence of *B. coli*. Ewing (1919) isolated a spore bearing organism which was able to produce acid and gas in lactose. Similar organisms have been isolated by Norton and Weight (1924), Sohn (1925), Hostettler and Gettrust (1926), and Koser and Shinn (1927).

Meyer (1918) repeatedly recovered an anaerobic spore forming organism giving gas from lactose broth. Levine (1918), Frost (1916), Kahn (1918), Muer and Harris (1924), Raab (1923), Meader and Bliss (1923), Norton and Barnes (1928), Spray and Laux (1930) and others also reported on the presence of anaerobic organisms interfering with the presumptive test.

The use of dyes

Brilliant green, gentian violet and crystal violet have been used more than any other dyes for the elimination of Gram

positive organisms. These dyes in high dilution are particularly effective against those organisms which are not decolorized by alcohol, but, on the other hand, show a minimum of effect on the Gram negative bacteria. Of the three dyes, brilliant green has probably been used more in water works practice.

Brilliant green was originally introduced into bacteriological technique for the isolation of the typhoid bacillus from feces and urine. Browning, Gilmour and Mackie (1913) found that this dye exerts a much more marked inhibitory effect on *B. coli* than on *B. typhosus* or the paratyphoid organisms. In the presence of brilliant green the typhoid bacillus is not outgrown by the colon organisms. This was further shown by Browning and Gilmour (1913), Krumwiede and Pratt (1914), Browning, Mackie and Smith (1914), Krumwiede, Pratt and McWilliams (1916), and Teague and Clurman (1916a). Teague and Clurman (1916b) used brilliant green for preserving typhoid stools for delayed examinations.

Bile-dye media

Bile or its salts, either with or without dyes, are extensively used in sugar broth media for their stimulating effect on the growth of the colon organisms. Jackson (1906) employed lactose bile for the presumptive test. He concluded that the bile broth prevented negative tests from the overgrowth of other bacteria as well as positive tests from mixtures of other gas formers. Levine (1921) found that in a medium containing 0.5 per cent peptone with crystal violet, a bile concentration less than 0.5 per cent stimulated growth of *B. coli* while more than that amount exhibited a marked inhibitory action. Similar results were obtained when bile salts were used (Levine, 1922). The amount of bile to be added varied with the concentration of peptone. In the presence of bile or its salts a higher concentration of dye is necessary, due to absorption and inactivation. According to Dunham, McCrady and Jordan (1925) the following deductions can be made as to the use of brilliant green bile broth: (1) Its use eliminates the large number of confirmatory plates on which

no growth occurs; (2) spore forming organisms are eliminated; (3) brilliant green bile appears to extend its selective inhibitions to attenuated forms of the colon group. Other workers who have reported favorably on the use of brilliant green bile broth are Ruchhoft (1926), Dunham and Schoenlein (1926), Hale (1926), Hinman (1927), and Jordan (1927). Hall and Ellefson (1918 and 1919) used gentian violet without bile for the elimination of spurious presumptive tests. The use of gentian violet has also been advocated by Wagner and Monfort (1921) and Stearn (1923). Dominick and Lauter (1929) used methylene blue without bile. On the other hand Howard and Thompson (1925) found that brilliant green bile broth is highly inhibitory and useless except to restrict the growth of the colon group. This applied not only to the use of whole bile but also to bile salts. Poe (1930) reported that crystal violet alone, would eliminate most anaerobes. With most strains of *B. welchii* the addition of bile actually stimulated their growth. The inhibitive power of the crystal violet lactose broth was destroyed by the addition of bile, as far as the growth of anaerobes was concerned.

Choice of dyes

It is rather unfortunate that gentian violet was ever used in bacteriological media to restrict the growth of certain organisms. Gentian violet is a term which does not refer to any definite chemical compound but rather to a mixture of dyes of a certain group (Committee, 1922). Grüber apparently originated the term. It is not recognized in the dye or textile industry. Such dyes in the mixtures sold as gentian violet all belong to the pararosaniline series but at the present time the different manufacturers and dealers nearly all sell different mixtures of these dyes under the name of gentian violet. On the other hand, crystal violet, which is one of the components of gentian violet is a definite chemical compound. A definite chemical formula can be assigned to this dye and, although commercial samples of it are not necessarily pure and may vary in their composition, it should be used in preference to gentian violet.

Experimental

Brilliant green is used to a far greater extent for its inhibitory action on the growth of Gram-positive organisms than crystal violet. It is not clear just why this dye has been selected for this purpose in preference to the latter. Browning, Gilmour and Mackie (1913) in the course of systematic investigations on the antiseptic action of benzol derivatives found that a marked difference existed between the action of dyes which are members of the diamidotriphenyl-methane group (brilliant green) and those of the triamidotriphenyl methane series (crystal violet). Both groups exert a powerful action on Gram-positive organisms. In addition, the diamidotriphenyl methane dyes, of which brilliant green is a member, are also fairly actively bactericidal toward the coli-typhoid group, whereas these organisms are comparatively insusceptible to the triamidotriphenyl methane group (crystal violet).

Comparative studies on dyes incorporated in media and inoculated with several organisms strengthen the findings of Browning, Gilmour and Mackie, that brilliant green is more bactericidal than crystal violet towards the colon organisms. In table 1 are results obtained when various dilutions of crystal violet and brilliant green are inoculated with *B. coli*, *B. aerogenes*, *Staphylococcus albus* and *Staphylococcus aureus*, and *B. subtilis*. The tubes were examined at the end of forty-eight hours for turbidity. With crystal violet it can be seen that *B. coli* is relatively more susceptible to its action as compared with brilliant green. On the other hand crystal violet exerts a more toxic effect against Gram-positive organisms than brilliant green. In other words a greater range of safety exists between Gram-positive and Gram-negative organisms when crystal violet is used. More crystal violet can be used than brilliant green without exerting a more toxic action on *B. coli*. This means that with crystal violet a better chance exists for the elimination of those Gram-positive organisms, responsible for false tests, than when brilliant green is used.

The amount of dye to be incorporated varies with the amount

TABLE 1

Comparison of bactericidal action of crystal violet and brilliant green

Medium employed:

Peptone, Difco..... 5 grams

 K_2HPO_4 5 grams KH_2PO_4 1 gram

Lactose..... 5 grams

Dye, sufficient quantity

Distilled water, to make 1000 cc.

DYE DILUTION	CRYSTAL VIOLET					BRILLIANT GREEN				
	<i>B. coli</i>	<i>B. aerogenes</i>	<i>Staph. albus</i>	<i>Staph. aureus</i>	<i>B. subtilis</i>	<i>B. coli</i>	<i>B. aerogenes</i>	<i>Staph. albus</i>	<i>Staph. aureus</i>	<i>B. subtilis</i>
50,000	—	—	—	—	—	—	—	—	—	—
100,000	—	+	—	—	—	—	—	—	—	—
150,000	+	+	—	—	—	—	+	—	—	—
200,000	+	+	—	—	—	—	+	—	—	—
250,000	+	+	—	—	—	—	+	—	—	—
300,000	+	+	—	—	—	—	+	—	—	—
350,000	+	+	—	—	—	—	+	—	—	—
400,000	+	+	—	—	—	—	+	—	—	—
450,000	+	+	—	—	—	—	+	—	—	—
500,000	+	+	—	—	—	—	+	—	—	—
1,000,000	+	+	—	—	—	—	+	—	—	—
1,500,000	+	+	—	—	—	—	+	—	—	—
2,000,000	+	+	—	—	—	—	+	—	—	—
2,500,000	+	+	—	—	—	+	+	—	—	—
3,000,000	+	+	—	—	—	+	+	—	—	—
3,500,000	+	+	—	—	—	+	+	—	—	—
4,000,000	+	+	—	—	—	+	+	—	—	—
4,500,000	+	+	—	—	—	+	+	—	—	—
5,000,000	+	+	—	—	—	+	+	—	—	—
6,000,000	+	+	—	—	—	+	+	—	—	—
7,000,000	+	+	—	—	—	+	+	—	—	—
8,000,000	+	+	—	—	—	+	+	—	—	+
9,000,000	+	+	—	—	—	+	+	—	—	+
10,000,000	+	+	—	—	—	+	+	—	—	+
11,000,000	+	+	—	—	—	+	+	—	—	+
12,000,000	+	+	—	—	+	+	+	+	—	+
14,000,000	+	+	—	—	+	+	+	+	—	+
16,000,000	+	+	—	—	+	+	+	+	—	+
18,000,000	+	+	+	—	+	+	+	+	—	+
20,000,000	+	+	+	—	+	+	+	+	—	+
22,000,000	+	+	+	—	+	+	+	+	—	+
24,000,000	+	+	+	—	+	+	+	+	—	+

All readings were made at the end of forty-eight hours.

of peptone and other organic matter added to the media. As the amount of peptone is increased the amount of dye also is increased. In other words, the concentration of dye necessary to produce the desired results varies directly with the amount of peptone.

Using the medium as given in table 1, it was found that a dye concentration of 1:700,000 did not affect the growth of the colon organisms, and, at the same time was sufficient to eliminate the growth of organisms giving false presumptive tests.

Crystal violet broth

Preparation of crystal violet solution. Place 1 gram of crystal violet in a glass stoppered bottle of 150 cc. capacity and add 50 cc. of 95 per cent alcohol. Shake thoroughly and then add 50 cc. of distilled water. Shake well again until all of the dye is dissolved. Pipette 20 cc. of this 1 per cent solution into a liter volumetric flask. Add sufficient water to make 1000 cc. This gives a 1:5000 solution of crystal violet.

The medium for the presumptive test is now prepared as follows:

Peptone, Difco.....	15	grams
K ₂ HPO ₄	15	grams
KH ₂ PO ₄	3	grams
Lactose.....	15	grams
Crystal violet (1:5000).....	21.5	cc.

Distilled water to make the quantity = 2000 cc.

Mix ingredients and stir until solution is effected. Distribute the medium into test tubes, 20 cc. to each tube. Sterilize in an Arnold sterilizer or in the streaming steam of an autoclave for ten minutes. Only one heating is sufficient to sterilize. Autoclaving under pressure will decompose some of the dye making the medium unfit for use. It is not necessary to adjust the reaction of the medium as this is controlled by the buffer salts.

The tubes are inoculated with a 10 cc. portion of water sample, giving a final volume of 30 cc. This brings the final volume of the two liters of medium to three liters. The formula after the addition of the water sample becomes:

Peptone, Difco.....	15	grams
K ₂ HPO ₄	15	grams
KH ₂ PO ₄	3	grams
Lactose.....	15	grams
Crystal violet (1:5000).....	21.5	cc.
Distilled water to make.....	2000	cc.
Water sample.....	1000	cc.
	<hr/>	
	3000	cc.

It makes no difference which dilutions are run as long as the above proportions are not disturbed. All that is necessary is to increase or decrease the amount of distilled water used in preparing the medium.

As an example, if 1 cc. amounts are to be inoculated into tubes, this may be calculated as follows:

Amount of medium per tube.....	cc.
Amount of inoculum per tube.....	4
	1
	<hr/>
Total.....	5
$\frac{1}{5} : \frac{x}{3000} \quad 5x = 3000$	
$x = 600 \text{ cc.} = \text{the inoculum}$	
$3000 - 600 = 2400 \text{ cc.} = \text{the medium}$	

Prepare the above medium using sufficient distilled water to make 2400 cc. Distribute 4 cc. amounts in test tubes. Then, on inoculation with 1 cc. quantities of water sample, the above proportions of the various ingredients will be the same as in the tubes holding 10 cc. amounts of water sample.

Inoculating tubes of crystal violet broth with 50 strains of *B. aerogenes* and 180 strains of *B. coli* yielded the results shown in table 2.

TABLE 2
Results obtained by inoculating crystal violet broth medium with pure cultures of *B. coli* and *B. aerogenes*

ORGANISM	NUMBER OF STRAINS	10 PER CENT OR MORE GAS	CORRELATION
			per cent
<i>B. aerogenes</i>	50	50 positive	100
<i>B. coli</i>	180	180 positive	100

It is seen that perfect correlation is obtained by inoculating tubes of the medium with pure cultures of *B. coli* and *B. aerogenes*. A dye concentration of 1:700,000 is not inhibitory to the growth of these two organisms.

A NEW MEDIUM FOR THE CONFIRMATORY TEST

The majority of the solid media now in use for the detection of *B. coli* in water were originally used for the isolation of *Bacillus typhosus* and *Bacillus dysenteriae* from infected feces and urine. Changes have been made in their composition and mode of preparation adapting their uses to water works practice. Very few of the media now extensively in use were compounded solely for water examinations. This accounts for the large number of modifications of Endo and other differential media. This is a point that should be kept in mind.

Drigalski and Conradi (1902) used crystal violet to eliminate other organisms interfering with the isolation of *B. typhosus* from stools. Litmus was added to the medium to identify the presence of the acid producing colon organisms. The well known medium of Endo (1903) is composed of basic fuchsin decolorized by the use of sodium sulfite. Modifications of the original formula have been reported by Kendall and Walker (1910), Kendall and Day (1911), Kinyoun and Deiter (1912), Harding and Ostenberg (1912), Robinson and Rettger (1916), Levine (1918), and others adapting the medium to special investigations. Holt-Harris and Teague (1916), incorporated the dyes methylene blue and eosin in an agar base containing lactose and sucrose and were able to isolate the typhoid and dysentery organisms on this preparation. This medium has been modified and simplified by Levine (1918 and 1921), adapting its use to water works laboratories.

Differentiation of the coli and aerogenes groups

The consensus of opinion among bacteriologists at the present time is that the coli and aerogenes divisions of the colon group should be differentiated. This was advocated, as early as 1918, by Rogers (1918) and Levine (1918). These organisms are no

longer held to have the same sanitary significance in reporting on the potability of a water supply. It has been shown by Rogers and his associates (Rogers, Clark and Davis, 1914; Rogers, Clark and Evans, 1914 and 1915; Rogers, Clark and Lubs, 1918), that *B. coli* is characteristic of the intestinal tract of man and animals while *B. aerogenes* is very rarely isolated from human or animal excreta, but is found chiefly in soil and on grains. Therefore these two organisms should not be considered the same from a sanitary standpoint. Among others who believe the two organisms should be separated may be mentioned Wood (1919), Levine (1921), Wilson (1922), Hinman (1925), Clark (1925), Spray (1927), and Chen and Rettger (1920).

Rogers, Clark and Davis (1914), Rogers, Clark and Evans (1914 and 1915), and Rogers, Clark and Lubs (1918) in the course of extensive and systematic investigations on the coli and aerogenes organisms were able to separate the members into two main groups by means of their gas ratios. The members of the *B. coli* division produced carbon dioxide and hydrogen in approximately equal proportions whereas those of the *B. aerogenes* section produced more carbon dioxide than hydrogen. *B. coli* is termed the low ratio organism while *B. aerogenes* belongs to the high ratio group.

By the use of the hydrogen electrode, Clark and Lubs (1915 and 1917) followed the changes in the hydrogen ion concentration in various cultures of *B. coli* and *B. aerogenes*. Simple conditions were found whereby the metabolism was so controlled that the pH of cultures of one group were made to diverge widely from those of the other group. It was further found that the differences in the final hydrogen ion concentrations of cultures of *B. coli* and *B. aerogenes* correlated perfectly with their gas ratios. This principle was made use of by Clark and Lubs (1915 and 1917), and Clark (1918) in the so-called methyl red test.

Other differential tests for the separation of the coli and aerogenes divisions of the colon group have been advocated. Voges and Proskauer (1898) recommended the so-called V. P. test which depends on the formation of acetyl methyl carbinol by *B. aerogenes* but not by *B. coli*. Koser (1918) used uric acid

as the only source of nitrogen and found that *B. aerogenes* was able to utilize this compound. *B. coli* cannot use uric acid as a nitrogen source. Another test recommended by Koser (1923 and 1924) is the citrate test giving a positive growth with *B. aerogenes*. On the other hand *B. coli* fails to grow in this medium. Simmons (1926) added agar and brom-thymol blue to Koser's citrate solution and used it as a solid plating medium.

It can be seen that with the exception of Simmon's citrate agar all of the media named above are liquid media in which it is not possible to make a study of the colonies of the growing organisms. In no case can the growth of *B. coli* be studied on solid media. It was considered of great advantage to possess a solid streaking medium which would differentiate the two main subgroups of the colon group, and at the same time permit a study of the organisms growing in colonies.

Experimental

Erythrosine, methylene blue and brom-cresol-purple were the dyes selected to be incorporated in a buffered lactose agar base. The combination of erythrosine and methylene blue gives a bright metallic appearance to the colonies of *B. coli* but not to those of *B. aerogenes*. *B. coli* changes the color of the brom-cresol-purple from purple to orange while *B. aerogenes* is unable to affect the color of the indicator. The medium gives two tests in one, namely, the presence or absence of a metallic sheen and a positive or negative color change in the agar surrounding the colonies.

The medium is similar to one previously reported by the writer (Salle, 1927) but has been modified to give sharper reactions and more satisfactory results.

Erythrosine, methylene blue and brom-cresol-purple change in color from purple to orange at about pH 5.6. A medium in order to give satisfactory results must be so prepared that *B. coli* will grow and produce its maximum acidity below pH 5.6. This is achieved by the addition of buffer substances to the medium. In table 3 the results of a preliminary test with five different kinds of broth containing increasing amounts of buffer

TABLE 3

The effect of an increase in the buffer content on the final pH of the medium

CULTURE NUMBER	MEDIA									
	(1)*		(2)		(3)		(4)		(5)	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
	pH	pH	pH	pH	pH	pH	pH	pH	pH	pH
1	4.55	4.51	4.78	4.70	4.92	4.80	5.07	4.90	5.17	5.16
2	4.62	4.60	4.80	4.72	4.97	4.78	5.02	4.92	5.14	5.07
3	4.46	4.38	4.77	4.63	4.89	4.75	4.95	4.84	5.12	5.07
4	4.33	4.29	4.56	4.41	4.68	4.55	4.82	4.65	5.02	4.87
5	4.62	4.55	4.82	4.77	4.95	4.85	5.04	4.94	5.17	5.14
6	4.60	4.56	4.92	4.85	5.00	4.87	5.14	5.00	5.26	5.17
7	4.48	4.40	4.70	4.62	4.80	4.75	4.87	4.82	5.04	4.94
8	4.43	4.45	4.75	4.68	4.85	4.80	4.92	4.89	5.06	5.11
9	4.45	4.41	4.68	4.63	4.85	4.70	4.94	4.84	5.12	5.09
10	4.53	4.50	4.84	4.75	4.94	4.95	5.09	4.97	5.19	5.16
11	4.45	4.41	4.68	4.63	4.89	4.80	4.95	4.84	5.14	5.07
12	4.33	4.33	4.62	4.46	4.72	4.62	4.78	4.60	4.90	4.90
13	4.45	4.41	4.65	4.60	4.77	4.70	4.80	4.72	5.04	4.97
14	4.58	4.53	4.80	4.72	4.89	4.82	5.04	4.92	5.17	5.11
15	4.63	4.58	4.85	4.78	5.00	4.92	5.02	4.94	5.16	5.11
16	4.50	4.46	4.68	4.67	4.80	4.72	4.87	4.77	5.04	4.97
17	4.51	4.45	4.77	4.68	4.94	4.80	5.00	4.85	5.14	5.11
18	4.63	4.63	4.82	4.77	4.99	4.90	5.09	4.94	5.21	5.17
19	4.53	4.50	4.85	4.78	4.99	4.92	5.12	5.06	5.26	5.17
20	4.36	4.31	4.58	4.50	4.70	4.63	4.78	4.72	4.97	4.90
21	4.62	4.56	4.80	4.72	4.94	4.85	5.06	4.97	5.17	5.11
22	4.62	4.55	4.85	4.75	5.02	4.78	5.09	4.90	5.21	5.16
23	4.53	4.46	4.78	4.70	4.95	4.85	5.06	4.95	5.16	5.14
24	4.45	4.43	4.63	4.58	4.84	4.75	4.92	4.82	5.07	5.11
25	4.53	4.46	4.73	4.68	4.87	4.80	4.92	4.92	5.11	5.07
26	4.46	4.43	4.68	4.63	4.84	4.77	4.92	4.85	5.11	5.06

* (1) Peptone, Difco, 5 grams; lactose, 5 grams; distilled water to 1000 cc.

(2) Peptone, Difco, 5 grams; K_2HPO_4 , 1 gram; lactose, 5 grams; distilled water to 1000 cc.(3) Peptone, Difco, 10 grams; K_2HPO_4 , 1 gram; lactose, 5 grams; distilled water to 1000 cc.(4) Peptone, Difco, 10 grams; K_2HPO_4 , 2 grams; KH_2PO_4 , 0.5 gram; lactose, 5 grams; distilled water to 1000 cc.(5) Peptone, Difco, 10 grams; K_2HPO_4 , 3 grams; KH_2PO_4 , 0.6 gram; lactose, 5 grams; distilled water to 1000 cc.

substances are summarized. Pure cultures of *B. coli* were used. The final hydrogen ion concentrations of the cultures were determined by means of the hydrogen electrode. The tubes were incubated at 37°C. and the results recorded at the end of twenty-

TABLE 4

Final hydrogen ion concentrations of cultures of B. coli

Medium: Peptone, 10 grams; K_2HPO_4 , 3 grams; KH_2PO_4 , 0.6 grams; water, 1000 cc.

CULTURE NUMBER	pH IN DIFFERENT MEDIA AT DIFFERENT TIMES							
	1 per cent lactose		0.6 per cent lactose		0.5 per cent lactose		0.4 per cent lactose	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
1	4.99	4.94	5.06	4.97	5.09	4.99	5.12	5.16
2	5.06	4.97	5.07	5.07	5.17	5.07	5.26	5.19
3	4.94	4.85	4.97	4.90	4.99	4.94	5.14	5.19
4	5.04	4.90	5.09	4.92	5.22	5.02	5.60	6.29
5	4.72	4.62	4.75	4.62	4.85	4.77	4.97	4.90
6	5.02	4.94	4.99	4.94	5.04	4.95	5.12	5.04
7	5.06	4.94	5.09	4.99	5.14	4.95	5.24	5.26
8	4.99	4.92	5.04	4.90	5.17	5.04	5.38	5.56
9	5.00	4.97	5.04	4.92	5.17	5.09	5.65	6.09
10	5.06	5.04	5.14	5.00	5.21	5.16	5.61	6.27
11	4.82	4.62	5.02	4.66	5.00	4.90	5.63	6.32
12	4.84	4.62	4.97	4.65	5.04	4.77	5.29	5.33
13	4.87	4.84	4.92	4.85	5.02	4.94	5.21	5.28
14	5.02	4.97	4.99	4.97	5.14	5.00	5.22	5.22
15	4.99	4.87	5.04	4.90	5.16	5.02	5.60	6.24
16	5.07	5.04	5.09	5.06	5.19	5.07	5.56	6.00
17	4.89	4.85	4.95	4.87	5.11	4.94	5.11	5.07
18	4.90	4.89	4.94	4.90	4.97	4.95	4.97	5.12
19	4.97	4.94	5.00	4.94	5.07	4.99	5.21	5.24
20	5.07	4.95	5.11	4.97	5.21	5.00	5.44	5.63
21	5.02	4.92	5.07	4.97	5.16	4.99	5.50	5.88
22	4.85	4.85	4.94	4.90	5.04	4.99	5.14	5.11
23	4.70	4.67	4.82	4.73	4.85	4.80	4.99	4.95
24	5.02	4.92	5.07	4.92	5.21	4.99	5.56	6.10
25	4.97	4.94	4.99	4.97	5.06	4.99	5.14	5.12

four and forty-eight hours. It is quite evident that as the amount of buffer is increased the final pH of the medium is correspondingly increased. These results are in harmony with the statement by Clark (1915) that the greater the buffer effect of the medium the lower the final hydrogen ion concentration attained.

For a given amount of sugar *B. coli* will form more acid than *B. aerogenes*, provided the greatest amount added is just sufficient for *B. coli* to produce its maximum hydrogen ion concentration. The amount of lactose that is just sufficient for *B. coli* to produce its maximum acidity is inadequate for *B. aerogenes* to produce its limiting hydrogen ion concentration. In table 4 are shown results obtained by inoculating lactose broth with cultures of *B. coli*, using 1, 0.6, 0.5, and 0.4 per cent lactose respectively. Formula 5, (table 3) was used. Readings were made at the end of twenty-four and forty-eight hours of incubation at 37°C. It is seen that as the amount of lactose is decreased the final hydrogen ion concentration is also decreased until a concentration is reached (0.4 per cent lactose) which is insufficient for *B. coli* to produce its characteristic limiting pH in the above medium. In order to sharply differentiate *B. coli* from *B. aerogenes* the amount of lactose added must be just sufficient for *B. coli* to produce its limiting hydrogen ion concentration. An excess may permit *B. aerogenes* to produce the same pH and the medium will be of little value. The results shown in table 3 suggested 0.5 per cent lactose as the most suitable quantity.

In table 5 are shown results with *B. aerogenes* employing the same medium and lactose concentrations as used for *B. coli* (table 3). It is seen that when results of like concentrations of lactose are compared *B. coli* cultures produce a lower pH than tubes inoculated with *B. aerogenes*. This is just what is desired as a sharp differentiation between these two organisms. The desired result is, therefore, possible.

The medium which was finally selected and which is now in use has the following composition:

Peptone, Difco.....	10	grams
K ₂ HPO ₄	3	grams
KH ₂ PO ₄	0.6	gram
Agar.....	20	grams
Distilled water.....	1000	cc.

No adjustment of the reaction is required. This is controlled by the buffer substances. The ingredients are mixed and boiled

until the agar is dissolved. The loss of water due to evaporation is restored. The agar is not filtered, but distributed directly into 250 cc. Erlenmeyer flasks, 100 cc. to each flask. When ready

TABLE 5
Final hydrogen ion concentration of cultures of *B. aerogenes*
Medium: Peptone, 10 grams; K_2HPO_4 , 3 grams; KH_2PO_4 , 0.6 gram; water, 1000 cc.

CULTURE NUMBER	pH IN DIFFERENT MEDIA AT DIFFERENT TIMES							
	1 per cent lactose		0.6 per cent lactose		0.5 per cent lactose		0.4 per cent lactose	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
1	5.68	5.26	5.61	5.88	5.73	6.54	5.88	6.70
2	5.61	5.24		6.32	5.73	6.42	6.04	6.71
3	5.41	5.34	5.63	6.31	5.73	6.42	6.29	6.85
4	5.55	5.24	5.65	6.15	5.78	6.59	5.82	6.58
5	5.14	5.78	5.77	6.58	6.19	6.91	6.27	6.97
6	5.12	5.66	5.73	6.53	5.83	6.75	6.24	6.88
7	5.16	5.51	5.97	6.81	6.00	6.76	6.48	6.95
8	5.70	5.22	5.60	5.93	5.65	6.41	5.77	6.36
9	5.77	5.39	5.44	5.90	5.80	6.42	5.90	6.66
10	5.58	5.36	5.63	6.19	5.75	6.32	6.19	6.91
11	5.48	5.14	5.51	5.95	5.66	6.63	5.82	6.68
12	5.12	5.75	5.88	6.49	6.27	6.76	6.48	6.85
13	5.58	5.39	5.63	6.15	5.70	6.29	6.19	6.81
14	5.12	5.38	5.85	6.58	5.71	6.46	6.51	6.93
15	5.31	5.44	5.56	6.04	5.61	6.31	6.19	6.75
16	5.28	5.11	5.60	6.09	5.82	6.49	6.22	6.91
17	5.75	5.36	5.66	6.15	5.71	6.80	5.93	6.78
18	5.17	5.51	5.73	6.54	6.22	6.64	6.44	7.05
19	5.16	5.36	5.83	6.49	6.14	6.71	6.39	6.83
20	5.50	5.22	5.56	6.10	5.58	6.26	5.77	6.46
21	5.40	5.06	5.58	6.15	5.68	6.31	5.87	6.61
22	5.48	5.38	5.80	6.54	6.15	6.68	6.22	6.86
23	5.68	5.43	5.50	6.20	5.78	6.49	6.00	6.80
24	5.34	5.17	5.56	6.24	5.73	6.58	6.36	7.05
25	5.53	5.46	5.73	6.46	5.99	6.63		
26	5.43	4.99	5.61	6.09	5.77	6.37	6.12	6.59

to use, 0.5 gram lactose is added to each flask and the agar melted in an Arnold sterilizer or in boiling water. After the agar is melted the following dyes are added to the contents of each flask: erythrosine (2 per cent aqueous), 2 cc.; methylene blue (1 per cent aqueous) 0.2 cc.; and brom cresol purple (1 per cent

alcoholic) 2 cc. The agar is rotated in the flask to obtain an uniform mixture and then poured into sterile petri dishes.

Preparation of dye solutions

Methylene blue, 1 per cent. Dissolve 1 gram of methylene blue in 100 cc. of distilled water. This may be conveniently carried out by placing the dye in a glass stoppered bottle, adding the distilled water and shaking vigorously until completely dissolved.

Brom cresol purple, 1 per cent. Place 1 gram of the indicator in a mortar, add 25 cc. of 95 per cent alcohol and triturate until completely dissolved. Then add sufficient distilled water to make 100 cc.

Erythrosine, 2 per cent. Place 2 grams of the dye in a mortar and add about 50 cc. of distilled water. Then add 5 per cent KOH solution, drop by drop, until the dye is completely dissolved. Triturate well after each addition in order to avoid adding a large excess of alkali. Finally make up the volume to 100 cc. with distilled water.

At the time this work was started it was not appreciated that different brands of dyes or even different lots of the same manufacturer's product could vary so greatly in their physical and chemical properties. It makes very little difference which brands of methylene blue and brom cresol purple are used but the difficulty lies with the erythrosine. With the exception of a small quantity of pre-war erythrosine on hand at the time no other brand has been found satisfactory. Work was, accordingly, started on the preparation of a satisfactory dye with the result that a product has been developed that is far superior to any of those tested and which has given very excellent results.

Preparation of erythrosine

A. Fluorescein U. S. P. sodium salt (National Aniline and Chemical Company).....	20 grams
Distilled water.....	120 cc.

Dissolve the fluorescein in the distilled water contained in a 400 cc. beaker.

B. Iodine U. S. P.....	40 grams
Potassium iodide, U. S. P.....	40 grams
Distilled water.....	340 cc.

Grind the iodine and the potassium iodide together in a mortar until finely divided. Rinse out the mortar with small portions of distilled water until the contents is all dissolved. Collect the solution into a liter beaker. Heat the mixture to 75°C. to dissolve all of the iodine. Then add to it, with constant stirring, the fluorescein solution (A). The erythrosine immediately precipitates out. Allow to stand for five minutes, again heat to 75°C. and pour out the contents into an enameled pan containing two liters of distilled water. Bring the contents of the pan to a boil, remove from the fire and allow to stand for five minutes for the precipitate of erythrosine to settle out. The scum that forms on the surface may be broken up and made to settle out by vigorously stirring while the mixture is boiling. Carefully pour off the supernatant liquid, containing free iodine, in order to not disturb the precipitate. Add two more liters of distilled water to the precipitate and again bring to a boil. After allowing to stand for five minutes carefully pour off the supernatant fluid. Repeat the operation for the third time but use only one liter of distilled water. Add 125 cc. of 5 per cent KOH solution to dissolve the precipitate of erythrosine. Pass the clear solution through a 5-inch Buchner funnel with the aid of suction. Dilute the filtrate with sufficient distilled water to make about one liter. Bring to a boil and add about 20 cc. of concentrated HCl with constant stirring to precipitate the erythrosine. Allow to stand for five minutes. Pour off most of the supernatant liquid and collect the precipitate on a 5-inch Buchner funnel using suction. Rinse out the pan with several portions of distilled water, dry the precipitate well with suction, finally drying thoroughly in the incubator or in a warm place. When dry, pulverize in a mortar and preserve in a well stoppered bottle.

On this medium the colon organisms produce brilliant metallic

colonies, slightly raised, with the surfaces usually flat or slightly concave. The surfaces are rarely convex. The colonies remain confined and show little tendency toward confluence. The agar surrounding the colonies will be changed in color from a purple to orange tinge. *B. aerogenes* strains rarely, if at all, produce metallic colonies and then only in the central portions. The

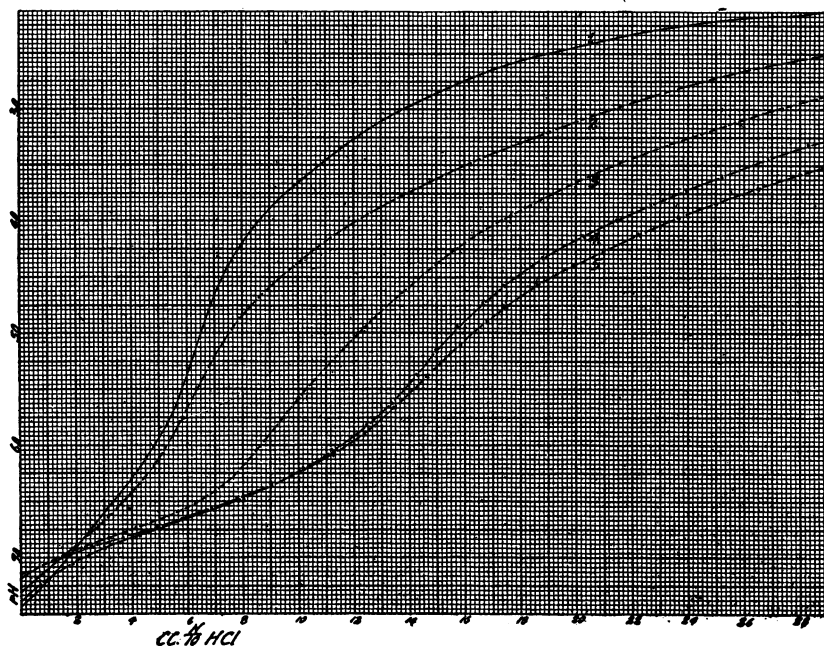


FIG. 1. TITRATION CURVES OF MEDIA GIVEN IN TABLE 3

pH readings by hydrogen electrode, and titration figures in cubic centimeters of $N/10$ HCl for 100 cc. of medium are used.

colonies are raised, convex, very moist and show great tendency to run together. There is no change in the color of the agar.

In figure 1 are shown titration curves of the five media given in table 3. One hundred cubic centimeters of the media were titrated with $N/10$ HCl and the pH readings followed by means of the hydrogen electrode. Titration curves are very useful in that they tell us at what pH range the media exert their maximum

buffer effect. They also help others to duplicate results. For instance, it makes no difference what brand of peptone is used as long as the titration curves are the same. Taking up the curve for medium 5, table 3, it is evident that it requires about one-half as much acid to change the pH of the medium from 6 to 5 as to change the pH from 7 to 6. *B. aerogenes* produces its maximum acidity (using 0.5 per cent lactose) at about pH 6.0; *B. coli* has its limiting hydrogen ion concentration at about pH 5.0. Between pH 6.0 and 5.0 a relatively large change is obtained for a given amount of acid as compared with the pH range between 7.0 and 6.0. This is just what is desired, since such a medium will afford a sharp differentiation of *B. coli* and *B. aerogenes*.

TABLE 6
Results obtained by streaking pure cultures of *B. coli* and *B. aerogenes* on the differential medium

ORGANISM	STRAINS	REACTION ON AGAR		CORRELA- TION
		Me- tallic colony	Color	
<i>B. coli</i>	180	+	Orange	per cent 100
<i>B. aerogenes</i>	50	-	Purple	100

Results

In table 6 are shown results obtained by streaking on the surface of the medium 180 strains of *B. coli* and 50 strains of *B. aerogenes*. The results show perfect correlation.

The solid medium here described incorporates two tests in one operation, namely, the presence or absence of a metallic sheen to the colonies and a positive or negative color change in the agar. In water examinations when speed is very essential, the agar dye preparation will shorten the period to complete an analysis by twenty-four hours.

Instead of fishing metallic colonies from an Endo or an E. M. B. plate and making tests for the presence of fecal *B. coli* (which requires twenty-four hours more time) the results may now be

obtained on the plate. In laboratories where it is the custom to carry the test only to the partially confirmed stage, more satisfactory conclusions may now be drawn with the aid of this medium, since two tests are better than one.

Results of water examinations

More than 100 water examinations were carried out using Standard Methods broth and crystal violet broth media. Five tubes of lactose broth and crystal violet broth each containing 10 cc. of water sample were used for each sample of water ex-

TABLE 7
Results of water examinations using standard broth and crystal violet broth

		NUMBER OF TUBES
Samples examined.....	101	
Perfect correlation between standard and crystal violet broth.....	62	
Samples giving higher results in standard broth.....	20	33
Samples giving higher results in crystal violet broth....	11	18
Samples in which standard broth tubes did not confirm for members of the coli-aerogenes group.....	18	33
Samples in which crystal violet broth tubes did not confirm for members of the coli-aerogenes group....	1	1

amined. Readings were made at the end of forty-eight hours. The results are given in table 7.

The results tabulated in table 7 bring out the superiority of crystal violet broth over Standard lactose broth. Of 101 samples analyzed 62 showed perfect correlation. Twenty samples in standard broth gave 33 more positive tubes than crystal violet broth. However, on confirmation 18 of the above samples including 33 tubes gave either no growth or negative results for members of the coli-aerogenes group. Eleven samples including 18 tubes gave higher results in crystal violet broth all of which confirmed, with the exception of one tube.

The results clearly show that gas formation in crystal violet broth in practically 100 per cent of the tubes is due to the presence of members of the coli-aerogenes group. Other organisms are

eliminated by the dye in almost all cases, giving sharper and more clear cut results on the confirmatory plates. A considerable saving in time and materials is thus accomplished by incorporating crystal violet in the medium.

Of the 20 samples mentioned, twenty-four hours additional time was required to complete an analysis. This time would have been saved by the use of crystal violet broth. Also the majority of the 20 samples were obtained from chlorinated supplies where the treatment did not destroy anaerobic and aerobic spore formers capable of fermenting lactose with the production of acid and gas. None of these organisms produced positive results in crystal violet broth.

The dye broth will show its superiority particularly with the chlorinated samples which tend to give a large percentage of false tests in Standard broth. Of the 101 samples examined only a small number were from chlorinated supplies. If the number of chlorinated samples had been greater the results would have been even more striking.

DISCUSSION

A system for the bacteriological examination of water is proposed which it is believed will prove superior to any now in use.

Comparative tests of brilliant green and crystal violet have shown the superiority of the latter for water examinations. Brilliant green has enjoyed widespread popularity for water examinations but it is not clear just why this dye has been selected in preference to crystal violet.

A crystal violet broth medium is described which in 101 samples examined has been shown to give practically 100 per cent results for members of the coli-aerogenes group. With the use of such broth much time will be saved due to the elimination of those organisms capable of giving acid and gas in standard lactose broth but which do not confirm for the members of the colon group.

An agar dye medium for the confirmatory test is described which incorporates two tests in one operation. In reality, results on this plate medium are equivalent to a partially confirmed

and a confirmed test for the presence of *B. coli*. The *B. coli* organisms produce bright metallic colonies while *B. aerogenes* colonies do not produce a metallic appearance, except in very rare cases. The amount of buffer and lactose in the medium are so controlled that brom-cresol-purple will change in color from purple to orange by the growth of *B. coli* but not by *B. aerogenes*. In those rare instances in which colonies of *B. aerogenes* give a metallic lustre the absence of a change in the color of the agar will distinguish them from *B. coli*.

Comparative tests on 101 samples, using standard broth and crystal violet broth have shown the superiority of the latter in water analysis. Much time and materials will be saved due to the elimination of these organisms giving false tests in water examinations. Also, it is no longer necessary to make confirmatory tests on colonies picked from the plate. Two tests are now incorporated in one operation thereby shortening the period of a complete water analysis by twenty-four hours.

SUMMARY

1. Comparative tests using the five organisms, *B. coli*, *B. aerogenes*, *B. subtilis*, *Staphylococcus aureus* and *Staphylococcus albus* have shown the superiority of crystal violet over brilliant green as a bacteriostatic agent.

2. A crystal violet broth medium for the presumptive test is described: containing peptone (Difco), 15 grams; K_2HPO_4 , 15 grams; KH_2PO_4 , 3 grams; lactose, 15 grams; crystal violet (1:5000 solution), 21.5 cc.; distilled water, to make 2000 cc.

3. Production of acid and gas in this medium in practically 100 per cent of the samples examined is due to members of the coli-aerogenes group.

4. An agar-dye differential medium is described: containing peptone, (Difco), 10 grams; K_2HPO_4 , 3 grams; KH_2PO_4 , 0.6 gram; lactose, 5 grams; agar, 20 grams; distilled water, 1000 cc.; erythrosine (2 per cent aqueous), 20 cc.; methylene blue (1 per cent aqueous), 20 cc. Brom cresol purple (1 per cent alcoholic) 2 cc.

5. Results on this medium are equivalent to a partially con-

firmed and a confirmed test. In other words, two tests are obtained in one operation, thus shortening the period to complete an analysis by twenty-four hours. *B. coli* and *B. aerogenes* are sharply differentiated because of distinct differences in their carbohydrate metabolism.

6. Results of water examinations are detailed showing the superiority of the method over that given in "Standard Methods." The method gives, not only more clear cut results due to elimination of interfering non-colon organisms, but also a saving of at least twenty-four hours in the time necessary to complete an examination.

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