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The study of media employed in, or suggested for, the bacteriological analysis of water

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THE STUDY OF MEDIA
EMPLOYED IN, OR SUGGESTED FOR,
THE BACTERIOLOGICAL ANALYSIS OF WATER

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INTRODUCTION

The presence of members of the Escherichia-Aerobacter group in water has long been recognized as an efficient measure of the safety of the water. This necessitated the use of some medium or media which would show the presence of these organisms even if they were in mixed cultures. Standard Methods have attempted to point out and make a survey of any such media in order to determine the best methods to ^{be} used in routine water analysis.

The Standard Methods Procedure, since its inception in 1904, has been criticised for certain weaknesses; lack of specificity, permitting of false presumptive tests, and encouragement of the growth of weak Escherichia coli which are reported to be of no significance. As a result there have been other media suggested; Brilliant green lactose peptone bile, Methylene blue-erythrosine-brom cresol purple broth, Crystal violet broth, Lactose broth-buffered, Ferrocyanide citrate agar, Methylene blue, erythrosine, brom cresol agar, Bile salt agar, and Sulfite glucose agar.

It is the purpose of this thesis to study the behavior of certain organisms expected in water on some of these media.

REVIEW of LITERATURE

Standard Methods

At a meeting of the American Public Health Association in Montreal, Canada, in 1894, the Committee on the Pollution of Water Supplies closed its report with the suggestion of a cooperative investigation into the bacteriology of water supplies. This suggestion was approved by the association and a sub-committee was appointed to make a survey of bacteriological methods then in use. The sub-committee undertook to determine methods of laboratory practise to be adopted in routine water analysis. Because of the wide variation in the general laboratory practise, they called for a convention of American Bacteriologists to be held in New York City, in 1895. Here the variations in practise were discussed and another committee was appointed to make definite recommendations as to the practises to be adopted. This committee reported in 1897 at the Philadelphia Meeting of the American Public Health Association.

In this report (54) they state their aim thus: "The recommendations thus do not indicate the previous procedures in all details of any single member of the committee, but are a concord of what has appeared best in the methods and techniques of all the members and of bacteriologists generally.--- The committee freely admits that there may be other and better methods than those here detailed. It has on the other hand, striven to recommend what in the present state of our knowledge would seem to be best and most likely to gain

acceptance.--It does not demand of bacteriologists in general--that these and only these methods shall be employed. It does ask that where new species are being studied for publication the procedure here recommended be given a fair trial.--It publishes them in the hope that by this act it will direct attention to the urgent need now existing for full and accurate description of species of bacteria in which the items have been determined by methods common to the main body of workers, and as a consequence are capable of verifications and control.--In a science so rapidly developing as bacteriology, it need scarcely be said that any attempt to present the best technical procedures can only apply to the existing state of science; and that much will be added and much corrected in the near future." This report was published in 1898 and was widely used as a standard method for laboratory procedure.

In 1899 a committee was appointed to extend the standard procedures to include all lines of investigation involved in the analysis of water. They undertook to discover the standard practise not only in bacteriological but also in the chemical, physical, and microscopic examination of water. Several progress reports were made by this committee.

In 1901 the committee was instructed to revise the 1897 report of the Bacteriological Committee, in order to remove from it those features not found to be of general use, and to include such new methods as later development had justified.

In 1905 the first volume of Standard Methods of Water

Analysis was published under the title, "Report of the Committee on Standard Methods of Water Analysis to the Laboratory Section of The American Public Health Association." In this report much more prominence was given to the test for the Escherich-Aerobacter group than to the test for organic matter which was determined by chemical tests. The use of tests for Esch. coli in examining water had been first suggested by Theobald Smith in a report, in 1893, on the use of dextrose broth for isolating Esch. coli. The committee also stated that with chemical methods it is impossible to separate that portion of the organic material which is of no sanitary significance from that which is associated with pollution. In this edition, as in the 1897 report, they expressed the hope that while a system of standard methods, if used improperly, might slow up progress, every effort should be made to continue to improve the technic of water analysis.

In 1907, it was decided that Standard Methods needed revision. Two committees were appointed, one for the chemical and the other for the bacteriological methods. In 1912, a second edition of Standard Methods (52) was published by them. Previous to this time glucose broth had been used for the detection of the Escherichia-Aerobacter group, but in this edition it was suggested that a lactose bile broth consisting of 10 per cent fresh dry ox-bile, 1 per cent lactose, and 1 per cent peptone be used for the presumptive test as indicative of the colon group as a whole. The procedures recommended for testing water samples were; isolation by direct plating in litmus-lactose agar for polluted waters or for

relatively pure waters a preliminary cultivation in glucose broth followed by the same method of isolation, and identification by various tests for Escherichia or Aerobacter.

In the preface (5^o) this statement occurred: "In order to study the various bacteria of fecal origin a study has been made of the Escherichia coli group, and this group has been subdivided into four divisions. The quantitative tests for the B. coli group have been considered, and the dilution method in fermentation tubes has been recommended. In quantitative tests for general gas producing bacteria in water or sewage the use of liver broth has been suggested, and a number of comparative studies set forth in the report concerning the rejuvenation of attenuated forms in this method. Tests for B. sporogenes and E. typhi in drinking water are considered, and the report of the committee ends with the consideration of isolation of specific disease germs from water supplies.--".

The committee which had published the second edition **still was continued** and it was understood that a revised report should be made in about five years. The two committees together with committees from the American Chemical Society and the Society of American Bacteriologists prepared and published the third edition in 1917. The bacteriological section was entirely rewritten and dealt with the isolation and identification of the Escherichia-Aerobacter group only, since the committees felt that the isolation and identification of other species of bacteria did not come within the

province of a standard method for water testing.

The procedure given(53) for the isolation of the Escherichia-Aerobacter group was: presumptive test, by gas production in lactose broth; partially confirmed test, by development of typical colonies on Endo's or litmus-lactose agar plates which had been streaked with a loopful of fluid from the presumptive lactose broth tubes; completed test, by gas production in lactose broth tubes inoculated from the typical colonies on the confirmatory plates, by aerobic growth on nutrient agar slants inoculated from the same agar plate colonies, and by evidence of non-spore-formation as shown by a microscopic examination made from the agar slant cultures.

The following methods were given for further identification of fecal and non-fecal members of the Escherichia-Aerobacter group:

| | | | | |
|------------------------|---------------|----------------------------|-------------------------------------|------------------------------------|
| methyl red | + | - | - | - |
| Voges Proskauer | - | + | + | + |
| gelatin liquefaction | - | - | - | + |
| adonite fermentation | - | + | - | + |
| sacchrose fermentation | usually - | + | + | + |
| indol production | usually + | usually - | usually - | usually - |
| | B.coli, fecal | .aerobac- enes fecal | B.aerobac- enes non- fecal | B.cloacae fecal or non-fecal |

In 1920 another revision of the methods was published in which there was little change in the bacteriological section. In 1923 a fifth edition was published in which the chemical section was entirely rewritten. In the bacteriolog-

ical section the most important modification was the elimination of litmus-lactose agar and the addition of eosin methylene blue agar in its place. The committee expressed the belief that the whole section needed rewriting.

In 1925 a sixth edition was published jointly by the American Water Works Association and the American Public Health Association, with the American Chemical Association cooperating on the chemical section. In this edition the bacterial section was still not revised and was basically the same as in the third edition.

In 1933 a seventh edition (54) was published. There were several additions made: for unfinished waters a method was given as standard using parallel planting in both lactose broth and brilliant green bile for a presumptive test, with confirmation in brilliant green bile of the positive tests in lactose broth. In the appendix several non-standard methods were given. These included for primary planting; methylene blue-erythrosine-brom cresol purple broth, crystal violet broth, buffered lactose broth, and ferrocyanide citrate agar. A methylene, blue, erythrosine, brom cresol purple agar and a cellibiose test were given for differentiation of *Escherichia* and *Aerobacter*. Several methods used by English Bacteriologists were also included.

False Presumptive Tests.

False presumptive tests, that is water samples giving positive presumptive tests, but failing to be confirmed for the Escherichia-Aerobacter group, have long been found in testing waters. Originally, when glucose broth was used as the presumptive medium there were many such false tests due to the large number of species of bacteria able to produce acid and gas in dextrose broth. It was observed that there were fewer species which could produce acid and gas in lactose broth and, therefore, lactose broth was adopted, in 1917, as the presumptive medium and has been used as such since that time. During this period many false presumptive tests have been found in analyzing water and it has been observed that there are several other species of water bacteria beside the Escherichia-Aerobacter group which can either singly or in combinations produce acid and gas from lactose.

Meyer (35) found an aerobic spore-forming organism which was able to produce acid and gas in lactose broth. He was able to isolate this organism at least eight times from tap water. Ewing (9) confirmed this by isolating an aerobic spore-bearing organism from a false presumptive lactose broth tube. Greer (11) (12) also found this organism in raw and chlorinated water and infrequently in sewage. More of this type of bacteria were found in treated than in raw waters. Hinman and Levine (17) and Lisk (31) also have reported the finding of aerobic spore-bearing bacilli which could produce gas from lactose. Ginter (10) found an aerobic spore-bearing organism

which formed gas from lactose and produced a coli-like growth with sheen when streaked on Endo's agar. Koser and Shin (24) in describing such an aerobic spore-bearing organism which had been isolated by them stated that this organism greatly resembled *Aerobacter aerogenes* on Endo's or eosin methylene blue agar plates. Hinman (16) reported that with chlorinated water 80 per cent of the samples examined gave false presumptive tests due to the presence of spore-bearing organisms which were not inhibited or killed by the chlorine.

An anaerobic spore-forming organism later found to be identical with *Clostridium welchii* was isolated in England. This organism produced acid and gas from lactose broth and was present in both sewage and water. Winslow and Belcher (65) and later Greer (11) found this organism present in water. Greer (12) found *Cl. welchii* present in sewage, and in raw and treated waters. The organism was often present in larger numbers than was *Escherichia coli*, especially in treated waters. Raab (42) reported that with treated waters 90 per cent of the positive presumptive tests were not due to the *Escherichia-Aerobacter* group and that most of the false tests were due to *Cl. welchii*. He even suggested that with this type of water attention might well be given to developing a test for anaerobes as an index of pollution. Spray and Laux (51) also have reported the finding of anaerobic lactose-fermenting organisms in water which was known to be contaminated with sewage.

There are a few other species of bacteria occasionally found in water which are capable of producing both acid and

gas in lactose broth but these due to their scarcity are of **no great importance** in testing for the purity of water.

Not all false presumptives are due to bacteria producing gas from lactose. Many false presumptive tests are due to two species working together to produce a fermentation from lactose which neither could produce alone. This is called synergism. Sears and Putnam (49) found that several pairs of organisms working together could produce acid and gas in lactose broth. They found the following pairs produced 5 per cent or more gas during five days incubation in lactose broth:

| | |
|------------------------|--------------------------------|
| Staphylococcus aureus | with Salmonella schottmulleri, |
| Staph. aureus | with Proteus vulgaris, |
| Streptococcus faecalis | with Sal. schottmulleri, |
| Strep. faecalis | with Sal. morganii. |

They found some difference in the amount of gas with various strains of the same organism, some strains even failing to act as one of a synergistic pair. In general they reported that a synergistic pair consisted of an acid-forming organism which decomposed the lactose into a simpler substance, and an organism which attacked this simpler substance producing gas. They found that both organisms must be present at the same time in order to produce both acid and gas.

Dunham, McCrady, and Jordan (8) reporting on combinations of organisms stated that symbiotic fermentations result from complexes which usually contain at least one organism capable of forming acid from lactose, Gram positive, non-motile, non-sporing cocci, usually streptococci, which grow on ordinary culture media, and another symbiout capable of producing gas in dextrose broth but not in lactose.

While studying European Baker's Yeast, Castellani found

two or more yeasts present and several bacterial species which could produce acid but no gas in the sugars employed. These mixtures were able to produce acid and gas from many more sugars than any of the individual species could. He also found that taking pure laboratory cultures of the above species and using them in combinations he could obtain more active fermentations than with any one of the single species. He concluded that in such synergistic combinations the most important condition necessary was that the second organism be able to produce acid and gas from the carbon compound, usually glucose, which the first organism was able to form from lactose.

Holman and Meekinson (18) reported that the following pairs of organisms produced both acid and gas from lactose:

| | |
|------------------------|--------------------------------|
| Staphylococcus aureus | with Salmonella schottmulleri, |
| Staph. aureus | with Proteus vulgaris, |
| Streptococcus faecalis | with Sal. schottmulleri, |
| Strep. faecalis | with Proteus vulgaris. |

They also found that the two species must be in close association in the media if any synergistic results were to be obtained. Thompson (57) and Ritter (43) both reported symbiotic pairs as the cause of many false presumptives. Leitch (27) studied a number of non-lactose-fermenting bacteria isolated from water in combinations with other species of bacteria. He found that acid and gas were produced from lactose broth with these combinations and concluded that such combinations were common in water and probably were the cause of false presumptive tests.

Thompson (57) stated that 90 per cent of all false presumptive tests were due to the above causes; spore-bearing

organisms, and symbiotic pairs. However, the other 10 per cent were due to Escherichia-Aerobacter organisms being present and giving a positive presumptive test but being lost in the process of confirmation. He thought that this type were by far the most important of any of the false presumptive tests, since in these cases the test should actually have been positive for the Escherichia-Aerobacter group indicating pollution. Butterfield (4) stated that in his experience the reason for false presumptive tests was not other gas-producing organisms but the failure of Esch. coli present in the presumptive medium to be confirmed.

Young and Greenfield (68) found in soil stored in such a manner that it was exposed to rain and air, but to no other source of contamination, that both Escherichia coli and Pseudomonas fluorescens were present during the fall of the year. Later in the winter only Esch. coli were found and these in greatly reduced numbers. In the spring and early summer Ps. fluorescens was again present and overgrew the Esch. coli. In a test where the soil was kept a hundred per cent saturated with water the Esch. coli were so overgrown by the Ps. fluorescens that the former could not be recovered from the soil after a short time. They concluded that the amount of moisture present had a great effect on the inhibitory action of Ps. fluorescens for Esch. coli growth in soils. The effect of soil moisture may be indicated by the work of Thompson (57) and Grear (12) who have reported the greatest number of false presumptive water tests to be during the months from October to April, a period of great moisture in the soil.

Greer (13) found that *Pseudomonas aeruginosa* tended to overgrow *Escherichia coli*. This overgrowth usually occurred after 24 hours incubation, the *Esch. coli* being present and active up to 20 hours incubation in lactose broth. After this time *Ps. aeruginosa* often inhibited any gas production and frequently no *Esch. coli* could be recovered upon transfer to another medium. Similarly Greer found that *Ps. aeruginosa* did not have any inhibitory effect on *Clostridium welchii* until after the *Cl. welchii* had produced gas from the lactose, but that then it did frequently inhibit the further growth of *Cl. welchii*. Ritter (43) found that *Ps. fluorescens* and *Ps. aeruginosa*, both, overgrew and sometimes masked *Esch. coli* on Endo's agar plates. Lewis (30) found that in an old sterilized culture of *Ps. fluorescens* there was an inhibitory substance which when added to a nutrient broth culture of certain bacteria could easily inhibit further growth, but that the *Escherichia-Aerobacter* organisms were not inhibited by the addition of 20 percent of this sterilized broth culture of *Ps. fluorescens*.

Prescott and Baker (41) discovered that the Streptococci found in water outgrew *Escherichia coli* when both were incubated in dextrose broth. *Esch. coli* were found in 12 hours, after this time, however, the Streptococci outgrew and inhibited the growth of the former organism. Winslow and Hunnewell (67) observed that when both *Esch. coli* and *Strep. faecalis* were present the former was responsible for dextrose broth fermentation, but were later outgrown by the latter organism. These investigators also had found colonies on litmus-lactose plates

which, while they resembled *Esch. coli*, were Streptococci. In polluted water they observed that these Streptococci seemed to outgrow and kill themselves so that not nearly the number expected were ever found. Greer (13) reported that in a mixture of the above mentioned types the *Esch. coli* were active up to 24 hours, but were outgrown later by the *Strep. faecalis*, especially when the latter outnumbered the former in the original planting.

Smith and Smith (50) reported that they found that *Esch. coli* was able to produce acid but no gas from lactose in the presence of *Salmonella schottmulleri*. In studying this inhibitory action they found that removal of the *Sal. schottmulleri* organisms with kaolin or by Berkfeldt filter destroyed the inhibitory agent in the broth, and that the addition of the organisms left on the filter to a fresh lactose broth culture of *Esch. coli* had no effect on the growth of the latter. Heat also destroyed the inhibitory agent.

It had been reported that when both *Clostridium welchii* and *Escherichia coli* were present in lactose broth, the former outgrew the latter giving a false presumptive test. Greer (13) found a great variety of results when these two types were both present in lactose broth. He concluded that the final result depended on several factors: the number of organisms present in the inoculating material, the age of such material, variations in strain, and the relative aerobic or anaerobic conditions. Greer also found that *Esch. coli* consistently outgrew any aerobic, lactose-fermenting, spore-bearing organism.

Thompson (57) suggested that the *Escherichia-Aerobacter*

group might be present and yet not be confirmed ^{due} to its own production of sufficient acid to bring the pH to a point lower than the lethal pH for the group.

In spite of all the results obtained and investigations carried on, false presumptive tests are still a great problem and experiments are being conducted to find out still more definite facts about these false tests and to provide some means of eliminating more of them. Various investigators have suggested many media to be used for this purpose. Immediately following is a historical review of some of the media recommended for water analysis.

Media

Brilliant green bile: Bile and bile salts were early used in an attempt to find a medium which would differentiate between the colon bacteria (*Escherichia coli* and *Eberthella typhosa*). MacConkey in an article (53) published in 1908 stated that bile salts were first used in 1889 by Th. Limbourg who used the sodium salt of cholalic acid in a peptone-pancreas extract medium for the study of feces. In the same article MacConkey mentioned that Leuberscher in 1890 first used pure bile and found it a good medium for *Salmonella schottmulleri*.

In this early period there were many conflicting results obtained, but nearly all investigators found a bile medium good for growth or isolation of *E. typhosa*. A medium that was often used in these experiments was that of MacConkey (7), which consisted of 1 per cent glucose, 0.5 per cent sodium taurocholate, 2 per cent peptone, and water. Jordan and co-workers (22) published an article on the typhoid organism in which they concluded that the modified MacConkey's medium used by them for isolating the organism from sewage was good for this purpose, but that it also had no restraining action on the ordinary sewage bacteria.

Jackson (20) was one of the first in this country to use a lactose bile medium for water analysis work. He found that most species of bacteria other than the *Escherichia-Aerobacter* group were killed or inhibited in this medium. By tests he proved that the cholic acid radical was the agent responsible for this inhibiting action. Therefore, he concluded that

a concentration of sodium glycocholate equal to the concentration of this salt in fresh bile was necessary to make an efficient medium. Previous to this erroneous results had been obtained using "Smith Solution" which consisted of broth and sodium taurocholate. In heavily polluted waters the Esch. coli were over grown by other bacterial species. Jackson concluded that these results were due to the low concentration of bile salt. He found that with his own medium which consisted of 1 per cent lactose and bile salt in the same concentration as in fresh ox-bile all tubes containing 25 per cent gas were positive for Esch. coli, and that no mixtures of organisms except those containing Esch. coli gave as high as 20 per cent gas. There was no overgrowth of the Escherichia-Aerobacter organisms by other species in this medium.

Sawin (48) corroborated Jackson's results finding that with the lactose-bile medium the percentage of gas was about 39 per cent whenever Escherichia-Aerobacter organisms were present and never that high in their absence. Sawin, as did other early investigators, tried to determine for each bacterial species a definite percentage of gas which would hold for the one species only. The value which he tried to show was specific for Esch. coli was 39 per cent.

Others did not agree with the findings of Jackson. Weston and Tarbett (63) found that a lactose-bile medium was little better than the dextrose broth which was standard at the time. They found in studying raw and unfiltered water that 24 out of 63 samples were positive in dextrose broth and negative in lactose-bile broth. However, of these only 30 per cent were

at all like Esch. coli on litmus-lactose agar confirmation plates. Weston and Tarbett concluded that glucose was not as specific as the lactose-bile broth, but that the lactose-bile broth might inhibit some coli-like forms.

The investigators in the period up to 1912 agreed for the most part that gas formation in lactose-bile broth was a more definite proof of the presence of coli than gas formation in dextrose broth. In the 1912 edition of Standard Methods (52) a lactose-bile broth was recommended as the standard medium to be used as a presumptive test for the colon group as a whole.

Some workers still pointed out that there were less gas-formers recovered with the lactose-bile broth than with plain lactose broth. It was also suggested that with a bile agar less Escherichia coli were found than with the standard litmus-lactose agar. In 1913 Jordan (21) found that from one third to one half of the viable Esch. coli were inhibited by fresh ox-bile added to lactose broth or to nutrient agar, the amount of inhibition varying with the strain of Esch. coli and the type of bile used. He found no difference in inhibition between attenuated and fresh strains, thus disproving some contentions that the Esch. coli inhibited were weak, attentuated forms of no sanitary significance. He did find that, while bile inhibited some Esch. coli, 25% gas production in lactose-bile broth was an almost positive proof of their presence.

Following this there were many investigators who obtained irregular results when using lactose-bile media.

Obst (39) decided that the freshness of the bile accounted for some of these irregular results. She found that bile carefully collected and held at 1°C. could not safely be used after one weeks storage. Dried bile was found to be expensive and to vary in composition. She concluded that a plain lactose broth was much easier to obtain and was equally efficient. She found that more gas producing organisms, (especially at high dilution, were isolated with lactose broth than with lactose-bile broth.

Bunker (3) stated that with relatively pure waters, containing few Esch. coli, the organism could be recovered from lactose broth containing 10 per cent bile, 5 per cent bile, 2 per cent bile, and no bile. Upon storing the water, Esch. coli could be obtained over a longer period from the medium containing no bile. In heavily polluted waters the exact opposite was true, Esch. coli recovery after storage of the water being best in the 10 per cent bile-lactose broth. He concluded that with gross pollution lactose allowed overgrowth of Esch. coli by other strains and was not suitable for such waters. However, with slight pollution lactose broth gave a much more delicate test for Esch. coli than the lactose-bile broth did. He used a weak dilution of bile since he believed that the dilution to be most effective would be that found normally in the intestines. He concluded that both bile and lactose should be included in the presumptive test.

Nevertheless, due to the great number of experiments in

which lactose was found to give a greater number of gas-formers than the lactose-bile broth, the third edition of Standard Methods (55) recommended the use of plain lactose broth in the place of lactose-bile broth.

Following this the use of bile was still carried on experimentally by many workers. A Japanese investigator, Koyania, (25) brought out the idea that bile from different animals had different effects. He found ox-bile had no effect on the growth of *Esch. coli* or *L. typhosa*. Ritter (44) confirmed Bunker's work that the use of both lactose and lactose-bile was efficient in identifying *Esch. coli*, acid and gas production in both lactose broth and lactose-bile broth was confirmed as caused by *Esch. coli* in 75 per cent of all cases, and 97 per cent of the cases where the gas tubes were positive in 24 hours.

Salter (47) found that *Esch. communior* was stimulated by use of 0.5 per cent less bile salts but the same organism was inhibited by a higher concentration up to 1 per cent. He also found that the type of bile salt used, and the variety of bile, altered the results. This would tend to explain many of the earlier conflicting results.

Levine (29) summed up the previous work thus: "1. Lactose-bile is a more reliable presumptive test but a greater proportion of the colon group may be detected in preliminary enrichment in lactose broth. 2. If the proper concentration of bile salts could be determined, the bile medium would probably be preferable. For the present, considering the difficulty of obtaining bile of constant composition, or

the chemically pure salts, and in view of our insufficient knowledge as to the optimum concentration of bile salts, it seems best to employ lactose broth since a more uniform medium may thus be obtained in different laboratories. It is very probable that if a standardized evaporated bile were available a concentration of 1 to 2 per cent in lactose peptone water would be superior to lactose broth. "

Previous to this various investigators, Hall and Eilefson (15), and Bronfenbrenner and co-workers (2) had suggested the use of various aniline dyes to make the media more selective. These investigators had used gentian violet and rosolic acid. In 1916, Teague and Churman (56) used brilliant green, the commercial salt of tetraethyl-diamine-triphenyl-carbinol, in a medium for inhibiting *Esch. coli* and isolating *Eberthella typhosa*. They found that brilliant green in 1/3000 per cent concentration added to nutrient agar made a good medium for *E. typhosa*, and that some *Esch. coli* strains grew on this medium. Salter (47) found that a concentration of 1/6000,000 to 1/10,000,000 brilliant green inhibited *Esch. communior* to some extent but not wholly.

Muer and Harris (36) used brilliant green lactose bile as a presumptive test for water. They found that a dilution of 1/10,000 of the dye prevented growth of *Clostridium welchii*, *Cl. sporagenes*, and several other anaerobes, but had no restraining action on *Esch. coli*. They found that *Esch. coli* was not entirely inhibited by a dilution of 1/200, but was completely inhibited by a dilution of 1/100. They found *Esch. coli* in more cases with brilliant green lactose bile than

with plain lactose broth. Colonies isolated from this new medium were nearly pure Esch. coli. Brilliant green added to lactose broth without bile made a very unsuccessful medium for isolating Esch. coli. Their medium consisted of 5 per cent ox-tall, 1 per cent peptone, 1 per cent lactose, and 0.1 per cent brilliant green.

Winslow and Dolloff (10) found that a medium containing 5 per cent sodium chelate, was more favorable than lactose broth since more gas was formed at all the pH values employed (5-8) in 24 hours in the coils than in 48 hours in plain lactose broth. They tested the toxicity of gentian violet, rosolic acid, and brilliant green. In lactose, brilliant green was toxic in a dilution of 1/100,000 to 1/1,000,000. In lactose bile, this extreme toxicity was lost and 1/1000 dilution of brilliant green was toxic to most of the Escherichia-Aerobacter group and 1/100 to Acro-trochones.

McCrary (11) found that brilliant green lactose bile broth inhibited practically all spore-forming bacteria but was not as sensitive in detecting all of the Escherichia-Aerobacter group as lactose broth. Jordan and McCrary (8) found that the use of a 1/10,000 brilliant green lactose bile as a confirmatory medium eliminated the need of using the Endo plates. Lactose broth gave more presumptive tests but a much lower percentage of confirmations than the brilliant green lactose bile. The latter medium inhibited some weak Esch. coli but also did away with the need of many Endo plates by eliminating false presumptive tests due to spore-forming bacteria.

Most of the later workers who have used this medium have reached similar conclusions. Hale (14) stated similarly that: brilliant green bile eliminates all aerobic and nearly all anaerobic spore-forming organisms, 12 per cent more tubes were confirmed for lactose broth than for brilliant green bile, brilliant green bile was specific for Escherichia-Aerobacter in relatively pure waters, but not so specific in highly polluted waters. He recommended the use of brilliant green bile in water work, stating that in raw and polluted waters it eliminates attenuated forms of Escherichia-Aerobacter group which he considered were of no sanitary significance, and that in pure waters it is very specific for the Escherichia-Aerobacter group.

Jordan (23) reviewed the whole situation. He concluded that:

1. Lactose broth was too nonspecific.
2. Brilliant green bile was specific but not too selective.
3. Bile was inhibitory in 10 per cent concentration but in 1 to 2 per cent concentration it accelerated Escherichia-Aerobacter group.
4. Brilliant green bile made a specific medium for the Escherichia-Aerobacter group, while it was inhibitory for many other types of water bacteria.

He suggested for water work a parallel planting into both lactose broth and brilliant green bile for a presumptive test, and the planting of all lactose positive tests which

were brilliant green negative into secondary brilliant green bile tubes for confirmation. McCrady (33) in criticising this, stated that he considered a primary planting in lactose broth with confirmation in brilliant green bile would be a better test for the Escherichia-Aerobacter group.

As a result of all this study with brilliant green bile, the 1933 edition of Standard Methods (54) included it as a medium which can be used as outlined by Jordan(37) , for "unfinished waters".

Farr and Caldwell (40) using a dilution of 1/75,000 of brilliant green with 3 per cent bile found that brilliant green bile in comparison with lactose broth gave fewer positive presumptive tests but a greater per cent of the presumptive tests were confirmed. Contrary to other investigators, they found that brilliant green bile did not inhibit anaerobes.

Butterfield (4) disagreed with most of the previous findings. He reported, using the same medium as Farr and Caldwell, that 16 per cent less Esch. coli were recovered than were recovered with lactose broth. In effluent waters the per cent of Esch. coli lost went up to 60. In the parallel planting method of Jordan there was only 5 per cent difference between the two media, but this varied greatly with the source of the water. He also disapproved of the parallel planting method because it confirmed the Escherichia-Aerobacter group with the use of only two characteristics, namely the production of acid and gas from lactose in the presence of brilliant green and bile, while Standard Procedure uses four

characteristics: non-spore -formation, aerobic growth, and the fermentation of lactose with the production of acid and gas.

Ruchhoft and co-workers (45) found that brilliant green bile as a confirmatory medium was as efficient as eosin methylene blue agar. Winslow (64) concluded a review of this medium by stating that on the average it gives about 4/5 as many positives as Standard Methods. It gives more positive results with the more polluted waters and fewer with less polluted waters. He considered that ,while brilliant green bile gives results which differ from those obtained with Standard Methods, these results may give a more accurate estimate of the pollution.

So far the use of bile in water works practise has not been very sucessful, but with the addition of brilliant green to lactose bile, the medium which had previously been considered best, it has seemed to be quite selective, but not too selective, for the Escherichia-Aerobacter group. Since its addition to Standard Methods investigators have again begun to study it critically to see if they can either find it to be the best medium so far known for Escherichia-Aerobacter determination, or else show that it is not reliable and can again discard it.

Dominick and Lauter: Salle (46), in 1929, reported an agar which he had used. This was a lactose agar to which had been added erythrosine, brom cresol purple, and methylene blue. The amount of lactose was so limited that while there was enough to satisfy the Escherichia coli organisms, there was

not enough for the aerobacter aerogenes organisms. The Esch. coli attacked the lactose and produced a color change with the brom cresol purple, from purple to orange or yellow, and also a metallic sheen. Because of the limiting amount of lactose the Aero. aerogenes could not produce these color changes.

Later in the same year Dominick and Lauter reported on a fluid medium containing the same materials. They were searching for a medium whose use would be less time consuming than Standard Methods, but as accurate. They found that the Aero. aerogenes group were responsible for the presumptive tests which were not confirmed and caused useless labor and a waste of time. They tried a methylene blue-erythrosine-brom cresol purple broth and found that the results were accurate, there being 100 per cent correlation between the confirmed lactose tubes of Standard Methods and the presumptive tubes with their new medium. Dominick and Lauter found that both their medium and brilliant green bile gave results in the presumptive test comparable with the confirmed test of Standard Methods. They suggested the use of this methylene blue, erythrosine, brom cresol purple broth as a completed test which needed no confirmation.

Later workers tried to use it as a substitute for lactose broth in the presumptive test of water analysis. Leahy (26) compared it with Standard Methods lactose broth and found that 98 per cent of the positive presumptive tests in Dominick and Lauter medium were confirmed as Escherichia-Aerobacter, while only 67 per cent of the positive presumptive lactose broth tubes were confirmed. With the Dominick and Lauter medium all positive presumptive tests were obtained in

24 hours, while with the lactose broth many positive presumptive tests were delayed to 48 hours. Howard (19) compared the Dominick and Lauter medium with brilliant green bile and with lactose broth. In raw waters there was no difference between the media as to the per cent of tests confirmed. In filtered waters lactose broth gave slightly better results. In chlorinated waters lactose broth gave a lower per cent of positive presumptive tests, but a much lower per cent of confirmed tests, than the Dominick and Lauter medium. Knolte and Kramer (38) found in a similar study that the Dominick and Lauter medium was very superior in "finished waters", superior in filtered water, and nearly the same as lactose broth in raw waters.

Butterfield (4) stated that, while most of the previous reports had been favorable, he found the Dominick and Lauter medium to be very unsatisfactory since it inhibited many Escherichia-Aerobacter strains in all of the water dilutions except the lowest. Stewart (55) at nearly the same time reported that this medium gave an accurate presumptive test in 24 hours and also a partial confirmation because of the color change and was, therefore, a time-saving medium.

In spite of the fact that this medium was still the subject of much controversy, it was included in the 7th, edition of Standard Methods (54) as a non-standard medium. In 1934 Lauter (64) stated that, in an effort to save time and expense he had been using this medium for a period of nearly 5 years as a supplement to Standard Methods in testing filtered water.

He found by confirmation with Standard Procedure that 99 per cent of all positive presumptive tests in the Dominick and Lauter medium were confirmed and that most of these presumptive tests were positive in 24 hours. The test could be completed with 99 per cent accuracy in 24 hours with the use of only the one medium. He recommended the Dominick and Lauter medium as a direct inoculation medium, especially for filtered waters.

MacConkey's bile salt agar: MacConkey (32), in 1905, published the formula for a bile salt medium which he used to stimulate the growth of *Eberthella typhosa*. This consisted of 1 per cent glucose, 0.5 per cent sodium taurocholate, and 2 per cent peptone in distilled water. He used this as a fluid medium and by adding agar made a plating medium. In 1907, he (33) reviewed the previous use of bile salts in media, and reported that most investigators had found bile salts to be stimulating to *E. typhosa* and other bacteria characteristic of fecal pollution. He also mentioned that Conrad had found that a nutrient substance and a simple solution of bile salts made a good medium for the growth of *Escherichia coli*. MacConkey had been experimenting with bile and bile salts since 1897, when he had originated a bile salt-potato juice medium. From this medium he had developed a bile salt-sugar (lactose or glucose) broth and suggested this as an efficient medium for testing for fecal pollution in water. In 1907 he reported the formulae for a bile salt- lactose agar and a glucose-bile salt solution, both similar in composition to the formula al-

ready given . He explained that acid production from the lactose by the *Escherichia-Aerobacter* organisms caused a precipitation of the bile salts, thus forming a haze around each colony. He later modified this lactose-bile agar by adding neutral red as an indicator, since Grunbaum and Hume had pointed out that the neutral red indicator showed the acid-producing colonies better than did the haze of precipitated bile salts.

MacConkey found that no bacteria characteristic of fecal pollution were inhibited by this medium, but that soil and air bacteria were in general inhibited. *Eberthella typhosa*, *Salmonella schottmulleri*, and *Escherichia coli* grew well, but *Streptococci* often found in polluted waters varied in their ability to grow on this medium.

Salter (47) confirmed MacConkey's results by finding that *Escherichia communior* was stimulated by the use of 1 per cent to 0.5 per cent of bile salts, but was inhibited by higher concentrations of the salts. This varied with the type of bile salts used. Mac Conkey's bile salt agar is still used in England and is included in the last edition of Standard Methods of water analyses as an example of English water works practice.

The above media depend for their effectiveness upon the addition of substances either stimulating to the *Escherichia-Aerobacter* group, or inhibiting to other common water species of bacteria. A second group of selective media suggested for water analysis consisted of synthetic media in which the sources of nitrogen and carbon were simple substances. This type of medium inhibited many species of water bacteria.

Tonney and Noble: In 1918 Ayers and Rupp (1) suggested a synthetic plating agar consisting of 1 per cent lactose, 0.4 per cent sodium ammonium phosphate, 0.2 per cent acid potassium phosphate dissolved in distilled water, and used in equal portions with a 3 per cent agar solution. They used basic fushsin as an indicator of acid production. They found that the Escherichia-Aerobacter species and a few molds were the only organisms that would grow on this medium. The molds were not at all similar to the Escherichia-Aerobacter colonies. They concluded, therefore, that their medium was very specific for the Escherichia-Aerobacter group and they used it in working with water samples since they believed that this group could use much simpler substances as sources of carbon and nitrogen than could the other water organisms. Cannon (5) confirmed the results of Ayers and Rupp. He found that their medium was very specific for the Escherichia-Aerobacter group and was very good for determining the number of Esch. coli present.

In 1928 Noble (37) suggested a new synthetic medium, his so-called ferrocyanide-citrate agar. This consisted of the same ingredients as the Ayers and Rupp medium with the addition of ferric citrate and potassium ferrocyanide. He used this medium for making direct counts of the Escherichia coli and Aerobacter aerogenes organisms present in water. He published several favorable reports on his progress with the use of this medium.

Beginning in 1931 there appeared a series of articles by Tonney and Noble giving results obtained with this medium and

modifications of it. In using the medium for direct plating of water they found that the typical colonies, when studied by comparison with other tests such as the methyl red, Voges-Proskauer, sodium citrate, and eosin methylene blue agar, consisted of only *Esch. coli* and *Aero. aerogenes* organisms. As used for a direct counting method this medium shortened the time and labor in determining the purity of water.

From results of their investigation, Tonney and Noble (59) reached the conclusion that *Escherichia coli* were from a definitely fecal source while *Aerobacter aerogenes* were more likely to be from a non-fecal source. In another study they (60) discovered that in the cases observed, *Aero. aerogenes* increased in water at a much faster rate than *Esch. coli*. Since on the ferrocyanide-citrate agar the two types could be differentiated, Tonney and Noble concluded that it was a very efficient medium to be used in water analysis. It is interesting to note that in these experiments the work of early investigators was also confirmed by finding that the *Escherichia-Aerobacter* group were of the most sanitary significance of any of the species of water bacteria since they survived longer than any other water types.

In 1932 Tonney and Noble (61) corroborated their earlier findings by showing that water giving a high *Esch. coli* count on their medium came from a badly polluted source, as shown by a sanitary survey, while water giving a low *Esch. coli* but a high *Aero. aerogenes* count showed potential or past pollution but no heavy pollution at the time of sampling. Levine (28)

also emphasized the importance of differentiation between *Esch. coli* and *Aero-aerogenes* because he considered the former but not the latter to be of definite fecal source.

Other workers did not agree with Tonney and Noble and many obtained different results. Hale (63) stated that he found there was as much proof for the importance of *Aero. aerogenes* in a test for pollution in water as had been offered against its importance by Tonney and Noble. He, therefore, did not recommend the differentiation of the two sections of the *Escherichia-Aerobacter* group. He also found even if such differentiation were advisable that the ferrocyanide-citrate agar was no better than Endo's or eosin-methylene blue agar for separating *Esch. coli* and *Aero. aerogenes*. Nolte and Kramer (38) found in a comparison of three media that the Tonney and Noble medium was not good for raw water containing few organisms, and that with "finished or unfinished waters" it was the least efficient of the three media used. Many other workers have found it difficult to prepare this ferrocyanide-citrate agar to differentiate between the *Esch. coli* and *Aero. aerogenes* colonies upon the medium.

Ruchoft and associates (45) used the ferrocyanide-citrate agar for direct count of the *Escherichia-Aerobacter* group in water and found that while this count was slightly lower than the count obtained by Standard Methods, (Phelps index), it was very satisfactory and gave reliable results. The Tonney and Noble medium is at present given in Standard Methods of Water Analysis (54) as one of the non-standard methods for use in water analysis or in research work.

EXPERIMENTAL

Media and Methods

The media used in this study were lactose broth, eosin methylene blue agar, and Endo's medium of the Standard Methods, the Dominick and Lauter medium, brilliant green lactose peptone bile, the ferrocyanide citrate agar of Tonney and Noble, and MacConkey's bile salt agar. The brilliant green lactose peptone bile and Dominick and Lauter medium were prepared according to the directions given in the 7th edition of Standard Methods of Water Analysis(54); the MacConkey's bile salt agar according to the formula in the Manual of Dehydrated Culture Media and Reagents (34); the ferrocyanide citrate agar according to directions given by Tonney and Noble in the Journal of the American Water Works Association (58). The composition of the media follows:

Lactose Broth

| | |
|-----------------------------------|----------|
| beef extract | 3 gm. |
| peptone | 5 " |
| lactose | 5 " |
| Distilled water | 1000 ml. |
| brom thymol blue (alcoholic sol.) | 5 " |

Tube in 7 ml. amounts in fermentation tubes and sterilize at 15 lbs. for 15 mi.

The brom thymol blue solution was added in order to note the change in pH value in the medium during incubation.

Endo's Agar

| | |
|--|----------------------|
| dibasic potassium phosphate (K_2HPO_4) | 3.5 gm. |
| peptone | 10. " |
| lactose | 10. " |
| agar | 15 " |
| distilled water | 1 liter(used 500 ml) |

Tube in 100 or 50 ml. amounts in Blake bottles and sterilized at 15 lbs. for 20 min. At time of use to every 100 ml. of double strength agar add 100 ml. sterile distilled water, 0.5 gm. sodium sulfite (anhydrous), and 1 ml. of a 10 per cent filtered alcoholic solution of basic fuchsin.

Eosin Methylene Blue Agar

| | |
|--|--------|
| peptone | 10 gm. |
| dibasic potassium phosphate (K_2HPO_4) | 2 " |
| agar | 15 " |
| lactose (20% sol.) | 50 " |
| eosin (2% aqueous sol.) | 20 " |
| methylene blue(0.5% aqueous sol.) | 20 " |
| distilled water | 1000 " |

Place in Blake bottles in 150 ml. amounts. Sterilize at 15 lbs. for 20 min.

Brilliant Green Lactose Peptone Bile

| | |
|--------------------|---------|
| peptone | 10 gm. |
| lactose | 10 " |
| distilled water | 500 ml. |
| dehydrated ox-bile | 20 gm. |
| distilled water | 200 ml. |

Dissolve the peptone and lactose in the 500 ml. of water and dissolve the ox-bile in the rest of the water. Add the two solutions to each other and make the volume up to 975 ml. Adjust the pH to 7.4 (this was usually quite acid and NaOH was added to bring the media this pH value). Add 13.3 ml. of a 0.1 per cent water solution of brilliant green and make the total volume up to 1000 ml. Tube in 7 ml. amounts and sterilize at 15 lbs. for 15 min.

Methylene Blue-Erythrosine-BromCresolPurple Broth of Dominick and Lauter

| | |
|---|---------|
| lactose | 7.5 gm. |
| peptone | 12.5 " |
| beef extract | 1.25 " |
| dipotassium phosphate($K_2HPO_4 \cdot 3H_2O$) | 3.6 " |
| acid potassium phosphate(KH_2PO_4) | .5 " |
| erythrosine | 0.01 " |
| brom cresol purple | 0.016" |
| methylene blue | 0.025" |
| distilled water to make one liter | |

If less than 1 ml. is used in inoculating dilute this medium to 1600 ml.

Tube in 10 ml. amounts and sterilize at 15 lbs. for 15 min. In actual practise a 1 per cent water solution of the dyes was made and 1 ml. of 1% erythrosine, 1.6 ml. of 1% brom cresol purple, and 2.5 ml. of 1% methylene blue used.

MacConkey's Bile Salt Agar

| | |
|---------|-------|
| ox-bile | 5 gm. |
| lactose | 10 " |
| peptone | 20 " |
| agar | 15 " |

MacConkey's Agar (cont'd.)

neutral red 0.05 gm.
distilled water 1 liter

The pH should be adjusted to 7, before the neutral red is added. Tube in Blake bottles in 100 ml. amounts and sterilize 20 min. at 15 lbs. In actual practise a 0.5% water solution was made and 10 ml. of this neutral red solution used.

Ferrocyanide-citrate Agar of Tonney and Noble

Stock agar

A specially prepared agar made by Digestive Ferments Company was made up to a concentration of 1.75% distilled water. This according to their directions should be run into sterile cotton plugged bottles and sterilized in the Arnold for 15 min. In this work the distilled water was sterilized in a flask in the autoclave for 15 min. at 15 lbs. When ready for use the proper amount of agar to make a 1.75% solution was added and the whole placed in the Arnold for 15 min. After being sufficiently cooled the agar was used immediately.

Solution "A" 1 liter

| | | |
|---|-------|-----|
| anhydrous sodium sulphite (Na_2SO_3) | 4.6 | gm. |
| acid potassium phosphate (KH_2PO_4) | 16.8 | " |
| sodium ammonium phosphate ($\text{NaNH}_4\text{H}_2\text{PO}_4$) | 50.4 | " |
| Sterile distilled water | 170.0 | ml. |
| 20% lactose, sterile | 420.0 | " |
| basic fuchsin (10 gm. in 100 ml. of 95% alcohol) | 8.4 | " |
| 0.625% potassium ferrocyanide, sterile ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$) | 400.0 | " |

This solution will keep for four days if kept on ice.

Solution "B"

1.11% ferric citrate, sterile.

This will keep at room temperature except for evaporation. Sterile in these solutions means heated in an Arnold sterilizer for 15 min. only. In actual practice the water for the various sterile solutions was measured out into small flasks and sterilized in the autoclave for 15 min. at 15 lbs. This was stored at room temperature for not over three days before use. Then the lactose, potassium ferrocyanide, and ferric citrate were added to their respective flasks and heated in the Arnold for 15 min. At time of using, 9 ml. of Solution "A" should be added to each 100 ml. of melted agar which has been cooled to 75°C. and the whole agitated. Then 3.6 ml. of solution "B" should be added. In this work, approximately 500 ml. of the agar was used at one time. This was all made in one flask and proportional amounts of "A" and "B" added. Then the agar was further cooled

and used immediately.

In plating with the Tonney and Noble agar, 1 ml. of a suspension of the organism and 5 ml. of sterile distilled water were added to each petri dish. Then approximately 16 ml. of the agar was added and the whole thoroughly mixed. When the agar was firm about 5 ml. of a melted 1.75% solution of the special stock agar was poured over the surface of the plate to form an agar cover.

Organisms Employed

The organisms employed in this work were: ten strains of *Escherichia coli*, isolated from feces; ten strains of *Aerobacter aerogenes*, and one *Streptococcus faecalis*, isolated from water; three strains of *Esch. acidilactici*, one *Esch. neapolitana*, two *Aero. cloacae*, one *Aero. oxytocum*, eight *Staphylococcus aureus*, three *Staph. albus*, two *Staph. citreus*, two *Micrococci*, three *Chromo. bacteria*, three *Pseudomonas aeruginosa* and one *Ps. fluorescens*, two *Proteus* species, four *Salmonella schottmulleri*, one *Alcaligenes faecalis*, all from the stock collection of the Department of Bacteriology and Physiology, Massachusetts State College. The complete list follows:

| | | |
|-----------------------------------|------|------------------------|
| <i>Staphylococcus aureus</i> | # 51 | M. S. C. |
| " " | #52 | Yale phage susceptible |
| " " | #53 | " " " |
| " " | #54 | " " " |
| " " | #55 | Walker Yale |
| " " | #56 | Brown #60 |
| " " | #57 | Brown #60A. |
| " " | #58 | Valley Yale |
| <i>Staphylococcus albus</i> | #59 | M. S. C. |
| " " | #60 | M. S. C. |
| " " | #61 | Brown #61 A |
| <i>Staphylococcus citreus</i> | #62 | Brown #62 U |
| " " | #63 | M. S. C. |
| <i>Microoccus flavus</i> | #65 | A. M. N. H. |
| " <i>varians</i> | #66 | Bradley |
| <i>Serratia Marcescens</i> | #88 | M. S. C. |
| <i>Flavobacterium aurantiacum</i> | #90 | M. S. C. |

| | | |
|---------------------------|-------------|---------------------------|
| Chromobacterium violaceum | #91 | M. S. C. |
| Pseudomonas aeruginosa | #94 | M. S. C. |
| " " | #95 | M. S. C. |
| " " | #96 | White Delaware University |
| " flourescens | #97 | M. S. C. |
| Escherichia acidilactici | #134 | Stiles |
| " " | #136 | Clague |
| " " | #137 | F. |
| " neapolitana | #139 | A. T. C. |
| Aerobacter cloacae | #146 | Jordan |
| " " | #147 | A. T. C. |
| " oxytocum | #151 | MacLinn |
| Prtoeus americanus | #152 | Yale |
| " vulgaris | #153 | M. S. C. |
| Salmonella schottulleri | #166 | N.Y.C. Board of Health |
| " " | #167 | M. S. C. |
| " " | #168 | Bradley |
| " " | #170 | Conn. State |
| Alcaligenes faecalis | #192 | M. S. C. |
| Streptococcus faecalis | #200 | from water |
| Escherichia coli | #1- # 10 | all fecal |
| Aerobacter aerogenes | #101- # 110 | all from water. |

Methods of Inoculating Media

In these experiments fresh (18-24)Hour) agar slants inoculated from stock cultures were used as a source of inoculum. In most cases a fairly heavy suspension of the organisms was made in sterile normal saline. Endo's plates were streaked with a small loop of the saline suspension. With the poured Tonney and Noble plates dilution of the saline suspension were made. With the pure cultures, one 4 mm. loop from a hundred ml. saline suspension (made by adding a bit of the culture on an inoculating needle to 100 ml. of sterile saline) was added to a 100 ml. water blank and 1 ml. of the latter used to make the plate. With the mixed cultures 0.5 ml. of a saline suspension of each culture was placed in a sterile test tube and one small loop of this mixed suspension added to a 100 ml. water blank, and 1 ml. of this was used to make the plate.

With the MacConkey's agar a loop of the saline suspension of the pure single cultures was used. With the combination of organisms a loop of the mixed saline suspension (described above) was used.

With the fluid media a small amount of the saline suspensions was added by means of a 1 ml. pipette. This amount was measured in drops. In the single culture studies, three drops were added to each tube. With the culture combinations one drop of the suspension of each culture used was added to each tube. Three drops were approximately equal to 0.25 ml.

Part I :Pure Cultures .

The list of organisms previously mentioned contains certain species which could possibly be confused with members of the Escherichia-Aerobacter group in the presumptive test for the group, as well as certain species which have been reported in the literature as being able to cause inhibition of the tests for the Escherichia-Aerobacter group, and certain non-members of the group which, when grown in symbiosis, have been responsible for false presumptive tests in water analysis. The object of the experiment here reported was two-fold : first, to observe the ability of certain media to eliminate interfering organisms; and second, to observe whether certain non-members of the Escherichia-Aerobacter group could be confused with members of the group in any of the media employed.

Each of the strains listed was inoculated into each of the media in the manner described above. Incubation was for 24 and 48 hours at 37°C., at which time results were recorded as shown in tables 1 and 2 . The strains of one species did not vary from each other except in intensity of reactions; as for example, strains 57 and 58 of Staphylococcus aureus were able to produce as vigorous growth on MacConkey's agar in 24 hours as other strains of this organism could in 48 hours. Therefore, the results of the various strains of each species are combined in the tables and only one reaction is given for each species. Analysis of tables 1 and 2 show the following data:

Lactose broth: The medium supported the growth of all organisms in 48 hours, as was to be expected, although several

species, not of the Escherichia-Aerobacter group, failed to show evident growth in 24 hours. Aside from the Escherichia-Aerobacter group, Streptococcus faecalis was the only organism which gave gas and which growing alone would give a false presumptive test. Aero. cloacae and Strep. faecalis required 48 hours for the production of gas.

Brilliant green bile: This medium did not support the growth of any Staphylococci or Micrococci. In 48 hours there was some growth with some strains of Staph. aureus. Only members of the Escherichia-Aerobacter group and Strep. faecalis produced gas. Gas production by Strep. faecalis and Aero. cloacae was delayed to 48 hours.

The Dominick and Lauter medium: This broth failed to support any growth of Staphylococcus citreus, Micrococcus varians, Micro. flavus. Only members of the Escherichia-Aerobacter group and Strep. faecalis produced gas. There was no gas production with Aero. cloacae in 48 hours. Endo's agar plates streaked from the negative Dominick and Lauter's broth tubes showed that the organism was present, but was inhibited from producing gas.

In the brilliant green bile and the Dominick and Lauter broth the failure of growth was verified by streaking on agar slants after 48 hours incubation. Occasionally slight growth was obtained indicating that failure to grow on the two former media had been due to inhibition of the organisms, rather than to their complete destruction.

Endo's medium: Reactions of the Escherichia-Aerobacter group were apparently normal except that Aero. oxytocum resembled Escherichia rather than Aerobacter. Strep. faecalis and

Alc. faecalis resembled members of the *Escherichia-Aerobacter* group, and at 48 hours *Salmonella schottmulleri*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Proteus americanus* gave results which might have been confused with those of the *Escherichia-Aerobacter* group. No growth was obtained with the Gram-positive *Staphylococci*, and *Micrococci*, *Flavobacterium aurantiacum*, *Chromobacterium violaceum*, or *Ps. fluorescens*.

Eosin methylene blue agar: Results were nearly identical with those of the Endo's medium, the principal exception being that, while on Endo's agar, *Aero. oxytocum* and *Sal. schottmulleri* resembled *Escherichia*, on eosin methylene blue agar these species resembled *Aerobacter*. The species which failed to grow on Endo's medium, failed to grow on eosin methylene blue agar.

Tonney and Noble medium: On this medium only members of the *Escherichia-Aerobacter* group, *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, and *Proteus vulgaris* gave growth at the end of 48 hours. *Aero. cloacae* and *Aero. oxytocum* at 24 hours resembled *Escherichia* but in 48 hours they were more typical of *Aerobacter*. *Ps. fluorescens* and *Alc. faecalis* might have been confused with *Escherichia*.

MacConkey's agar: Reactions were normal for all of the *Escherichia-Aerobacter* group except that at 24 hours *Aero. cloacae* resembled *Escherichia*. *Salmonella schottmulleri*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus americanus*, and *Alcaligenes faecalis* resembled *Aerobacter* after 48 hours. A few species failed to grow and of these the most important were *Micrococci*, *Staphylococcus citreus*, and *Ps. fluorescens* which might be significant in interfering with the presumptive test.

Part II: Antibiotic Combinations.

In the literature several organisms have been reported as interfering in various ways with the presumptive test due to the Escherichia-Aerobacter group. Among the organisms are:

Pseudomonas { flourescens
 { aeruginosa

Proteus { vulgaris
 { americanus

Salmonella schottmulleri

Staphylococcus { aureus
 { albus
 { citreus

Streptococcus faecalis .

Combinations of these organisms with Esch. coli, Aero. aerogenes, and the less common members of the group were studied. Inoculation and incubation were as previously indicated. Results are shown in tables 3 and 4. Several strains of each organism were employed, but the results for any one species were uniform, and therefore were combined and recorded in the tables as if one strain only of each had been used. Analysis of tables 3 and 4 show the following data:

Lactose broth: There was no apparent inhibition of gas production by Escherichia coli or Aerobacter aerogenes. There was no interference of gas production by Aero. oxytocum or Esch. acidilactici. Esch. neapolitana and Aero. cloacae failed to produce gas in any of the combinations except with Streptococcus faecalis. In the cultures of Esch. neapolitana and Aero. cloacae which were inhibited by an associated organism, the presence of Escherichia and Aerobacter were verified by

streaking onto Endo's and eosin methylene blue agar. The streak plates showed their presence in every case.

Brilliant green^{pile} broth: Gas was produced in all cultures, but in a few instances its production was delayed to 48 hours

The Dominick and Lauter broth: Acid and gas were produced in all cultures. In a few cases gas production was delayed to 48 hours.

Tonney and Noble medium: There was no interference with the characteristic growth of the members of the Escherichia-Aerobacter group.

MacConkey's agar: The results were similar to those obtained on the Tonney and Noble medium. In a few instances there would be difficulty in differentiating between Escherichia and Aerobacter but all species would be recognized as members of the group.

24 hours. Staphylococcus with Pseudomonas was negative in 24 hours. The other combinations contained traces of gas in 24 hours.

Endo's agar and eosin methylene blue agar were streaked from cultures of combinations giving gas in lactose broth, to determine whether the resulting growth on the Endo's and eosin methylene blue agar could be confused with that of the Escherichia-Aerobacter group. Results are shown in table 6. It was observed that the Staphylococcus-Salmonella and Strep. faecalis-Proteus combinations on both media gave reactions which could have been interpreted as those of the Escherichia-Aerobacter group. The same results were obtained on plates streaked from the Dominick and Lauter and brilliant green bile media. The combination of Staphylococcus with Pseudomonas which was positive in the Dominick and Lauter broth, when streaked on Endo's agar, produced a growth which could be confused with Aerobacter.

Tonney and Noble medium: No results were obtained which would in any way be confused with positive results for the Escherichia-Aerobacter group.

MacConkey's agar: The combinations of Staphylococcus with Pseudomonas, and Strep. faecalis with Proteus gave results which could be confused with the growth of the Escherichia-Aerobacter group. None of the other combinations gave results which might be confusing.

The following abbreviations are used in the tables:

| | |
|----------|---|
| L. B. | Lactose broth |
| B. G. B. | Brilliant green lactose peptone bile |
| D. L. | Methylene blue, erythrosine, from cresol purple broth - Dominick and Lauter medium |
| Endo's | Endo's agar |
| Em M. B. | Eosin - methylene blue agar |
| T. N. | Ferrocyanide citrate agar. Tonney and Noble |
| MacC. | MacConkey's bile salt agar. |

These symbols are used to indicate the results obtained:

| | |
|----------------|---|
| Ac. | Acid production |
| Alk. | Production of an alkaline reaction in the medium |
| G. | Gas production |
| G ₂ | Indicating that gas at 24 hours was less than 10 per cent. |
| Gr. | growth |
| C. | growth resembling Esch. coli growth |
| A. | growth resembling Aero. aerogenes growth |
| CA, or AC. | Intermediate between Escherichia and Aerobacter, the first letter indicating the predominating type of growth |
| -- | no growth |

Table 1 : Growth in 24 hours of pure cultures of different bacteria on media employed in water analysis .

| Culture | Media Employed | | | | | | |
|--------------------|----------------|--------|-------|--------|--------|------|-------|
| | L.B. | B.G.B. | D.L. | Endo's | E.M.B. | T.N. | MacC. |
| Esch. coli | Ac.G. | G. | Ac.G. | C. | C. | C. | C. |
| Aero. aerogenes | Ac.G. | G. | Ac.G. | A. | A. | A. | A. |
| Esch. acidilactici | Ac.G. | G. | Ac.G. | C. | C. | C. | C. |
| Esch. neapolitana | Ac.G. | G. | Ac.G. | C. | C. | C. | C. |
| Aero. cloacae | Ac. | Gr. | Ac. | A. | A. | C. | A. |
| Aero. oxytocom | Ac.G. | G. | Ac.G. | C. | A. | C. | C. |
| Sal. schottmulleri | Gr. | Gr. | Ac. | Gr. | A. | -- | A. |
| Staph. aureus | Ac. | -- | Gr. | -- | -- | -- | Gr. |
| Staph. albus | Ac. | -- | Gr. | -- | -- | -- | Gr. |
| Staph. citreus | -- | -- | -- | -- | -- | -- | -- |
| Micro. flavus | -- | -- | -- | -- | -- | -- | -- |
| Micro. varians | -- | -- | -- | -- | -- | -- | -- |
| Strep. faecalis | Ac. | Gr.G. | Ac.G. | Gr. | CA. | -- | Gr. |
| S. marscens | -- | Gr. | Ac. | Gr. | Gr. | -- | Gr. |
| Flavo. aurantiacum | -- | Gr. | Ac. | -- | -- | -- | -- |
| Chromo. violaceum | -- | Gr. | Gr. | -- | -- | -- | -- |
| Ps. aeruginosa | Alk. | Gr. | Ac. | Gr. | A. | C. | A. |
| Ps. flourescens | Alk. | Gr. | Gr. | -- | -- | -- | -- |
| Proteus vulgaris | Gr. | Gr. | Gr. | Gr. | Gr. | -- | A. |
| Proteus americanus | Gr. | Gr. | Gr. | Gr. | -- | -- | A. |
| Alc. faecalis | Alk | Gr. | Ac. | C. | Gr. | C. | A. |

Table 2: Growth in 48 hours of pure cultures of different bacteria in media employed in water analysis.

| Culture | Media Employed | | | | | | |
|---------------------------|----------------|--------|---------|--------|--------|------|-------|
| | L.B. | B.G.B. | D.L. | Endo's | E.M.B. | T.N. | MacC. |
| <i>Esch. coli</i> | Ac.G. | G. | Ac.G. | C. | C. | C. | C. |
| <i>Aero. aerogenes</i> | Ac.G. | G. | Ac.G. | A. | A. | A. | A. |
| <i>Esch. acidilactici</i> | Ac.G. | G. | Ac.G. | C. | C. | C. | C. |
| <i>Esch. neapolitana</i> | Ac.G. | G. | Ac.G. | C. | C. | C. | C. |
| <i>Aero. cloacae</i> | Ac.G. | G. | Ac. | A. | A. | CA. | A. |
| <i>Aero. oxytocum</i> | Ac.G. | G. | Ac.G. | C. | A. | CA. | CA. |
| <i>Sal. schottmulleri</i> | Gr. | Gr. | Ac. | C. | A. | -- | A. |
| <i>Staph. aureus</i> | Ac. | Gr. | Gr.(Ac) | -- | -- | -- | Gr. |
| <i>Staph. albus</i> | Ac. | -- | Gr. | -- | -- | -- | Gr. |
| <i>Staph. citreus</i> | Ac. | -- | -- | -- | -- | -- | -- |
| <i>Micro. flavus</i> | Gr. | -- | -- | -- | -- | -- | -- |
| <i>Micro. varians</i> | Ac. | -- | -- | -- | -- | -- | -- |
| <i>Strep. faecalis</i> | Ac.G. | G. | Ac.G. | C. | AC. | -- | Gr. |
| <i>S. marscens</i> | Gr. | Gr. | Ac. | Gr.(A) | Gr. | -- | A. |
| <i>Flavo. aurantiacum</i> | Gr. | Gr. | Ac. | -- | -- | -- | Gr. |
| <i>Chromo. violaceum</i> | Gr. | Gr. | Gr. | -- | -- | -- | Gr. |
| <i>Ps. aeruginosa</i> | Alk. | Gr. | Ac. | Gr.(A) | A. | C. | A. |
| <i>Ps. flourescens</i> | Alk. | Gr. | Ac. | -- | -- | -- | Gr. |
| <i>Proteus vulgaris</i> | Gr. | Gr. | Ac. | Gr. | Gr. | Gr. | A. |
| <i>Proteus americanus</i> | Gr. | Gr. | Ac. | A. | Gr.(A) | -- | A. |
| <i>Alc. faecalis</i> | Alk. | Gr. | Ac. | C. | Gr. | C. | A. |

Table 3: Growth in 24 hours of potentially antibiotic combinations on media employed in water analysis.

| Cultures | Media Employed | | | | |
|---------------------------------------|----------------|--------|-------------|------|-------|
| | L. B. | B.G.B. | D.L. | T.N. | MacC. |
| Esch.coli with Pseudomonas | Ac.G. | G. | Ac.G. | C. | A. |
| Esch.coli with Proteus | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.coli with Salmonella | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.coli with Staphylococcus | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.coli with Strep.faecalis | Ac.G. | G. | Ac.G.Gr.(C) | | C. |
| Aero.aerogenes with Pseudomonas | Ac.G. | G. | Ac.G. | A. | A. |
| Aero.aerogenes with Proteus | Ac.G. | G. | Ac.G. | A. | A. |
| Aero.aerogenes with Salmonella | Ac.G. | G. | Ac.G. | A. | A. |
| Aero.aerogenes with Staphylococcus | Ac.G. | G. | Ac.G. | A. | A. |
| Aero. aerogenes with Strep.faecalis | Ac.G. | G. | Ac.G. | A. | A. |
| Esch.acidilactici with Pseudomonas | Ac.G. | G. | Ac.G. | C. | AA |
| Esch.acidilactici with Proteus | Ac.G. | G. | Ac.G. | Gr. | C. |
| Esch.acidilactici with Salmonella | Ac.G. | G. | Ac.G. | C. | CA. |
| Esch.acidilactici with Strep.faecalis | Ac.G. | G. | Ac.G. | Gr. | C. |
| Esch.neapolitana with Pseudomonas | Ac. | G-. | Ac.G- | C. | CA. |
| Esch.neapolitana with Proteus | Ac. | G-. | Ac.G- | C. | C. |
| Esch.neapolitana with Salmonella | Ac. | G-. | Ac. G- | C. | C. |
| Esch.neapolitana with Strep.faecalis | Ac.G- | G. | Ac.G. | Gr. | C. |
| Aero.cloacae with Pseudomonas | Ac. | G-. | Ac.GG | A. | A. |
| Aero.cloacae with Proteus | Gr. | G-. | Ac.G- | A. | A. |
| Aero.cloacae WITH Salmonella | Gr. | G-. | Ac.G- | A. | A. |
| Aero.cloacae with Strep.faecalis | Ac.G. | G-. | Ac.G. | -- | A. |
| Aero.oxytocum with Pseudomonas | Ac.G. | G. | Ac.G. | CA. | CA. |
| Aero.oxytocum with Proteus | Ac.G. | G. | Ac.G. | CA. | C. |
| Aero.oxytocum with Salmonella | Ac.G. | G. | Ac.G. | CA. | C. |
| Aero.oxytocum with Strep.faecalis | Ac.G. | G. | Ac.G. | C. | C. |

Table 4: Growth in 48 hours of potentially antibiotic combinations in media employed in water analysis.

| Cultures | Media Employed | | | | |
|---------------------------------------|----------------|--------|--------|------|-------|
| | L.B. | B.G.B. | D.L. | T.N. | MacC. |
| Esch.coli with Pseudomonas | Ac.G. | G. | Ac.G. | C. | A. |
| Esch.coli with Proteus | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.coli with Salmonella | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.coli with Staphylococcus | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.coli with Strep.faecalis | Ac.G. | G. | Ac.G. | C. | C. |
| Aero.aerogenes with Pseudomonas | Ac.G. | G. | Ac.G. | A. | A. |
| Aero.aerogenes with Proteus | Ac.G. | G. | Ac.G. | A.A | A. |
| Aero.aerogenes with Salmonella | Ac.G. | G. | Ac.G. | A. | A. |
| Aero.aerogenes with Staphylococcus | Ac.G. | G. | Ac.H. | A. | A. |
| Aero. aerogenes with Strep.faecalis | Ac.G. | G. | Ac.G. | A. | A. |
| Esch.acidilactici with Pseudomonas | Ac.G. | G. | Ac.G. | C. | A. |
| Esch.acidilactici with Proteus | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.acidilactici with Salmonella | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.acidilactici with Strep.faecalis | Ac.G. | G. | Ac.G. | C. | C. |
| Esch.neapolitana with Pseudomonas | Ac. | G. | Ac.G. | C. | CA. |
| Esch.neapolitana with Proteus | Ac. | G. | Ac.G. | C. | C. |
| Esch.neapolitana with Salmonella | Ac. | G. | Ac.G. | C. | C. |
| Esch.neapolitana with Strep.faecalis | Ac.G. | G. | Ac.G. | C. | C. |
| Aero.cloacae with Pseudomonas | Ac. | G. | Ac. G. | A. | AA |
| Aero.cloacae with Proteus | Ac. | G. | Ac.G. | A. | A. |
| Aero.cloacae with Salmonella | Ac. | G. | Ac.G. | A. | A. |
| Aero.cloacae with Strep.faecalis | Ac.G | G. | Ac.G. | C. | A. |
| Aero.oxytocum with Pseudomonas | Ac.G. | G. | Ac.G. | CA. | CA. |
| Aero.oxytocum with Proteus | Ac.G. | G. | Ac.G. | CA. | C. |
| Aero.oxytocum with Salmonella | Ac.G. | G. | Ac.G. | CA. | C. |
| Aero.oxytocum with Strep.faecalis | Ac.G. | G. | Ac.G. | C. | C. |

Table 5 : Growth of combinations of organisms not of the Escherichia-Aerobacter group on media employed in water analysis.

| Cultures | 24 hours | | | | | Media Employed | | | | |
|---------------------------------|----------|--------|-------|------|---------|----------------|--------|------|------|-------|
| | L.B. | B.G.B. | D.L. | T.N. | MacC. | L.B. | B.G.B. | D.L. | T.N. | MacC. |
| Staphylococcus with Pseudomonas | Ac. | Gr. | Ac. | Gr. | Gr.(CA) | | | | | |
| Staphylococcus with Proteus | Ac.G- | Gr. | Ac.G- | -- | Gr. | | | | | |
| Staphylococcus with Salmonella | Ac.G- | Gr. | Ac.G- | Gr. | Gr. | | | | | |
| Strep.faecalis with Proteus | Ac.G- | G. | Ac.G- | -- | A # | | | | | |
| Strep.faecalis with Salmonella | Ac. | Gr. | Ac.G- | | | | | | | |
| | 48 hours | | | | | | | | | |
| Staphylococcus with Pseudomonas | Ac. | Gr. | Ac.G. | Gr. | Gr.(CA) | | | | | |
| Staphylococcus with Proteus | Ac.G. | Gr. | Ac.G. | Gr.# | Gr. | | | | | |
| Staphylococcus with Salmonella | Ac.G. | Gr. | Ac.G. | Gr. | Gr. | | | | | |
| Strep.faecalis with Proteus | Ac.G. | G. | Ac.G. | -- | A. # | | | | | |
| Strep.faecalis with Salmonella | Ac.G. | Gr. | Ac.G. | | | | | | | |

This result was obtained only with those combination cultures containing Proteus vulgaris as one of the pair; those cultures containing Proteus Americanus produced a growth which was not significant.
which w

#2 This was the result when Proteus vulgaris was one of the pair; there was no growth when Proteus americanus was used in these combinations on this media.

Table 6: Endo's and eosin methylene blue plates streaked from the combination cultures (of table 5) in lactose broth, brilliant green bile, and the Dominick and Lauter medium.

Media Employed

| Cultures | Media Employed | | | |
|--|----------------|--------|----------|--------|
| | 24 hours | | 48 hours | |
| Streaked from L.B. | Endo's | E.M.B. | Endo's | E.M.B. |
| Staphylococcus with Pseudomonas | Gr. | Gr. | A. | Gr. |
| Staphylococcus with Proteus | Gr. | Gr. | Gr.(A) | Gr. |
| Staphylococcus with Salmonella | C. | Gr. | CA. | Gr.(A) |
| Strep.faecalis with Proteus | C. | Gr.(A) | C. | Gr.(A) |
| Streaked from B.G.B. | | | | |
| Staphylococcus with Pseudomonas | Gr. | Gr. | A. | Gr. |
| Staphylococcus with Salmonella | C. | Gr. | CA. | Gr. |
| Strep.faecalis with Proteus | Gr.(C) | A. | C. | AC. |
| Streaked from D.L. | | | | |
| ² Staphylococcus with Pseudomonas | Gr. | Gr. | A. | Gr. |
| Staphylococcus with Proteus | Gr. | CA. | Gr. | Gr.(A) |
| Staphylococcus with Salmonella | C. | CA. | Gr. | A. |
| Strep.faecalis with Proteus | C. | A. | C. | AC. |

DISCUSSION

The foregoing results have shown the ability of Standard Methods lactose broth to support the growth of common water-borne organisms which might in one way or another confuse the interpretation of presumptive tests for the Escherichia-Aerobacter group in water analysis. Brilliant green bile demonstrated its ability to eliminate certain acid-forming bacteria, particularly the Streptococci and Micrococci, which are a significant part of any combination giving a false presumptive test. It has been stated by Dunham, McCrady, and Jordan (8) that in any combination of organisms, none of which produce gas from lactose when growing alone, but which do produce gas when growing in symbiosis, one of such a combination must be able to produce acid from lactose. The Dominick and Lauter medium gave results which were comparable with those of brilliant green bile. Some of the Streptococci, however, were able to grow in the Dominick and Lauter medium.

Of the solid media employed the Tonney and Noble medium was emphatically the best, due to the fact that it did not support the growth of many of the organisms, particularly Gram-positive species, which might otherwise be confused with, or interfere with, the Escherichia-Aerobacter group. The medium has the additional advantage, claimed for it by its originators, that it does, in a general way, distinguish between Escherichia and Aerobacter.

In studying the associative action of various bacteria, antibiosis was demonstrated only in combinations of Pseudomonas, Proteus, or Salmonella with the less common member of the Escher-

ichia-Aerobacter group. These antibiotic effects were observed only in the lactose broth, so that any of the other media employed would be efficient in eliminating this factor.

Of the symbiotic relationships studied only that of *Staphylococcus* with *Pseudomonas* failed to produce gas in lactose broth. The Dominick and Lauter medium gave a positive presumptive test in all of the combinations in 48 hours, whereas, brilliant green bile permitted a false presumptive test only in the case of *Strep. faecalis* with *Proteus*. The Tonney and Noble medium entirely eliminated all of the false presumptive tests, while MacConkey's agar permitted a false test only in the case of *Strep. faecalis* with *Proteus*.

It would appear from the results that, of the fluid media employed, the brilliant green bile is the most satisfactory, since it entirely eliminated the antibiotic effects and permitted a false presumptive test with only one combination. The Tonney and Noble medium proved more satisfactory than MacConkey's bile salt agar, but only slightly so. The Tonney and Noble medium would seem to be worthy of further study. Its preparation is laborious and so its use is not practical except in a laboratory doing a considerable number of water analyses.

A fact worthy of mention here is that *Pseudomonas fluorescens* failed to grow on the solid media employed, although other Gram-negative species were able to grow. This was probably due to the fact that the optimum temperature for this organism is lower than that used for incubation in these studies. In a preliminary experiment using various incubation temperatures, *Ps. fluorescens* was able to grow on the solid media

when the incubation temperature was 30°C. Another fact that might be noted is that several strains of the Escherichia-Aerobacter group were weak and slow growing. Aero-cloacae was particularly slow growing and failed to produce gas in the Dominick and Lauter medium. Some investigators have reported that inhibitory media, such as the Dominick and Lauter medium and brilliant green bile were not reliable since they could inhibit the less sturdy members of the Escherichia-Aerobacter group and thus fail to show the presence of existing pollution. However, the sanitary significance of such weak strains of the Escherichia-Aerobacter group is still an open question.

In the present experiments it has been impossible to duplicate water conditions. As has been reported by Sears and Putman (49) and Greer (13) the results obtained with either symbiotic or antibiotic combinations vary with the strains used and with the number of organisms of each species added. In water these factors would vary from the conditions in the experiments and different results might be obtained. However, the results here reported indicate the probable reactions with the media studied.

Certain aerobic and anaerobic spore-forming organisms, especially *B. asterosporus* and *Cl. welchii*, have been reported as giving false presumptive tests. It was not possible, however, to include them within the scope of this work.

1. Certain media employed in the bacteriological analysis of water have been studied with a number of species of bacteria commonly encountered in water. Among these are species which have been reported as interfering with tests for the Escherichia-Aerobacter group, and others which in symbiotic combinations have been reported as giving false presumptive tests in water samples.

2. The media used were: lactose broth, brilliant green lactose peptone bile, the Dominick and Lauter medium, Endo's agar, eosin methylene blue agar, the Tonney and Noble ferrocyanide-citrate agar, and MacConkey's bile salt agar.

3. Of the fluid media, brilliant green bile indicated its ability to eliminate organisms which might interfere with true presumptive tests, and others which might produce false presumptive tests. The Dominick and Lauter medium was partially effective in both respects but not so much so as the brilliant green bile.

4. Of the solid media the Tonney and Noble medium seems to offer much promise for elimination of organisms which interfere with presumptive tests or of those which produce, symbiotically, false presumptive tests. The Tonney and Noble medium appears to have verified the claims made for it by its originators, that it can differentiate between Escherichia and Aerobacter.

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