A STUDY OF THE PRESUMPTIVE TEST IN WATER ANALYSIS, COMPARING STANDARD LACTOSE BROTH WITH SCHREINER'S MODIFICATION USING BASIC FUCHSIN.

by

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Review of Literature

Although methods of bacteriological water analysis in the United States are very well standardized due to the cooperative work which resulted in the procedures recommended in Standard Methods of Water Analysis [1], new methods, and especially new media, are constantly being developed. Repeated attempts have been made to find a medium favorable to the coli-aerogenes group of bacteria, and unfavorable to other organisms present in water.

Since it has long been recognized that Escherichia coli (Bacterium coli) in water is significant as an index of the probable presence of disease germs, the test for the coli-aerogenes group of bacteria in water has been made in various ways. The litmus-lactose-agar plate was used by Sedgwick and Mathews[2], in 1892. When it was found that growth from the sample in a liquid medium gave a more delicate test, preliminary enrichment broth came into use,

and nutrient broth with the addition of 1.0 per cent dextrose, was used until 1906. Lactose broth replaced dextrose broth in the presumptive test, when it was shown that many bacteria not in the coli-aerogenes group, and therefore not fermenting lactose, gave a positive presumptive test in dextrose broth. Various modifications of the standard lactose broth have been made. Lactose bile was recommended in the 1912 edition of Standard Methods of Water Analysis, but as various investigations showed that the selective action of bile salts inhibited the weaker strains of Escherichia coli (Bacterium coli) present, this medium is no longer recommended. However, work is still being done, using different concentrations of bile salts. Other variations, never widely used, include phenol broth; the Eykman test, which involves incubation at 46° C; media containing neutral red as an indicator; aesculin media, and liver broth. In the 1917 and subsequent editions of Standard Methods, lactose broth has been recommended as the enrichment medium.

Present day attempts to improve the presumptive test, by eliminating the non-confirming gas formers, but inhibiting none of the coli-aerogenes organisms, are largely concerned with the use of dyes in the enrichment broth. Anilin dyes as inhibitory agents have been used in various media, many of them for the isolation of the typhoid group of organisms. Loeffler added malachite green to agar, for this purpose; Conradi-Drigalsky

medium contains crystal violet in a dilution of 1 to 100,000 to inhibit the Gram-positive contamination when testing for Gram-negative organisms; similarly, an egg medium by Petroff, used for the isolation of Mycobacterium tuberculosis (Bacillus tuberculosis), contains gentian violet. Brilliant green agar, (Krumwiede) containing graduated amounts of a 0.1 per cent aqueous solution of brilliant green, is used in the isolation of Eberthella typhi (Bacillus typhosus) and Salmonella paratyphi (Bacillus paratyphosus A). Brilliant green is also used for the same purpose in liquid media, as peptone water or dextrose broth.

The work of Churchman,[3,4] in 1912 gave an impetus to this development. He observed in trying to remove Bacillus subtilis from Serratia marcescens (Bacillus prodigiosis) that Bacillus subtilis failed to grow in the presence of gentian violet. Further work, on many organisms, showed that, with a 1-100,000 dilution of gentian violet, the Gram-positive group usually failed to grow, but that Gram-negative organisms were not inhibited. Interesting variations include one strain of Salmonella enteritidis (Bacillus enteritidis) which failed to grow in gentian violet media and was found to differ in agglutination from those which did grow. Krumwiede and Pratt[5] (1914) added to agar aqueous solutions of gentian violet in such amounts that final concentrations of the dye were

1-500,000, 1-100,000 and 1-50,000. The agar, when slanted in Petri dishes, and inoculated with fresh broth cultures, showed the same grouping: inhibition of Gram-positive organisms, but a quantitative variation in reaction to dyes, more marked with some species than with others. The relation of hydrogen ion concentration and dye was studied by Stearn and Stearn [6] (1924). Working with gentian violet and basic fuchsin as basic dyes, and acid fuchsin and ponceau as acid dyes, these authors inoculated broth containing dye with lactose fermenting organisms, determined the pH, and correlated it with the amount of precipitation of the dye. From the results, they concluded that "the behavior of bacteria may be closely related to the chemistry of their protein content". Gentian violet and basic fuchsin were found to act in the same manner in their experiments.

Hall and Ellefson [7] (1918) found that the addition of gentian violet to lactose broth, as an enrichment medium, eliminated many of the anaerobic gas-formers. They also found that, altho larger proportions of gentian violet gave a larger proportion of confirmations, these increased concentrations also tended to diminish the number of Escherichia coli (Bacterium coli) isolated, by inhibiting the weaker strains. Concentrations of gentian violet above 1-100,000 were

inhibitive. Wagner and Monfort [8] 1921, prepared broth from 0.2 per cent lactose, 2 per cent peptone, and 0.00½ per cent gentian violet, and claimed results superior to those with standard 1% or 0.5% lactose broth. Hinman [9] (1924), using this broth, reported that most non-confirming lactose fermenting organisms found in the water of Iowa City, Iowa, were inhibited and that the positive presumptive tests increased on prolonged incubation (more than two days).

Muer and Harris [10] (1920), used brilliant green in bile media. They reported that 1-30,000 checked the development of Clostridium welchii (Bacillus welchii) and Escherichia coli (Bacterium coli) will grow in 1-350. Their medium suggested for enrichment contains 5% dried oxgall, 1% peptone, 1% lactose, and .01% brilliant green. Howard and Thompson [11] (1925), in reporting on some comparative studies, found brilliant green broth highly inhibitory "and not much difference between sodium taurocholeate broth and brilliant green lactose bile, finding both slightly inhibitory". From brilliant green bile, they report fewer positive presumptive tests, but 96% confirmations. They conclude that as a confirmatory medium, brilliant green bile may be safely substituted.

Bronfenbrenner, Schlesinger and Soletsky [12] (1920), in using china blue-Rosalic acid as an indicator, found that the

rosalic acid had bactericidal power against Gram-positive bacteria, but almost all Gram-negative bacteria grew readily in media containing twenty-five times the amount inhibitive to the Gram-positive organisms.

Winslow and Dolloff[13] (1922), comparing rosalic acid, gentian violet and brilliant green, in lactose broth and in lactose bile, containing 5% of sodium choleate, found all three dyes inhibitive in one part in 1000 in lactose bile. Rosalic acid was the same in lactose broth, but gentian violet was 5 to 50 times as toxic in lactose broth, and brilliant green 200 to 1000 times as toxic.

Crystal violet was added, in 1-100,000 dilution to eosin methylene blue plates, by Skinner and Murray[14] (1924). They claimed inhibition of spreading colonies and more distinctive appearance of colonies of Escherichia coli (Bacterium coli) and of Aerobacter aerogenes (Bacterium aerogenes).

In the Water and Sewage Laboratory of the Kansas State Board of Health, it was observed that many positive presumptive tests did not confirm. With the idea in mind of developing a medium with which confirmation would be unnecessary, but which would not be inhibitory to the coli-aerogenes group, Schreiner[15] (1921), then bacteriologist, modified the enrichment broth by the addition of basic fuchsin. For this

purpose, he used a saturated alcoholic solution, prepared by the Standard Methods of Water Analysis recommendation for Endo medium. The amount best suited to the purpose, he found to be 4 c.c. for 5 liters of the regulation lactose broth recommended in Standard Methods of Water Analysis. This modification was adopted by the Water and Sewage Laboratory in 1921, and has been used since that time.

Experimental Work.

The present work was undertaken to study the effects of basic fuchsin, in the enrichment broth, on the results in routine water analysis. The following points were studied:

1. A comparison of results from fuchsin broth and standard broth, as to positive tubes and confirmations.

2. A study of results from all analyses made during three years, in the Water and Sewage Laboratory, with special regard to:

   a. Number and percentage of positive tubes from various kinds of water;

   b. Number and seasonal variation of non-confirming tubes

c. A consideration of the importance of non-confirming tubes in interpreting bacterial analysis of water;

d. A study of the cause of gas production in tubes not confirming.

Routine Work

Methods

For all routine work, lactose broth is prepared in 5 liter lots, and basic fuchsin added after the other ingredients are dissolved and the reaction adjusted. The stock solution of the dye is prepared by saturating 95% alcohol with basic fuchsin crystals, and decanting the supernatant solution. Certified basic fuchsin as recommended for use in Endo's medium, is used; dyes were obtained from Henry Heil Chemical Company, Coleman and Bell Co., and National Aniline and Chemical Company. The media is tubed in Dunham fermentation tubes, using two sizes of tubes, and corresponding sizes of fermentation vials, for different amounts of the water samples. Sterilization is in the autoclave, at 15 pounds for 15 minutes.

Some explanation of the samples routinely tested is necessary. The public water supplies of the state are

divided into two main classes: ground waters, such as wells and springs; 220 in number, tested twice a year, and surface waters, 64 in number, tested once a week. Samples of the former are all of the same type, as that group is not treated; of the latter, samples are of the raw water, from the coagulation basin, the filter effluent, and the tap effluent, which has been chlorinated. Other samples include those listed as "Public Health", which are from private wells, cisterns and swimming pools; in addition, there are ices, and bottled waters.

In planting samples for the presumptive test, three tubes are made from each dilution, these dilutions depending on the source of the water. For all ground waters, filter effluents, tap effluents and ices, 10 cubic centimeter, 1 cubic centimeter and .1 cubic centimeter amounts are used; for samples from coagulation basins, 1 cubic centimeter and .1 cubic centimeter, and for raw water, 1 cubic centimeter, .1 cubic centimeter, .01 cubic centimeter and .001 cubic centimeter. The partially confirmed test, from Standard Methods, using Endo plates, is employed routinely, but when there is doubt, the culture is retested in lactose broth. Readings from the presumptive tubes are made at 24 hour and 48 hour periods, the positives of the first reading

being recorded in red ink, and the final reading in black ink. Active bubbling is reported as positive, experience having shown that with fuchsin broth any amount of gas usually indicates coli-aerogenes group organisms. The Standard Methods practice of confirming only one tube, of the highest dilution showing a positive reading, is used for samples of raw water and samples from coagulation basins, but for other samples, where more than one is positive, tubes from two dilutions are selected. Thus the number of tubes to be confirmed far exceeds the number of positive samples.

Results.

Altho the medium was originally designed to eliminate false positives in the presumptive test, it does not give 100% confirmations for the waters of Kansas. Since it was observed that the non-confirming tubes were more numerous at certain seasons, and from certain sources, a tabulation was made of results from the routine analyses made during the years 1924, 1925 and 1926. These results are shown in Table 1. From samples showing two tubes not confirming, the tubes were counted separately. These were but a small part of the whole number. The samples listed

as"railroad" were ground waters or surface tap effluents, on which five 10 cubic centimeter portions were run, to comply with Treasury Department standards. Of the 28 non-confirming tubes from ice samples, 23 were from natural ices.

TABLE 1.

Tabulation of samples run routinely in fuchsin broth in the Water & Sewage Laboratory.

	Surface Waters	Ground Waters	Ice	Rail-road	Public Health	Total
Samples run in 1924	8699	1,759	331	11	332	11,132
Tubes +	7899	974	194	9	530	9,606
Tubes not confirming	713	79	12	0	13	817
Per cent of tubes not confirming	9.03	8.11	6.20	0.00	2.45	8.51
Samples run in 1925	9396	1609	389	22	692	12,108
Tubes +	7303	990	177	9	988	9,467
Tubes not confirming	345	49	9	0	10	413
Per cent of tubes not confirming	4.72	4.94	5.08	0.00	1.01	4.36

TABLE 1-Cont'd.

	Surface Waters	Ground Waters	Ice	Rail-road	Public Health	Total
Samples run in 1926	6916	1415	297	33	677	9,338
Tubes +	5277	1033	121	12	1001	7,444
Tubes not confirming	238	26	7	1	6	278
Per cent of tubes not confirming	4.51	2.51	5.78	8.33	0.59	3.73
Samples run in 1924, 1925 & 1926	25011	4783	1017	66	1701	32578
Tubes +	20479	2997	492	30	2519	26517
Tubes not confirming	1296	154	28	1	29	1508
Per cent of tubes not confirming	6.33	5.14	5.70	3.33	1.15	5.68

TABLE 2.

Non-confirming tubes from surface waters run in fuchsin broth, tabulated as to source and month.

1924	Raw Water	Coagulation Basin sample	Filter Effluent	Tap Effluent	Total
Jan.	18	16	11	6	51
Feb.	42	35	32	7	116
Mar.	56	38	51	10	155
April	35	31	26	9	101
May	25	25	28	8	86
June	11	9	11	7	38
July	3	2	4	3	12
Aug.	9	9	5	2	25
Sept.	9	7	9	7	32
Oct.	31	17	13	15	76
Nov.	2	2	0	1	5
Dec.	7	2	5	2	16
Total from each source	248	193	195	77	713
Per cent from each source	34.8	27.1	27.3	10.8	

TABLE 2-cont'd.

	Raw Water	Coagulation Basin Sample	Filter Effluent	Tap Effluent	Total
1925.					
Jan.	21	17	27	10	75
Feb.	9	8	20	3	40
Mar.	26	16	17	7	66
April	9	8	6	4	27
May	9	5	8	3	25
June	2	6	7	3	18
July	3	4	2	4	13
Aug.	1	3	0	1	5
Sept.	7	5	7	0	19
Oct.	6	6	13	6	31
Nov.	3	1	7	2	13
Dec.	3	2	8	0	13
Total from each source	99	81	122	43	345
Per cent from each source	28.7	23.5	35.4	12.5	

TABLE 2-Cont'd.

	Raw Water	Coagulation Basin Sample	Filter Effluent	Tap Effluent	Total
1926					
Jan.	9	3	34	6	52
Feb.	8	2	28	6	44
Mar.	13	5	21	9	48
April	15	5	18	4	42
May	1	1	3	1	6
June	0	0	0	2	2
July	0	1	0	0	1
Aug.	0	0	1	1	2
Sept.	0	0	0	1	1
Oct.	1	0	1	0	2
Nov.	2	1	10	2	15
Dec.	4	5	9	5	23
Total from each source	53	23	125	37	238
Per cent from each source	22.4	9.6	52.5	15.5	

As the non-confirming tubes are of the greatest interest, a special study of them was made. The chronological distribution is shown by the curves in Figure 1. As surface and ground waters were the largest in number, but different in values, separate curves of those groups were made. The striking thing is the abrupt drop, during April, of the false positives, to the low number maintained during the summer, until an increase appeared in October, which in turn fell until January. This general curve is true of samples from all sources, showing that conditions are much the same, irrespective of the source of supply. Figure 2 gives the percentage for each year, and shows that the yearly number corresponds to the peaks of the curves in Figure 1.

Among the surface samples, which represent all stages of water purification, some comparisons may be made. Table 2 shows the non-confirmations from surface samples listed as to source and month.

By far the greater number of non-confirming tubes had not shown gas until 48 hours incubation. There is no apparent difference in the amount of gas production between the confirming and non-confirming tubes. The false positives do not constitute a large number of

FIGURE 1

Monthly distribution of non-confirming tubes from routine analyses in fuchsin broth for the years 1924, 1925, 1926.

——— Total non-confirming tubes

– – – – – Surface water

..... Ground water

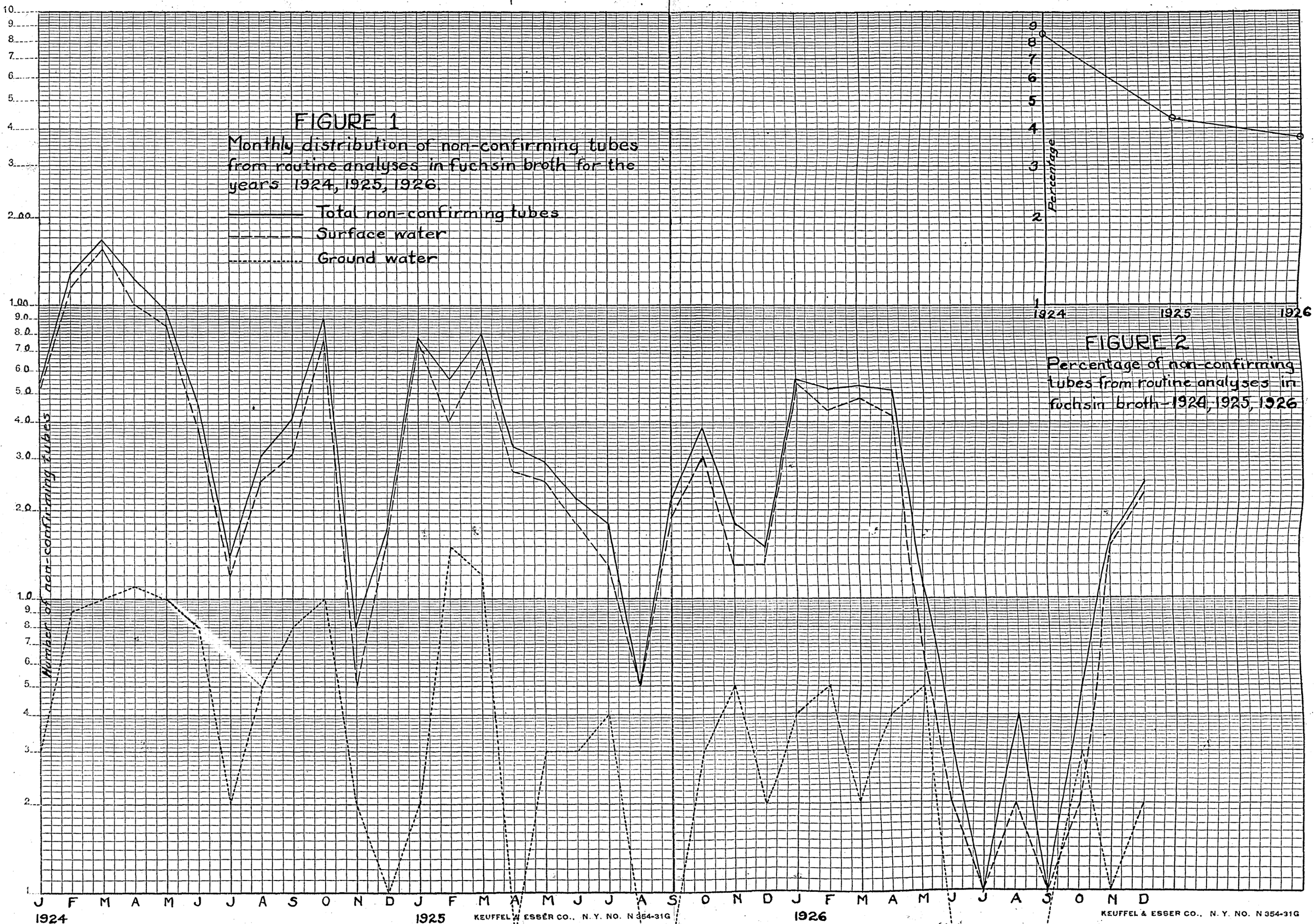

FIGURE 2

Percentage of non-confirming tubes from routine analyses in fuchsin broth-1924, 1925, 1926

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the positive tubes of any one sample but are about equally distributed between samples confirming in other dilutions and those having no confirming tubes. Those showing false positives but no coli-aerogenes organisms almost always have only one tube of the highest dilution positive after 48 hours. Those with other tubes confirming, usually fail to confirm in the highest dilution, this frequently being a single 48 hour positive, with confirming tubes from 24 hours incubation. Thus, the coli index, in the majority of the cases, would be but slightly changed, if no confirmations were made.

Comparison of Media

Methods

In order to compare results in fuchsin broth, directly with those obtained from standard lactose broth, a series of samples were run in the two media simultaneously. When the broth was prepared, a portion was tubed before the addition of the dye, thus the composition of the two media varied only in the fuchsin content. Samples were planted in both media during the process of the routine work, similar dilutions and incubations being used. The only variation was in the confirmations. Endo plates are used routinely with fuchsin broth, Endo and eosin methylene blue plates were used, at different

times, for standard broth tubes. Also, each positive tube was streaked for confirmation, from the standard broth, instead of the smaller number selected, as described, from fuchsin broth tubes. The 514 samples run were tested at different times during three years, and were picked at random from the routine work.

Results.

In making comparisons, the coli index, as indicated from the confirmations, was used where the results differed. The coli index was figured from the tables prepared by McCrady, as given in "Elements of Water Bacteriology", by Prescott and Winslow,[2] (1924), fourth edition. The results of these tests are given in Table 3.

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TABLE 3.

Results of samples run in fuchsin broth and standard broth simultaneously.

	Surface Water				Ground Water	Total for each result	
	Raw Samples	Coagulation Basin Samples	Filter Effluents	Tap Effluents		Number	Percentage
Both media negative	5	4	14	35	116	172	33.5
Coli index the same from both media	4	3	17	2	27	53	10.3
Total with presumptive tests the same	7	7	31	37	143	225	43.8
Coli index higher in standard broth	6	4	27	10	44	91	17.7
Coli index higher in fuchsin broth	10	1	17	13	46	87	16.9
Coli index negative; false positives in standard broth	6	2	16	57	23	104	20.2
Coli index negative; false positives in fuchsin broth	0	0	2	2	3	7	1.4
Total no. of samples run	29	14	93	119	259	514	

It will be observed from Table 3 that in the majority of the analyses, the number of coli-aerogenes organisms is the same with either method. For 43.8% of the samples, the results from the presumptive test were identical, for 65.4% of the samples, the results after confirming, were the same. A comparison of the samples, giving more coli-aerogenes organisms in one medium, based on the difference shown by the coli index for each sample, shows such individual variations as 75 per 100 cubic centimeters of sample, in fuchsin broth, to 4 in standard broth, or such similarities as 9 in fuchsin broth to 9 in standard broth. Similar differences are noticed in samples showing more coli-aerogenes organisms in standard broth. For the ground waters, as well as the total number of samples, one medium shows little advantage over the other, in number of isolations.

Samples of raw water and those from coagulation basins of surface supplies, show few differences. Filter effluents show a slightly higher percentage of confirmations in standard broth, but in tap effluents the order is reversed. The most striking result in this group is the large proportion of false positives in standard broth, with no coli-aerogenes group organisms present. There

were a number of non-confirming tubes of standard broth, from samples showing Escherichia coli (Bacterium coli) which are not shown as such in Table 3, but are listed according to the coli index. These tubes were found in 24.0% of the ground waters showing Bacterium coli, 56.0% of the filter effluents, and 36.0% of the tap effluent samples. A count of the tubes of standard broth shows 15.5% of the tubes from ground waters and 43.2% of the tubes from surface waters not confirming. The tap samples alone showed 88.3% of false positive tubes. The difference between these numbers and those of fuchsin tubes not confirming, is too great to need comment.

Study of Causes of Non-Confirming Tubes

Introduction

The reasons generally given for positive presumptive tests which fail to confirm, are anaerobic spore formers, or symbiotic complexes. Sears and Putnam[16] (1923) showed that, for example, Streptococcus fecalis and Salmonella schotmülleri (Bacillus paratyphosus B) when grown together in lactose broth would bring about vigorous gas production. Synergism is the term used by Holman & Meekison[17] (1926) for this phenomenon, in which one of a pair of bacteria splits a test substance and

forms acid, and the other forms gas from the monosaccharid. They show that the various results depend on such factors as the stage of growth and functional activity of the bacteria, and the pH and its regulation by bacterial products. It was demonstrated by Leitch [13] (1925) that gas producing complexes could be isolated from water, one of the group forming acid from lactose, and being usually a Gram-positive coccus, the other forming gas from dextrose, these being in every case bacilli.

Methods

To test this theory as an explanation for non-confirming tubes, in the winter of 1926 and 1927, cultures were picked from Endo or eosin methylene blue plates not showing confirmation. These had been inoculated with material from both standard and fuchsin tubes. As the plates were not streaked for individual colonies, the first cultures were necessarily made from all of the colonies appearing. Standard lactose broth tubes were used thruout. Incubation was at 37°C., until gas appeared, or for 15 days if no gas was formed. Tubes showing gas were streaked on Endo plates, and colonies picked into standard broth. Different combinations, as well as individual colonies, for a control, were cultured. If any of

these tubes showed gas before 15 days, they were again streaked on Endo plates, and colonies picked as before.

Results

Of thirty such cultures picked, 23 formed gas in 48 hours, but 7 of them produced no growth, subsequently on Endo plates, and all cultures, or combinations, failed to continue producing gas, after one or two more generations. Of another group, five cultures from two different sources continued to show gas. One source was a filter effluent, apparently giving a negative result from a fuchsin tube streaked on Endo. The other was a filter effluent, showing non-confirming positives in standard broth, and no fermentation in fuchsin broth. It was finally apparent that the gas producer was one organism, which from subsequent tests was identified as Aerobacter aerogenes (Bacterium aerogenes). These tests are shown in Table 4. The first three cultures are from the same source, as are the last two.

TABLE 4

Cultural Characteristics of Organisms from Apparently Non-Confirming Tubes

	Gram	Spores	Motility	Lactose	Saccharose	Indol	Methyl red	Voges-Proskauer
1	-	-	-	+	+	+	-	-
2	-	-	-	+	+	+	-	+
3	-	-	-	+	+	+	-	+
4	-	-	slightly	+	+	-	-	+
5	-	-	slightly	+	+	-	-	+

In the winter of 1927-1928, work to demonstrate symbiotic complexes was again started. Attempts were made to isolate organisms corresponding to those shown by Leitch to form gas in symbiosis. Cultures picked from confirmatory plates yielded organisms fermenting dextrose, and a smaller number forming acid from lactose. These cultures were all Gram-negative rods; Gram-positive coccus forms were not isolated. In no case could gas be formed in lactose broth by a combination of these organisms, and the same organisms re-isolated. As before, in certain cases a lactose fermenting culture manifested itself in a mixed culture appearing negative on Endo, after several transfers. These cultures could be classified as Escherichia coli (Bacterium coli) and Aerobacter aerogenes (Bacterium aerogenes), as shown by the tests in Table 5. Included in the table in one culture, A35, which was first isolated as a dextrose fermenter, but later was alone responsible for gas production from lactose, particularly when it was inoculated with a culture forming acid from lactose.

TABLE 5

Cultural Characteristics of Organisms from Non-Confirming Tubes

	Gram	Spores	Motility	Lactose	Saccharose	Indol	Methyl red	Voges-Proskauer
1	-	-	-	+	+	-	-	+
2	-	-	-	+	-	+	+	-
3	-	-	-	+	-	+	+	-
4	-	-	-	+	+	+	-	+
5	-	-	-	+	-	+	+	-
A35	-	-	slightly	+	+	-	-	+

As a matter of interest in this connection, Endo plates were prepared, substituting dextrose for lactose. During February and March, when the largest number of non-confirming tubes were appearing, parallel inoculations were made on these dextrose plates, and the routine Endo plates. Since so few 24 hour tubes are negative, 48 hour positives were used, for the most part. As would be expected, tubes positive on Endo were also positive on dextrose plates. In 67 cases, the culture was negative on Endo. In 41 cases, or 61.2% of these, it was positive on dextrose plates; in 26 cases, or 38.8%, it was negative on dextrose plates.

Since it appeared from these cases that the characteristics of coli-aerogenes organisms on Endo plates were masked or changed by the presence of other water organisms, seven tubes from the presumptive test, giving typical colonies on Endo plates, were streaked on successive days. They were incubated at 37° C. for the entire time of testing. The cultures showed colonies giving the appearance of both Escherichia coli (Bacterium coli) and Aerobacter aerogenes (Bacterium aerogenes) and a few non-lactose-fermenting colonies. On the 11th day, one culture ceased to show colonies, but two days later, positive

colonies again appeared. After the fourteenth day, another culture ceased growing, and after the sixteenth day, one other did not show colonies. In both of these cases, after colonies had ceased to appear on Endo plates, transfers of one half cubic centimeter were made from the original tube to lactose broth tubes. These sub-cultures were positive, and showed typical colonies on Endo plates. Five cultures were still growing at the end of 22 days, when the experiment was discontinued. The colonies on Endo plates were faded, but on reculturing, typical colonies grew. The cultures seemed to contain both Escherichia coli (Bacterium coli) and aerobacter aerogenes (Bacterium aerogenes), neither outgrowing the other.

Discussion.

One other possible cause for non-confirming tubes, is the occasional presence on Endo plates of cultures of the Pseudomonas fluorescens (Bacillus fluorescens liquefaciens) type. By picking and restreaking from such cultures, coli-aerogenes organisms can be isolated, from seemingly negative growth. That this type of overgrowth is responsible for some of the seasonal variation in confirmations is suggested by the work of Young & Greenfield[19] (1923), in which inoculated soil was exposed for six

years. During periods of much rainfall, October and the early spring months, they report that the Escherichia coli (Bacterium coli) was masked by Pseudomonas fluorescens (Bacillus fluorescens liquefaciens) altho Escherichia coli (Bacterium coli) was present, as shown by its isolation at the end of the period.

Apparently these several factors are responsible for the non-confirming tubes, namely: symbiotic complexes, a change in appearance of coli-aerogenes organisms by the metabolic products of associated water bacteria, and a masking of the Escherichia coli (Bacterium coli) by the seasonal growth of Pseudomonas fluorescens (Bacillus fluorescens liquefaciens).

In connection with the spontaneous appearance of Escherichia coli (Bacterium coli) and Aerobacter aerogenes (Bacterium aerogenes) in cultures, may be mentioned the work of Stearn[20] (1923), in which she showed that the prolonged cultivation of seven strains of lactose-fermenting water-borne bacteria in gentian violet broth, brought about an increase in the growth, motility and fermentative action of the bacteria, so that at the end of five months incubation, strains with diverse characteristics at the beginning of the experiment gave similar

reactions, all of B. coli communior A. However, incubation was for a longer period of time than that in our own experiments and she was dealing with pure cultures, whereas this work was of necessity with mixed cultures.

As for symbiotic complexes, it is quite possible that the formation of gas for one or two generations, as shown in 23 cases, might have been from this phenomenon, and that altered conditions resulting from the metabolism of the various bacteria involved, caused changes which inhibited gas formation. In the later attempts, the failure to isolate Gram-positive coccus forms would suggest that they might be inhibited by the dye, altho the presence of dextrose-fermenting, non-lactose fermenting organisms in 61.2 per cent of 67 non-confirming tubes, would suggest that the phenomenon had been present. However, instead of explaining the seasonal appearance of non-confirming positive tubes by the activity of symbiotic complexes, it may be that the small number of these non-confirming presumptive tests is due to an inhibition by the dye, of one or more of the organisms involved.

There was no evidence of bacteriophage in plates, or from the broth of non-confirming tubes, transferred to broth tubes of pure cultures.

Altho but little work has been done to test the inhibitory powers of basic fuchsin, it apparently acts much the same in inhibiting the Gram-positive organisms, as brilliant green, gentian violet, and rosalic acid. These dyes are closely related chemically, all being classed as phenyl-methane dyes; brilliant green is a diamino tri-phenyl methane, gentian violet and basic fuchsin triamino tri-phenyl methanes, and rosalic acid a hydroxy tri-phenyl methane. That some of the growth occuring in standard broth is inhibited in fuchsin broth, is indicated by the clearness of many of the fuchsin tubes in which gas is not formed. Furthermore, some colonies picked from agar plates, as prepared for the total count, will grow readily in standard broth, but show no clouding in fuchsin broth. That anaerobic organisms are inhibited is suggested by the large number of chlorinated tap effluent samples which give false positives in standard broth, but negative presumptive tests in fuchsin broth. Very often, these standard broth tubes will show no aerobic growth when streaked on Endo plates or eosin methylene blue plates, but all positive tubes of fuchsin broth show aerobic colonies. Failure to show aerobic growth might indicate the presence of organisms intolerant of the dye,

as Pseudomonas aerüginosa (Bacillus pyocyaneus).

It is interesting to note that Thompson[21](1927), reporting non-confirming positive presumptive tests from raw Lake Ontario water, notes that the condition appears to be seasonal, as he finds the majority of such results, during the period October to AApril. However, he attributes the failures to a lethal H-ion concentration, and obtained a larger number of confirmations by increasing the buffering capacity of the presumptive medium by the addition of dipotassium phosphate. In a discussion by Gorman, of a paper by Noble[22](1928), the antibiosis of Escherichia coli (Bacterium coli) and Clostridium Welchii (Bacillus Welchii) in the Chicago water, as causing an apparent seasonal diminution of Escherichia coli (Bacterium coli) is suggested. This decline occurred in the winter and spring months.

Conclusions.

1. Basic fuchsin, used in a final concentration of 1-1250 of a saturated alcoholic solution in lactose broth, as suggested by Schreiner, has been found a satisfactory modification of that medium for routine water analysis in Kansas.

2. Results from 32,578 samples show 94.32% confirmations.

3. There is a marked seasonal variation in the non-confirming tubes showing gas, the maximum being from November to May, and the minimum from May to November.

4. Comparative results of 514 samples run in standard broth with and without fuchsin, show similar results as regards isolation of coli-aerogenes organisms in 65.4% of the cases, a higher number of isolations from standard broth in 17.7% of the cases, and a higher number of isolations from fuchsin broth in 16.9% of the cases.

5. The selective action of basic fuchsin is most marked in chlorinated surface tap effluent samples.

6. Attempts to show the cause for non-confirming positive tubes gave evidence to show that no one factor was responsible. In different cases the gas might be due to: a. coli-aerogenes organisms masked on confirmatory plates by Pseudomonas fluorescens (Bacillus fluorescens liquefaciens); b. coli-aerogenes organisms altered in appearance on

confirmatory plates by the metabolic activities of other organisms present; and c. symbiotic complexes, as suggested by the formation of gas for one or two generations by twenty-three non-confirming samples, which showed no subsequent growth; and the presence of dextrose-fermenting organisms in lactose-negative cultures, suggesting the symbiotic association of these types with lactose-splitting organisms, and consequent production of gas from lactose. There was no evidence of bacteriophage.

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