STUDIES ON PRIMARY AND SELECTIVE MEDIA FOR COLIFORM ORGANISMS

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Charles Willis Darby

1943

This is to certify that the

thesis entitled

Studies on Primary and
Selective Media
For Coliform Organisms

presented by

Charles Willis Darby

has been accepted towards fulfilment
of the requirements for

M. S. degree in Bacteriology

Major professor

Date March 9, 1943

STUDIES ON PRIMARY AND SELECTIVE MEDIA FOR COLIFORM ORGANISMS

By
Charles Willis Darby

A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology and Hygiene

1943

THESIS

PREFACE

Because the academic requirements for the Master of Science Degree were fulfilled on a part-time basis this work was extended over a period of four years. After the completion of the first part of the problem, Professor W. L. Mallmann suggested that it would be highly desirable to have this work published. Permission was obtained from the Graduate Council for the publication of Studies on Media for Coliform Organisms, which was published in the Journal of the American Water Works Association, April, 1939.

Studies were then started to devise an improved selective medium for the bacteriological examination of water. The laboratory and field results of these investigations were presented as a second paper, Uses of a Lauryl Sulfate Tryptose Broth for the Detection of Coliform Organisms. This paper was published in the American Journal of Public Health, February, 1941.

A study of these papers will show the necessity for early publication so that other workers would have the opportunity to check the new media and methods under practical field conditions.

The Graduate Council kindly consented to allow the published papers to be included, in reprint form, as a portion of this thesis.

TABLE OF CONTENTS

INTRODUCTION

A study designed to develop selective media for cultivating a specific group of bacteria naturally divides itself into three phases.

The first phase is to develop a practical basic medium that will supply an ideal environment for growth. The experimental work covering this phase of the problem is presented in Part I, Studies on a Basic Medium for Coliform Organisms.

Finding an ideal selective agent to retard or eliminate undesirable organisms is the second major phase. The experimental work dealing with this phase of the problem is presented as Part II, Studies on Bacteriostatic Agents.

The third and final phase is to combine the basic medium and the selective agent into a selective medium. This medium must then be subjected to laboratory and field trials for a final evaluation. These studies are presented in Part III, Studies on Selective Media for Coliform Organisms.

Part I

Studies on a Basic Medium for Coliform Organisms

Introduction

Soon after the introduction of quantitative laboratory technics, bacteriologists began to compare the various media then available for the growth of different groups of bacteria. From these studies evolved numerous media, especially a great variety for the more fastidious organisms.

Because the coliform (1) group of bacteria grow in a wide variety of the more simple media, relatively little attention has been given towards developing more suitable media to support their growth.

Winslow (2), in 1905, demonstrated that the type of medium and the period of incubation would make radical differences in bacteria counts from water and sewage.

The first report on investigations concerning media and technics to be used for water analysis was published in 1898 (3). A permanent Committee on Standard Methods was formed in 1901 (5). From 1901 to 1925 numerous changes were made in standard methods, both in the content and preparation of the media used and in the methods of executing these tests in the laboratory. The latest major changes were made in the report of the American Committee on Standard Methods (1925).

As early as 1891, Reinsch (6) demonstrated that the reaction of the medium would cause a marked difference in

bacteria counts from the same water samples. In 1922 Bunker and Schuber (7) demonstrated the value of having a definite standard hydrogen-ion concentration for all media used in water analysis. They recommended a pH of 6.8-7.2 for ordinary solid and liquid media.

In 1904 Gage and Adams (8) demonstrated that marked differences in counts resulted from using different peptones in the basic medium. The same authors demonstrated the importance of using distilled water in the preparation of standard media.

A definite step toward uniformity was made when the time-consuming and inconstant meat infusions were replaced by Liebig's or any comparable meat extract (4).

The pioneers in water analysis first relied on agar or gelatin plates for the primary isolation of coliform organisms. As early as 1892, Mathews (9) used litmus-lactose-agar plates for this purpose.

In 1893 Smith (10) demonstrated the value of a primary enrichment medium of dextrose broth. By seeding definite amounts of water into a series of tubes a quantitative test was developed using only a broth medium. That this method was qualitatively superior to the direct plating method was demonstrated by Irons in 1901 (11).

Soon after the introduction of dextrose broth as a presumptive medium, numerous workers found that only 60 to 70 per cent of the tubes showing gas in this medium actually contained coliform organisms. Although these reports were published and

a great deal of discussion resulted, it was not until the 1923 edition of Standard Methods that lactose was substituted for dextrose in the presumptive test.

Although various enrichment media have been devised in recent years, standard lactose broth has remained unchanged since 1925. This medium contains the following per liter: beef extract, 3 grams; peptone, 5 grams; and lactose, 5 grams. The final hydrogen-ion concentration should be between 6.8 and 7.0.

In practically all of the papers that have appeared in the literature comparing media used in water analysis, the same standards have been used for comparison. They have taken total counts after one, two or more days' incubation or gas production in 24 to 48 hours or longer as a measure of efficiency.

The first appended publication, Studies on Media for Coliform Organisms, presents the data obtained in an effort to devise a more efficient basic medium to be used in the presumptive test for water analysis.

Reprinted from JOURNAL OF THE AMERICAN WATER WORKS ASSOCIATION
Vol. 31, No. 4, April, 1939

Studies on Media for Coliform Organisms

By C. W. Darby and W. L. Mallmann

THERE has been considerable doubt in the minds of the writers as to the value of standard lactose broth as an enrichment medium for culturing coliform organisms from water and other sources when the organisms have been attenuated or when they appeared in minimal numbers. The fact that coliform organisms frequently appear in fermentation tubes after incubation periods longer than 48 hours or in tubes incubated at temperatures below 37°C. would seem to indicate that the prolonged lag phase of growth might be caused by an unfavorable environment in the nutrient medium. Little effort has been made to check the efficiency of this medium by the development of better enrichment media for comparative tests. More frequently this medium has been used as a standard to measure the value of new selective media in an attempt to produce a new medium with selected properties for the coliform organism with enrichment value equal to that of the present standard lactose broth.

The appearance on the market of new peptones which are being used extensively in the growth of such pathogenic bacteria as the diphtheria bacillus and gonococcus offers an opportunity for the application of these nutrients to the growth of other organisms such as the coliform group. In this laboratory Bacto-tryptose, a peptone, which is being used extensively for the cultivation of organisms that grow scantily on Bacto-peptone media, seems particularly adaptable to this application.

In most studies on the elaboration of a nutrient medium it has been customary to measure the response of the organisms to the medium by using a loopful of organisms to each tube of material

A record of research contributed by C. W. Darby, B.A., and W. L. Mallmann, Ph.D., Michigan Engineering Experiment Station, East Lansing, Michigan. This study was made by Mr. Darby in partial fulfillment of the requirements for the master's degree.

tested and then measuring the maximum growth obtained at the end of 24 to 48 hours incubation. The medium giving the greatest amount of growth at this time has been selected as the best. In most instances, perhaps, this procedure has obtained its end, particularly if the investigator desires large quantities of organisms.

It is the authors' contention, however, that the medium giving the greatest number of organisms in 24 or 48 hours incubation is not necessarily the best diagnostic medium. It would seem that the best diagnostic medium is one that will allow the development of the desired organism when minimal numbers are present in the material to be examined. In waters of questionable sanitary quality it is conceivable that the use of standard lactose broth may not obtain such results.

TABLE 1

Viability of Esch. Coli in Different Media Before and After Lethal Exposure to Irradiation

MEDIA	UNTREATED	IRRADIATED	HEATED
Nutrient agar	570,000	20	27
Nutrient agar/blood	570,000	65	102
Nutrient agar/glucose	600,000	45	105
Nutrient agar/yeast	610,000	25	27
Infusion agar	610,000	38	189
Tomato juice milk powder agar	540,000	69	237

It is not within the province of this paper to show the practical significance of the increasing numbers obtained but it is our thesis that a diagnostic medium should produce a maximum number of the organisms present from the material examined whether it be the diphtheria bacillus from a throat swab or coliform organisms from a water supply. The value of a diagnostic medium lies in those of its properties which promote a rapid growth of the dormant viable cells in the material examined. There should be a continuance of rapid reproduction until the cells appear in such abundance that they can be recognized by their physiological action on the medium or so that they can be transplanted to differential media.

Many media do not present a suitable environment for the viable cells to reach the reproductive stages and thus fail to give positive cultures. Many viable cells of *Escherichia coli* fail to reproduce on certain media as shown by Curran and Evans (2) in table 1. It will

be observed from this table that on the tomato juice milk powder agar 237 cells survived heat treatment; whereas on the nutrient agar only 27 cells survived. That the latter medium is best suited for demonstrating surviving organisms is quite apparent.

It is a well established fact that when organisms are transferred from one environment to another, particularly if the new environment differs radically from the previous one, many viable cells fail to reproduce due to their inability to adjust themselves to the new conditions or to adjust their immediate surroundings to their needs. Because of this it is customary in transferring many organisms to use mass seedings, hoping that a sufficient number of viable cells will reproduce and thus assure a positive culture.

In single-cell isolation work Wright and Hendrickson (20) found that by incubating the single cell in a drop of broth in a moist chamber, rather than in a tube of nutrient medium, they were able to grow as high as 90 per cent of the cells isolated. It would appear from this work that the cell must adjust its environment to its own needs and that by the use of a minimum rather than a large volume of broth the cell is able to adjust itself more rapidly and more surely. A number of years ago, the junior author of this paper spent several months trying to single-cell several strains of *Salmonella pullorum* using many types of enrichment media without success. Then one night in a demonstration of single-cell culture, five out of six cells grew in plain nutrient broth. Following this discovery, using the same batch of medium, single-cell cultures were prepared for all the desired strains of *Sal. pullorum* with a very high percentage of the single-cells growing. No explanation was found as to why this particular batch of medium gave results when previous batches and other media failed. But it appears quite obvious that environmental conditions were such that each viable single cell found its surroundings conducive to immediate growth without considerable adjustment prior to reproduction.

Reproduction begins after a cell has overcome its stationary phase and has taken in sufficient food material. As soon as the cell has undergone fission the resulting two young cells begin growth and ultimately reproduction. A new critical period in the life of the organism begins with the completion of fission. The work of Salter (11), Schultz and Ritz (12), Reichenbach (10), Sherman and Albus (13, 14), Stark and Stark (17), Heiberg (4), and Sherman and Cameron (15) shows that young cells are much more susceptible to

adverse conditions such as heat, 2 per cent sodium chloride, 0.5 per cent phenol, and dilute crystal violet, than cells found in the lag stages of a logarithmic growth phase. Huntington and Winslow (6) show that during the lag stage and the early logarithmic stage of growth the young cells exhibit all the characteristics of physiological youth comparable to those exhibited in multi-cellular organisms. This means that a good medium must produce an environment such that no inimical conditions exist during the critical period of lag and early logarithmic stages of growth. Otherwise the mortality of the cells may be such that no growth results.

It is our thesis in the light of past experience and the studies cited that a culture medium for diagnostic use should be measured not by gross growth obtained at the completion of the logarithmic stage

TABLE 2

Influence of Bacto-peptone in Various Concentrations in the Base Medium on Lag Phase of Growth of Esch. Coli, No. 161*

CONCENTRATION OF BACTO-PEPTONE	NUMBER OF BACTERIA PER ML.					
	Initial	4 hours	6 hours	12 hours	24 hours	48 hours
per cent						
0.5	15	800	2,900	52,000,000	413,000,000	408,000,000
1.0	20	1,200	4,700	30,000,000	704,000,000	731,000,000
2.0	18	1,260	4,400	32,000,000	1,300,000,000	872,000,000
3.0	16	770	1,750	8,300,000	1,940,000,000	1,244,000,000

* Base medium consists of: Bacto-beef extract 0.3 per cent, lactose 0.5 per cent.

but by the behavior of the organism during the lag and early logarithmic stages of growth. Therefore in the studies presented we have worked with minimal numbers of organisms that have been attenuated by age and we have studied the behavior of these organisms primarily during the lag and early logarithmic growth stages.

Procedure

Growth rates were determined in broth media containing various concentrations of the ingredients that enter into a nutrient medium to ascertain the amounts giving the maximum rate of reproduction during the early stages of the logarithmic growth phase of the organism. For these studies a laboratory strain of *Esch. coli* (No. 161

was selected. This culture was kept on an agar slant and, after the full development of the culture, it was kept in the refrigerator. Storage in the refrigerator over a period of months tended to attenuate the culture sufficiently to increase the stationary phase of the organism and thus give a better picture of the value of the medium studied in the recovery of the viable cells present. In all experiments organisms were taken directly from these slant cultures and introduced directly into the medium under investigation.

Small seedings were used to intensify the difficulties of the organisms to overcome unsuitable environments and thus increase the marginal differences in growth rates of the different types of media used.

Considerable care was exercised in the preparation of the media and in the technics of growing the cultures and the determinations of the numbers present in order to eliminate as far as possible all variables other than the one variable under study.

Experimental Background

Peptone. In an earlier study of bacteriostatic action of dyes, it was observed (table 2) that a concentration of 3 per cent Bacto-peptone in a medium caused a decreased growth in the lag phase of growth of *Esch. coli*. The fact that this concentration acted as an inhibitory agent seemed to indicate that a concentration of 0.5 to 1 per cent was best, not because the organisms grew best at these concentrations, but that in more dilute amounts it was less toxic. It would appear that its use in an enrichment medium such as lactose peptone broth certainly would not favor the development of isolated cells or groups of cells that were partially attenuated by chlorination. It appears quite evident that a more satisfactory source of nitrogenous food should be found.

A comparison of Bacto-tryptose and Bacto-peptone showed that much more rapid growth occurred with the former peptone (table 3). The two media are not exactly comparable because the constituents and their concentrations differ; however, the medium containing the Bacto-tryptose was far superior at all stages of growth through a 12-hour incubation period. The data are presented to show the marked difference between the two media.

The most effective concentration of tryptose was determined by varying the amounts of tryptose from 1 to 3 per cent in the same base medium. In table 4 data are presented for growth rates of

Esch. coli in varying concentrations of tryptose in a base medium of 2 per cent bile brilliant green lactose broth while for the growth rates in table 5 a phosphate buffered medium was used. In the bile brilliant green medium, 3 per cent tryptose gave the most rapid growth while in the buffered medium there was little choice between 2 and 3 per cent tryptose, although the 2 per cent medium showed slightly higher rates of growth. In similar experiments 2 and 3 per cent tryptose showed about the same effect on growth rates, although in general the 3 per cent concentration appeared to be slightly the better medium. In concentrations up to 4 per cent, the highest concentration tested, Bacto-tryptose showed no inhibitory effect such as that observed with Bacto-peptone.

TABLE 3

Comparison of Bacto-peptone and Bacto-tryptose Media by Means of Growth Rates of Esch. Coli, No. 161

MEDIUM	NUMBER OF BACTERIA PER ML.				
	Initial	3 hours	6 hours	9 hours	12 hours
Lactose broth*	86	1,560	573,000	31,300,000	232,000,000
Tryptose broth†	97	5,300	12,700,000	585,000,000	1,050,000,000

* Lactose broth: dehydrated Difco lactose broth.

† Tryptose broth: Bacto-tryptose 3.0 per cent, lactose 0.5 per cent, NaCl 0.5 per cent.

The concentration of 2 per cent was selected as optimum as the increased activity induced by 3 per cent tryptose did not appear to warrant the additional expense involved in practical use of the medium. It is possible that in practical applications, a one per cent concentration may be satisfactory.

Hydrogen ion concentration. Although the pH range for the growth of *Esch. coli* appears to be quite wide, it was felt that the influence of pH should be carefully checked to determine the effect on early growth rates. Media consisting of 3 per cent Bacto-tryptose and 0.5 per cent lactose were adjusted to pH values of 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8 and tested as in previous experiments. No pH values below 6.8 were tested as acid will cause a hydrolysis of the lactose during sterilization. The results are presented in table 6. It should be observed that at the end of 6 hours incubation at pH 6.8 the number of organisms was nearly double the number at

TABLE 4

Influence of Bacto-tryptose in Various Concentrations in a 2 per cent Brilliant Green Lactose Broth Base Medium on Lag Phase of Growth of Esch. coli, No. 161*

CONCENTRATION OF BACTO-TRYPTOSE	pH	NUMBER OF BACTERIA PER ML.								
		Initial	2 hours	3 hours	4 hours	6 hours	8 hours	12 hours	24 hours	48 hours
per cent										
1.0	7.4	30	55	700	850	11,130	302,000	205,000,000	600,000,000	86,000,000
2.0	7.4	33	62	904	2,450	17,000	869,000	398,000,000	980,000,000	95,000,000
3.0	7.4	36	52	1,380	4,800	36,100	2,444,000	839,000,000	1,420,000,000	132,000,000
Standard 2 per cent bile—B. G. broth Difco		33	37	470	530	6,200	133,000	78,000,000	440,000,000	33,000,000

* Base medium consists of: Bacto-ox-bile 2 per cent, brilliant green 0.00133 per cent, lactose 0.5 per cent.

TABLE 5

Influence of Bacto-tryptose in Various Concentrations in the Base Medium on Lag Phase of Growth of Esch. Coli, No. 161*

CONCENTRATION OF BACTO-TRYPTOSE	SAMPLE	NUMBER OF BACTERIA PER ML.			
		Initial	3 hours	6 hours	12 hours
per cent					
1	1	84	3,800	333,000	1,330,000,000
	2	86	3,300	397,000	995,000,000
	3	88	2,700	402,000	1,150,000,000
	Average	86	3,300	377,000	1,158,000,000
2	1	102	4,500	1,712,000	1,760,000,000
	2	90	3,600	646,000	1,450,000,000
	3	89	3,400	1,144,000	1,510,000,000
	Average	94	3,800	1,168,000	1,587,000,000
3	1	78	2,000	1,262,000	1,950,000,000
	2	96	2,900	994,000	2,000,000,000
	3	79	3,760	1,400,000	1,780,000,000
	Average	84	2,900	1,218,000	1,953,000,000

* Base medium consists of: lactose 0.5 per cent; K_2HPO_4 0.4 per cent; KH_2PO_4 0.15 per cent; NaCl 0.5 per cent; adjusted to pH 6.8 before sterilization.

pH 7.0 and that at increasing pH values the amount of growth decreased until at pH 7.8 the growth was only approximately one-fourth that obtained at pH 6.8.

In a number of other similar experiments, the maximum growth appeared at pH 6.8 during the early periods of the lag phase of growth. It will also be observed from these data that with the higher pH values, greater amounts of growth appeared at 24 and 48 hours incubation. This would be expected as the limiting acid concentrations caused by the fermentation of the lactose would be approached sooner in the growth cycle of the organisms in the media which were adjusted to lower pH values. A reaction of pH 6.8 was selected as the optimum value for the growth of *Esch. coli.*

TABLE 6

Influence of the Hydrogen Ion Concentration of lactose-bacto-tryptose Broth on Lag Phase of Growth of Esch. Coli, No. 161*

pH	NUMBER OF BACTERIA PER ML.				
	Initial	6 hours	12 hours	24 hours	48 hours
6.8	38	31,800	182,000,000	920,000,000	880,000,000
7.0	35	17,800	177,000,000	1,150,000,000	1,670,000,000
7.2	41	17,200	229,000,000	1,218,000,000	1,040,000,000
7.4	30	14,100	185,000,000	1,250,000,000	1,428,000,000
7.6	31	8,800	240,000,000	1,993,000,000	2,510,000,000
7.8	35	7,900	281,000,000	2,015,000,000	2,630,000,000

* The base medium consists of: Bacto-tryptose 3 per cent and lactose 0.5 per cent.

Walker and Winslow (19), Mallmann and Gallo (7), Slanetz and Rettger (16), and others have demonstrated that the addition of a phosphate buffer to a medium caused an increase in the total number of bacteria produced as compared to a similar medium without buffer. The use of buffered media for fermentation experiments was suggested by Bronfenbrenner and Schlesinger (1). The increased number of bacteria was believed by Mallmann and Gallo (7) to be due not only to the buffering effect on the acid produced by the organisms, but also to the need of phosphate in the metabolism of the organisms. The use of a buffered lactose peptone broth as a presumptive medium for the coliform organisms was recommended by Thompson (18).

In a study of buffers, Perry and Hajna (9) recommended a buffer mixture of 0.4 per cent K_2HPO_4 and 0.15 per cent KH_2PO_4 for the growth of coliform organisms. As these amounts give a pH of 6.8 in the medium before sterilization, this mixture was used. In all instances where the pH values were other than 6.8, the medium was adjusted to this value by the addition of normal NaOH. In table 7, the data for three series of these tests are presented. It will be observed that in all instances the growth during the early stages of reproduction and the later stages of the logarithmic phase, was much greater for the buffered medium. In media prepared by the Difco Laboratories, there was little choice between the buffered and non-buffered media during the early growth period but in most instances in the late logarithmic phase the buffered medium was superior. In general, the data showed that the addition of a buffer to the medium,

TABLE 7

Influence of a Buffer Mixture in the Base Medium on the Lag Phase of Growth of Esch. Coli, No. 161*

BUFFER	pH	SERIES NO. 1				
		Number of bacteria per ml.				
		Initial	6 hours	12 hours	24 hours	48 hours
None	7.0	35	17,800	177,000,000	1,150,000,000	1,670,000,000
Present	7.0	32	81,100	578,000,000	1,850,000,000	2,790,000,000
		SERIES NO. 2				
		Initial	3 hours	6 hours	9 hours	12 hours
None	6.8	19	725	1,217,000	470,000,000	997,500,000
Present	6.8	16	890	3,362,000	840,000,000	1,555,000,000
		SERIES NO. 3				
		Initial	2 hours	4 hours	6 hours	8 hours
None	6.8	95	350	28,700	2,050,000	88,200,000
Present	6.8	108	430	40,700	2,570,000	139,700,000

* Buffer mixture: K_2HPO_4 0.4 per cent; KH_2PO_4 0.15 per cent.
Series No. 1, base medium: Bacto-tryptose 3.0 per cent, lactose 0.5 per cent.
Series No. 2, base medium: Bacto-tryptose 2.0 per cent, lactose 0.5 per cent, NaCl 0.5 per cent.
Series No. 3, base medium: same as No. 2.

although not always causing an increase in reproduction during the early stages, did not hinder the rate of growth and in the late stages of the logarithmic phase the buffer caused a marked increase in the number of organisms.

TABLE 8

Influence of Sodium Chloride in a Base Medium on the Lag Phase of Growth of Esch. Coli, No. 161*

CONCENTRATION OF NaCl	BUFFERED MEDIUM* Number of bacteria per ml.				
	Initial	6 hours	12 hours	24 hours	48 hours
per cent					
None	32	81,100	578,000,000	1,850,000,000	2,790,000,000
0.5	35	106,000	1,068,000,000	2,500,000,000	1,970,000,000
	NON-BUFFERED MEDIUM†				
None	35	17,800	177,000,000	1,150,000,000	1,670,000,000
0.5	39	67,000	535,000,000	1,755,000,000	1,610,000,000

* Base medium consists of: Bacto-tryptose 3 per cent, lactose 0.5 per cent, K_2HPO_4 0.4 per cent; KH_2PO_4 0.15 per cent.

† Base medium consists of: Bacto-tryptose 3 per cent, lactose 0.5 per cent.

TABLE 9

Influence of Sodium Chloride in the Base Medium on the Lag Phase of Growth of Esch. Coli, No. 161*

CONCENTRATION OF NaCl	pH	NUMBER OF BACTERIA PER ML.				
		Initial	2 hours	4 hours	6 hours	8 hours
per cent						
0.5	6.8	108	430	40,700	2,570,000	139,700,000
1.0	6.8	90	540	53,100	2,090,000	153,200,000
2.0	6.8	109	250	20,600	650,000	42,000,000

* Base medium consists of: Bacto-tryptose 2 per cent, lactose 0.5 per cent, K_2HPO_4 0.4 per cent, KH_2PO_4 0.15 per cent.

Sodium Chloride. Dunham (3) reports that the addition of sodium chloride to eosin-methylene blue medium causes a marked increase in the number of positive coliform organisms isolated from lactose presumptive tubes showing gas. Mooney and Winslow (8) observed that *Salmonella pullorum* would not grow in peptone-glucose broth medium when aerated but when 0.5 per cent NaCl was added, growth was rapid. For these reasons the influence of NaCl was tested for

the development of the coliform organisms in a tryptose medium. In table 8 the data on the effect of 0.5 per cent NaCl in both a buffered and non-buffered medium are presented. It will be observed that in both media the addition of NaCl caused a marked increase in growth both in the early stages of growth and even in the decreasing logarithmic phase. In table 9 data showing the effect of 0.5, 1.0, and 2.0 per cent NaCl in a medium are presented. There was little difference between 0.5 and 1.0 per cent but 2.0 per cent showed a marked inhibitory effect on the rate of growth at

TABLE 10

Influence of Lactose in the Basic Medium on Lag Phase of Growth of Esch. Coli, No. 161*

CONCENTRATION OF LACTOSE	SAMPLE	NUMBER OF BACTERIA PER ML.				
		Initial	2 hours	4 hours	6 hours	8 hours
per cent						
0.25	1	86	240	8,220	626,000	78,000,000
	2	97	240	7,200	804,000	52,500,000
	Average	91	240	7,710	715,000	65,250,000
0.5	1	95	230	7,000	558,000	70,000,000
	2	108	250	9,400	470,000	66,600,000
	Average	102	240	8,200	514,000	68,300,000
1.0	1	92	220	9,400	640,000	53,800,000
	2	100	280	9,100	710,000	82,000,000
	Average	96	250	9,250	675,000	67,900,000
2.0	1	86	250	9,200	404,000	30,500,000
	2	97	240	8,150	506,000	36,200,000
	Average	91	245	8,675	455,000	33,350,000

* Basic medium consists of: Bacto-tryptose 2 per cent, NaCl 0.5 per cent, KH_2PO_4 0.15 per cent, K_2HPO_4 0.4 per cent; pH 6.8 before sterilization.

all stages of the logarithmic phase up to 8 hours incubation. In the light of these data, the addition of 0.5 per cent NaCl seemed advisable.

Lactose. Hershey and Bronfenbrenner (5) found that a concentration of 3.0 per cent lactose in a synthetic medium encouraged greater production of carbon dioxide and growth of slow lactose-fermenting organism than did a concentration of 0.5 per cent. To determine the influence of the concentration of lactose an experiment was set up using 0.25, 0.5, 1.0, and 2.0 per cent lactose. The

results are presented in table 10. No difference was observed in the rate of growth for concentrations of 0.25, 0.5 and 1.0 per cent lactose. With 2.0 per cent lactose a decrease in growth was evident after 4 hours incubation.

Mallmann and Gallo (7) found that *Esch. coli* even in a buffered medium seldom utilizes more than 0.5 per cent glucose and seldom more than 0.3 per cent in an unbuffered peptone broth. Inasmuch as no difference was found in concentrations varying from 0.25 to 1.0 per cent and because actual utilization of lactose is generally not more than 0.5 per cent, there appeared no reason for a change from the present accepted concentration.

TABLE 11

*Comparative Growth Rates of Strains of Escherichia coli and Aerobacter aerogenes on Tryptose lactose Broth**

CULTURE	NUMBER OF BACTERIA PER ML.			
	Initial	4 hours	8 hours	12 hours
Esch. coli- 161	72	44,200	92,000,000	1,400,000,000
Esch. coli—165	211	16,500	37,000,000	990,000,000
Esch. coli—166	207	58,000	28,000,000	1,430,000,000
Esch. coli—171	253	41,900	74,000,000	1,100,000,000
Aer. aerogenes—140	225	24,400	20,160,000	740,000,000
Aer. aerogenes—131	245	46,500	46,200,000	1,100,000,000
Esch. coli—167	182	37,500	103,000,000	1,530,000,000

* The medium consists of: Bacto-tryptose 3 per cent, lactose 0.5 per cent, K_2HPO_4 0.4 per cent, KH_2PO_4 0.15 per cent, NaCl 0.5 per cent; medium adjusted so pH 6.7 after sterilization was obtained.

These results do not in any way fail to confirm the work of Hershey and Bronfenbrenner (5) since their observations were made at the end of two days incubation and the above-mentioned observations were limited to 8 hours incubation.

For comparative purposes and uniformity of data, only one strain of the coliform group was used, namely *Esch. coli*, No. 161. To check the effectiveness of the tryptose lactose medium in growing the coliform organisms from their stationary phases, five strains of *Escherichia coli* and two strains of *Aerobacter aerogenes* were tested. The results are presented in table 11. No cases of marked retardation were observed although there was a difference in the rates of reproduction during the early growth phases.

Comparison of Lactose Peptone and Tryptose Lactose Broths

Throughout all of the studies presented in this paper, comparisons of the tryptose lactose medium were made with standard lactose peptone broth. In table 12 three comparisons are presented. In these comparative tests, as well as in all of the other tests, wherein these two media were compared, the tryptose lactose medium was far superior to standard lactose peptone broth at all stages of growth. In all instances, the periods of stationary and lag phases were much

TABLE 12

Comparison of Standard Lactose Broth and Lactose Tryptose Buffer Broth for Rate of Growth of Esch. coli, No. 161

MEDIUM	pH	SERIES NO. 1				
		Number of bacteria per ml.				
		Initial	3 hours	6 hours	9 hours	12 hours
Standard*.....	6.55	51	960	79,000	7,750,000	229,500,000
Tryptose†.....	6.8	64	5,885	1,440,000	970,000,000	2,205,000,000
		SERIES NO. 2				
Standard.....	6.8	29	49	2,300	62,000	360,000,000
Tryptose.....	6.8	49	420	80,300	115,000,000	2,070,000,000
		SERIES NO. 3				
		Initial	2 hours	4 hours	6 hours	8 hours
Standard.....	6.8	25	41	455	25,000	1,235,000
Tryptose.....	6.8	27	72	1,785	170,000	13,600,000

* Standard: Bacto-lactose broth, A.P.H.A.

† Tryptose, medium consists of: Bacto-tryptose 2 per cent, lactose 0.5 per cent, K_2HPO_4 0.4 per cent, KH_2PO_4 0.15 per cent, NaCl 0.5 per cent.

shorter in the tryptose lactose broth, indicating very clearly that this medium allowed the rapid development of the viable cells by the fact that it offers a much better environment for the early reproduction of the organisms. This, in turn, means that more viable cells will reproduce so that the colon index will be higher in this medium than in standard lactose peptone broth particularly if the coliform organisms present have been attenuated.

The physiological by-products of metabolism parallel roughly the rate of reproduction of the organisms. In tables 13 and 14 data

are presented to show the time of appearance of gas. Three media are presented, the proposed tryptose lactose broth, the same medium minus buffer, and standard lactose peptone broth. An examination of the data shows that gas appeared sooner in the tryptose media than in the standard lactose peptone broth and that the quantity

TABLE 13

Influence of the Medium on the Rate of Gas Production of Esch. Coli, No. 161 (Series No. 1)

MEDIUM	TUBE NUMBER	TIME OF APPEARANCE OF GAS AND GROWTH									
		9.5 hours		10.5 hours		11.5 hours		12.5 hours		13.5 hours	
		Visible growth	Gas	Visible growth	Gas	Visible growth	Gas	Visible growth	Gas	Visible growth	Gas
1	1	+	+	+	10	+	40	+	60	+	90
	2	+	+	+	15	+	40	+	60	+	80
	3	+	+	+	10	+	40	+	60	+	80
	4	+	+	+	15	+	40	+	70	+	80
	5	+	+	+	20	+	50	+	70	+	90
2	1	+	+	+	2	+	20	+	40	+	60
	2	+	+	+	20	+	60	+	70	+	70
	3	+	+	+	40	+	75	+	80	+	90
	4	+	+	+	25	+	60	+	70	+	80
	5	+	10	+	50	+	75	+	80	+	90
3	1	±	−	+	−	+	−	+	5	+	20
	2	±	−	+	−	+	+	+	5	+	10
	3	±	−	+	−	+	−	+	10	+	15
	4	±	−	+	−	+	−	+	5	+	10
	5	±	−	+	−	+	+	+	10	+	20

Medium 1 consists of: Bacto-Tryptose 2 per cent, lactose 0.5 per cent, NaCl 0.5 per cent, K_2HPO_4 0.4 per cent, KH_2PO_4 0.15 per cent.

Medium 2 consists of: Bacto-Tryptose 2 per cent, lactose 0.5 per cent, NaCl 0.5 per cent.

Medium 3: Bacto-lactose-peptone broth.

Inoculum: 580 organismo per tube.

of gas was far greater in the former media. The non-buffered tryptose broth showed more gas than the buffered medium at the same early periods due to the fact that the fall in pH was more rapid in the non-buffered medium; hence there would be less conversion of the carbon dioxide to carbonates. As would be expected from a

examination of the growth curves of the various media, visible turbidity appeared earlier in the tryptose media.

The value of growth rates, particularly during the lag and early logarithmic phases for the evaluation of a nutrient medium rather than maximum growths is clearly demonstrated. For example, in

TABLE 14

Influence of the Medium on the Rate of Gas Production of Esch. Coli, No. 161 (Series No. 2)

MEDIUM	TUBE NUMBER	TIME OF APPEARANCE OF GAS AND GROWTH									
		11 hours		13 hours		14 hours		15 hours		24 hours	
		Visible growth	Gas	Visible growth	Gas	Visible growth	Gas	Visible growth	Gas	Visible growth	Gas
1*	1	+	−	+	−	+	−	+	10	+	90
	2	+	−	+	−	+	−	+	10	+	90
	3	+	−	+	−	+	−	+	+	+	100
	4	+	−	+	−	+	5%	+	5	+	90
	5	+	−	+	−	+	5%	+	5	+	90
2	1	+	−	+	−	+	5	+	20	+	70
	2	+	−	+	−	+	+†	+	5	+	70
	3	+	−	+	−	+	10	+	5	+	60
	4	+	−	+	−	+	+	+	10	+	60
	5	+	−	+	−	+	+	+	20	+	70
3	1	−	−	±	−	±	−	+	−	+	10
	2	−	−	±	−	±	−	+	−	+	20
	3	−	−	±	−	±	−	+	−	+	20
	4	−	−	±	−	±	−	+	−	+	10
	5	−	−	±	−	±	−	+	−	+	10

* Medium 1 consists of: Bacto-tryptose 2.0 per cent, lactose 0.5 per cent, NaCl 0.5 per cent, K_2HPO_4 0.4 per cent, KH_2PO_4 0.15 per cent.

Medium 2 consists of: Bacto-tryptose 2.0 per cent, lactose 0.5 per cent, NaCl 0.5 per cent.

Medium 3: Bacto-lactose-peptone broth.

† Bubble of gas is indicated by +.

Inoculum per tube: 40 organisms.

table 6, in measuring the significance of pH in the growth of *Esch. coli*, the medium of pH 6.8 gave a count of 31,800 in 6 hours incubation while the medium at pH 7.8 gave a count of 7,900. At the end of 48 hours incubation the medium at pH 7.8 showed a count of 2,630-000,000; whereas the medium at pH 6.8 showed only 880,000,000

organisms. If the two media were measured by total growth, the medium at pH 7.8 is superior but if the media were measured by the initial number of viable cells that reproduce, the medium at pH 6.8 is far superior. It is quite evident that the medium at pH 7.8 does not offer a favorable environment for the development of the viable cells present and hence such a medium would be an unsatisfactory diagnostic or enrichment medium.

Another example of the value of this procedure is shown in table 8 in the influence of sodium chloride in the medium on growth rates. The amount of growth in both the salt and non-salt media is approximately the same at the end of 48 hours incubation whereas at the end of 6 hours incubation the salt media show a much higher count. If the media were measured at the end of 48 hours incubation, it would appear that salt had no appreciable value in the medium; whereas at the end of 6 and 12 hours incubation, it is quite evident that salt increases growth rates materially. Repeatedly during this study similar experiences have occurred, demonstrating the value of the growth rate method for the development of a culture medium designed for diagnostic purposes.

It is realized that the medium presented in this paper differs radically from the present standard lactose peptone medium, but it is the opinion of the writers that an enrichment medium used as a presumptive medium for the detection of the coliform organism should allow the development of all viable cells present in the material to be examined. This medium based on studies made with minimal numbers of viable cells shows that a much greater rate of reproduction occurs during the lag and early logarithmic phases of growth with a materially shorter stationary phase than standard lactose peptone broth would show. That it does allow the development of more viable cells than standard lactose peptone broth was demonstrated this past summer on swimming pool samples.

The use of this medium on routine water analysis has not as yet been tested on a wide scale. At the present writing it is being used in seven water purification plant laboratories in parallel with standard lactose peptone broth for comparative purposes. The results of this study will be presented at a later date.

The medium is being presented at this time to give water analysts an opportunity to make comparative tests so that it will be possible to determine whether or not its use would be worthwhile for routine use in place of the standard lactose peptone broth.

Summary

1. Bacto-tryptose was found to be superior to Bacto-peptone in a base medium for all growth phases of *Esch. coli*.

2. A concentration of 2 per cent Bacto-tryptose in the medium was found to give the greatest rate of reproduction during the lag and early logarithmic growth phases.

3. The addition of phosphate buffer to the medium caused a much greater growth in the late logarithmic phase and a slightly greater increase during the lag phase than the non-buffered medium.

4. The rate of growth during the lag phase was greatest at a pH of 6.8.

5. The concentration of lactose appeared to have no influence on the rate of growth during the lag and early logarithmic phases.

6. The addition of 0.5 per cent sodium chloride to the medium caused a marked increase in the rate of reproduction during the lag and early growth phases.

7. A 2 per cent Bacto-tryptose lactose buffered broth was far superior to standard lactose peptone broth for the growth of coliform organisms during the lag and early logarithmic phases. The increase in numbers persisted into the late logarithmic and decreasing logarithmic phases of growth.

8. The medium is suggested as an enrichment medium for the growth of coliform organisms. The formula follows:

Bacto-tryptose	2	per cent
Lactose	0.5	per cent
K_2HPO_4	0.4	per cent
KH_2PO_4	0.15	per cent
NaCl	0.5	per cent

pH before sterilization: 6.8

In a subsequent paper the results showing the use of this medium as a presumptive medium for the detection of the coliform organisms in water supplies will be presented.

EDITOR'S NOTE. This paper has been published at the request of the Executive Committee of the Water Purification Division in the hope that the media therein suggested will be extensively tested elsewhere so that experiences may be summarized in a discussion before the Water Purification Division.

References

1. BRONFENBRENNER, J., AND SCHLESINGER, M. J. Carbohydrate Fermentation by Bacteria as Influenced by the Composition of the Media. Proc. Soc. Exp. Biol. and Med. **16:** 44 (1918).
2. CURRAN, H. R., AND EVANS, F. R. The Importance of Enrichments in the Cultivation of Bacterial Spores Previously Exposed to Lethal Agencies. J. Bact. **34:** 179 (1937).
3. DUNHAM, H. G. Personal communication.
4. HEIBERG, B. Die Thermoresistenz bei jungen und alten Bakterien und "jungen" and "alten" Bakteriophagen. Zeitschr. Hyg. u. Infektionskr. **114:** 425 (1932).
5. HERSHEY, A. L., AND BRONFENBRENNER, J. The Influence of the Composition of the Medium on the Metabolism of Some Slow Lactose-Fermenting Bacteria of Intestinal Origin. J. Bact. **32:** 519 (1936).
6. HUNTINGTON, EVELYN, AND WINSLOW, C.-E. A. Cell Size and Metabolic Activity of Various Phases of the Bacteria Culture Cycle. J. Bact. **33:** 123 (1927).
7. MALLMANN, W. L., AND GALLO, FRANK. The Influence of Phosphates on the Metabolism of Bacteria. Mich. Acad. Sci. Arts, and Letters **14:** 617 (1931).
8. MOONEY, G., AND WINSLOW, C.-E. A. The Metabolic Activity of Various Colon Group Organisms at Different Phases of the Culture Cycle. J. Bact. **30:** 427 (1935).
9. PERRY, C. A., AND HAJNA, A. A. A Modified Eiijkmann Medium. J. Bact. **26:** 419 (1933).
10. REICHENBACH, H. Die Absterbeordnung der Bakterien und ihre Bedeutung fur Theorie und Praxis der Desinfektion. Zeitschr. f. Hyg. **69:** 171 (1911).
11. SALTER, R. C. Observations on the Rate of Growth of Bacillus Coli. Jour. Infect. Dis. **24:** 260 (1919).
12. SCHULTZ, J. H., AND RITZ, H. Die Thermoresistenz junger und alter Coli-Bacillen. Centralb. f. Bakt. I Abt., Orig. **54:** 283 (1910).
13. SHERMAN, J. M., AND ALBUS, W. R. Physiological Youth in Bacteria J. Bact. **8:** 127 (1923).
14. Ibid. The Function of Lag in Bacterial Cultures. J. Bact. **9:** 303 (1924)
15. SHERMAN, J. M., AND CAMERON, G. M. Lethal Environmental Factors within Natural Range of Growth. J. Bact. **27:** 341 (1934).
16. SLANETZ, C. A., AND RETTGER, L. F. The Influence of Phosphate Buffer in Carbohydrate-free and in Glucose Containing Media. J. Bact **15:** 297 (1928).
17. STARK, C. N., AND STARK, P. The Relative Thermal Death Rates of Young and Mature Bacterial Cells. J. Bact. **18:** 333 (1929).
18. THOMPSON, R. E. Irregularities in the Test for B. Coli. in Water. J. Bact. **13:** 209 (1927).
19. WALKER, H. H., AND WINSLOW, C.-E. A. Metabolic Activity of the Bacterial Cell at Various Phases of the Population Cycle. J. Bact. **24:** 209 (1932).
20. WRIGHT, W. H., AND HENDRICKSON, A. A. Studies on the Progeny of Single-cell Isolations from the Hairy-root and Crown-gall Organisms Jour. Agri. Res. **41:** 541 (1930).

Part II

Studies on Bacteriostatic Agents

It is not within the province of this thesis to review completely the work that has been done in the field of bacteriostasis The major problem was to investigate the bacteriostats under controlled laboratory tests with the hope that some of the newer dyes or compounds at our disposal might be more effective than those in current use. The experimental data are presented, not as a completed study of any one series of dyes or other bacteriostatic agents, but merely as experimental data that may be of practical use in future investigations. The practical possibilities of each set of data will be discussed with each experiment.

In 1926 Churchman (27) gave the following definition for the term "bacteriostasis." "At least four phases of bacterial inhibition have been observed in the case of bacteria exposed to the action of gentian violet and related dyes: cessation of motility; inhibition of reproduction; suspension of animation; and inhibition of sporulation. Anilin dyes may show these four types of inhibition without killing. The difficulty of distinquishing between death and inhibition of growth has led the writer to use the term 'bacteriostasis.' In the cases of triphenymethane dyes, gram-positive bacteria are, as a rule, much more susceptible than the gram-negative."

Although bacteriologists were using bacteriostatic agents as early as 1902 (23), Churchman (24), in 1912, was the first

worker to present a comprehensive paper on this subject. He studied the action of gentian violet on a large collection of bacterial species. He divided these organisms into violet negative and violet positive groups, and demonstrated the practical importance of this work. In the same year Churchman presented a second paper (25), in which he demonstrated that by the use of gentian violet it was possible to differentiate between closely related bacterial species. In this paper he made the following prophetic statement. "---that a substance will be found possessing a similar selective affinity between such bacteriologically troublesome organisms as Bacillus typhosus and Bacillus coli does not seem to be out of the question."

In 1923 Churchman (26) demonstrated that this inhibition of gram-positive bacteria by gentian violet could be accomplished by adding the dye to the medium (extrinsic bacteriostasis) or the organisms could be stained with it before being planted on plain agar (intrinsic bacteriostasis). He also demonstrated that selective bacteriostasis is not necessarily associated with selective penetrability. Organisms that are heavily stained may grow, and others, apparently unstained by the dye, were inhibited. At this time he pointed out that the bacteriostatic action may be due to changes effected by the dye at the surface of the organisms.

Stearn and Stearn (28), in 1924, offer an elaborate chemical explanation for the mechanism of bacteriostasis. Their paper includes bacteriostatic titers obtained by other workers in this

in this field. Any investigator interested in bacteriostasis should review the material presented in this paper.

Dubos (29), in 1929, and Ingraham (30), in 1933, point out that the bacteriostatic action of gentian violet and related compounds is closely associated with the ability of these compounds to poise the oxidation-reduction potential of the medium. Ingraham definitely shows that during the lag phase of growth neither the dye nor the organisms are altered.

The possibility of inhibiting gram-negative bacteria and allowing gram-positive bacteria to grow was first recognized by Churchman in 1923 (26, 31). He demonstrated that in suitable concentrations acid fuchsin would exhibit the reverse action of gentian violet on gram-positive and gram-negative organisms. This phenomenon has been called "reverse bacteriostasis." In 1939 Bryan, Devereux, Hirschey and Corbett (32) demonstrated the value of sodium azide as an inhibitory agent for gram-negative bacteria. Mallmann (46), in 1940, demonstrated the value of sodium azide for the isolation of streptococci from sewage. In 1940 Snyder and Lichstein (33) and in 1941 Lichstein and Snyder (34) presented the value of sodium azide in inhibiting the growth of gram-negative bacteria when culturing feces for the isolation of streptococci. In 1941 Mallmann, Botwright and Churchill (35) published a paper on the selective bacteriostatic effect of slow oxidizing agents. In this study they demonstrated that both potassium dichromate and sodium azide exhibited the property of "reverse bacteriostasis."

In 1913 Browning, Gilmour and Mackie (23) presented a technic for the isolation of typhoid bacilli from feces. They demonstrated that brilliant green had the ability to inhibit Escherichia coli but allow Eberthella typhosa to grow. They stated that Conradi and Drigalski, Loffler, and Savage were using this selective action of brilliant green as early as 1908. Savage was using it in his studies for the isolation of the Gartner's bacillus from fecal material. In 1927 Bakieten and Rettger (36) used brilliant green in a buffered broth as an enrichment medium for typhoid and paratyphoid organisms. Mallmann, Thorp, and Semmes (37), in 1928, demonstrated the value of a brilliant green liver infusion medium for the isolation of Salmonella pullorum from infected chicks. The currently popular selective media, bismuth sulfite agar and Bacto-S. S. medium depend, to a large extent, upon the selective action of brilliant green. Currently, the use of brilliant green as an enrichment broth has been supplanted by tetrathionate broth (44).

For a complete discussion of the chemical composition and physical properties of the dyes studied in this paper the reader is referred to Conn (45).

In any series of experiments designed to determine the bacteriostatic properties of a compound there are several factors that must be carefully controlled and recorded if the data are to be of any value for future work. The importance of stating the source, serial number, certification number and any

other available data concerning the particular bacteriostat under study has been emphasized by Conn (38). The value of stating definite pH values has been shown by Stearn and Stearn (39, 40), McCalla and Clark (41) and Reed and Genung (42). The importance of the age of the culture or cultures used was stressed by Churchman in 1926 (43). That the constituents of the medium may vary the bacteriostatic titers of dyes was shown by Reed and Genung (42) and other workers in this field. Stearn and Stearn (40) point out the importance of the previous environment of the organisms under study. Other important factors that should be carefully recorded are (1) time and temperature of incubation; (2) concentration of the bacteriostat; (3) time and temperature of sterilization (if bacteriostat is subjected to heat); (4) additional organic compounds added to the medium (serum or blood, for example); (5) whether the medium is broth or agar in nature; (6) size or numbers of inoculum; (7) possible antibiotic or symbiotic effects of mixtures of bacteria; (8) the possibility of oxidation or reduction of the bacteriostat by the particular microorganisms under study; (9) the particular growth requirements of the bacterium under study; (10) the exact technic used in carrying out any series of experiments and (11) the stability of the medium under study.

The results of the experimental work carried out on various bacteriostats and mixtures of bacteriostats are recorded in the following series of five experiments.

Experiment 1

Dyes. The following dyes were used in this experiment:

(1) Ethyl purple 6B. No. 6734. National Aniline and Chemical Co., Inc. NBg-4.

(2) Brilliant green. Schultz No. 499. C. I. No. 662. Total dye content 94%. National Aniline and Chemical Co., Inc.

(3) Acid violet. Schultz No. 530. Lot No. 8393. National Aniline and Chemical Co., Inc.

Basic Medium. In this experiment the following basic medium was used: Bacto-beef extract, 0.3%; Bacto-peptone, 0.5%; sodium chloride, 0.5% and agar, 2.0%. The pH was adjusted to 7.2 and sterilization effected by autoclaving for 20 minutes at 15 pounds pressure. All dyes were prepared aseptically and added to the basic medium after autoclaving. Twenty ml. amounts were poured into standard Petri dishes (63.5 sq. cm.) and the plates incubated at 37°C. for 24 hours to insure sterility.

Technic. The plates were marked on the bottom so that four equal areas were obtained for seeding. The inoculum was placed upon the agar surface as a single streak using one loopful from a standard 4 mm. platinum loop. All plates, including controls were run in duplicate. After seeding, the plates were incubated for 30 hours at 37°C.

Dilutions of Dyes. The following dilutions were made of each dye: 1 in 25,000; 50,000; 75,000; 100,000; 150,000; 200,000; 500,000; and 1 in 1,000,000.

Cultures. Twenty-four hour broth cultures of the following organisms were used in this study: Salmonella paratyphi A, Salmonella pullorum, Eberthella typhosa, Shigella gallinarum, Staphylococcus aureus, Escherichia coli, and Bacillus cereus.

The results, showing the bacteriostatic titers obtained in experiment I, are tabulated in table I. If the growth compared favorably with that obtained on the control plates, it was recorded as 4. A growth of approximately one-half that of the control was recorded as 2, and only a few scattered colonies as 1.

Table I-A
Bacteriostatic Titers of Selected Dyes in a Nutrient Agar Base

Cultures	Dilutions 1 in								Dyes
	25T	50T	75T	100T	150T	200T	500T	1M	
S. paratyphi B	3	3	3	3	3	3	3	4	Brilliant green
	3	3	4	4	4	4	4	4	Ethyl purple
	4	4	4	4	4	4	4	4	Acid violet
S. paratyphi A	3	3	3	3	3	3	3	4	Brilliant green
	3	3	4	4	4	4	4	4	Ethyl purple
	4	4	4	4	4	4	4	4	Acid violet
S. pullorum	3	3	3	3	3	3	4	4	Brilliant green
	-	1	2	3	3	4	4	4	Ethyl purple
	4	4	4	4	4	4	4	4	Acid violet
E. typhosa	3	3	3	3	3	3	3	4	Brilliant green
	3	3	4	4	4	4	4	4	Ethyl purple
	4	4	4	4	4	4	4	4	Acid violet

4 = growth same as controls
2 = " 1/2 that of controls
3 = " 3/4 that of controls
1 = few scattered colonies
- = no growth
T = thousand
M = million

Table I-B
Bacteriostatic Titers of Selected Dyes in a Nutrient Agar Base

Cultures	Dilutions 1 in								Dyes
	25T	50T	75T	100T	150T	200T	500T	1M	
S. gallinarum	-	-	-	-	-	-	2	3	Brilliant green
	1	3	3	3	3	4	4	4	Ethyl purple
	4	4	4	4	4	4	4	4	Acid violet
Staph. aureus	-	-	-	-	-	1	1	1	Brilliant green
	-	-	-	-	-	-	1	1	Ethyl purple
	1	2	2	4	4	4	4	4	Acid violet
E. coli	-	-	-	-	-	1	3	3	Brilliant green
	2	3	3	3	3	4	4	4	Ethyl purple
	4	4	4	4	4	4	4	4	Acid violet
B. cereus	-	-	-	-	-	-	-	-	Brilliant green
	-	-	-	-	-	-	1	2	Ethyl purple
	1	3	3	4	4	4	4	4	Acid violet

4 = growth same as controls
3 = " 3/4 that of controls
2 = " 1/2 that of controls
1 = few scattered colonies
T = thousand
M = million

Summary of Experiment 1

1. Acid violet. The dilutions used in this experiment were too high to demonstrate if this dye has any practical value. In the dilutions of 1 in 25,000 and 1 in 50,000 it was demonstrated that this dye produced some bacteriostatic effect on the gram-positive bacteria.

2. Ethyl purple. This experiment has shown that selected members of the genera Salmonella and Eberthella grew in all dilutions. S. pullorum was completely inhibited at a dilution of 1 in 25,000, and very little growth was obtained at the 1 in 50,000 dilution. S. gallinarum and E. coli grew in all dilutions of ethyl purple. The gram-positive representatives were inhibited in high dilutions.

3. Brilliant green. The Salmonella organisms grew in all dilutions of this dye. S. gallinarum was inhibited completely at 1 in 200,000. E. coli and Staph. aureus were completely inhibited at 1 in 150,000 and B. cereus at 1 in 1,000,000 dilution.

Practical Applications

1. More experimental work should be done with ethyl purple, especially on its toxicity for coliform organisms. The inhibition of S. gallinarum by brilliant green (1 in 75,000) and the excellent growth of this organism on ethyl purple (1 in 75,000) compared with the complete inhibition of S. pullorum on ethyl purple (1 in 25,000) might be another method of differentiation of these closely related organisms. This work should include the study of numerous strains of each genus.

Experiment 2

Purpose. Experiment 2 is designed to determine the bacteriostatic titers of the dyes studied in experiment 1 in a broth medium.

Dyes. The following dyes were used (1) brilliant green, (2) gentian violet, (3) ethyl violet and (4) ethyl purple 6B. The gentian violet used had the following identification: National aniline and Chemical Company; 81% dye content; C. I. No. 681; Certification No. N. C. 6. The other dyes were from the same lots as used in experiment 1.

Cultures. Twenty-four hour broth cultures of the following bacteria were used: E. typhosa, E. coli, S. paratyphi B, B. cereus and Staph. aureus.

Basic Medium. The dyes were added to the following basic medium: Bacto-beef extract, 0.3%; Bacto-peptone, 0.5% and sodium chloride, 0.5%. The pH was adjusted to 7.2. The dyes were now added to make the following dilutions: 1 in 25,000, 50,000, 75,000, 100,000, 150,000, 200,000, 500,000 and 1 in 1,000,000. Ten ml. amounts were dispensed into test tubes, plugged, and autoclaved for twenty minutes at 15 pounds pressure.

Technic. All dilutions were run in duplicate. The broth tubes were seeded with a standard 4 mm. loop. These broth tubes were then incubated for 48 hours at 37°C. The results of this experiment are tabulated in Tables II-A and B.

Conclusions. The bacteriostatic titers of the dyes studied are apparently higher in broth than in an agar medium.

Note. It will be noted that in this experiment both ethyl violet and ethyl purple 6B were used. Conn (45) states that ethyl purple 6B is a synonym for ethyl violet.

Table II-A
Bacteriostatic Titers of Selected Dyes in a Broth Medium

Cultures	Dilutions 1 in								Dyes
	25T	50T	75T	100T	150T	200T	500T	1M	
E. typhosa	+	+	+	+	+	+	+	+	Brilliant green
	-	-	-	+	+	+	+	+	Gentian violet
	-	+	+	+	+	+	+	+	Ethyl violet
	-	+	+	+	+	+	+	+	Ethyl purple
E. coli	-	-	-	-	-	-	+	+	Brilliant green
	-	-	-	+	+	+	+	+	Gentian violet
	-	+	+	+	+	+	+	+	Ethyl violet
	-	-	+	+	+	+	+	+	Ethyl purple
S. paratyphi B	+	+	+	+	+	+	+	+	Brilliant green
	-	-	+	+	+	+	+	+	Gentian violet
	-	+	+	+	+	+	+	+	Ethyl violet
	+	+	+	+	+	+	+	+	Ethyl purple

T = thousand
M = million
- = no visible growth
+ = visible growth

Table IL-B

Bacteriostatic Titers of Selected Dyes in a Broth Medium

Cultures	Dilutions 1 in								Dyes
	25T	50T	75T	100T	150T	200T	500T	1M	
B. cereus	-	-	-	-	-	-	-	-	Brilliant green
	-	-	-	-	-	-	-	-	Gentian violet
	-	-	-	-	-	-	-	-	Ethyl violet
	-	-	-	-	-	-	-	-	Ethyl purple
Staph. aureus	-	-	-	-	-	-	-	-	Brilliant green
	-	-	-	-	-	-	-	-	Gentian violet
	-	-	-	-	-	-	-	-	Ethyl violet
	-	-	-	-	-	-	-	-	Ethyl purple

T = thousand
M = million
- = no visible growth
+ = visible growth

Experiment 3

Purpose. This experiment was carried out to determine the bacteriostatic titers of a selected group of dyes in a Bacto-tryptose agar medium.

Dyes. The following dyes were studied: (1) Brilliant green. Schultz No. 499. C. I. No. 662. Total dye content 93%. Cert. No. N Bg 7. National Aniline and Chemical Co. (2) Ethyl violet. Schultz No. 518. Lot No. 3330. National Aniline and Chemical Co. (3) Iodine green. The Coleman and Bell Co. (4) Trypan Blue. Vital stain. No. 3417. National Aniline and Chemical Co. (5) Methyl violet 2B. Schultz No. 515. C. I. No. 680. Total dye content 81%. Cert. No. N. MV-4. National Aniline and Chemical Co. (6) Basic fuchsin. Total dye content 83%. Cert. No. N. F. 30. National Aniline and Chemical Co. (7) Pyronine G. Schultz No. 568. Lot. No. 2179. (8) Fast Green. F. C. F. The Coleman and Bell Co.

Basic Medium. The dyes were added in suitable amounts to the following basic medium: Bacto-tryptose, 2.0%; NaCl, 0.5%; K_2HPO_4, 0.4%; KH_2PO_4, 0.15%; dextrose, 0.1% and agar, 2.0%. The pH of this medium was 6.8 before sterilization. The medium containing the appropriate dye was sterilized by autoclaving for 20 minutes at 15 pounds. Twenty ml. portions were dispensed into Petri dishes, allowed to cool and incubated to check for sterility. The following dilutions of the various dyes were used: 1 in 10,000; 1 in 50,000; 1 in 150,000; and 1 in 500,000.

Technic. The method of seeding the plates and interpretation of the results were the same as those used in experiment 1. Incubation was carried out at 37°C. for 30 hours to be comparable with experiment 1.

Cultures. The following cultures were used in this experiment: S. enteritidis, S. gallinarum, S. gallinarum (1040), S. paratyphi B, S. typhimurium, S. paratyphi A, S. pullorum, S. pullorum (Coburn), Shigella dysenteriae, E. typhosa (Hopkins), E. coli (161), Pasteurella avicida, Aerobacter aerogenes (131), B. cereus and Alcaligenes fecalis.

Results. The following dyes exhibited very little bacteriostatic action: trypan blue, fast green, F. C. F., pyronine G, and iodine green. Trypan blue had no effect on the growth of the organisms tested in a dilution of 1 in 10,000 in the basic medium. Fast green exhibited a very slight action (a No. 3 growth) on all the organisms in the 1 in 10,000 dilution. Pyronine G completely inhibited B. cereus at a dilution of 1 in 50,000 and partially (No. 2 reading) at 1 in 150,000. The gram-negative organisms all grew in the 1 in 10,000 dilution, but there was some evidence of toxicity (a No. 3 growth was recorded for the gram-negative group). Iodine green had the same action on the gram-negative group as was exhibited by pyronine G. It inhibited B. cereus completely at the 1 in 10,000 dilution and partially (a No. 1 reading) at 1 in 50,000. The bacteriostatic titers of the other dyes tested are presented in tables III A, B, C and D.

Discussion. In general the bacteriostatic titers obtained in a tryptose base were lower than those obtained in a Bacto-

peptone base. One reason for this was the increased amount of peptone in the tryptose medium. The second factor of importance is the composition of the tryptose. Also, in this experiment, the effects of a greater amount of dye were studied (a dilution of 1 in 10,000 as compared to a dilution of 1 in 25,000 in experiment 1. Brilliant green inhibited S. pullorum in a dilu- of 1 in 25,000 and almost completely at 1 in 50,000 (a No. 1 reading) in the nutrient agar base. S. pullorum grew in the tryptose base at a dilution of 1 in 10,000. Brilliant green inhibited E. coli at a dilution of 1 in 150,000 in a nutrient agar base, whereas in the tryptose base a concentration of 1 in 50,000 was required. It is particularly important to note that in the tryptose medium E. coli and E. typhosa were inhibited at the same concentration of brilliant green. Neither a peptone or a tryptose base with brilliant green, would be suitable for the isolation of S. gallinarum or S. dysenteriae. Brilliant green still retains a high degree of selectivity for gram-positive bacteria.

With ethyl purple, the other dye used in both experiments 1 and 3, there was a uniform reduction in bacteriostatic titers when the tryptose base was used.

Table III

The Bacteriostatic Titers of Selected Dyes in a Tryptose Agar Medium

B--Methyl Violet 2B

Cultures	Dilution of dye 1 in			
	10T	50T	150T	500T
S. enteritidis	3	3	3	4
S. gallinarum	3	3	3	4
S. gallinarum No. 1040	3	3	3	3
S. paratyphi B	3	3	4	4
S. typhimurium	3	3	4	4
S. paratyphi A	3	3	4	4
S. pullorum	3	3	3	4
S. pullorum - Coburn	3	3	3	4
S. dysenteriae	2	3	3	4
E. typhosa	1	3	3	4
E. coli	1	2	3	4
P. avicida	2	3	3	4
A. aerogenes	3	3	3	4
Al. fecalis	4	4	4	4
B. cereus	-	-	-	-

T = thousand
4 = growth same as control
3 = growth 3/4 control
2 = growth 1/2 control
1 = few colonies
- = no growth

Experiment 4

Purpose. This experiment was carried out to determine the bacteriostatic titers of a selected group of compounds in a Bacto-tryptose broth medium.

Dyes and Compounds. The following selected group of compounds was used in this experiment. (1) Brilliant green. Schultz No. 499, C. I. 662. Total dye content 93%. National Aniline and Chemical Co. (2) Methyl violet 2B. Schultz No. 515, C. I. No. 680. Total dye content 81%. National Aniline and Chemical Co. (3) Acid fuchsin C. I. No. 692. Total dye content 60%. National Aniline and Chemical Co. N. R.-10. (4) Thionin Schultz No. 348. 2nd Ed. C. I. No. 920, NT-2. National Aniline and Chemical Co. (5) Ethyl purple 6B, National Aniline and Chemical Co. No. 6734. (6) Rosalic acid. (7) Sodium azide, Pract. (8) Pyronin--Schultz No. 469, 4th Ed. National Aniline and Chemical Co. Cert. No. N. P. 3. (9) Erythrosin--Fisher Scientific Co. (10) Nigrosine (Water Soluble). (11) Acriflavine hydrochloride. (12) Elon--No. 36092. (13) Toluidin Blue Cert. No. HU-1. Fisher Scientific Co.

Basic Medium. The dyes and other compounds tested were added to the following basic medium: Bacto-tryptose, 2.0%; dextrose, 0.1%; NaCl, 0.5%; KH_2PO_4, 0.15% and K_2HPO_4, 0.4%. The recorded concentrations of the compounds were added to the basic medium and dispensed, in 10 ml. amounts, into test

tubes. Sterilization was effected by autoclaving for 20 minutes at 15 pounds pressure. The pH of the medium, after sterilization, was 6.8. All tubes were checked for sterility by incubating them at 37°C. for 48 hours.

Technic. All tests were run in duplicate. The tubes were seeded with a 24-hour culture of the appropriate organism, each tube being seeded with one loopful, using a 4 mm. platinum loop. Growth was determined by gross observable turbidity, using unseeded tubes of the same dye-dilution as controls. The readings were made after 48 hours incubation at 37°C.

Cultures. The following cultures were used in this experiment: E. coli, Staph. aureus, B. cereus, S. pullorum, S. paratyphi A, S. typhimurium (230), S. typhimurium (226), S. gallinarum, E. typhosa, and S. dysenteriae.

Results. The following compounds showed very little bacteriostatic action and these data are presented below:

(1) Acid fuchsin--all cultures grew in a dilution of 1 in 5,000.

(2) Thionin--B. cereus and E. typhosa were inhibited in a dilution of 1 in 10,000. All of the other cultures grew at this dilution.

(3) Rosalic acid--Staph. aureus was inhibited in the 1 in 10,000 dilution but grew in the 1 in 50,000 dilution. B. cereus was inhibited in both the 1 in 10,000 and 1 in 50,000 dilutions, but grew in the 1 in 100,000 dilution. All

of the other cultures grew in the 1 in 10,000 dilution.

(4) Erythrosin--All cultures grew in the 1 in 10,000 dilution.

(5) Nigrosine--All cultures grew in the 1 in 10,000 dilution.

(6) Elon--B. cereus was inhibited in a 1 in 5,000 dilution, all other cultures grew in this dilution.

(7) Toluidine blue--B. cereus and S. dysenteriae were inhibited in a dilution of 1 in 10,000. All other cultures grew in this dilution.

The experimental data obtained from the other compounds tested are shown in the table IV series.

Discussion. In a tryptose broth medium brilliant green loses much of its selective action for gram-positive bacteria and all of its selective action for E. coli. Using gentian violet or ethyl violet in a tryptose medium does not markedly effect the bacteriostatic properties of these dyes. In a tryptose broth base, sodium azide exhibits "reverse bacteriostasis" but the differential titers are too close for practical use.

Table IV-B

The Bacteriostatic Titers of Selected Compounds
in a Tryptose Broth Medium

Cultures	Compounds and Dilutions in Thousands											
	Sodium Azide					Acriflavine hydrochloride				Pyronin		
	5*	10	50	100	500	5	10	50	100	10	50	100
E. coli	-	+	+	+	+	-	-	+	+	+	+	+
S. aureus	+	+	+	+	+	-	-	-	+	-	-	-
B. cereus	-	-	+	+	+	-	-	+	+	-	-	-
S. pullorum	-	+	+	+	+	-	-	+	+	+	+	+
S. paratyphi A	-	-	+	+	+	+	+	+	+	+	+	+
S. typhimurium (230)	-	+	+	+	+	+	+	+	+	+	+	+
S. typhimurium (226)	-	+	+	+	+	+	+	+	+	+	+	+
S. gallinarum	-	-	+	+	+	+	+	+	+	+	+	+
E. typhosa	-	+	+	+	+	-	-	+	+	+	+	+
S. dysenteriae	-	+	+	+	+	-	-	-	-	-	+	+

* = Dye dilution of 1 in 5,000
- = No growth
+ = Growth in 48 hours

Experiment V

Purpose. This is a continuation of experiment IV to show the bacteriostatic action of a mixture of bacteriostatic agents. In this work only five cultures were used. The results are presented in the table V series. The sodium lauryl sulfate was Duponal W. A. paste. 3.33 grams in 100 ml. were used as a 1% solution.

Discussion. The addition of sodium lauryl sulfate to brilliant green in a tryptose base had very little action. The bacteriostatic action of the sodium lauryl sulfate was not affected by the brilliant green. (Table V-A)

The addition of sodium lauryl sulfate to methyl violet 2B reduced, to some extent, the toxicity of methyl violet for gram-negative bacteria, but had no apparent effect on the gram-positive organisms. (Table V-B)

Sodium lauryl sulfate, when added to acriflavine hydrochloride, reduced the toxicity of this compound for gram-negative bacteria. The mixture of acriflavine and ethyl purple gives a greater toxicity, than either compound acting independently for both gram-positive and gram-negative bacteria. This did not occur when brilliant green and methyl violet were mixed. (Table V-E)

Table V-E

The Action of Mixtures of Bacteriostats

Bacteriostats	Dilution 1 in	Cultures: S. aureus	E. coli	S. pullorum	E. typhosa	S. gallinarum
Ethyl purple	25T	-	+*	-	-	-
Brilliant green	25T					
Ethyl purple	25T	-	-	-	-	-
Brilliant green	50T					
Sodium azide	10T	+	-	-	+*	-
Sodium azide	10T	-	-	-	+*	-
Na lauryl sulfate	10T					
Brilliant green	25T	-	+*	+*	-	+
Methyl violet	50T					
Brilliant green	50T	-	+	+	+*	+
Methyl violet	50T					
Brilliant green	100T	-	+	+	-	+
Methyl violet	50T					
Brilliant green	50T	+	+	+	+	+
Erythrosin	50T					

T = thousand
- = no growth
+ = growth in 48 hours
+* = no growth in 24 hours, growth in 48 hours

Part III

Studies on Selective Media for Coliform Organisms

Any selective medium designed to be used in water analysis must be based on a thorough knowledge of the bacterial flora of various types of water. The basis of any method of water analysis is to determine the presence or absence of coliform organisms. The whole technic is based on the thesis that when fecal contamination occurs, coliform bacteria will be present in the water. Elaborate technics have been devised to perfect a medium that will eliminate any organism, or group of organisms, other than the coliform group, that might possibly give a positive test by the technic employed.

An exhaustive study has been made by Greer and Greer and associates of the various types of organisms, other than coliform, found in water supplies. (12, 13, 14, 15, 16)

A second very complete discussion of both coliform and non-coliform lactose fermenting bacteria was published by Levine (17). A comprehensive review of the coliform bacteria was published by Parr in 1939 (1).

From a review of the cited references it was found that numerous non-coliform organisms have been isolated which have the ability to ferment lactose. Species of the genus Clostridium have probably been incriminated more than any other in false positive presumptive tests. Bergey (18) lists 18 species of this genus that have the ability to ferment lactose with acid and gas production. A few spore-forming and non-spore-forming

gram-positive, aerobic rods have been described which have the ability to ferment lactose. Organisms such as B. aerosporus (an aerobic spore-former) and Houston's "leather" bacillus, described by Greer (12) have the ability to produce gas from lactose. Greer also refers to many undescribed species that are capable of lactose fermentation. Many streptococci and staphylococci have the ability to ferment lactose without the production of gas. This action makes possible a synergistic fermentation of lactose. Members of the genus Proteus, for example, have the ability to produce acid and gas from dextrose, but not lactose. In a mixed bacterial population growing in lactose broth it is possible to have the lactose hydrolyzed by one species and the resulting dextrose fermented with gas production by a second species. This synergistic action probably causes many false positive presumptive tests.

Other non-coliform lactose-fermenting bacteria are members of the genera Erwinea and Klebsiella. These organisms, however, are rarely found in water and are of little significance in water bacteriology.

With the inception of a definite technic for water analysis, bacteriologists began to devise methods for the elimination of these non-coliform organisms. In part II of this thesis the early work concerning bacteriostatic agents has been reviewed. Greer (15) has presented a review of the development of the various selective media used in water analysis. The first selective agent used for this purpose was bile, which was added to lactose broth to inhibit the growth of the gram-positive bacteria.

It was found, however, that the concentration of bile necessary to obtain the desired results was inhibitory to the coliform bacteria. Various media containing gentian violet as the selective agent were devised for use in water analysis. Crystal violet lactose broth, formate ricinoleate broth, fuchsin lactose broth, and brilliant green bile 2% lactose broths are now recommended by "Standard Methods of Water Analysis" (19) for the confirmation of positive presumptive tests.

Of these selective media the brilliant green bile medium is the least toxic and most efficient in the elimination of false positive tests. The efficiency of this medium has been reported by Muer and Harris (20) and Dunham and Schoenlein (21). Jordan (22) reported that this medium was slightly superior to plain lactose broth for the determination of coliform organisms in water.

To warrant consideration in field tests any new selective medium should be superior to brilliant green bile 2% lactose broth.

The following experimental data are presented as preliminary work to the development of the selective medium presented in reprint form and appended to this thesis.

A Comparison of Brilliant Green and Ethyl Purple in a Bile Medium

In part II it has been shown that ethyl purple (ethyl violet) has high bacteriostatic action on gram-positive organisms, with relatively little toxicity for *E. coli*. This study was designed to determine the effect of 2% bile on the selective action of brilliant green and ethyl purple.

The basic medium contained the following: beef extract, 0.3%; Bact-peptone, 0.5%; Bact-oxgall, 2.0%; lactose, 1.0%. The pH was 7.0 before sterilization. The dyes were added to obtain the concentrations indicated in tables VI and VII. The media were dispensed, in 10 ml. amounts, into tubes containing fermentation inserts. The tubes were then autoclaved for 20 minutes at 15 pounds pressure. One 4 mm. loop of the 24-hour broth culture was seeded into the broth as indicated in the tabulated data. Incubation was carried out at 37° C.

An examination of the data presented in tables VI and VII shows that although the ethyl purple bile medium was slightly less toxic for the coliform organisms, it was also less efficient in preventing the growth of the gram-positive bacteria. It was concluded from these studies that an ethyl purple bile medium would not be as efficient as the brilliant green bile medium.

Ethyl Purple and Brilliant Green Bile in Tryptose Broth

In the appended reprint, Studies on Media for Coliform Organisms, the value of tryptose broth for the growth of coliform organisms was definitely demonstrated. Although the bacteriostatic titers in Part II indicated that brilliant green would not be a suitable bacteriostat in a tryptose base, the combination of brilliant green and bile had not been studied. The technic used in this study was similar to that used in the preceding study. Fourteen different media were tested using different combinations of organisms that would give true and false positive presumptive tests in selective and non-selective types of lactose broth.

Table VI

Brilliant Green - Oxgall Medium

Cultures	Dilutions of Dye 1 in						Hours Incubation
	5T	10T	25T	50T	100T	200T	
E. coli (1)	+ G	G G	G G	G G	G G	G G	24 48
E. coli (161)	+ G	G G	G G	G G	G G	G G	24 48
E. coli (2)	- G	G G	G G	G G	G G	G G	24 48
E. coli (3)	- G	G G	G G	G G	G G	G G	24 48
S. paratyphi B	+ +	+ +	+ +	+ +	+ +	+ +	24 48
E. typhosa	+ +	+ +	+ +	+ +	+ +	+ +	24 48
Staph. aureus	- -	- -	- -	- -	- -	+ -	24 48
B. cereus	- -	- -	- -	- -	- -	- -	24 48

- = no growth
+ = growth
G = gas production
T = thousand

The "Mount Clemens" organism was an unidentified gram-positive rod incriminated in false positive presumptive tests from the Mount Clemens water plant. This organism had apparently lost its ability to produce gas from lactose. The results of these tests are presented in the table VIII series.

The table IX series presents a comparison of brilliant green bile tryptose, standard brilliant green bile and the ethyl purple tryptose medium. In this series a more comprehensive group of organisms was used. The data obtained in these studies are presented in tables IX-A and B.

A study of the data presented in the table VIII and IX series justifies the following conclusions. (1) Ethyl purple in dilutions of 1 in 100,000 to 1 in 200,000 exhibits slightly less toxicity for coliform organisms than does the standard brilliant green bile 2% medium. (2) The ethyl purple in a 1 in 200,000 dilution in tryptose broth inhibited the clostridia in all tests in the VIII series. In the IX series this medium failed to inhibit the clostridia in two instances (culture No. 2 in 116 hours and culture No. 13 in 48 hours). (3) The ethyl purple tryptose medium inhibited all of the aerobic gram-positive organisms tested. (4) Brilliant green, even in high concentrations, in a 2% bile-tryptose medium failed to inhibit the clostridia and mixtures of clostridia tested in this series. (5) The brilliant green 2% bile-tryptose medium inhibited all of the aerobic gram-positive organisms tested. (6) The results obtained using the standard brilliant green bile medium were about the same as those obtained using the ethyl purple tryptose medium.

Table VIII-A

A Study of the Selective Action of Ethyl Purple and Brilliant Green Bile in Tryptose Broth

Medium	Hours incubation 37°C	Cultures									
		1	2	3	4	5	6	7	8	9	10
		Mt. Clemens Gram + rod	E. coli	S. typhi murium	Clostridium from Horse feces	Clostridium sp.	Cl. chauvei	B. cereus	S. aureus	A. aerogenes	3 + 5
Tryptose 3.0% Lactose 1.0% Ethyl purple 1 in 25,000	8	-	-	-	-	-	-	-	-	+	+
	24	-	-	+	-	-	-	-	-	60	+
	48	-	5	+	-	-	-	-	-	80	+
	150	-	50	+	-	-	-	-	-	70	+
Ethyl purple 1 in 50,000	8	-	-	+	-	-	-	-	-	+	+
	24	-	10	+	-	-	-	-	-	100	+
	48	-	60	+	-	-	-	-	-	100	+
	150	-	50	+	-	-	-	-	-	100	+
Ethyl purple 1 in 75,000	8	-	+	+	-	-	-	-	-	+	+
	24	-	50	+	-	-	-	-	-	100	+
	48	-	60	+	-	-	-	-	-	100	+
	150	-	50	+	-	-	-	-	-	100	+

\+ = visible growth
\- = no growth
No. = % of gas in insert

Table VIII-B

A Study of the Selective Action of Ethyl Purple and Brilliant Green Bile in Tryptose Broth

Medium	Hours Incubation	Cultures									
		1	2	3	4	5	6	7	8	9	10
Tryptose 3.0% Lactose 1.0% Ethyl purple 1 in 100,000	8	-	1	+	-	-	-	-	-	1	+
	24	-	40	+	-	-	-	-	-	100	+
	48	-	50	+	-	-	-	-	-	100	+
	150	-	40	+	-	-	-	-	-	100	+
Ethyl purple 1 in 125,000	8	-	+	+	-	-	-	-	-	+	+
	24	-	60	+	-	-	-	-	-	100	+
	48	-	75	+	-	-	-	-	-	100	+
	150	-	60	+	-	-	-	-	-	100	1
Ethyl purple 1 in 200,000	8	-	2	+	-	-	-	-	-	2	+
	24	-	75	+	-	-	-	-	-	100	+
	48	-	80	+	-	-	-	-	-	100	+
	150	-	70	+	-	-	-	-	-	90	+
Tryptose 3.0% Oxgall 2.0% Lactose 1.0% Brilliant Green 0.00133%	8	-	1	+	-	-	-	-	-	1	+
	24	-	50	+	-	-	-	-	-	95	+
	48	-	50	+	-	+	-	-	-	100	+
	150	-	40	+	5	15	-	-	-	80	+

+ = visible growth
- = no growth
No. = % of gas in insert

Table VIII-C

A Study of the Selective Action of Ethyl Purple and Brilliant Green Bile in Tryptose Broth

Medium	Hours Incubation	Cultures									
		1	2	3	4	5	6	7	8	9	10
Tryptose 3.0% Oxgall 2.0% Lactose 1.0% Brilliant green 0.0014%	8	-	1	+	-	-	-	-	-	1	+
	24	-	50	+	-	-	-	-	-	100	+
	48	-	60	+	-	+	-	-	-	100	+
	150	-	50	+	-	5	-	-	-	90	+
Brilliant green 0.0015%	8	-	1	+	-	-	-	-	-	1	+
	24	-	50	+	-	-	-	-	-	95	+
	48	-	60	+	-	5	-	-	-	100	+
	150	-	40	+	-	10	-	-	-	90	+
Brilliant green 0.0016%	8	-	1	+	-	-	-	-	-	1	+
	24	-	65	+	-	-	-	-	-	95	+
	48	-	75	+	-	5	-	-	-	100	+
	150	-	50	+	-	10	-	-	-	90	+
Brilliant green 0.002%	8	-	1	+	-	-	-	-	-	1	+
	24	-	50	+	-	-	-	-	-	60	+
	48	-	70	+	-	-	-	-	-	70	+
	150	-	50	+	-	-	-	-	-	80	+

+ = visible growth
- = no growth
No. = % of gas in insert

Table VIII-D

A Study of the Selective Action of Ethyl Purple and Brilliant Green Bile in Tryptose Broth

Medium	Hours Incubation 37°C.	Cultures									
		1	2	3	4	5	6	7	8	9	10
		Mt. Clemens Gram + rod	E. coli	S. typhimurium	Clostridium (horse feces)	Clostridium sp.	Cl. chauvei	B. cereus	S. aureus	A. aerogenes	3 + 5
Brilliant green Bile 2%-Difco Ref. No. 302596 4%	8	-	1	+	-	-	-	-	-	1	+
	24	-	40	+	-	-	-	-	-	30	+
	48	-	40	+	-	-	-	-	-	30	+
	150	-	40	+	+	-	+	-	-	40	+
Beef extract 0.3% Bacto-peptone 0.5% Lactose 1.0% NaCl 0.5%	8	+	1	+	+	-	-	+	+	+	+
	24	+	30	2	10	-	-	+	+	50	1
	48	+	40	2	40	-	-	+	+	60	1
	150	+	40	1	70	-	-	+	+	60	1
Tryptose 3.0% Oxgall 2.0% Lactose 1.0% Brilliant green 0.00133%	8	-	1	-	-	-	-	-	-	1	+
	24	-	50	+	-	-	-	-	-	80	+
	48	-	50	+	-	-	-	-	-	100	+
	150	-	50	+	-	2	-	-	-	90	+

\+ = visible growth
\- = no growth
No. = % of gas in insert

Table IX-A

A Study of Selective Media on Coliform and Non-Coliform Organisms

Medium	Hours Incubation 37° C.	Cultures									
		1	2	3	4	5	6	7	8	9	10
		Mt. Clemens Gram + rod	Clostridium from Horse feces	Pseud. aeruginosa	S. citreus	B. ruminatus	B. cereus	B. subtilis	S. pullorum	Clostridium sp. from water	Cl. oedematis maligni
Tryptose 3.0% Lactose 1.0% Oxgall 2.0% Brilliant gr. 0.00133%	24	-	-	+	-	-	-	-	+	-	-
	48	-	-	+	-	-	-	-	+	-	-
	116	-	G	+	-	-	-	-	+	-	-
Tryptose 3.0% Lactose 1.0% Ethyl purple 1 in 200,000	24	-	-	+	-	-	-	-	+	-	-
	48	-	+	+	-	-	-	-	+	-	-
	116	-	G	+	-	-	-	-	+	-	-
Brilliant Green Bile 2%--Difco No. 299191	24	-	-	+	-	-	-	-	+	-	-
	48	-	+	+	-	-	-	-	+	-	-
	116	-	+	+	-	-	-	-	+	-	-

- = no growth
+ = growth
G = gas production

Table IX-B

A Study of Selective Media on Coliform and Non-Coliform Organisms

Medium	Hours Incubation 37°C.	Cultures									
		11	12	13	14	15	16	17	18	19	20
		Cl. chauvei	E. coli	2 + 8	2 + 3	8 + 9	3 + 9	8 + 10	3 + 10	8 + 11	3 + 11
Tryptose 3.0% Lactose 1.0% Oxgall 2.0% Brilliant gr. 0.00133%	24	-	G	+	+	+	+	+	+	+	+
	48	-	G	G	+	+	+	+	+	+	+
	116	-	G	G	G	+	+	+	+	+	+
Tryptose 3.0% Lactose 1.0% Ethyl purple 1 in 200,000	24	-	G	+	+	+	+	+	+	+	+
	48	-	G	G	+	+	+	+	+	+	+
	116	-	G	G	+	+	+	+	+	+	+
Brilliant gr. Bile 2% Difco No. 299191	24	-	G	+	+	+	+	+	+	+	+
	48	-	G	+	+	+	+	+	+	+	+
	116	-	G	+	G	+	+	+	+	+	+

- = no growth
+ = growth
G = gas production

This medium failed to inhibit the clostridia in two tubes as shown in table VIII-D and did not inhibit culture No. 2 in table IX-A. A mixture of clostridia and Pseudomonas aeruginosa produced gas in this medium, as demonstrated in table IX-B. (7) These studies have demonstrated that the ethyl purple tryptose and standard brilliant green bile media are equally efficient in inhibiting the growth of non-coliform lactose fermenting organisms. Neither medium is 100% efficient in its designed selective action.

Growth Curve Studies of Selective Media for Water Analysis

The preceding studies have shown that standard brilliant green bile and ethyl purple tryptose broth are equally efficient in their selective action. The next step was to determine the relative toxicity of these media for coliform organisms. The technic used to demonstrate toxicity of selective media has been outlined in the appended reprint Studies on Media for Coliform Organisms. The test organism for these studies was E. coli (161).

The tabulated data showing the media studied and the bacteria counts after definite time periods are presented in tables X, XI, XII-A and XII-B. A study of these data justify the following conclusions. (1) In two of the three growth curves the ethyl purple medium was superior to the standard brilliant green bile medium. In the data presented in table XII the standard brilliant green medium was superior. (2) In testing the selective media now recommended by standard methods it was found that in using E. coli: Bacto crystal violet lactose broth was most toxic; Bacto fuchsin

Table X

No. Viable Bacteria per ml.**

Medium	0*	4	6.5	8.5	10	12	14	24
Beef Extract .3% Bacto Peptone 0.5% Lactose 1.0%	30	770	20,000	180,000	3,070,000	22,100,000	144,000,000	334,000,000
Brilliant Green Bile 2%--Difco 4 gm. per 100 cc.	28	80	3,800	74,000	330,000	330,000	164,000,000	270,000,000
Tryptose 2.0% KH_2PO_4 0.5% Ethyl purple 6B (1 in 200,000)	28	590	-	260,000	3,500,000	3,500,000	238,000,000	550,000,000

* Hours incubation at 37°C.
** E. coli (161)

Table XI

No. Viable Bacteria per ml.**

Medium	0*	3	5	24
Tryptose 2.0% Lactose 1.0% Ethyl purple (1 in 200,000)	14	79	1620	530,712,000
Tryptose 2.5% Lactose 1.0% Ethyl purple (1 in 200,000)	22	150	3640	628,000,000
Tryptose 3.0% Lactose 1.0% Ethyl purple (1 in 200,000)	19	224	6120	1,176,606,000
Brilliant green Bile 2%-Difco #299191	18	85	2930	284,310,000
Tryptose 4.0% Lactose 1.0% Ethyl purple (1 in 200,000)	20	198	4780	736,290,000

* Hours incubation at 37°C.
** E. coli (161)

Table XII-A

Toxicity of Selective Media for *E. Coli*

Medium	No. of Viable Bacteria per ml.**				
	0*	3	6	9	12
Bacto Brilliant Green Bile 2%	75	380	92,000	17,700,000	403,000,000
Bacto Crystal Violet Lactose Broth	78	80	2,560	6,100	60,000
Bacto Formate Ricinoleate Broth	34	116	41,500	5,510,000	140,000,000
Bacto Fuchsin Lactose Broth	90	450	23,800	825,000	31,200,000
Bacto Lactose Broth	86	1,560	573,000	31,300,000	232,000,000

* Hours incubation at 37°C.
** E. coli (161)

Table XII-B

Toxicity of Selective Media for *E. Coli*

Medium	No. of Viable Bacteria per ml.**				
	0*	3	6	9	12
Tryptose 2.0% Lactose 0.5% NaCl 0.5% K_2HPO_4 0.4% KH_2PO_4 0.15% Ethyl purple 1 in 200,000	96	520	21,200	360,000	8,900,000
Tryptose 2.0% Lactose 0.5% NaCl 0.5%	97	5,300	12,700,000	585,000,000	1,050,000,000
Tryptose 2.0% Lactose 0.5% NaCl 0.5% K_2HPO_4 0.4% KH_2PO_4 0.15%	99	5,000	6,500,000	830,000,000	1,510,000,000

* Hours incubation at 37°C.

** *E. coli* (161)

lactose broth second; Bacto formate ricincleate broth third; and Bacto brilliant green bile 2% was least toxic. (3) Ethyl purple (1 in 200,000) in a basic medium containing 3% tryptose was superior to basic media containing 2.0, 2.5, or 4.0% tryptose. (4) Although ethyl purple, in a dilution of 1 in 200,000, compares favorably with the other selective media tested, it does exert a marked toxicity for coliform organisms.

To establish definitely the toxicity of ethyl purple in a tryptose base another series of growth curves was run. As in the preceding studies, E. coli (161) was used as the test organism. These data are shown in tables XII-A and B.

In 1938, Cowles (see appended reprint) demonstrated the value of sodium lauryl sulfate (Drene Shampoo) as a selective agent incorporated in lactose broth. The toxicity of the lauryl sulfate was also determined by means of a growth curve. The results of this study are presented in table XIII-B.

A study of the data presented in tables XIII-A and B justifies the following conclusions: (1) Ethyl purple, even in a dilution of 1 in 500,000 is toxic for E. coli; (2) sodium lauryl sulfate (as Drene Shampoo), in the dilution used, did not inhibit the growth of E. coli.

Because of its non-toxic action on coliform organisms, sodium lauryl sulfate gave promise of being an ideal selective agent. If this compound had the ability to suppress non-coliform lactose-fermenting organisms it would be far superior to any other medium now being used in water analysis.

Table XIII-A

Toxicity of Ethyl Purple and Sodium Lauryl Sulfate for E. coli

Basic Medium*	No. of viable bacteria per ml:**				
	0**	3	6	9	12
Ethyl purple 1 in 25,000	16	3	2	4	6
Ethyl purple 1 in 50,000	23	13	17	48	82
Ethyl purple 1 in 75,000	19	13	51	380	460
Ethyl purple 1 in 100,000	17	35	373	2,000	7,000
Ethyl purple 1 in 125,000	18	38	980	7,300	90,000

*The basic medium contained: tryptose, 2.0%; lactose, 0.5%; K_2HPO_4, 0.4%; KH_2PO_4, 0.15%; NaCl, 0.5%; pH - 6.8.

**Hours incubation at 37°C.

***E. coli (161)

Table XIII-B

Toxicity of Ethyl Purple and Sodium Lauryl Sulfate for E. coli

Basic medium	No. of viable bacteria per ml.**				
	0*	3	6	9	12
Ethyl purple 1 in 150,000	18	47	1,360	10,300	127,000
Ethyl purple 1 in 175,000	20	113	4,600	212,000	7,590,000
Ethyl purple 1 in 200,000	21	216	17,300	1640,000	97,000,000
Ethyl purple 1 in 500,000	24	410	201,000	51,660,000	1,400,000,000
Control	30	1,410	3,310,000	352,000,000	1,630,000,000
1 ml. Drene per liter	22	1,070	974,000	764,000,000	1,880,000,000

*Hours of incubation at 37°C.
**E. coli (161)

Table XIV

The Bacteriostatic Action of Sodium Lauryl Sulfate

Medium	Cultures											
	Serratia indica	Aerobacter aerogenes	Pseudomonas arruginosa	Escherichia coli	Salmonella paratyphi B	Eberthella typhosa	Bacillus cereus	Bacillus subtilis	Bacillus mycoides	Bacillus ramosus	Bacillus vulgatus	Staphylococcus aureus
Basic Medium	+	G	+	G	+	+	+	+	+	+	+	+
Basic Medium 1 ml. Drene liter	+	G	+	G	+	+	-	-	-	-	-	-

Basic Medium: tryptose, 2.0%; lactose, 0.5%; NaCl, 05%; K_2HPO_4, 0.4%; KH_2PO_4, 0.15%.

- = no growth
+ = growth
G = gas production

Incubation at 37°C. for 48 hours

Table XIV shows the data obtained in a preliminary bacteriostatic study of sodium lauryl sulfate in a buffered tryptose-lactose broth. This preliminary study indicates that this compound has the ability to suppress the growth of aerobic gram-positive bacteria.

The second appended reprint, Uses of a Lauryl Sulfate Tryptose Broth for the Dectection of Coliform Organisms, completes the studies of part III of this thesis.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. W. L. Mallmann for his assistance and active participation in these studies.

REPRINTED FROM

American Journal of
Public Health
And The Nation's Health

Volume 31 February, 1941 Number 2

Uses of a Lauryl Sulfate Tryptose Broth for the Detection of Coliform Organisms

W. L. Mallmann, Ph.D., F.A.P.H.A., and C. W. Darby, D.V.M.

Published by the
American Public Health Association
1790 Broadway, New York, N. Y.

Reprinted from AMERICAN JOURNAL OF PUBLIC HEALTH, Vol. 31, No. 2, February, 1941

Uses of a Lauryl Sulfate Tryptose Broth for the Detection of Coliform Organisms*

W. L. MALLMANN, PH.D., F.A.P.H.A., AND
C. W. DARBY, D.V.M.

Section of Bacteriology, Michigan Agricultural Experiment Station, East Lansing, Mich.

AN attempt has been made to develop improved methods of procedure for the isolation of the coliform group from water, both qualitatively and quantitatively. These studies were undertaken primarily because of the fact that, seemingly in various water supplies in various parts of the United States, organisms were passing through water purification systems that caused intestinal upsets. In many instances these water supplies were meeting all bacteriological requirements of a safe water supply. In all instances the method of bacteriological analysis was based on the standard procedure of the American Public Health Association.

Coupled loosely with these epidemics were observations by a number of workers that longer incubation periods and special technic demonstrated the presence of organisms called, for want of a better term, "slow lactose fermenters."

Darby and Mallmann [1] showed in a laboratory study that the use of a new nutrient, tryptose, caused many so-called "slow lactose fermenters" to produce gas in greater quantities in a shorter period of time. This was due to the fact that this substance with a few other chemical agents allowed a more rapid growth of the coliform organisms and also caused a larger number of the bacteria initially present to grow. Thus an enrichment medium is available that grows a higher percentage of the bacteria initially present in the water in a shorter period of time than the present standard lactose broth.

Many attempts have been made to introduce into the primary lactose broth tubes a selective agent which would prevent the growth of Gram-positive bacteria and thus make the primary presumptive tests more significant. In all cases where dyes have been used, such as crystal violet, brilliant green, fuchsin, and others, not only the Gram-positive bacteria are inhibited but a marked toxicity is also exhibited for the Gram-negative organisms. In using the selective agent in the primary medium many coliform bacteria are inhibited and lower colon indices result. The Standard Method Committee of the American Public Health Association has never accepted any of these for primary use for this reason.

* Journal Article No. 490 n.s. from the Michigan Agricultural Experiment Station. Read before the Laboratory Section of the American Public Health Association at the Sixty-ninth Annual Meeting in Detroit, Mich., October 10, 1940.

TABLE 1

The Bacteriostatic Titers of Surface Tension Depressants

Compounds	Test Organisms *	Dilutions of Compounds 1–1T	1–5T	1–10T	1–20T	1–30T	1–40T	1–50T
Aerosol — M.A.	*E. coli*	+	+	+	+	+	+	+
	Streptococcus	+	+	+	+	+	+	+
	S. aureus	+	+	+	+	+	+	+
	B. megatherium	+	+	+	+	+	+	+
Nacconol N.R.S.F.	*E. coli*	+	+	+	+	+	+	+
	Streptococcus	—	—	—	—	—	+	+
	S. aureus	—	—	+	+	+	+	+
	B. megatherium	—	—	—	—	—	+	+
Duponol W.A. Paste	*E. coli*	+	+	+	+	+	+	+
	Streptococcus	—	—	—	—	—	+	+
	S. aureus	—	—	+	+	+	+	+
	B. megatherium	—	—	—	—	—	+	+
Santomerse #1	*E. coli*	+	+	+	+	+	+	+
	Streptococcus	—	—	+	+	+	+	+
	S. aureus	—	+	+	+	+	+	+
	B. megatherium	—	—	—	+	+	+	+
Igepon T	*E. coli*	+	+	+	+	+	+	+
	Streptococcus	—	+	+	+	+	+	+
	S. aureus	+	+	+	+	+	+	+
	B. megatherium	+	+	+	+	+	+	+

* Incubation 37° C. for 48 hours

In 1938, Cowls [2] demonstrated that the addition of sodium lauryl sulphate to lactose broth gave a medium selective for the coliform group.

Before adopting sodium lauryl sulfate in these studies the writers tried a number of wetting agents with the object of selecting the best preparation. In Table 1 are presented a few compounds to show the selective properties of these preparations. It will be observed that Nacconol N.R.S.F. and Duponol W.A. Paste are equal in selectivity, both as to organisms and degree of action. It will also be observed that two other wetting agents, namely, Aerosol M.A. and Igepon T. have no selective action. In Table 2 are presented the surface tensions of these compounds. The data show that the surface tension depressing action is about the same for all of these products. It is apparent that the reduction in surface tension alone is not the cause of the selective action.

To check the toxic properties, growth curves were made to determine the effects of various concentrations of the wetting agents on the rate of growth of the coliform organisms. In Table 3 are presented the bacterial counts which were obtained. The wetting agents

TABLE 2

The Surface Tensions of Several Wetting Agents in a Tryptose Broth Base

Compound	Surface Tension in Dynes * at the Following Dilutions 1–100	1–1T	1–5T	1–10T	1–20T	1–30T	1–40T	1–50T
Aerosol A.Y.	33.37	36.66	—	—	—	—	—	—
Nacconol N.R.S.F.	36.19	36.66	37.60	37.14	38.54	40.89	41.37	44.19
Duponol W.A.	33.37	39.95	39.01	39.95	39.49	42.30	43.24	47.00
Santomerse #1	36.66	36.66	—	—	—	—	—	—
Igepon T	36.66	36.66	—	—	—	—	—	—
Base Medium	55.94	—	—	—	—	—	—	—

* Taken at 25° C. from autoclaved samples.

TABLE 3

The Effect of Surface Tension Depressants on the Growth of Escherichia Coli in a Basic Tryptose Medium

Compound	Concentration of Compound (Per cent)	Number of Bacteria per cc. Initial	3 Hours	6 Hours	10 Hours
Nacconol N.R.S.F. 1-100	1	56	5,700	1,260,000	480,000,000
Duponol W.A. Paste 1-100	1	39	5,900	1,290,000	480,000,000
Base Medium *	—	67	15,700	10,200,000	1,280,000,000
Nacconol N.R.S.F. 1-10,000	0.01	64	24,200	5,860,000	990,000,000
Duponol W.A. Paste 1-10,000	0.01	78	29,500	15,020,000	1,040,000,000
Base Medium *	—	81	37,700	18,360,000	990,000,000
Bacto Formate Ricinoleate Broth		71	3,000	340,000	59,000,000

* Base medium consists of Bacto-tryptose—2%; lactose—0.5%; NaCl—0.5%; K_2HPO_4—0.4%; KH_2PO_4—0.15%; pH after sterilization—6.8%

show some toxicity at concentrations of 1:100, but at concentrations of 1:10,000 no such effect is shown. These products therefore make possible the addition of a selective agent to a primary medium without the attending toxic effects on the organisms desired.

An ideal medium would appear thus to be the tryptose lactose broth plus 1:10,000 dilutions of Duponol W.A. Paste (sodium lauryl sulfate) or Nacconol N.R.F.S.

To check the value of this medium in laboratory work the Difco Laboratories kindly supplied the base broth medium. The following laboratories collaborated in these studies: Detroit Board of Health, Water Purification Plants at Detroit, Highland Park, Wyandotte, Flint, Saginaw, and Bay City, Mich.

The same medium was used in all tests. Data were submitted to our laboratory for comparative study.

A comparison was made by testing three media in parallel, namely, standard lactose broth, lactose tryptose broth, and lauryl sulfate tryptose lactose broth. The results on tap waters for 6 plants are presented in Table 4. Using lactose broth, 238 tubes showed gas but, upon confirmation with brilliant green bile broth, only 4 tubes were confirmed. Lauryl sulfate tryptose lactose broth, on the other hand, showed gas in only 3 tubes and all of these tubes confirmed.

TABLE 4

A Comparison of Standard Lactose Broth, Tryptose Broth and Lauryl Sulfate Tryptose Broth as Primary Media for Tap Water Samples

Source of Samples	No. Tubes Tested	No. of Tubes Showing Gas L.B.	T.B.	L.S.T.B.	No. Tubes Confirmed L.B.	T.B.	L.S.T.B.
Flint	498	61	4	0	0	1	0
Wyandotte	240	25	4	0	0	0	0
Detroit-Springwells	126	16	2	0	0	0	0
Detroit W.W.P.	96	34	18	0	0	0	0
Saginaw	204	59	35	0	0	0	0
Bay City	384	43	5	3	4	3	3
Total	1,584	238	68	3	4	4	3

[1] L.B. denotes lactose broth.
[2] L.S.T.B. denotes lauryl sulfate tryptose broth.

TABLE 5

A Comparison of Standard Lactose Broth and Lauryl Sulfate Tryptose Broth as Primary Media for Tap Water Samples—Highland Park, Mich.

Period of Examination	No. of Tubes Tested	No. of Tubes Showing Gas		No. Tubes Confirmed	
		L.B.[1]	L.S.T.B.[2]	L.B.	L.S.T.B.
Mar. 6, 1939–May 28, 1939	585	59	2	0	0
May 29, 1939–Aug. 8, 1939	825	38	0	0	0
Aug. 9, 1939–Feb. 20, 1940	2,100	10	0	0	0
Total	3,510	107	2	0	0

[1] L.B. denotes lactose broth.
[2] L.S.T.B. denotes lauryl sulfate tryptose broth.

An interesting observation is the fact that only 68 tubes showed gas in tryptose broth, although no selective agent had been added. The writers are not prepared at the present time to offer any explanation of this selective action. In Table 5 are presented the data for tap water tested at the Highland Park Filtration Plant over a period of one year. These data are presented in groups to show the effect of seasonal change on the number of tubes showing gas in lactose broth that do not confirm. An examination of this table shows that of 3,510 tubes tested, 107 produced gas in lactose broth but none confirmed. In lauryl sulphate tryptose broth 2 tubes showed gas but failed to confirm. These data are very similar to the ones presented from the various laboratories in Table 4.

When comparative tests were made on polluted raw water the results were slightly different. For example, in Table 6, which shows a comparison of the tests at Flint, Saginaw, and Detroit, it will be observed that 454 tubes showed gas in lactose broth of which only 32 failed to confirm. On the other hand, 469 tubes showed gas in lauryl sulfate tryptose broth and 43 failed to confirm. As judged by these data, it would appear that the latter medium fails to hold back organisms which are not members of the coliform groups. In all cases confirmations were made with brilliant green bile lactose broth.

Because of the failure for confirmation on tubes from raw water our study with the various plants was discontinued until further information could be obtained. In order to determine the type of organisms which were failing to be inhibited by the lauryl sulfate, Mr. Dahljelm of the Highland Park Filtration Plant kindly consented to send us tubes of lauryl sulfate tryptose broth that failed to confirm on either eosin-methylene-blue agar or brilliant green bile broth. As soon as these tubes were received at our laboratory they were plated on various media to isolate the

TABLE 6

A Comparison of Standard Lactose Broth, Tryptose Broth and Lauryl Sulfate Tryptose Broth as Primary Media for Polluted Raw Water Samples

Source of Sample	No. Tubes Showing Gas			No. Unconfirmed Tubes		
	L.B.	T.B.	L.S.T.B.	L.B.	T.B.	L.S.T.B.
Flint	193	211	212	3	16	20
Saginaw	98	91	91	2	1	3
Detroit-Springwells	83	85	85	21	18	17
Detroit-W.W.P.	80	83	81	6	6	3
Total	454	470	469	32	41	43

[1] L.B. denotes lactose broth.
[2] L.S.T.B. denotes lauryl sulfate tryptose broth.

organisms present. Various typical colonies were picked and transferred to suitable media for examination. In all instances where tryptose lactose broth was used we were able to isolate from these tubes members of the coliform group. In the following tables, a number of such samples are reported. In Tables 7, 8, 9, and 10, samples which failed to confirm in lauryl sulfate tryptose broth are reported. It will be observed that in each instance members of the coliform group were isolated from tubes which failed to confirm on either brilliant green bile broth or E.M.B. agar.

TABLE 7

A Comparison of Standard Lactose Broth and Lauryl Sulfate Tryptose Broth on Raw Water—Highland Park, Mich., July 5, 1939—9 A.M.

		Amounts of Water Tested									
		10 cc.		10 cc.		10 cc.		10 cc.		10 cc.	
Medium	Incub. Time	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.
Lact. broth	24 hr.	0		0		0		0		0	
	48 hr.	0		10	—	0		50	—	0	
L.S. Tryp. broth	24 hr.	0 [1]		0		0		0 [2]		0 [3]	
	48 hr.	5	—	5	+	5	+	10	—	10	—

[1] Aerobacter
[2] Aerobacter
[3] Aerobacter

TABLE 8

A Comparison of Standard Lactose Broth and Lauryl Sulfate Tryptose Broth on Raw Water—Highland Park, July 7, 1939—3 P.M.

		Amounts of Water Tested									
		10 cc.		10 cc.		10 cc.		10 cc.		10 cc.	
Medium	Incub. Time	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.
Lact. broth	24 hr.	0		0		0		0		0	
	48 hr.	10	—	10	—	0		30	+	0	—
L.S.T. broth	24 hr.	0		0 [1]		0		0 [2]		0 [3]	
	48 hr.	0		4	—	0		10	—	10	—

[1] Aerobacter
[2] Not checked
[3] Aerobacter

TABLE 9

A Comparison of Standard Lactose Broth and Lauryl Sulfate Tryptose Broth on Raw Water—Highland Park, July 10, 1939—9 A.M.

		Amounts of Water Tested									
		10 cc.		10 cc.		10 cc.		10 cc.		10 cc.	
Medium	Incub. Time	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.
Lact. broth	24 hr.	0		0		0		0		0	
	48 hr.	0		10	—	0		0		0	
L.S.T. broth	24 hr.	0 [1]		0		0 [2]		0		0	
	48 hr.	10	—	10	+	10	—	10	+	30	+

[1] Escherichia
[2] Aerobacter

TABLE 10

A Comparison of Standard Lactose Broth and Lauryl Sulfate Tryptose Broth on Raw Water—Highland Park, July 7, 1939—3 P.M.

		Amounts of Water Tested									
		10 cc.		10 cc.		10 cc.		10 cc.		10 cc.	
Medium	Incub. Time	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.	Gas	Conf.
Lact. broth	24 hr.	0		0		0		0		0	
	48 hr.	10	—	10	—	0		30	+	0	
L.S.T. broth	24 hr.	0		0[1]		0		0[2]		0[3]	
	48 hr.	0		5	—	0		10	—	10	—

[1] Aerobacter
[2] Not checked
[3] Aerobacter

These observations demonstrated that apparently the confirmatory media either caused the organisms to lose their ability to ferment lactose or inhibited these organisms from growing. It also demonstrated that in many instances tryptose broth will allow organisms to grow and produce gas that otherwise failed in lactose broth.

In order to follow through more closely the results which might be obtained from the two methods under study, a laboratory procedure was set up for checking the water samples submitted to our laboratory. Due to the amount of work necessary in the confirmations which we made it was impossible to ask any of the water purification plants to carry on this type of survey.

In each instance the sample of water was plated in parallel on standard lactose broth and lauryl sulfate tryptose broth. At the end of the 48 hour period for gas formation, E.M.B. agar plates were smeared from all tubes showing gas on both media. After the proper incubation of these plates, observations were made for the type of colony. If typical colonies were not obtained, atypical colonies were fished and planted to lactose broth and tryptose lactose broth for confirmation. If gas failed to appear in the lactose broth tubes, transfers were then made to the tryptose broth. The selected results are presented in Table 11 for lactose broth with confirmation on E.M.B. agar plates, followed by checking the

TABLE 11

Comparative Confirmation Tests by Parallel Plantings from E.M.B. Plates to Standard Lactose Broth and Tryptose Broth

Sample No.	Gas in 10 cc. Portion Lact. Broth					E.M.B. from Lact. Broth					Lact. Broth from E.M.B.					Trypt. Broth from E.M.B.					Trypt. broth from Lact. Broth				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
43	3	3	3	3	—	+*	+	+	+		—	—	—	—		10	20	20	10	—	+	+	+	+	
44	5	3	3	3	—	+	+	+	+		20	—	—	—		30	30	20	15			+	+	+	
46	20	5	1	1	1	E[1]	+	+	+	—		2	—	—			30	5	—					+	
65	1	5	5	3	5	+	+	+	+	+	—	2	2	—	—	10	10	—	5	5	+	+		+	-
89	3	3	3	3	3	+	+	+	+	+	—	—	—	—	—	10	20	10	5	5	+	+	+	+	-
95	60	90	40	70	90	A[2]	Cl.[3]	+	+	E			—	—				2	40					+	+
96	40	15	30	10	40	+	Cl.	Cl.	Cl.	Cl.	—					—					—				
104	20	50	40	—	—	+	+	+			—	—	—			10	4	3			+	+	+		
140	—	3	3	3	5		+	+	+	+		—	—	—	—		3	5	3	5		+	+	+	-
187	2	—	10	—	2	+		A			—				—	3				3	+				
261	50	50	50	50	—	E	+	+	+			—	—	—			3	3	2				+	+	+

* + indicates atypical colonies.
[1] E indicates typical Escherichia colonies.
[2] A indicates typical Aerobacter colonies.
[3] Cl. indicates presence of Clostridia.

TABLE 12

Comparative Confirmation Tests from Lauryl Sulfate Tryptose Broth by Parallel Plantings from E.M.B. Plates to Standard Lactose Broth and Tryptose Broth

Sample No.	Per cent Gas in 10 cc. Lact. Broth					Per cent Gas in 10 cc. L.S.T. Broth					E.M.B. from L.S.T. Broth					Lact. Broth from E.M.B.					Trypt. Broth from E.M.B.				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
43	3	3	3	3	—	5	5	5	5	5	+*	+	+	+	+	—	—	—	—	3	10	10	10	10	10
44	5	3	3	3	—	5	5	—	10	5	+	+		+	+	10	2		—	2	30	30		25	25
46	20	5	1	1	1	5	5	5	10	5	+	A[1]	+	+	+	—		—	1	2	1		2	2	5
65	1	5	5	3	5	10	10	40	40	—	A	+	A	E[2]			—					5			
89	3	3	3	3	3	5	5	5	5	5	+	+	+	+	+	—	—	—	—	—	5	5	5	30	30
95	60	90	40	70	90	40	3	—	—	—	+	E				—					10				
96	40	15	30	10	40	—	—	—	—	—															
104	20	50	40	—	—	3	2	2	—	—	+	+	+			—	—	3			3	3	3		
140	—	3	3	3	5	5	5	5	5	3	+	+	+	+	+	—	3	3	10	10	1			70	20
187	2	—	10	—	2	—	5	5	—	—		+					—	—			1	3			
261	50	50	50	50	50	—	—	5	10	—			+	A				—				2			

* + indicates atypical colonies.
[1] A indicates typical Aerobacter colonies.
[2] E indicates typical Escherichia colonies.

colonies on lactose broth and tryptose lactose broth. It will be observed from these data that many instances have appeared wherein atypical colonies on E.M.B. agar plates obtained from lactose broth failed to produce gas in lactose broth. When transferred to tryptose broth, gas was produced in considerable quantities. Furthermore, when negative lactose broth tubes were transferred to tryptose lactose broth, gas production was obtained. These data thus confirmed the observations made at Highland Park that E.M.B. and brilliant green bile cannot be used as confirmatory media if it is desired to obtain confirmation on all coliform organisms which appeared in lactose broth. In Table 12 are presented the results for parallel planting of the samples shown in Table 11 on lauryl sulfate tryptose broth. It will be ob-

TABLE 13

Comparative Colon Indices Obtained by Parallel Plantings by Standard Methods and Lauryl Sulfate Tryptose Broth

Sample No.	Stand. Methods		L.S.T. Broth Method		
	Gas Index	Confirmed Index	Trypt. Broth Conf.	Gas Index	Trypt. Broth Conf.
43	8	0	8	10	10
44	8	2	8	8	8
46	10	2	6	10	10
49	4	0	0	0	0
65	10	4	8	8	8
75	2	0	2	0	0
89	10	0	10	10	10
95	10	4	4	4	4
96	10	0	0	0	0
103	2	0	2	6	6
104	6	0	6	6	6
140	8	0	8	10	10
187	6	2	4	4	4
200	10	0	4	10	10
230	2	2	2	2	2
234	6	2	6	8	8
261	8	2	8	4	4
Average	7.05	1.3	5.0	5.9	5.9

served that the same picture obtained as in the case of the lactose broth, except that a higher incidence of coliform organisms was obtained. It will also be observed that gas production was not obtained in the lauryl sulfate tryptose broth when coliform organisms were not isolated by the confirmation technic used.

In Table 13 are presented comparative colon indices obtained by parallel planting by standard methods and lauryl sulfate tryptose broth. It will be observed that the gas indices obtained in standard broth are much higher than the confirmed indices obtained by confirmation through E.M.B. agar and lactose broth. Where tryptose broth confirmation was made, the colon indices obtained compare favorably with the gas indices made from the standard lactose broth. The colon indices obtained from the parallel planting in the lauryl sulfate broth by confirmation in tryptose broth were the same as the gas indices obtained in this medium.

These data show that the confirmatory media used at the present time in standard methods act as suppressing agents to the coliform organisms and produce a lower colon index than would be obtained if more suitable confirmatory media were used.

These data also indicate that in lauryl sulfate tryptose broth gas production could serve not only as a presumptive test but also as a confirmatory medium for routine testing. It has been our observation that, when gas is produced in lauryl sulfate tryptose broth, confirmation is always obtained. These data should be checked further in routine laboratories, but until a satisfactory confirmatory medium for checking gas production in lauryl sulfate tryptose broth is developed, the results obtained would not check with those obtained in this research.

REFERENCES

1. Darby, C. W., and Mallmann, W. L. Studies on Media for Coliform Organisms. *J. Am. Water Works A.*, 31:689, 1939.

2. Cowls, P. B. A Modified Lactose Broth for Use in the Presumptive Test. *J. Am. Water Works A.*, 30:979, 1938.

Literature Cited

(1) Parr, L. W., Coliform Bacteria. Bact. Rev. 3 (1) : 1-48, 1939.

(2) Winslow, C. E. A. The number of bacteria in sewage and sewage effluents determined by plating upon different media and by a new method of direct microscopic enumeration. Jour. Infect. Dis. Sup. 1 : 209, 1905.

(3) Committee of Bacteriologists. 1898. Procedures recommended for the study of Bacteria, with especial Reference to Greater Uniformity in the Description and Differentiation of Species. Being the Report of a Committee of Bacteriologists to the Committee on the Pollution of Water-supplies of the American Public Health Association. Transactions of the American Public Health Association. 1897 Meeting. 23, 60. (4)

(4) Prescott, S. C. and Winslow, C. E. A. Elements of Water Bacteriology. 5th Ed. John Wiley and Sons, Inc. New York, 1931.

(5) Fuller, G. W. Report of Committee on Standard Methods of Water Analysis. Transactions of the American Public Health Association. 27 : 377, 1901 (4).

(6) Reinsch, A. Zur bakteriologischen Untersuchung des Trinkwassers. Centralblatt fur Bakteriologie, 10 : 415, 1891 (4).

(7) Bunker, G. C. and Schuber, H. The Reactions of Culture Media. Jour. Amer. Water Works Association. 9 : 63, 1922.

(8) Gage, S. D. and Adams, G. C. Studies of Media for the Quantitative Estimation of Bacteria in Water and Sewage. Jour. Infect. Dis. 1 : 358, 1904.

(9) Mathews, A. P. On Wurtz's Method for the Differentiation of Bacillus typhi abdominalis from Bacillus coli communis and

its Application to the Examination of Contaminated Drinking water. Technology Quarterly 6 : 241, 1893.

(10) Smith, T. A New Method for Determining Quantitatively the Pollution of Water by Faecal Bacteria. Thirteenth Annual Report of the State Board of Health of New York for 1892. 712, 1893. (4)

(11) Irons, E. E. Some Observations on Methods for the Detection of B. coli communis in Water. Transactions of the American Public Health Association, 1900 Meeting. 26 : 310, 1901.

(12) Greer, F. E. The Sanitary Significance of Lactose-Fermenting Bacteria not Belonging to the B. coli Group.

1. Groups Reported in the Literature and Isolated from Water in Chicago. Jour. Infect. Dis. 42 (5), 501-513, 1928.

2. Number of Lactose-Fermenting Organisms found in Chicago Water Supply. Jour. Infect. Dis. 42 (5): 514-524, 1928.

5. Factors Influencing the Survival of Microorganisms in Water. Jour. Infect. Dis. 42 (6): 545-550, 1928.

6. Sanitary Considerations. Jour. Infect. Dis. 42 (6): 551-555, 1928.

(13) Greer, F. E. and Myhan, F. V. The Sanitary Significance of Lactose-Fermenting Bacteria not belonging to the B. coli Group.

3. Bacterial Associations in Cultures Containing Lactose-Fermenting Bacteria. Jour. Infect. Dis. 42 (6): 525-536, 1928.

(14) Greer, F. E., Tonney, F. O. and Myhan, F. V. The Sanitary Significance of Lactose-Fermenting Bacteria not belonging to the B. coli Group.

4. Pathogenicity. Jour. Infect. Dis. 42 (6): 537-544, 1928.

(15) Greer, F. E., R. E. Noble, F. V. Nyhan and A. E. O'Neil. The Sanitary Significance of Lactose-Fermenting Organisms not belonging to the B. coli Group.

7. Mediums and Methods. Jour. Infect. Dis. 42 (6): 556-567, 1928.

(16) Greer, F. E. and R. E. Noble. The Sanitary Significance of Lactose-Fermenting Bacteria not belonging to the B. coli Group.

8. Conclusions. Jour. Inf. Dis. 42 (6): 568-574, 1928.

(17) Levine, M. Bacteria Fermenting Lactose and their Significance in Water Analysis. Iowa State College of Agriculture and Mechanic Arts Official Publication. 20 (31), Bul. 62, 1921.

(18) Bergey, D. H., R. S. Breed and E. G. D. Murray. Manual of Determinative Bacteriology, 5th Ed. Williams and Wilkins Co., Baltimore, 1939.

(19) Standard Methods of Water Analysis. Am. Pub. Health Assoc., 8th Edition, 257, 1936.

(20) Muer, T. C. and R. L. Harris. Value of Brilliant Green in Eliminating Errors due to Anaerobes in the Presumptive Tests for B. coli. Am. Jour. of Public Health. 10: 874-876, 1920.

(21) Dunham, H. G. and H. W. Schoenlein. Brilliant Green Bile Media. Stain Tech. 1 (3): 129-134, 1926.

(22) Jordan, H. E. Brilliant Green Bile for the Determination of the Colon-aerogenes Group. Jour. Am. Water Works Assoc. 18: 337, 1927.

(23) Browning, C. H., W. Gilmour and T. J. Mackie. The Isolation of Typhoid Bacilli from Faeces by means of Brilliant Green in Fluid Medium. Jour. Hyg. 13 (3): 335-342. 1913-14.

(24) Churchman, John W. The Selective Bactericidal Action of Gentian Violet. Jour. Exp. Med. 16 (2): 221-249, 1912.

(25) Churchman, John W. and W. Howard Michael. The Selective Action of Gentian Violet on Closely Related Bacterial Strains. Jour. Exper. Medicine 16 (6): 822-830, 1912.

(26) Churchman, John W. The Mechanism of Bacteriostats. Jour. Exper. Med. 37 (4): 543-554, 1923.

(27) Churchman, John W. Inhibiting Properties of Anilin Dyes. Stain Technology. 1 (1) : 27-32, 1926.

(28) Stearn, Allen E. and Esther W. Stearn. The Chemical Mechanism of Bacterial Behavior. III. The Problem of Bacteriostasis. Jour. Bact. 9 (5): 491-510. 1924.

(29) Dubos, Rene. The Relation of the Bacteriostatic Action of Certain Dyes to Oxidation--Reduction Processes. Jour. Exper. Med. 49 (4): 575-592, 1929.

(30) Ingraham, Mary A. The Bacteriostatic Action of Gentian Violet and its Dependence on the Oxidation--Reduction Potential. Jour. Bact. 26 (6): 573-598, 1933.

(31) Churchman, John W. The Reverse Selective Bacteriostatic Action of Acid Fuchsin. Jour. Exper. Med. 37 (1): 1-10, 1923.

(32) Bryan, C. S., E. D. Devereux, W. C. Hirschey and A. C. Corbett. The Use of Brilliant Green, Sodium Azide, and Dextrose in the Microscopic and Hotis Tests for Streptococcic Mastitis. North American Vet. 20: 41-46, 1939.

(33) Snyder, Marshall L. and Herman C. Lichstein. Sodium Azide as an Inhibiting Substance for Gram-negative Bacteria. Jour. Infect. Dis. 67: 113-115, 1940.

(34) Lichstein, Herman C., and Marshall L. Snyder. The Inhibition of the Spreading Growth of Proteus and other Bacteria to Permit the Isolation of Associated Streptococci. Jour. Bact. 42 (5): 653-664, 1941.

(35) Mallmann, W. L., W. E. Botwright and E. S. Churchill. The Selective Bacteriostatic Effect of Slow Oxidizing Agents. Jour. Infect. Dis. 69: 215-219, 1941.

(36) Rakieten, Morris L. and Leo F. Rettger. Brilliant Green and its use in an Enrichment Medium in the Isolation of Typhoid and Paratyphoid Organisms. Jour. Infect. Dis. 41 (2): 93-110. 1927.

(37) Mallmann, W. L., F. Thorp, Jr., and M. Semmes. A medium for the isolation of Salmonella pullorum and other members of the paratyphoid group from avian tissues. Jour. A. V. M. A. lxxiii, n.s. 26 (6): 825-838, 1928.

(38) Conn, H. J. Progress in the Standardization of Stains--Dyes for Bacteriostatic Work. Stain Tech. 5 (1): 1-2, 1930.

(39) Stearn, Esther and Allen E. Stearn. The Mechanical Behavior of Dyes, Especially Gentian Violet, in Bacteriological Media. Jour. Bact. 8 (6): 567-572, 1923.

(40) Stearn, Esther W. and Allen E. Stearn. Conditions and Reactions Defining Dye Bacteriostasis. Jour. Bact. 11 (5): 345-357. 1926.

(41) McCalla, T. M. and F. E. Clark. Dye Absorption by Bacteria at Varying H-ion Concentrations. Stain Tech. 16 (3): 95-100, 1941.

(42) Reed, Mary V. and Elizabeth F. Genung. The Titration of Dyes for their Bacteriostatic Action. Stain Tech. 9 (4): 117-128, 1934.

(43) Churchman, John W. Relation of Age of Bacteria to Bacteriostatic Properties of Anilin Dyes. Stain Tech. 1 (3): 103-104, 1926.

(44) Difco Manual of Dehydrated Culture Media and Reagents. Sixth Edition, revised 1931. reprinted 1941, Detroit, Michigan.

(45) Conn, H. J. Biological Stains. 3rd Ed. W. F. Humphrey Press, Inc., Geneva, New York, 1936.

(46) Mallmann, W. L. A New Yardstick for Measuring Sewage Pollution. Sewage Works Jour. 12 (5): 1940.

ROOM USE ONLY