STUDIES ON THE USE OF ETHYL VIOLET AS AN INHIBITORY AGENT IN A CONFIRMATION MEDIUM FOR THE DETECTION OF COLIFORM ORGANISMS

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THESIS

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STUDIES ON THE USE OF ETHYL VIOLET AS AN INHIBITORY AGENT IN A CONFIRMATION MEDIUM FOR THE DETECTION OF COLIFORM ORGANISMS

By

Frederick Just Post

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Since the early beginnings of water bacteriology attempts have been made to improve and make more exact the methods of determining the sanitary quality of a water supply. Diseases, which are frequently transmitted through polluted waters, have been successfully controlled to the point where they are no longer considered a menace. Much of the credit for this has been due to the constant effort of bacteriologists to make more stringent the criteria and limits of safety of pollution, especially through the agency of the coliform test.

Standard Methods for the Examination of Water and Sewage (1946), which is the accepted standard for bacteriological procedures in water analysis, defines coliform organisms as; "all aerobic and facultative anaerobic, gram negative, non-sporing bacilli which ferment lactose with gas formation." One of the permissible media allowed by the above "Standard Methods" for confirming the presence of these coliforms is brilliant green lactose bile broth, which is considered more toxic for gram positive organisms than for gram negative organisms. Many authors (Ruchoft, 1926; Hale, 1927; Salle, 1929; Mallmann and Hepler, 1936 and others) have found that brilliant green bile is toxic to many coliforms to the extent of completely inhibiting the weaker strains, lengthening the generation times or inhibiting the lactose fermenting powers of many others. There is, then, a constant search for a better medium to take its place; one that is less toxic and more nearly approaches optimum

conditions for the growth and multiplication of coliform organisms without removing its selectivity.

Following the earlier work of Jolliffe (1948), Bordt (1951), and Litsky, Mallmann and Fifield (1952), this dissertation concerns itself with the attempt to incorporate an aniline dye, ethyl violet (hexaethyl triamino triphenyl methane), in an enrichment medium to be used in confirming the presence of coliform organisms.

Purpose of the study. The purpose of this study was twofold: first, to develop the growth curves of a strain of Escherichia coli in various media containing varying amounts of ethyl violet dye in order to determine the most efficient combination; and second, to compare one dye-medium combination with brilliant green bile broth in the "Standard Methods" examination of various Michigan waters.

The test organism was grown in three media; "Standard Methods" lactose broth, tryptose lactose broth, and lauryl tryptose broth using concentrations of ethyl violet ranging from 1-10T to 1-800T (T = thousand). The growth cycle study was limited to the lag and early log phases which were considered more important in this type of study than the total number of organisms at 24 hours.

The comparison with brilliant green bile was carried out according to Standard Methods for the Examination of Water and Sewage (1946), at the Michigan Department of Health Laboratories, by inoculating duplicate tubes.

REVIEW OF LITERATURE

In reviewing the literature on growth curves of E. coli, little was found relating directly to the subject. Salter (1919), made some observations on the rate of growth of B. coli in lactose broth containing various concentrations of crystal violet and brilliant green dyes. He established the effect of the concentration of the dye by its effect on the generation time, or the rate of growth, for which he utilized the law of geometric proportions. He found that crystal violet decreased the growth rate at 1-1M (M = million) and its greatest effect was in the lag phase. Brilliant green did not inhibit at 1-6M but completely inhibited at 1-600T.

Mallmann and Darby (1939), utilized a technique using a minimal inoculum and subsequent sampling every two hours in order to determine the effect of various factors on the lag and early log phases. In this study they also developed a tryptose lactose broth medium that was far superior to lactose broth as an enrichment medium.

Later work by Litsky, Mallmann and Fifield (1952), using the above technique and medium, found that crystal violet and brilliant green were quite toxic to E. coli, the latter being more toxic at 1-1M, allowing an increase to 280 organisms, while crystal violet in the same concentration allowed 270T organisms in the same time period from the same minimal inoculum.

Prior to 1920 attempts at confirming the presence of coliform organisms were limited to the use of solid media while experiments

proceeded using dyes in the presumptive enrichment media. Meur and Harris (1920), first suggested the use of a lactose-bile-brilliant green medium for the presumptive test and Winslow and Dolloff (1922) determined the limiting concentrations of the bile and brilliant green.

Because of many conflicting reports on the results of using this broth, the American Water Works Association, Standard Methods of Water Analysis, Committee No. 1 studied this medium intensively and found it less suitable as a presumptive enrichment medium than lactose broth. Work by Jordan (1927) and others with optimum bile-brilliant green ratios, showed that it compared unfavorably with lactose broth as a presumptive medium. In this study, however, Jordan used the brilliant green bile broth as a confirmatory medium and found it very satisfactory as such. These results and others led to the inclusion of this medium in the 1933 "Standard Methods", and favorable results were later reported by France (1936), and Mallmann and Hepler (1936), although the latter noticed inhibition to some coliforms and the failure to suppress some spore formers both aerobic and anaerobic.

Again in 1941, Mallmann and Darby demonstrated that some organisms, when placed on eosin methylene blue agar or in brilliant green bile broth, lost their ability to ferment lactose or else were inhibited from growing.

Other liquid media, previously proposed as presumptive media, were examined for the possibility of obtaining a better confirmatory medium, just as occurred with brilliant green bile broth. Ruchhoft and Norton (1935), compared lactose broth, and lactose broth followed by brilliant

green 2 percent bile broth, formate ricinoleate broth, and MacConkey broth as confirmatory media. Carrying all tubes through the completed test, he found that the "Standard Methods" brilliant green bile medium compared unfavorably in that more coliform isolates were obtained from all of the confirmatory methods together than from any one alone. Most other confirmatory media have eventually been discarded because of toxicity, non-selectivity, or that they were not sufficiently sensitive for use.

McCrady (1939), in comparing MacConkey's broth and "Standard Methods" lactose broth for the presumptive test, found that lactose broth followed by brilliant green bile yielded the best results although brilliant green bile gave a few false positive tubes.

Richey (1941), and Wattie (1943), found brilliant green bile, on the whole, satisfactory, but Shane (1947), found that, by using lauryl sulfate broth as a presumptive medium, many of the false positive tubes occurring in the brilliant green bile confirmatory medium were eliminated.

A more complete and detailed evolution of sanitary water bacteriology and the methods and media used can be found in the book "Water Bacteriology" by Prescott, Winslow, and McCrady, (1946).

The first mention of ethyl violet as a bacteriostat found in the literature was in a paper by Petroff and Gump (1935), who used it as one of a series of 130 dyes and allied compounds tested for bacteriostatic action against several gram positive and negative organisms. This study utilized the method developed by Churchmann (1912), in his studies on gentian violet. The results were apparently disappointing

to the authors who found that ethyl violet, among others, was less toxic to gram positives than gentian violet but more than brilliant green. It is assumed that ethyl violet was not toxic to the gram negative organisms studied for it was not included among those compounds active against this group. Brilliant green was included and was about equally toxic for the gram negative organisms (including E. coli) as for the gram positives. It is interesting to note that these authors prepared the next higher homolog of ethyl violet, n-proply violet, and found it less toxic to gram positives and apparently non-toxic to gram negatives.

The above authors, however, did not appear to recognize the possibilities of ethyl violet for not until the work of Darby (1943), was this dye mentioned again. Using agar and broth containing ethyl violet, brilliant green and several other agents to determine bacteriostasis, he found that ethyl violet in agar showed marked inhibition to gram positives and very little to gram negatives. In tryptose broth the bacteriostatic titer was considerably higher. Using a tryptose broth base and comparing the growth cruves of E. coli with brilliant green bile broth, he found that the broths compared favorably when a 1-200T concentration of ethyl violet was used. This combination, however, showed slight toxicity to coliforms as did the brilliant green bile medium. He also found that ethyl violet was not 100 percent effective against gram positives.

In studying the growth curves of several enteric organisms, using a base medium of lauryl sulfate broth, Jolliffe (1948), found that ethyl

violet was less toxic than brilliant green or crystal violet but that all three were about equal with respect to inhibition of several gram positive organisms.

Bordt (1951), found that ethyl violet in a concentration of 1-80T in tryptose lactose broth inhibited the gas production of some coliforms and when the concentration reached 1-333T no inhibition was exhibited. His studies also indicated that this dye was more toxic to E. coli than to either Aerobacter aerogenes or E. freundii.

Litsky, Mallmann and Fifield (1952) found that ethyl violet showed markedly less toxicity to E. coli at 1-200T concentration than either brilliant green at 1-1M or crystal violet at 1-1M and that all three were inhibitory to Bacillus subtilis from 1-1M to 1-10M concentrations.

MATERIALS AND METHODS

Cultures. Escherichia coli, Cinncinnati strain 198 was obtained from D. Muentener of the Michigan State College Department of Bacteriology and was maintained and transferred daily on tryptose glucose extract agar (TGE) and incubated at 35 - 37C. The IMViC formula was the usual E. coli + + - -. This organism was used for all of the growth curve determinations.

Culture media. Three of the media used; brilliant green bile broth, lactose broth and lauryl sulfate broth, were prepared from the standard dehydrated Difco products and all media used were autoclaved at 15 pounds for 15 minutes.

The fourth medium, tryptose lactose broth, was prepared according to the formula suggested by Darby and Mallmann (1939). and is as follows:

Bacto-tryptose	2 %
Lactose	0.5%
K_2HPO_4	0.4%
KH_2PO_4	0.15%
NaCl	0.5%
pH before sterilization	6.8

The description of the dye used in this study is as follows: Ethyl Violet 6B, Lot no. 12552, CI*682, 57.5 percent dye content, manufactured by the National Aniline and Chemical Company. A stock solution of the dye was prepared by dissolving 0.17391 gms in 4 cc. of ethyl alcohol and making up to 100 ml. This gave a working solution of 1-1000 actual dye content. Sterility was determined by plating in TGE and incubating at 35-37C.

The media used in the tests were prepared and added to 250 ml flasks in such a manner that after autoclaving and addition of the predetermined volume of the dye solution, the total volume was 99 ml. In all the preliminary growth curve studies the dye concentrate was added aseptically to the desired medium after autoclaving and immediately before inoculation. Near the end of these experiments it was found desirable to determine the effects of autoclaving on the dye medium combination in which case the dye concentrate was added prior to sterilizing. During the comparison with "Standard Methods" brilliant green bile in the field, the dye was added to the medium, tubed in fermentation tubes, plugged, and autoclaved at 15 pounds for 15 minutes.

Growth studies. Cultures were maintained on TGE agar slants by transferring daily for at least five days before each trial. Stock cultures were refrigerated at 4C and transferred monthly. At the time of inoculation of the flasks, the slant containing the culture was washed with physiological saline and adjusted to a known density by visual comparison with a McFarland turbidity standard. Appropriate dilutions were made in physiological saline so that after the addition of 1 ml to the medium, each flask contained between 20 and 80 organisms per ml.

One ml samples were removed at time intervals of two hours, beginning immediately after inoculation and ending at six hours. The flasks were vigorously swirled, alternately clockwise and counterclockwise at least 80 times before sampling. Using the appropriate dilutions, the samples were plated in duplicate using TGE as the plating medium. The

flasks and plates were incubated at 35 - 37 C as recommended by Boniece and Mallmann (1950), and counted at the end of 24 hours using a Quebec colony counter.

Field comparison. This portion of the study was undertaken with the cooperation and at the laboratories of the State of Michigan Department of Health. It consisted of using the ethyl violet medium as a confirmatory test for the presence of coliforms by using it in parallel with the brilliant green bile medium test on various Michigan waters. Transplants were made from tubes of standard lactose broth showing gas to the brilliant green bile medium and the ethyl violet medium by pipetting 0.1 ml into each and incubating at 37 C for 48 hours. Tubes showing gas in both brilliant green bile and ethyl violet were recorded and discarded. Parallel tubes showing different results at the end of 48 hours were examined in the attempt to recover coliforms, from one or both, by using the "Standard Methods" completed test. This test consists of streaking on eosin methylene blue agar plates and incubating for 24 hours. Isolated colonies are inoculated into standard lactose broth and streaked on agar slants. Gram stains are made from the agar slant at 24 hours and examined if the lactose broth tube shows gas at 24 or 48 hours. If spores are present further isolation is undertaken and the completed test repeated again.

RESULTS AND DISCUSSION

All the growth curves in this study were determined in exactly the same manner; removing one ml samples, diluting and plating in duplicate. This technique was adapted from the work of Darby and Mallmann (1939), who did extensive work with growth curves in developing tryptose lactose broth and lauryl sulfate tryptose broth. These authors thought that studies on bacteriostats and inhibitory media should be made during the lag and early log phases, when the young cell, which has been placed in a new environment and is commencing to divide, is very susceptible to adverse conditions. It was assumed that the earlier the lag phase can be overcome the more efficient the medium in isolation work. In view of this concept the growth curve was studied only for the first six hours since the lag phase should be of considerably shorter duration to produce good results.

Escherichia coli was the organism of choice for these growth curves since Bordt showed evidence that ethyl violet was more toxic to this coliform than to A. aerogenes or the Intermediates.

The results of the growth determinations are given in Tables I, II, and III and Figures 1, 2, 3, and 4. A total of eight different dye concentrations was added to the three media under test in order to determine the relative effect of the concentration. For each group of flasks a control, containing no dye, was used to determine the uninhibited growth in that medium; these are included in each graph in order to make a comparison with the dye curve.

The first medium to be tested was "Standard Methods" lactose broth, (Table I and Figure 1), using concentrations of dye 1-10T, 1-50T, 1-100T, 1-200T, 1-400T, and 1-800T. An examination of Figure 1 shows that dye concentrations less than 1-100T were extremely toxic to E. coli and 1-100T still somewhat so. The slope of the growth curve does not approach that of the control until the dye concentration is 1-400T and here the lag phase shows a greater decline of numbers in the first two hours than the control due to the death of susceptible organisms, presumably at the time of dividing. At a dye concentration of 1-800T the two curves are very nearly alike. It is probable then, from these results, that the 1-400T concentration represents the maximum concentration that could be used, and since the initial lag phase drops slightly it would probably be less toxic if a concentration less than 1-400T were used.

Tryptose lactose broth was the next medium to be tested for its suitability as an ethyl violet base, Table II, and Figure 2. The first noticable difference in results from the lactose broth is that any particular dye concentration is less toxic in this medium. A concentration of 1-10T roughly corresponds to 1-50T in lactose broth and this similarity holds for each one tested. The results indicate that 1-200T is the maximum concentration that can be used, for there is no appreciable loss of organisms in the first two hours although the generation time in the lag phase is slower than in the control. The slope of the 1-400T concentration very nearly approaches that of the control so that the safest concentration would very probably lie about 1-300T since here one would expect a lag phase generation time only slightly slower than the control and the log phase rate about the same as that of the control.

TABLE I

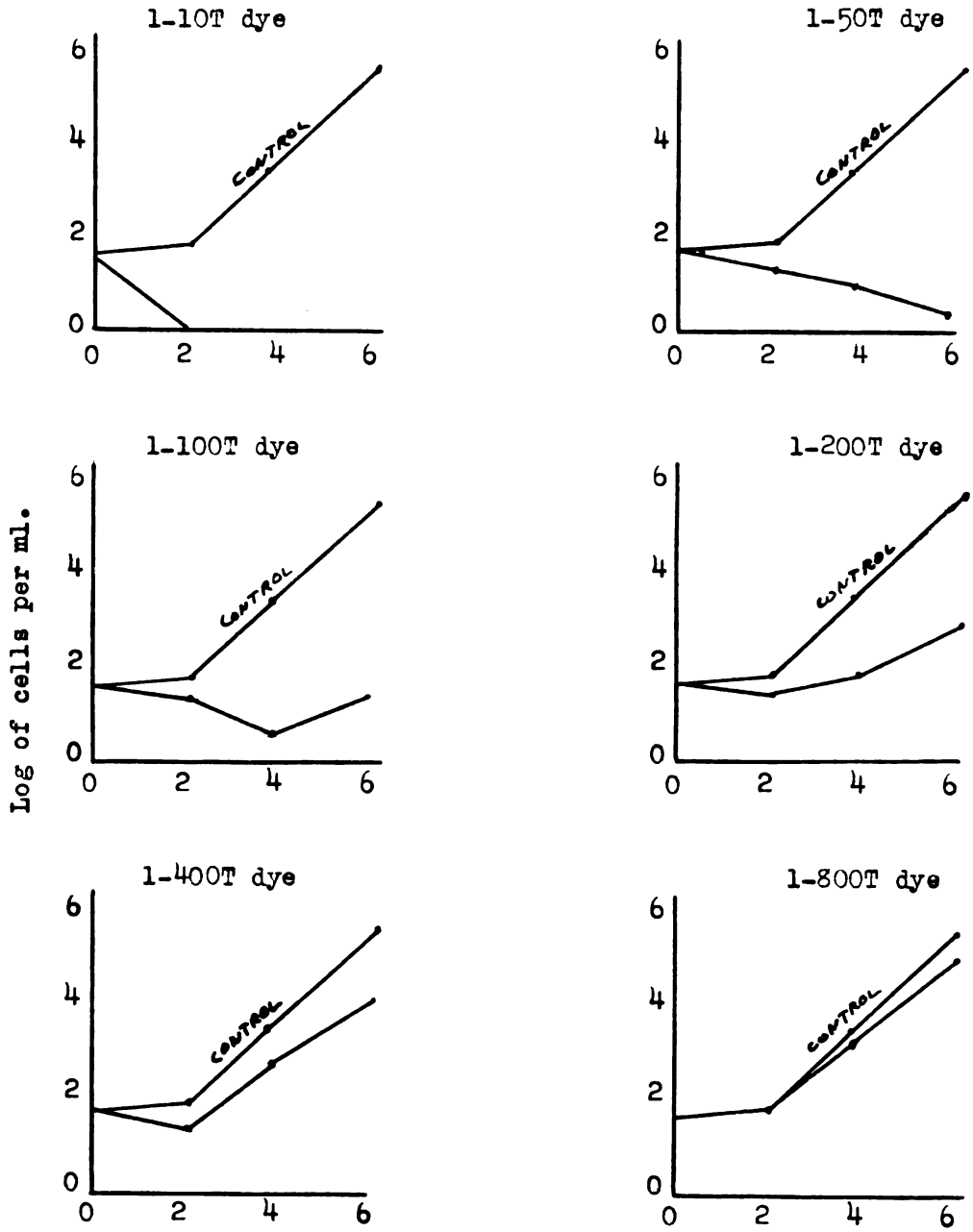
Populations of E. coli in varying concentrations of ethyl violet in lactose broth

		Number of bacteria per ml. Concentration of dye						
	Hrs	1-10T	1-50T	1-100T	1-200T	1-400T	1-800T	Control
Run # 1	0	14	33	43	52	72	59	78
	2	1	25	23	33	36	98	200
	4	0	16	14	160	720	2500	7300
	6	0	6	50	1000	6000	61,000	410,000
Run # 2	0	35*	167*	42	52	56	47	56
	2	1	80	34	42	31	56	77
	4	0	40	15	79	590	1600	3800
	6	0	27	30	730	6500	35,000	220,000
Run # 3	0			47	51	51	49	46
	2			38	40	45	40	52
	4			31	89	530	850	2400
	6			21	510	7800	33,000	240,000

*Taken from runs with excessive counts. However, it does show the trend.

Figure 1

Effect of various concentrations of ethyl violet on the growth curves of E. coli, in lactose broth*.



*Typical curves of at least 3 individual runs

TABLE II

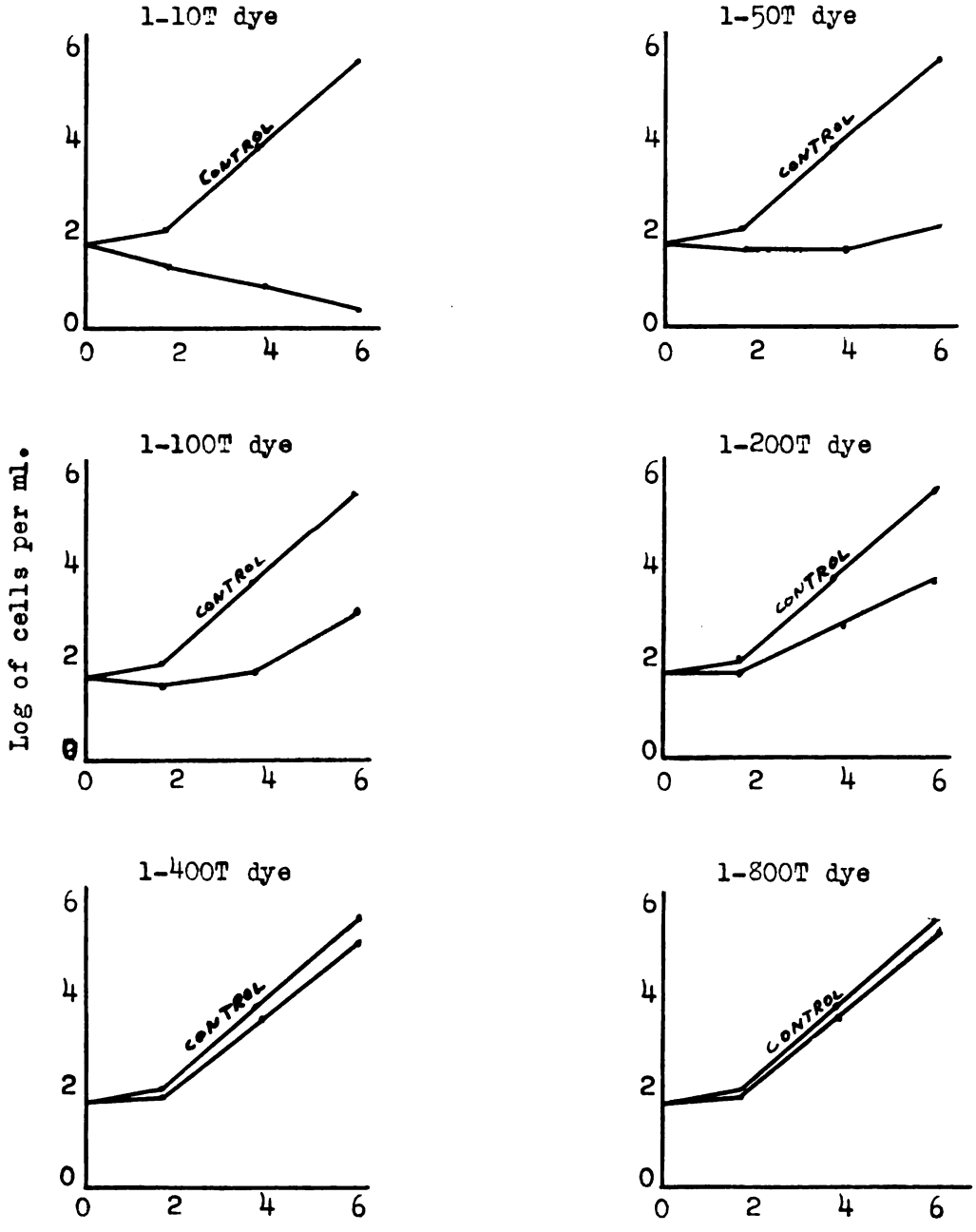
Populations of E. coli in varying concentrations of ethyl violet in tryptose lactose broth.

		Number of bacteria per ml. Concentration of dye						
	Hrs	1-10T	1-50T	1-100T	1-200T	1-400T	1-800T	Control
Run # 1	0	26	39	35	46	36	45	47
	2	15	31	26	43	62	59	85
	4	10	25	68	330	1550	2200	4700
	6	8	55	290	(7000)*	60,000	85,000	260,000
Run # 2	0	32	44	54	59	61	56	58
	2	15	37	42	50	71	75	110
	4	12	33	76	390	1450	2200	3900
	6	8	89	280	3600	42,000	30,000	240,000
Run # 3	0	70	70	75	68	75	70	70
	2	48	60	85	91	114	139	148
	4	34	57	147	690	3200	3700	4900
	6	24	124	450	7100	81,000	180,000	380,000

*Taken from plates showing only 4 and 9 organisms.

Figure 2

Effect of various concentrations of ethyl violet on the growth curves of E. coli, in tryptose lactose broth*.



*Typical curves of at least 3 individual runs.

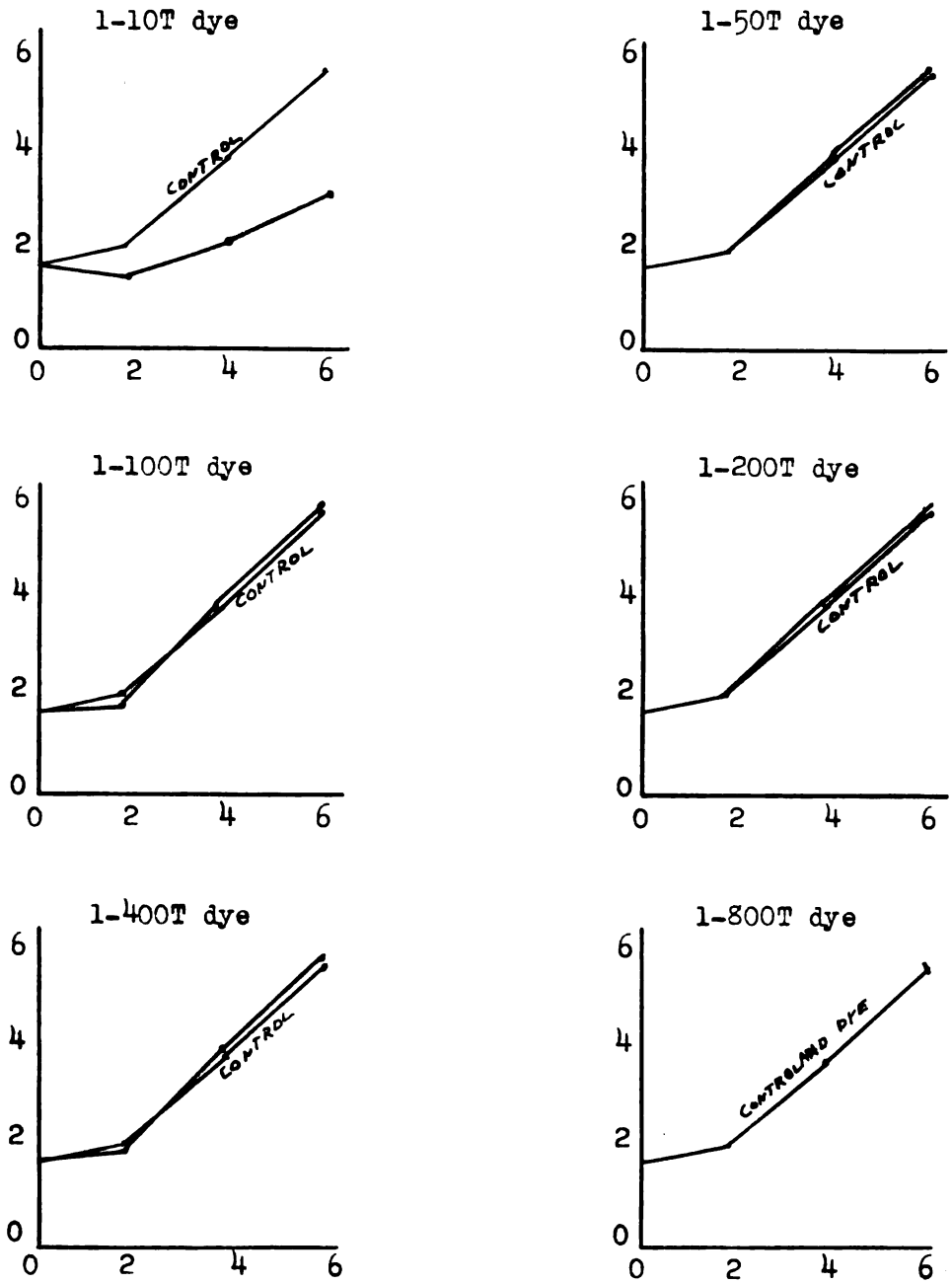
TABLE III

Populations of E. coli in varying concentrations of ethyl violet in lauryl tryptose broth.

		Number of bacteria per ml. Concentration of dye						
	Hrs	1-10T	1-50T	1-100T	1-200T	1-400T	1-800T	Control
Run #1	0	50	52	43	41	56	56	50
	2	59	78	90	103	83	103	101
	4	100	TNC	4600	5900	4200	3600	2500
	6	380	TNC	TNC	420,000	340,000	220,000	121,000
Run #2	0	46	39	37	43	43	47	51
	2	37	92	88	102	105	102	123
	4	105	6900	5600	5100	6700	3800	3600
	6	430	490,000	246,000	190,000	280,000	210,000	200,000
Run #3	0	51	46	44	37	41	51	44
	2	47	97	83	108	97	102	109
	4	106	7300	4000	6300	6700	7700	2800
	6	430	610,000	320,000	370,000	250,000	360,000	260,000

Figure 3

Effect of various concentrations of ethyl violet on the growth curves of E. coli, in lauryl sulfate broth*.



Time in Hours

*Typical curves of at least 3 individual runs.

Lauryl tryptose broth produced some unexpected curves as seen in Table III and Figure 3, since the only major difference between this broth and the previous one is the presence of lauryl sulfate. In four out of the six dye concentrations studied, the dye-medium combination actually resulted in an increase in organisms per ml over the control. This stimulation was not observed in the most concentrated nor the most dilute solutions used. In the latter the curves coincided. At 1-50T then, the dye-medium combination apparently offered a better medium, at least in this part of the growth curve, than the lauryl sulfate alone. At 1-10T the toxic properties of the dye are again strongly exerted, and although no attempt will be made at this time to explain this stimulation, it is of interest to note that the lauryl sulfate concentration in this medium is also 1-10T, possibly indicating a molecular combination of some sort.

Judging from the stimulation noted in the lag and log phases of the growth curves, lauryl tryptose broth was adopted as the base medium and 1-50T as the ethyl violet concentration for the comparison with brilliant green bile. However, in preparing the medium for use the dye was added before sterilizing. After the dye medium was sterilized and removed from the autoclave it was noted that 30 - 50 percent of the dye had precipitated out of the medium. Autoclaving was attempted using several lesser dilutions of dye but the precipitation occurred in each case. Upon being added to the base medium and allowing it to stand for 24 hours, a small portion of the dye precipitated out but not to the extent encountered after autoclaving. Also, considering the fact that lauryl

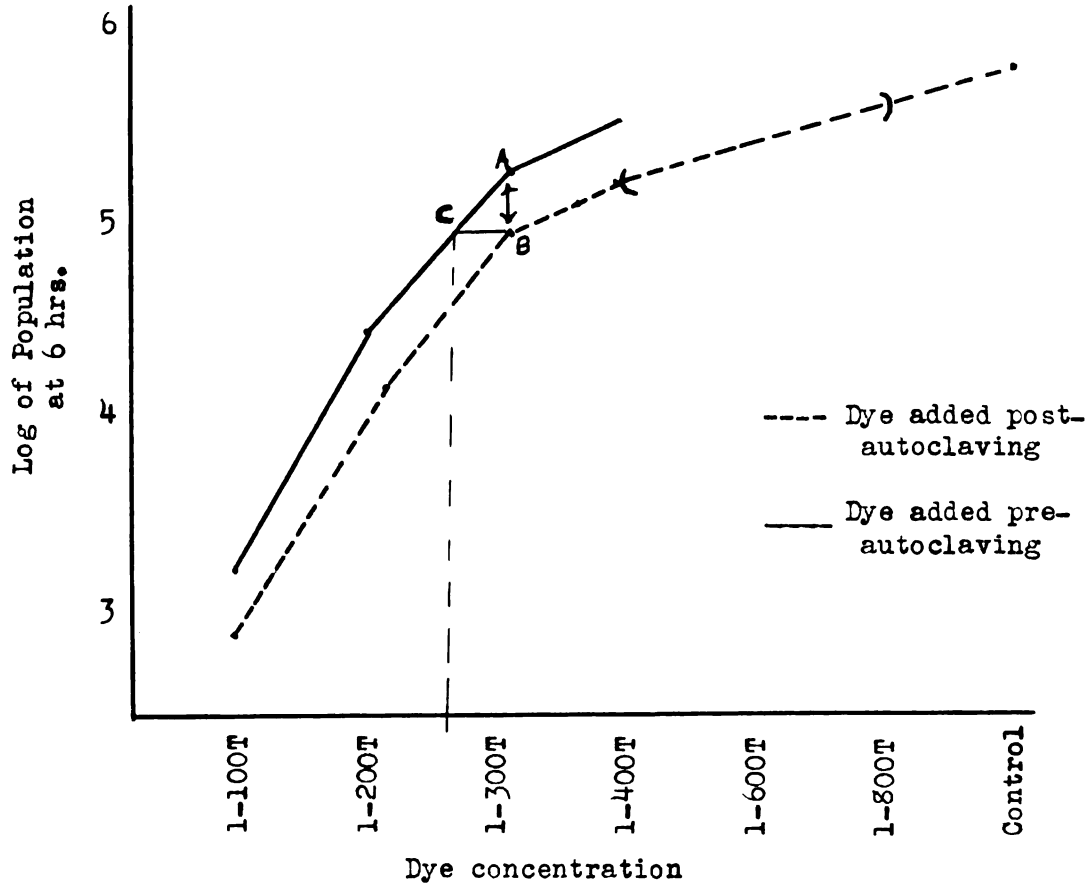
sulfate might be used as a presumptive medium and therefore its results as a confirmatory medium might be open to doubt, the medium was removed from consideration.

Turning back to the medium which showed the next best results, tryptose broth, it was decided that a 1-200T concentration of ethyl violet was too concentrated. The curve of the lag phase dropped slightly in the first two hours and the slope of the log phase was not quite steep enough to warrant its use, especially if weaker organisms might be encountered in field work. (Figure 2). It was thought that 1-400T was not concentrated enough, due to the similarities between it, the 1-800T concentration and the control. This was later borne out by other work (Figure 4). A 1-300T ethyl violet concentration was finally chosen since it lay midway between what were considered the extremes.

The medium was sterilized after the addition of the dye. After the autoclaved medium had stood for a few hours, a barely perceptible precipitate was noticed. An experiment was then set up to determine the effect of precipitation of the dye on the growth curves of E. coli. This test, incidentally, showed the toxicity curve from 1-100T to 1-400T and with interpolation, to 1-800T. The test consisted of making up media, as in the previous growth curve determinations, and dividing the flasks into two groups. One group had the appropriate dye concentrations added while the others were left alone; both groups were autoclaved simultaneously. Dye was added to the second group of flasks just prior to use. An examination of Figure 4 indicates that autoclaving does have some effect on the growth curve, and, while relatively slight, it is consistent.

Figure 4

Effect of autoclaving ethyl violet tryptose broth medium.*



Line AB - Difference in population by adding dye before autoclaving (A) and after (B).

Point C - Point at which population of medium adding dye before autoclaving is the same as point B in medium where dye is added after autoclaving.

Line CD - Concentration of dye-medium which, when autoclaved, gives approximately the same results as 1-300T dye added after autoclaving the medium.

(_ _ _) - Portion of the curve extrapolated from Table II.

*Typical curves of at least 3 individual runs.

Referring to Figure 4, points A and B on the graph show the differences in population when the dye was added prior to autoclaving and after autoclaving, respectively. Point C, therefore, represented the concentration of the dye added prior to autoclaving which gave the same results, in terms of population, as 1-300T, added after autoclaving. A line drawn to the abscissa gives approximately 1-250T as the concentration yielding point C. This graph also shows the relative toxicities of the various concentrations. The ethyl violet became slowly more toxic from 1-800T up to 1-400T, edged downward slightly faster to 1-300T and then suddenly became very rapidly toxic down to 1-100T. It is assumed that this curve approaches the vertical at higher concentrations. These population figures were taken at six hours and the curve would probably be flatter at shorter time intervals (with the possible exception of 4 hours). The extreme end of the curve, from 1-400T to 1-800T was interpolated from Table II. It can be seen that 1-300T lies near the top of the toxicity curve and may be a good choice.

Tryptose lactose broth with a concentration of ethyl violet prior to autoclaving of 1-250T, was finally chosen as the medium to be used in the field tests.

Comparison with brilliant green lactose bile (BGB). Preliminary studies indicated that all presumptive tubes showing gas in 24 hours would confirm in the 1-250T ethyl violet tryptose broth (EV) and were invariably coliforms, so it was decided to limit this portion of the study only to tubes inoculated from the same presumptive tube which did not agree on the presence of gas. The two parallel tubes of confirmatory

media were selected and attempts at recovering coliforms were made on both.

"Standard Methods for the Examination of Water and Sewage" require that all presumptive tubes showing gas in 24 or 48 hours to be inoculated into a confirmatory medium, such as BGB. Tubes of BGB showing gas in 24 to 48 hours are then streaked on eosin methylene blue agar (EMB) plates and incubated for 24 hours. Typical and/or atypical coliform colonies are picked and inoculated into lactose broth and then streaked on an agar slant. Gram stains are made from the agar slant at 24 hours and if gas appears in the lactose tube in 24 or 48 hours these preparations are examined for the presence of spores or spore-forming bacilli. If the latter are present, they are inoculated into formate ricinoleate broth and if gas appears, the procedure is repeated beginning with EMB. In this particular study spore-formers were eliminated by the dilution plate method rather than using formate ricinoleate broth.

This portion of the study was carried out on waters sent to the Michigan Department of Health from many parts of the state. As reflected in Table IV, over 80 percent of the samples were derived from wells, the rest coming from springs, city distribution systems, a lake and some from unknown sources. A total of 140 samples was studied during the months of June, July, and August. Each sample was prepared by placing 10 ml into each of 5 tubes of double strength lactose broth and 1 ml into one tube of single strength lactose broth for a total of six tubes per sample.

Of the total number of tubes in the samples 67.7 percent showed gas production, (Table V), or, were presumptive positive.

TABLE IV

Source and number of samples.

Source	Number
Wells	117
Springs	2
Municipal distribution systems	10
Lakes	1
Unknown	10
Total	140

TABLE V

The comparative confirmation of positive presumptive tubes by the use of brilliant green bile broth and ethyl violet tryptose broth

Medium	No. of tubes	Percent
Positive presumptives in lactose broth	569	67.7
Confirmed in BGB	474	83.3
Confirmed in EV	486	85.4
Total no. of tubes in 140 samples	840	

A study of Table VI indicates that EV is slightly less toxic and allows the production of gas faster than the BGB medium, since 68.2 percent of the presumptive tubes confirmed in 24 hours, while 62.2 confirmed in BGB in 24 hours. This represents an improvement of only six percent, but this increases slightly when broken down by times of presumptive tubes showing gas. Here, 63.4 percent of the positive presumptive tubes studied showed gas in 48 hours and of these 51.1 percent confirmed in EV in 24 hours; an increase of 7.5 percent over BGB. Of the presumptive tubes showing gas in 24 hours the two confirmatory media were more nearly alike indicating that the EV medium was less toxic to the less hardy organisms encountered in the 24 - 48 hour period than was BGB, while the hardier organisms encountered in the first 24 hours were about similarly affected by both media. The 48 hour group most likely contains the attenuated coliforms, coliforms whose lactose fermenting power has been lost or otherwise affected and the slow lactose fermenting spore-forming bacilli. As seen in Table VIII the 48 hour group was of predominate importance in the differences of the two media. Before leaving this topic it might be noted that gas in the 24 hour EV medium appeared in larger quantities at this time indicating that it might also appear earlier. In the 48 hour group the amount of gas was approximately the same.

Turning to the differences between EV and BGB in terms of samples and Most Probable Numbers (MPN), Table VII shows some interesting results. Of the samples studied 33 showed differences of MPN and of these, 21 gave EV a higher MPN than BGB while 12 gave lower MPN's. Further

TABLE VI

Time of confirmation related to time of becoming presumptive positive among all samples.

Time Confirmed in	Transferred to BGB from: Presumptive positive in 24 hrs.	Per-cent	Presumptive positive in 48 hrs.	Per-cent	Total	Per-cent	Transferred to EV from: Pres. pos. 24 hrs	Per-cent	Pres. pos. 48 hrs	Per-cent	Total	Per-cent
24 hrs.	194	93.4	160	44.3	354	62.2	201	96.7	187	51.8	388	68.2
48 hrs.	7	3.3	113	31.3	120	21.1	4	1.9	94	26.0	98	17.2
Not Confirmed	7	3.3	88	24.4	95	16.7	3	1.4	80	22.2	83	14.6
Totals	208	36.6	361	63.4	569		208	36.6	361	63.4	569	

TABLE VII

Distribution of most probable number (MPN) divergency among the samples

MPN		Number of Samples	Percent	Number of Samples that: Differ by 1 Tube	Differ by 2 Tubes	Differ by 3 Tubes	Differ by 4 Tubes or More	Total No. of Samples
*BGB *EV	Agree	107	76.5					
BGB EV	Disagree	33	23.5					
	EV High	21	63.6	17	3	1	0	21
	EV Low	12	36.4	11	0	1	0	12
Sub-totals		33		28	3	2	0	33
Total		140						

*BGB = brilliant green bile broth.
EV = ethyl violet (1-250,000) tryptose broth.

TABLE VIII

Time distribution of confirmations among the samples not agreeing in MPN.

Classif. of conf. tubes	From 24 hr. Pres.			From 48 hr. Pres.			Total conf. 24 hrs.	Total conf. 48 hrs.	Total	Percent	# of samples occurs in
	conf. 24 hrs.	conf. 48 hrs.	Total	conf. 24 hrs.	conf. 48 hrs.	Total					
# tubes conf. in BGB and not EV	0	0	0	3	11	14	3	11	14	12.1	12
# tubes conf. in EV and not BGB	2	2	4	5	17	22	7	19	26	22.4	21
Tubes conf. in both BGB	10	2	12	23	30	53	33	32	65	56.0	25
EV	11	1	12	21	32	53	32	33			
							33	32			
Totals	23	5	16	52	90	89	43	62	105		33
Percent			15.2			84.8	40.8	59.2			
# tubes conf. in neither									11		

breakdown shows that 28 of these samples differed by only one tube, 3 by two tubes and 2 by three tubes. Table XII adds a little more to this by indicating that these differences are fairly evenly distributed over the range of the number of positive tubes occurring in any one sample. In other words the occurrence of different MPN's was apparently not exclusively dependent on the number of coliforms present in the water. Some importance might be attached to the fact that seven samples showed EV with 2 tubes positive and BGB with only 1 tube positive, however, the rest were farily evenly distributed.

These 33 samples were analysed for time of confirmation. From the 24-hour positive presumptives no tubes confirmed in BGB that did not also confirm in EV, (Table VIII). The reverse, however, is not true, since a total of 4 tubes confirmed in EV which did not confirm in the corresponding BGB tube. A total of 12 tubes confirmed in both media from this group and a total of 11 tubes did not confirm at all. The importance of the 48-hour presumptive group is here emphasized; since 14 tubes confirmed in BGB and not in EV from this group while 22 confirmed in Ev and not BGB and 53 confirmed in both media. This gives a total of 16 tubes from the 24-hour positive presumptives and 89 from the 48-hour group. The major differences between the two media, then, are encountered with organisms that are slow lactose fermenters which may include attenuated coliforms and some aerobic and anaerobic spore-forming bacilli. Table IX is a condensation of the above; and from the 33 non-agreeing samples 40 tubes (in parallel) were studied with the intention of isolating coliforms from the media.

TABLE IX

Distribution of tube differences
among the non-agreeing samples

Parallel tubes in which:	No. of tubes showing difference in presence of gas	No. of Samples Represented
*EV + and BGB -	26	21
*EV - and BGB +	14	12
Totals	40	33

*Note: only one classification occurred in any given sample.

It is here noted that attempting to isolate coliforms from the presumptive medium by several different means would probably give a truer picture of the coliform population, however, this study has been confined to isolation according to the "Standard Methods" completed test and only from parallel tubes showing differences in gas production.

Before discussing the tabulated results of this test it might be of interest to note a few observations made on the cultures isolated during the test. Of the 33 samples tested only one gave typical colonies of E. coli and none of A. aerogenes on EMB. The remainder gave only atypical colonies. These were slow to develop being about one millimeter in diameter in 24 hours. After removing portions of colonies for the rest of the completed test, the plates were re-incubated up to 72 hours. Two samples gave atypical colonies at 24 hours which slowly developed sheens to become typical E. coli colonies by 48 hours. Further study indicated that these were probably coliforms which had lost some of the power to ferment lactose and had become slow lactose fermenters. Another sample yielded a large colony with a typical sheen at 48 hours (no sheen at 24 hrs.) and gave gas in lactose in 48 hours but when isolated in pure culture was a large gacillus probably of the B. aerosporus group. No further attempts at identification were made. IMViC reactions were determined on 6 to 8 atypical coliform cultures isolated from the samples and the predominate grouping seemed to be - + - +. However, no attempt was made to classify all of the cultures isolated. When the amount of gas in the inserts was compared it was found that EV produced up to two times as much gas, if from a 24 hour

presumptive, than BGB, although from the 48 hour presumptives the amounts were very similar.

An examination of Tables X and XI shows the results of the attempt to isolate coliforms from the parallel tubes of confirmatory media showing different results. In this classification 26 tubes gave gas in EV and none in BGB while 14 tubes gave gas in BGB and none in EV. The completed test showed that coliforms were present in 14 out of the 26 parallel tubes, in EV as well as the negative BGB tubes. Two sets of parallel tubes gave inconclusive results, either due to errors in technique or some failure in the isolation. The rest failed to yield any coliforms. The first group can be considered BGB false negatives occurring as a result of the toxicity of the medium or failure to supply an adequate growth medium. The last group can be termed EV false positives and may be due to one or several factors, such as, insufficient initial toxicity or after a large population of gram negative organisms has removed sufficient dye to allow them to grow.

The end result here, even though 10 EV false positives have occurred, is a net increase of 4 tubes of coliforms since 14 tubes have been uncovered by the Ev that would otherwise have been recorded as negative.

The next category contains those tubes which gave no gas in Ev but did in BGB. Of the 14 sets of parallel tubes studied only 3 yielded coliforms in both tubes of the set. These can be considered EV false negatives. This is a big increase in recovery of organisms over the same category with BGB, and is a strong indication that the EV medium is less toxic to those weaker organisms. Ten sets of parallel tubes did not yield any coliforms and these can be considered BGB false

TABLE X

Results of completed test on samples showing difference in MPN from parallel tubes.

No. of Tubes of EV + and BGB -	*completed from:	No. of Tubes (in parallel)
(BGB false negative)	Both BGB and EV	14
	BGB only	1
	EV only	1
(EV false positive)	neither	10
	Total	26
No. of Tubes of Ev - and BGB +	***completed from:**	
(EV false negative)	Both BGB and EV	3
	BGB only	1
	EV only	0
(BGB false positive)	neither	10
	Total	14
	Grand Total	40

*Note: completed means coliforms isolated.

TABLE XI

Occurrence of false tests as determined from Table X

	BGB	EV	Totals
False +	10	10	20
False -	14	3	17
Totals	24	13	37

positives. This is the same number as that found with the similar group with EV.

If the ethyl violet medium were less toxic and supplied a richer medium for the growth of attenuated coliforms, then it would be expected to recover more of these organisms than BGB. Table XI shows this to be the case. A richer medium, on the other hand, would tend to allow the growth of interferers, especially the slow lactose fermenting bacilli which form spores. Judging from Table XI it seems that, although the medium containing the ethyl violet dye was more nutritive, it allowed no more false positives among this group than the BGB. Furthermore it is suspected that the growth of these organisms causing the false tests in EV may be due to the removal of a portion of the dye by the much faster growing gram negative organisms normally present in water. The EV medium then, gave only 13 false tests to 24 for the BGB. This is a reduction of 54 percent for the sets of parallel tubes studied.

Table XII presents the number of tubes showing gas in each sample and the medium which gave the correct results as determined by the completed test. The ethyl violet was correct in 19 samples and BGB in 11. No pattern seems apparent as to the relationship of the number of tubes positive in a sample with the MPN, or whether EV gives a high or low MPN.

TABLE XII

Number of positive confirmatory tubes in each medium, by sample, and the medium showing the correct results by the completed test.

No. of Samples	No. tubes positive		Medium correct by the completed test		
	EV	BGB	EV	BGB	?
4	6	5	3	1	
1	6	4	1		
1	5	4		1	
2	4	3	2		
1	3	2	1		
1	3	0	1		
7	2	1	2	4	1
2	2	0		2	
2	1	0	1		1
3	0	1	3		
3	1	2	2	1	
1	2	3	1		
1	2	4	1		
2	3	4	1	1	
1	4	5			1
1	5	6		1	
33			19	11	3

SUMMARY AND CONCLUSIONS

Ethyl violet dye at a concentration of 1-50,000 in lauryl tryptose broth showed the least interference with the lag and log phases of *E. coli*, actually stimulating the organism, but could not be used due to excessive precipitation upon autoclaving or on standing.

Studies with ethyl violet in tryptose broth indicated that autoclaving has some effect on the activity of the dye and should be considered if further studies are made.

The medium decided upon for comparison with brilliant green bile is as follows:

Tryptose	2.0%
Lactose	0.5%
K_2HPO_4	0.4%
KH_2PO_4	0.15%
NaCl	0.5%
Ethyl Violet	1-250,000 final concentration added before sterilizing
pH	6.8 before sterilizing

Comparison with BGB indicated that the ethyl violet medium was less toxic to the weaker coliforms and equally toxic to the interferers. It also allowed faster development of gas in the fermentation tubes allowing them to confirm earlier.

It is therefore concluded that the 1-250T ethyl violet tryptose broth shows great promise in the field of coliform confirmatory media

particularly due to its reduced toxicity to the weaker organisms. It is suggested however, that further studies be undertaken to overcome the false positive tests encountered. One method of attack might follow an observation made by Shane, that the number of false positives in BGB was greatly reduced by using lauryl sulfate broth as a presumptive medium instead of lactose broth.

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