STUDIES ON THE EFFECT OF ETHYL PURPLE ON CERTAIN BACTERIA

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Much of our knowledge concerning differential and selective media for the isolation and identification of organisms, particularly those of the colon-typhoid-paratyphoid group, is based on the discovery by Churchman (1), in 1912, of the selective action of gentian violet on Gram positive organisms. This action on the Gram positive organisms was termed bacteriostasis. Churchman (2) further noted that when bacteria were exposed to gentian violet or related dyes, there were four phases of bacterial inhibition, namely; cessation of motility, inhibition of reproduction, suspension of animation, and inhibition of sporulation. Anilin dyes show these four types of inhibition without killing the bacteria although there is no doubt that some dyes actually kill some bacteria.

Several theories have been suggested to explain the selective bacteriostatic action of dyes. It is agreed that both chemical and physical factors are involved in the action of dyes but the exact mechanisms of these factors are not known. Changes in physical environment greatly influence the inhibitory action of dyes as shown by Churchman (3), in 1926, in his studies on the influence of age in relation to bacterial resistance to dye. He proved that older cultures are much less resistant to gentian violet than young ones. McCalla and Clark, in 1941, (4) demonstrated that at pH values higher than the iso-electric point, basic dyes are absorbed while at lower values acid dyes are absorbed. As the bacterial systems were made more acid, the hydrogen ion adsorption increased and the ability of the bacterial cell to take up crystal violet decreased. Ingraham (5) demonstrated by a series of experiments that the action of gentian violet was due to a

certain degree on the oxidation-reduction potential. The effectiveness of the dye depended on the initial oxidation-reduction potential. The dye is toxic only during the lag phase when the cells are adjusting the oxidation-reduction potential. She also showed that agents such as serum, bile, or dead cells which favor a reduction of potential are known to counteract the action of dyes. Fisher (6) pointed out in 1944 that the bacteriostatic action of triphenylmethane dyes may be connected with the respiratory metabolism of the micro-organism.

The paper is primarily devoted to the use of the various cultural media containing dyes for the enteric colon group of organisms. As early as 1902 Conradi and Drigalski (7) suggested the use of malachite green for inhibitory purposes. In 1913, Browning, Gilmore, and Mackie (8) confirmed Loeffler's work with brilliant green in which it was found that Escherichia coli was inhibited to a greater extent than Eberthella typhosa. Endo (9), in 1903, employed basic fuchsin, decolorized with sodium sulfite, for the isolation of various colon organisms. In 1926, Wilson and Blair (10) evolved a medium for the isolation of E. typhosa containing glucose, bismuth and sodium sulfite. Various modifications of this medium have been suggested in order to obtain better results. In 1936, Jones (11) suggested the use of brilliant green eosin lactose agar at concentrations that would inhibit E. coli but would allow the growth of E. typhosa. Eosin methylene blue agar for differentiating the colon from the typhoid-dysentary group of bacteria was proposed in 1916 by Holt-Harris and Teaque (12). Levine (13), in 1918, suggested a simplified medium using this same principle. S and S, a relatively new medium, has been developed by Difco laboratories in Detroit, Michigan.

The constant search for new types of media is the result of the many shortcomings of the present formulations. Some of these media are based on the principle that sufficient concentration of certain dyes be used to reduce the growth of the non-pathogenic gram negative organisms found in fecal material. However in the proper concentration the dye has a slight inhibitory effect on the pathogens so that when few organisms are present as in carrier cases growth fails thus giving false negatives. Other media allow the growth of such organisms as the E. coli and Aerobacter aerogenes as well as the pathogens but use lactose and acid indicators to differentiate the organisms. False negative results occur under these conditions also. The use of enrichment media before the differential and selective medium has enabled the workers to detect the pathogens when they occur in small numbers, but such a procedure has many disadvantages, such as a long period of time before results are obtained, and increased cost due to labor and materials.

During this period of experimentation with media for enteric pathogens, bacteriologists were also experimenting with media for growth of E. coli and A. aerogenes to determine the sanitary condition of water. The first medium to be used for the quantitative test for E. coli in water was glucose broth suggested by Theobald Smith in 1893 (14). In 1912 lactose bile broth became standard medium for the presumptive test. The use of lactose broth for the presumptive test was recommended in 1917 by the Standard Methods Committee for the examination of water and sewage. Hall and Ellelson (15), in 1919, suggested the addition of gentian violet to lactose broth for water examination. Brilliant green lactose medium was suggested, in 1920, by Muer and Harris (16). Jordan (17) as well as others have tested a

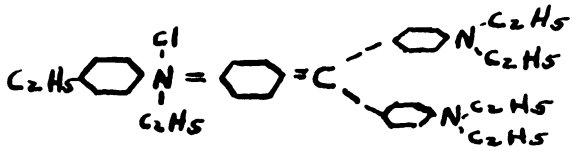
variety of media containing brilliant green. Horwood and Heifertz (18), Ruchhoft (19), Mallmann and Darby (20), Stark and England (21), and Ruchhoft and Norton (22) and others have found that presumptive media containing brilliant green and crystal violet are too toxic to the colon-aerogenes group thus giving a number of false negative results. Black and Klinger in 1936, (23) tested several media by the Butterfield (24) dilution method, the results of which were based on gas production. They found the media, ranked in order of decreasing sensitivity were, standard lactose broth, buffered lactose broth, fuchsin broth, methylene blue brom cresol purple broth, brilliant green bile broth, crystal violet broth and formate ricinoleate broth. It was found that brilliant green lactose bile broth equaled standard lactose broth in sensitivity with A. aerogenes and intermediate strains but not with E. coli.

Brilliant green lactose bile broth, Endo's medium, eosin methylene blue agar have been used for the partial confirmatory test. McCrady (25) has shown that many errors occur with use of E.M.B. and Endo's media. Mallmann and Darby (26) showed that some organisms placed in brilliant green lactose bile broth or streaked on E.M.B. agar caused the organisms to lose their ability to ferment lactose or inhibited these organisms from growing.

It is evident that the dyes now in use are somewhat toxic to gram negative bacteria. Although most of the enteric organisms do grow in the presence of these dyes, it is questionable upon initial isolation from fecal material and sewage contaminated water supplies whether all of the viable cells of these organisms grow.

Several years ago in this laboratory Darby (27) in a study of dyes found that ethyl purple showed less toxicity to some Gram negative organisms than did such dyes as brilliant green and crystal

violet. These studies were never completed. The limited data gathered at that time indicated that further studies might be fruitful. Accordingly the study was initiated to determine more completely the value of this dye as a bacteriostatic agent for the gram positive bacteria. The literature is void of any research concerning the use of ethyl purple in medium work. It has been used, however, as a stain for spirochetes, islets of Langerhans, and nervous tissue. The structural formula of ethyl purple 6B is:



PROCEDURE:

The work consisted of showing the effect of ethyl purple on a representative group of gram negative and gram positive organisms. Growth curve studies were carried out on the gram negative organisms. Ethyl purple was compared to brilliant green, crystal violet, and lauryl tryptose broth. The organism on which the growth curve studies were made were 24 hour agar cultures of E. coli, E. typhosa and Salmonella typhimurium.

In the first experiment in which lauryl tryptose broth and lactose broth were compared it was shown that the lag phase was not as long with lauryl tryptose broth. Since LTB was also shown to be superior to lactose broth in testing water samples by Darby and Mallmann (28) it was used as the control and base medium. In the experiments 100 ml. samples of the LTB were prepared to which was added just prior to inoculation varied concentrations of the dye. These

samples were then seeded with E. coli, S. typhimurium and E. typhosa so as to give approximately 0-30 organisms per ml. Duplicate plate counts were made on the samples at the time of seeding and at 2, 4, 6, and 24 hours. These plates were counted after 24 hours incubation. Two trials were made in each case. The results are shown in The Appendix. Each graph represents one trial.

The second phase of the experimental work was to determine the effect of ethyl purple on a group of gram positive organisms. Bacteriostatic studies were done on Micrococcus caseolyticus, Staphylococcus and Bacillus subtilis. The base medium was tryptose broth to which was added brilliant green, crystal violet or ethyl purple to obtain concentrations of 1-1 million, 1-2.5 million, 1-5 million, 1-8 million, 1-10 million and 1-25 or 50 million. One-tenth ml. and 1/10 ml. of 1-10, 1-100, 1-1,000, 1-10,000 and 1-100,000 dilution of a suspension of organisms from a 24 hour agar slant were planted into 5 tubes of each of the various concentrations of the dye. Plate counts were made on the .1 ml. amount to determine the approximate number of organisms inoculated in the tubes. The tubes were incubated and read at 24 and 48 hours for turbidity.

RESULTS:

I. Growth curve studies of S. typhimurium

Tables II, III, and IV show the growth curves of S. typhimurium in media containing brilliant green, crystal violet and ethyl purple respectively. These data show that with brilliant green and crystal violet there is marked inhibition at a dilution of 1-1 million where as the break appeared at 1-333 thousand concentration of ethyl purple. The reduction in growth rate when ethyl purple was at a concentration

of 1-125 thousand was similar to that of brilliant green at a level of 1-1 million. Graph I shows how closely the growth rate of S. typhimurium in the medium containing a 1-1 million concentration of ethyl purple compared to growth rate of the organism in the control medium. Graphs I and II demonstrate that the growth rate of S. typhimurium was much slower in media containing brilliant green or crystal violet. It can be concluded from the data that ethyl purple is not as toxic to this organism as brilliant green or crystal violet.

II. Growth curve studies of E. typhosa

The results of the growth curve experiment on E. typhosa can be found in table V, VI, and VII. Brilliant green at a concentration of 1-1 million was considerably more toxic than ethyl purple was at a concentration of 1-333 thousand. Ethyl purple proved to be less toxic at a concentration of 1-333 thousand than did crystal violet at a concentration of 1-500 thousand. The crystal violet at this concentration was so toxic, in fact, that in 24 hours the counts were not anywhere near as great as their control. Graphs III and IV show the difference in these curves. Therefore ethyl purple was obviously less toxic to E. typhosa than was brilliant green or crystal violet.

III. Growth curve studies of E. coli

The data on the effect of brilliant green, crystal violet, and ethyl purple on E. coli are presented in tables VIII, IX, and X. Brilliant green was more inhibitive to E. coli at a concentration of 1-1 million than was ethyl purple at a concentration of 1-125 thousand. At a concentration of 1-333 thousand ethyl purple was less toxic to the organism than was crystal violet at a level of 1-500 thousand. Graphs V and VI show that brilliant green or crystal violet caused slower growth of the organism than did ethyl purple.

IV. Bacteriostatic studies on Bacillus subtilis

Tables XI, XII, and XIII show the bacteriostatic effect of brilliant green, crystal violet and ethyl purple on B. subtilis. A study of these tables show that the organism failed to grow with any of the dyes at a concentration of 1-1 million. In a concentration of 1-2.5 million there was no growth with crystal violet but one positive tube with brilliant green and 2 positive tubes with ethyl purple. At a concentration of 1-5 million a high percentage of the tubes were positive with all dyes. Thus these data indicate that the bacteriostatic effect of these dyes were not significantly different. It might be noted that the initial inoculation in the different media was 45,000 organisms.

V. Bacteriostatic studies on Micrococcus caseolyticus

The bacteriostatic effect of brilliant green, crystal violet, and ethyl purple on M. caseolyticus may be found in tables XIV, XV and XVI. Crystal violet had the greatest bacteriostatic effect on this organism since there was no growth even in the highest dilution of 1-50 million. Ethyl purple was one hundred percent bacteriostatic up to and including a concentration of 1-5 million. The brilliant green, however was not as effective since one tube showed growth in as high a concentration of brilliant green as 1-1 million. At 1-5 million of brilliant green there was a high percentage of positive tubes. It is interesting to note that although there was a heavier inoculant of organisms than with B. subtilis ethyl purple and crystal violet were more bacteriostatic against M. caseolytic than with B. subtilis. Ethyl purple, therefore, showed excellent bacteriostatic properties against this organism.

VI. Bacteriostatic studies on Staphylococcus aureus

The results of the studies made on this Gram positive organism may be found in tables XVII, XVIII and XIX. In a 1-1 million concentration of the dyes there was no growth in any of the tubes. There was scattered growth in the 1-2.5 million concentration. Ethyl purple was slightly less toxic to the S. aureus than was brilliant green and crystal violet. However, ethyl purple demonstrated good bacteriostatic properties against this organism.

DISCUSSION:

The usual methods of measuring dye toxicity is by streaking agar plates and noting the total growth. By comparing the amount of growth the degree of toxicity can be determined. This procedure is adequate when the difference in toxicity of various substances are great. In these studies it was believed that the difference in toxicity would be too small to measure by this method. Since the difference although small might be significant the method of using an initial minimum inoculant and following growth curves over the early growth phase was used. This method gives a more accurate determination of the toxicity. Furthermore in the isolation of bacteria where growth must start from minimal numbers, and frequently from single cells, the effect of dye would be expected on the first generation of cells. If the dye were too toxic these few cells may not grow, therefore there would be no isolation obtained. This technique is fully justified by the results obtained. For example most of the growth curves show that there was much variation in the rate of growth up to and including the 6 hour seeding. However by the 24 hour period the organisms had adjusted themselves to the dye so that most of the population levels were similar. Thus

if the agar streak method had been used, the growth at 24 hours would have indicated no significant difference between the dyes.

The technique of using growth curves for measuring toxicity of dyes offers a new method for evaluating dyes and other inhibitory agents. Darby and Mallmann (29) used this technique successfully in the development of tryptose lactose broth for the detection of colon organisms.

Ethyl purple with the three test organisms, namely; E. typhosa, S. typhimurium, and E. coli, was found to offer less toxicity than did brilliant green and crystal violet as shown by the reduction in growth rates. On the other hand the bacteriostatic titers against three Gram positive organisms, namely; B. subtilis, M. caseolyticus, and S. aureus, were not significantly different for the crystal violet, brilliant green or ethyl purple broths.

It would appear that ethyl purple is an effective bacteriostat against gram positive organisms and is comparable to brilliant green and crystal violet. Since the dye could be used as a bacteriostatic agent against gram positive organisms and at the same time could be used in a dilution that would be less inhibitive to gram negative organisms than brilliant green or crystal violet, it could be used successfully in media work. The fact that ethyl purple with the test organism, S. typhimurium at a 1-1 million dilution allowed a growth rate during the first six hours equal to that of the lauryl tryptose broth control further indicates its usefulness. This is true especially since ethyl purple was one hundred per cent bacteriostatic at this concentration.

It has been demonstrated that the substitution of methyl groups in a hexa methyl tri amino tri phenol methane dye by ethyl groups causes a decreased toxicity toward Gram negative organisms but little or no change in toxicity toward Gram positive organisms.

It is also interesting to note that in brilliant green which contains four ethyl groups that a greater toxicity toward gram negative organisms is exhibited. No explanation for these findings are offered at this time.

SUMMARY:

It was shown that ethyl purple is less toxic to Gram negative organisms than is crystal violet or brilliant green. This difference was demonstrated by determining growth curves on S. typhimurium, E. typhosa, and E. coli. The experiments further indicate that at concentrations that have little or no toxicity to these Gram negative organisms, ethyl purple is bacteriostatic to such Gram positive organisms as B. subtilis, M. caseolyticus and S. aureus. It is suggested that further work should be done with media containing ethyl purple for water analysis and enteric isolations.

REFERENCES CITED:

1. Churchman, John W. The Selective Action of Gentian Violet on Closely Related Bacterial Strains. Jour. Exptl. Med. 16: 221-247. 1912.

2. Churchman, John W. Inhibitory Properties of Anilin Dyes. Stain Technol. 1(1): 27-32. 1926.

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

4. McCalla, T. M. and Francis E. Clark. Dye Adsorption by Bacteria at Varying Hydrogen Ion Concentrations. Stain Technol. 16(3): 95-100. 1941.

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

6. Fisher, E., O. Hoffman, E. Prado and R. Bone. On the Mechanism of Bacteriostasis with Triphenylmethane Dyes. Jour. Bact. 48: 439-466. 1944.

7. Conradi, H. and V. Drigalski. Ueber eim Verfahren zum Nachweir der Typhusbacillen. Zeitschrift fur Hygiene. 39: 283-300. 1902.

8. Browning, Gilmour and Mackie. The Isolation of Typhoid Bacilli from Feces by Means of Brilliant Green in Fluid Medium. Jour. Hyg. 13: 335-342. 1913.

9. Endo, S. Ueber ein Verfahren zum Nachweis der Typhus bacillen. Centralbl. f. Bakteriol. Originale. 35: 109-110. 1904.

10. Wilson, W. J. and M. Blair. Sodium Sulphite Affording an Enrichment and Selective Medium for the Typhoid-Paratyphoid Groups of Bacteria. Jour. Path. and Bact. 29: 310. 1926.

11. Jones, E. R. The Use of Brilliant Green Eosin Agar and Sodium Tetrathionate Broth for the Isolation of Organisms of the Typhoid Group. Jour. Path. and Bact. 42(2): 455-467. 1936.

12. Holt-Harris, J. E. and O. Teague. A New Culture Medium for the Isolation of Bacillus Typhus from Stools. Jour. Infect. Dis. 18: 596-600. 1916.

13. Levine, Max. Differentiation of B. coli and B. aerogenes on a Simplified Eosin Methylene Blue Agar Jour. Infect. Dis. 23: 43-47. 1918.

14. Smith, Theobald. Ann. Rept. State Bd. of Health of N. Y. 13: 712. 1893.

15. Hall and Ellefson. Further studies on Gentian Violet as a Means of Eliminating Spurious Presumptive Tests for B. coli in Water. Jour. Amer. Water Works Assoc. 6: 67. 1919.

16. Muer, T. C. and R. L. Harris. Value of Brilliant Green in Eliminating Errors Due to the Anerobes in the Presumptive Test for B. coli. Amer. Jour. Publ. Health. 1-: 874. 1920.

17. Jordan, Harry E. Brilliant Green Bile as the Detection of the Colon-Aerogenes Group. Jour. Amer. Water Works Assoc. 18: 337. 1927.

18. Horwood, M. P. and A. Heifertz. A Comparative Study of Certain Presumptive Test Media. Jour. Bact. 27: 57-58. 1934.

19. Ruchhoft, C. C. Comparative Studies of Standard Methods and the Brilliant Green Bile Medium on Lake Michigan Water at Chicago. Jour. Amer. Water Works Assoc. 16: 778. 1926.

20. Mallmann, W. L. and C. W. Darby. The Use of Sodium Lauryl Sulfate Lactose Tryptose Broth as a Primary Medium for Detection of the Coliform Group. Amer. Jour. Publ. Health. 31: 127-134.

21. Stark, C. N. and C. W. England. A Note on the Use of Crystal Violet in Presumptive Tests for Water Pollution. Jour. Bact. 23: 36. 1932.

22. Ruchhoft, C. C. and John E. Norton. Study of Selective Media for Coli-aerogenes Isolation. Jour. Amer. Water Works Assoc. 27: 1134. 1935.

23. Black, Luther A. and Mary E. Klinger. A Comparison of Media for the Detection of Escherichia-Aerobactor. Jour. Bact. 31(2): 171-179. 1936.

24. Butterfield, C. T. Experimental Studies of Natural Purification in Polluted Waters VII. The Selection of a Dilution Water for Bacteriological Examinations. Publ. Health Repts. 48: 681-691. 1933.

25. McGrady, M. H. A Practical Study of Procedures for the Detection of the Presence of Coliform Organism in Water. Amer. Jour. Publ. Health. 27: 1143-1258. 1937.

26. Mallmann, W. L. and C. W. Darby. Uses of Lauryl Sulphate Lactose Broth for the Detection of Coliform Organisms. Amer. Jour. Publ. Health. 31: 127-134.

27. Darby, C. W. Studies on Primary and Selective Media for Coliform Organisms. Thesis for Master of Science Degree. Michigan State College. 1943.

28. Darby, C. W. and W. L. Mallmann. Studies on Media for Coliform Organisms. Jour. Amer. Water Works Assoc. 31(4): 689-706. 1939.

29. Mallmann, W. L. and C. W. Darby. Uses of Lauryl Sulphate Lactose Broth for the Detection of Coliform Organisms. Amer. Jour. Publ. Health. 31: 127-134.

Table I - A Comparison of Growth Rates for Lactose Broth and Lauryl Tryptose Broth using Escherichia coli as a Test Organism.

Hours Incub.	Comparison of Lactose Broth to Lauryl Tryptose Broth.	
	Lactose Broth	Lauryl Tryptose Broth
	.011*	.009
0	.019	.045
2	.65	.95
4	7.3	49.00
6	300,000	600,000
24		

* Bacteria per ml. in thousands.

Table II - Growth Rates of Salmonella typhimurium in a Medium Containing Brilliant Green in Varying Concentrations.

Hours Incub.	Control	Dye Dilution							
		1-5m		1-1.6m		1-1m		1-625t	
		Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
0	.018	.02	.019	.014	.016	.016	.014	.018	.017
2	.013	.017	.016	.012	.014	.016	.017	.012	.008
4	.225	.16	.2	.16	.06	.019	.026	.014	.014
6	7.0	8.0	8.0	7.0	2.0	.022	.026	.017	.014
24	95,000	150,000	170,000	160,000	200,000	190,000	124,000	160	480

* Bacteria per ml. in thousands.

Table III - Growth Rates of Salmonella typhimurium in a Medium Containing Crystal Violet in Varying Concentrations.

Hours Incub.	Control	Dye Dilution							
		1-5m		1-1.6m		1-1m		1-625t	
		Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
0	.024	.028	.035	.03	.021	.023	.026	.023	.029
2	.050	.044	.03	.034	.03	.028	.029	.026	.026
4	.8	.65	.75	.425	.5	.4	.375	.25	.2
6	22.5	25.0	27.5	15.0	17.5	10.	10.	7.5	7.5
24	60,000	190,000	160,000	155,000	175,000	135,000	160,000	145,000	180,000

* Bacteria per ml. in thousands.

Table IV - Growth Rates of Salmonella typhimurium in a Medium Containing Ethyl Purple in Varying Concentrations.

Hours Incub.	Control	Dye Dilution							
		1-1m		1-333t		1-200t		1-125t	
		Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
0	.02*	.021	.015	.025	.020	.016	.016	.018	.019
2	.016	.022	.019	.019	.014	.016	.014	.02	.013
4	.37	.290	.33	.1	.09	.075	.055	.03	.02
6	3.1	2.0	2.8	.315	.310	.23	.14	.05	.06
24	160,000	200,000	190,000	150,000	140,000	140,000	65,000	5,000	45,000

* Bacteria per ml. in thousands.

Table X - Growth Rates of Escherichia coli in a Medium

Containing Ethyl Purple in Varying Concentrations

Hours Incub.	Control	Dye Dilution							
		1-333t		1-200t		1-125t		1-100t	
		Trial I	Trial II	Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
0	.012	.006	.012	.005	.008	.008	.008	.01	.006
2	.025	.018	.02	.01	.008	.006	.004	.006	.003
4	.4	.225	.175	.075	.035	.02	.015	.01	.009
6	20	3	3	.4	.3	.2	.2	0	0
24	1,000,000	1,000,000	1,000,000	1,000,000	1,250,000	500,00	750,000	80,000	120,000

* Bacteria per ml. in thousands.

Table XI - Bacteriostatic Action of Various Concentrations of Brilliant Green on Bacillus Subtilis

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	2	0	0	0	0	0
5m	24	5	0	0	0	0	0
	48	5	1	2	1	0	0
8.3m	24	5	5	5	4	0	0
	48	5	5	5	5	4	2
10m	24	5	5	4	4	1	0
	48	5	5	5	5	4	0
25m	24	5	5	5	5	4	1
	48	5	5	5	5	4	1
TLB	24	5	3	5	5	4	1
	48	5	3	5	5	5	1

* Initial inoculation of 45,000 organisms.
** Number of positive tubes out of five inoculated.

Table XII - Bacteriostatic Action of Various Concentrations of Crystal Violet on Bacillus subtilis

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
5m	24	5	5	5	5	1	0
	48	5	5	5	5	4	2
8.3m	24	5	0	0	0	0	0
	48	5	4	5	5	2	1
10m	24	5	5	5	0	0	0
	48	5	5	5	5	5	0
50m	24	5	5	5	5	4	0
	48	5	5	5	5	5	1
TLB	24	5	3	5	5	4	1
	48	5	3	5	5	4	1

* Initial inoculation of 45,000 organisms.
** Number of positive tubes out of five inoculated.

Table XIII - Bacteriostatic Action of Various Concentrations of Ethyl Purple on Bacillus subtilis

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	1	1	0	0	0
	48	0	1	1	0	0	0
5m	24	4	3	2	1	0	0
	48	5	5	3	1	0	0
8.3m	24	1	2	5	4	4	2
	48	1	2	5	4	4	2
10m	24	1	2	2	5	5	2
	48	1	2	2	5	5	2
50m	24	5	5	5	5	4	3
	48	5	5	5	5	5	4
TLB	24	5	3	5	5	4	1
	48	5	3	5	5	4	1

* Initial inoculation of 45,000 organisms.
** Number of positive tubes out of five inoculated.

Table XIV - Bacteriostatic Action of Various Concentrations of Brilliant Green on Micrococcus caseolyticus

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	1**	0	0	0	0	0
	48	1	0	0	0	0	0
2.5m	24	4	0	0	0	0	0
	48	5	0	0	0	0	0
5m	24	5	5	5	0	0	0
	48	5	5	5	4	2	0
8.3m	24	5	5	5	5	5	1
	48	5	5	5	5	5	5
10m	24	5	5	5	5	5	3
	48	5	5	5	5	5	4
25m	24	5	5	5	5	4	0
	48	5	5	5	5	5	5
TLB	24	5	5	5	5	5	4
	48	5	5	5	5	5	5

* Initial inoculant 175,000 organisms.
** Number of positive tubes out of five inoculated.

Table XV - Bacteriostatic Action of Various Concentrations of Crystal Violet on Micrococcus caseolyticus

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
5m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
8.3m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
10m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
50m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
TLB	24	5	5	5	5	5	5
	48	5	5	5	5	5	5

* Initial inoculant 175,000 organisms
** Number of positive tubes out of five inoculated.

Table XVI - Bacteriostatic Action of Various Concentrations of Ethyl Purple on Micrococcus caseolyticus

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	0	0	0	0	0	0
5m	24	0	0	0	0	0	0
	48	0	2	0	0	0	0
8.3m	24	5	4	0	0	0	0
	48	5	5	5	3	1	2
10m	24	1	0	1	0	0	0
	48	1	5	2	1	0	0
50m	24	5	5	5	5	5	5
	48	5	5	5	5	5	5
TLB	24	5	5	5	5	5	5
	48	5	5	5	5	5	5

* Initial Inoculant 175,000 organisms.
** Number of positive tubes out of five inoculated.

Table XVII - Bacteriostatic Action of Various Concentrations of Brilliant Green on Staphylococcus aureus

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	1	0	0	0	0	0
5m	24	5	0	0	0	0	0
	48	5	1	0	0	0	0
8.3m	24	5	0	0	0	0	0
	48	5	5	1	0	0	0
10m	24	5	5	5	5	2	0
	48	5	5	5	5	5	5
25m	24	5	5	0	0	0	0
	48	5	5	5	0	0	0
TLB	24	5	5	5	5	5	5
	48	5	5	5	5	5	5

* Initial inoculant was 2,300,000 organisms.
** Number of positive tubes out of five inoculated.

Table XVIII - Bacteriostatic Action of Various Concentrations of Crystal Violet on Staphylococcus aureus

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	0	0	0	0	1	0
5m	24	5	0	0	0	0	0
	48	5	4	1	0	0	0
8.3m	24	0	0	0	0	0	0
	48	5	1	0	0	0	0
10m	24	0	0	0	0	0	0
	48	5	0	0	0	0	0
50m	24	2	0	0	0	0	0
	48	5	0	0	0	0	0
Ethyl Purple 5m	24	0	0	0	0	0	0
	48	5	4	0	0	0	0
TLB	24	5	5	5	5	5	5
	48	5	5	5	5	5	5

* Initial inoculant is 2,300,000 organisms.
** Number of positive tubes out of five inoculated.

Table XIX - Bacteriostatic Action of Various Concentrations of Ethyl Purple on Staphylococcus aureus

Dye Dilutions	Hours Incub.	Dilutions of organisms*					
		Undil.	1-10	1-100	1-1t	1-10t	1-100t
		Number of positive tubes					
1m	24	0**	0	0	0	0	0
	48	0	0	0	0	0	0
2.5m	24	0	0	0	0	0	0
	48	1	1	2	0	0	0
5m	24	5	5	5	5	5	0
	48	5	5	5	5	5	0
8.3m	24	5	2	0	0	0	0
	48	5	5	2	1	1	0
10m	24	4	1	0	0	0	0
	48	5	5	3	0	0	0
50m	24	5	5	5	5	5	0
	48	5	5	5	5	5	1
TLB	24	5	5	5	5	5	0
	48	5	5	5	5	5	0

* Initial inoculum - 12,000,000 organisms per one-tenth milliliter.
** Number of positive tubes out of five inoculated.

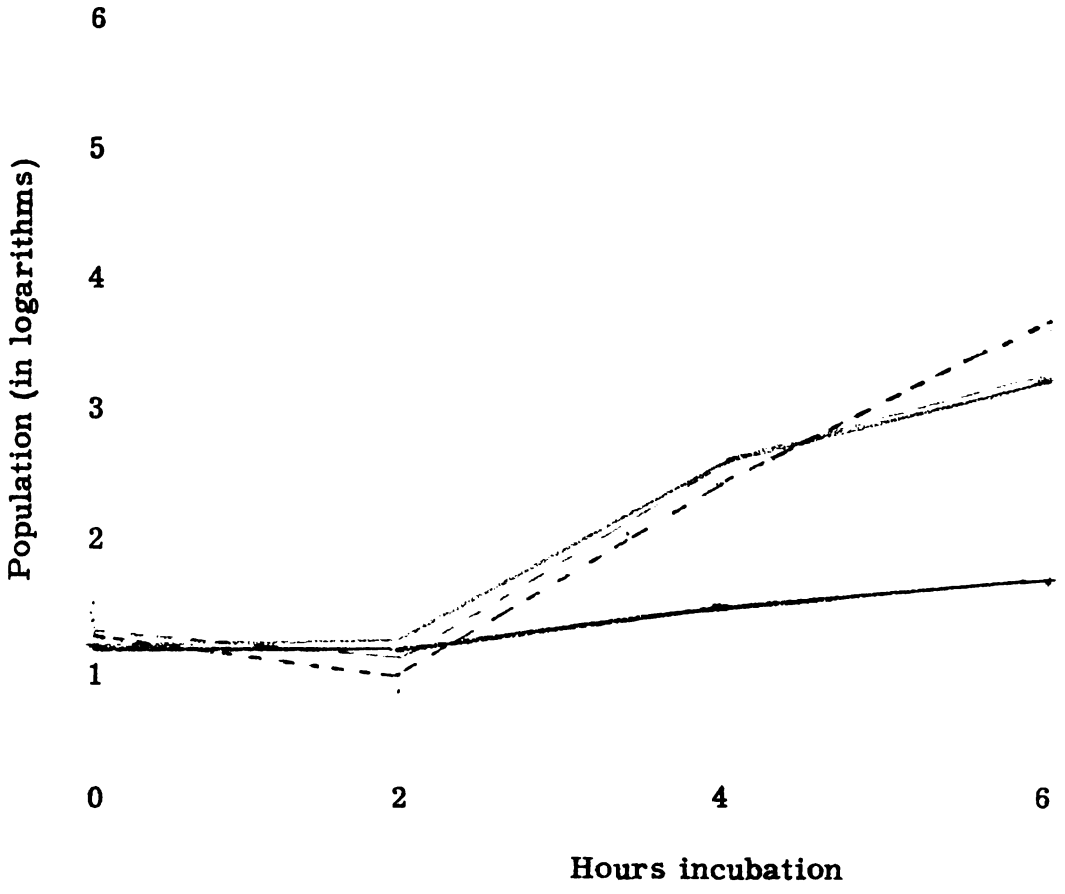
GRAPH I - S. typhimurium growth curves with 1-1 million concentration of ethyl purple and brilliant green.

---LTB control curve for B. G.

—Brilliant green curve 1-1M

---LTB control curve for E. P.

—Ethyl purple curve 1-1M



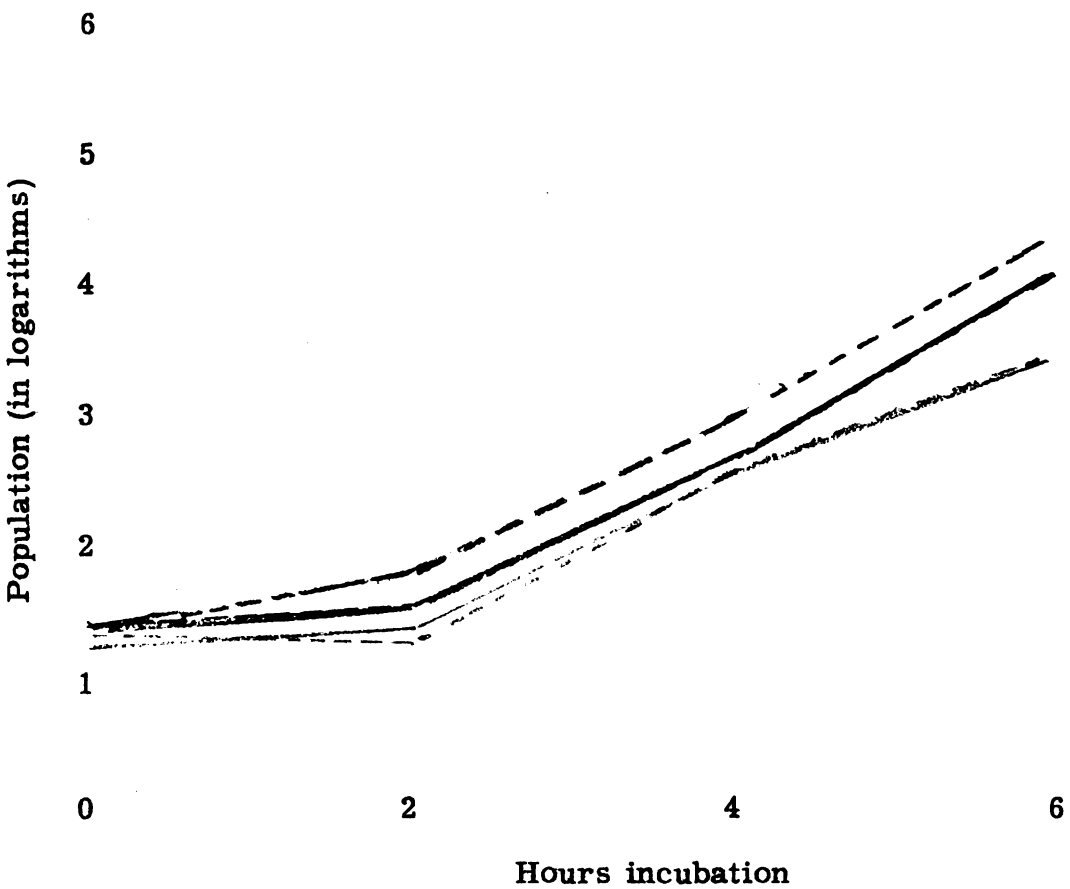
GRAPH II - S. typhimurium growth curve with 1-1M conc. of ethyl purple and crystal violet

---LTB control for C. V.

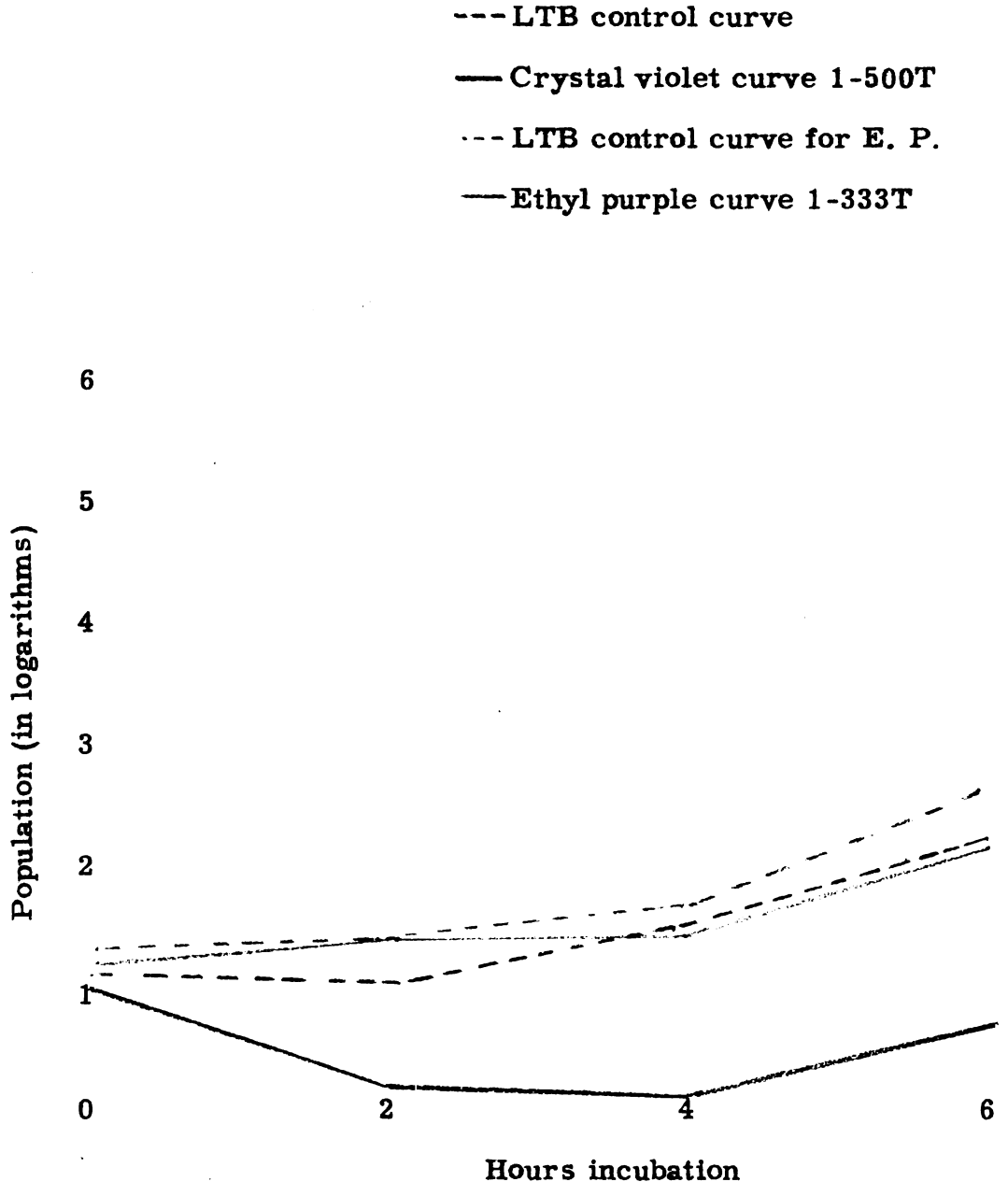
——Crystal violet curve 1-500T

---LTB control curve for E. P.

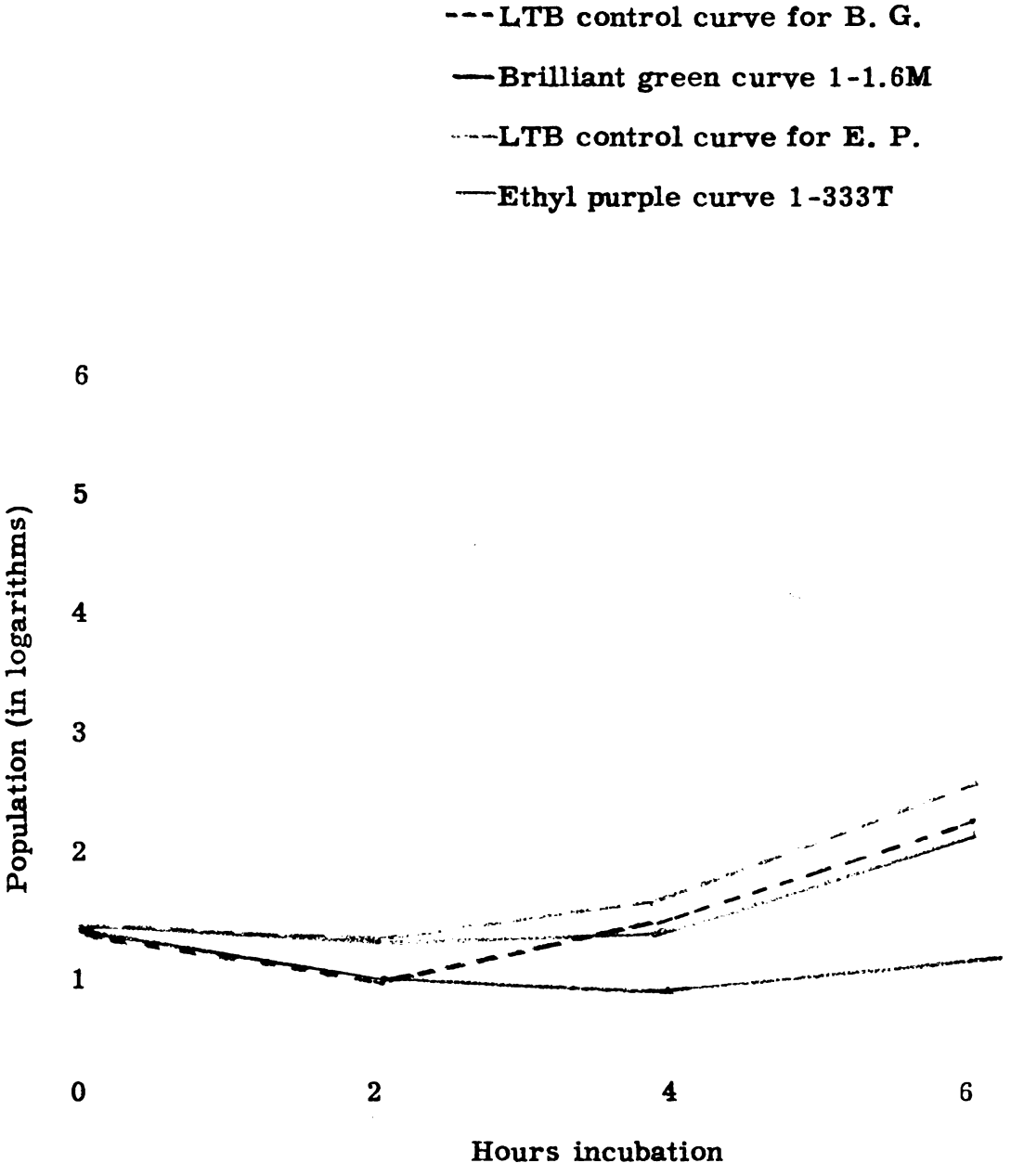
——Ethyl purple curve 1-333T



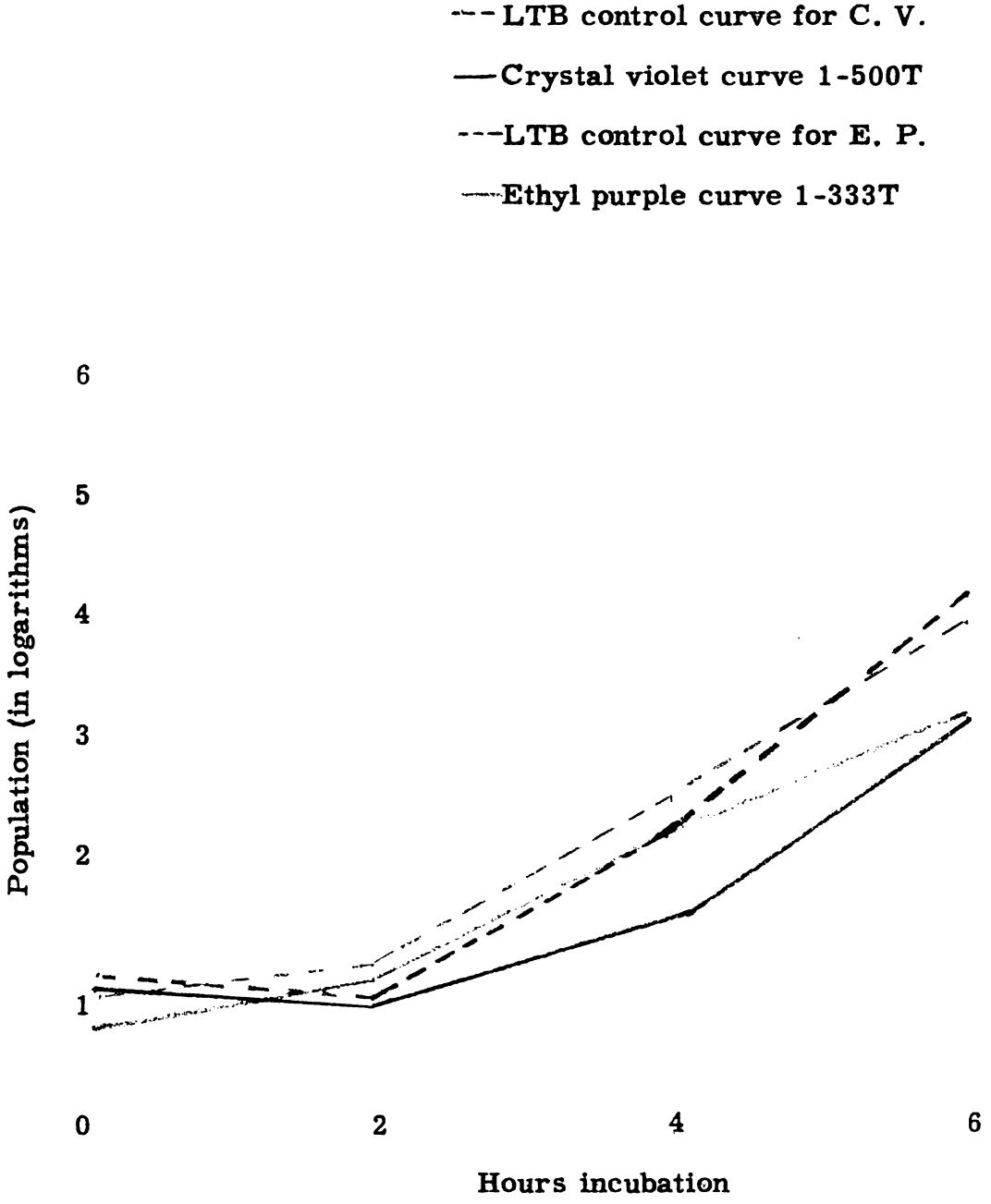
GRAPH III - E. typhosa growth curves with 1-333 thousand concentration of ethyl purple and 1-500 thousand concentration of crystal violet.



GRAPH IV - E. typhosa growth curves with 1-333 thousand concentration of ethyl purple and 1-1.6 million concentration of brilliant green.



GRAPH V - E. coli growth curves with 1-333T concentration of ethyl purple and 1-500T concentration of crystal violet.



GRAPH VI - E. coli growth curves with 1-333 thousand concentration of ethyl purple and 1-1 million concentration of brilliant green.