Toxicity of Brilliant Green for Certain Bacteria*

EDMUND K. KLINE, Dr.P.H., F.A.P.H.A.

Cattaraugus County Department of Health, Olean, N. Y.

THE purposes of this study are best described in the following resolution adopted during the Annual Meeting of A.P.H.A. in Washington in 1932.

Since the practice of incorporating dyes in culture media for the purpose of growth of certain kinds of bacteria is increasing, and since the value of such differential media is dependent upon the toxicity of these dyes; therefore, be it resolved by the Laboratory Section Council of the American Public Health Association that a committee be appointed to consider the possibility of establishing standards of toxicity for such dyes with especial reference to those used in media recognized by the Standard Methods of Water and Sewage Analysis.†

This paper reports a study of the toxic effect of various brilliant green dyes incorporated in a simple culture medium. Since primary interest concerns the ability of the dye to inhibit growth in various media the emphasis has been placed on bacteriostatic rather than bacteriocidal action.

Bacteriostasis is probably simply a manifestation of vital staining, wherein the reproductive mechanism of the organism is destroyed or at least rendered inoperative. All of the theoretical considerations and experimental evidence that have been advanced regarding the mechanism of staining reactions is applicable in some degree to the present problem. Excellent reviews of literature dealing with this are available.[1, 2]

DETERMINATION OF BACTERIOSTATIC TITER

There are several possible ways of determining experimentally the bacteriostatic powers of various lots of dyes.

1. Measuring the smallest inoculum that will initiate growth—A method previously used by Dunham and open to the drawback that there is no satisfactory way of measuring small inocula except by dilution methods and that these are subject to rather wide chance variations as the number of organisms in the inoculum becomes very small.

2. Measuring the rate of multiplication following a standard inoculum—The method used by Dunham and Schoenlein in their work on bile brilliant green media in 1926 for the Standard Methods Committee of the American Water Works Association.[3]

3. Measuring the lag phase in the presence of dye in relation to the normal lag phase in control media—A method for the quantitative determination of bacteriostatic action recently devised by Mary A. Ingraham, University of Wisconsin.[4]

---

* While this was read as a paper, it is also to be considered as a report from the Referee, presented at the request of the Standard Methods Committee, to the Laboratory Section of the American Public Health Association at the Sixty-third Annual Meeting in Pasadena, Calif., September 4, 1934.

† With the reorganization of the Standard Methods Committee this problem was assigned to the section on Diagnostic Methods and Procedures and the author was appointed *Referee* for this study. Dr. H. J. Conn, Chairman of the Biological Stain Commission and H. G. Dunham, of the Difco Laboratories, both of whom are pioneers in the field of dye standardization, have served as *Associate Referees*. Advice and assistance have also been received from Harry Jordan of Indianapolis, Dr. Max Levine of Iowa State College, and Frank E. Hale of the Mount Prospect Laboratories of New York City Bureau of Water Supply. The work has all been performed by the Referee in the laboratories of the Cattaraugus County Department of Health, Olean, N. Y.

TABLE I
COMPARATIVE BACTERIOSTATIC ACTION OF TWO BRILLIANT GREEN DYES

Dye Certification Number		CBg4						NBg5					
		Hours of incubation						Hours of incubation					
	Dilution of dye	24	48	72	96	120	168	24	48	72	96	120	168
a. Escherichia coli	1:200,000	++	++	++	++	++	++	++	++	++	++	++	++
	1:300,000	++	++	++	++	++	++	++	--	--	--	--	--
	1:400,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:500,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:600,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:700,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:800,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:900,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:1,000,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:1,250,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:1,500,000	-+	-+	-+	-+	-+	-+	--	--	--	--	--	--
	1:1,750,000	-+	--	--	--	--	--	--	--	--	--	--	--
	1:2,000,000	--	--	--	--	--	--	--	--	--	--	--	--
b. Aerobacter aerogenes	1:125,000	++	++	++	++	++	++	++	++	++	++	++	++
	1:150,000	++	++	++	++	++	+-	-+	-+	-+	-+	-+	-+
	1:200,000	++	++	++	++	++	++	++	--	--	--	--	--
	1:500,000	--	--	--	--	--	--	--	--	--	--	--	--
c. Citrobacter sp.	1:100,000	++	++	++	++	++	++	++	++	++	++	++	++
	1:150,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:200,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:250,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:300,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:350,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:400,000	++	++	+-	+-	--	--	--	--	--	--	--	--
	1:450,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:500,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:550,000	++	+-	+-	+-	+-	+-	--	--	--	--	--	--
	1:600,000	++	+-	+-	+-	+-	+-	--	--	--	--	--	--
d. Bacillus subtilis	1:5,000,000	++	++	++	++	++	++	++	++	++	++	++	++
	1:10,000,000	++	++	++	++	++	++	+-	+-	+-	+-	+-	+-
	1:20,000,000	++	++	++	++	++	++	+-	+-	+-	+-	+-	+-
	1:30,000,000	++	++	++	++	++	++	+-	+-	+-	+-	+-	+-
	1:40,000,000	++	++	++	++	++	++	+-	+-	+-	+-	+-	+-
	1:50,000,000	++	++	++	++	++	++	+-	+-	+-	+-	+-	+-
	1:60,000,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:80,000,000	++	+-	+-	+-	+-	--	--	--	--	--	--	--
	1:90,000,000	++	++	++	++	++	++	--	--	--	--	--	--
	1:100,000,000	--	--	--	--	--	--	--	--	--	--	--	--
e. Bacillus cereus	1:10,000,000	+	+	+	+	+	+	+	+	+	+	+	+
	1:20,000,000	+	+	+	-	-	-	-	-	-	-	-	-
	1:30,000,000	+	+	+	+	+	+	-	-	-	-	-	-
	1:40,000,000	+	+	+	+	+	+	-	-	-	-	-	-
	1:50,000,000	-	-	-	-	-	-	-	-	-	-	-	-

+ Signs indicate bacteriostasis (no growth of bacteria). – Signs indicate growth. Double columns indicate duplicate tubes.
All of these tests in lactose-peptone broth pH 6.9.

4. Measuring the concentration of dye that will inhibit gas formation—The method used by Hale in previous work for the Biological Stain Commission.
5. Measuring the concentration of the dye which will inhibit growth with a standard inoculum.

In devising a procedure to be used in the present studies it seemed that the last method was the simplest and most likely to be properly controlled. The variable factors which might influence the results included—composition and reaction of the media, concentration of the dye, activity of the organisms, composition of the diluent, and size of the inoculum.

OUTLINE OF METHOD

Medium—A base medium consisting of 1 per cent Bacto-peptone and 1 per cent Bacto-lactose was chosen. Unadjusted, it had a reaction of pH 6.9 after sterilization.

The final volume in the test might conveniently be 10 c.c. A number of test tubes were marked at 7½ and at 10 c.c. Ten grams of Bacto-peptone and 10 gm. of Bacto-lactose were dissolved in 750 c.c. of distilled water, tubed in 7½ c.c. amounts and sterilized in the autoclave. After the addition of the dye and the inoculum, sterile water was added to each tube to bring it to a volume of 10 c.c. so that the medium during the time of incubation had the concentration of the simple base medium.

Dye—The dye was used as an aqueous solution in distilled water prepared in volumetric flasks and added to the medium in such dilution that not more than 2 c.c. nor less than 0.2 c.c. was added to each tube.

Inoculum—After the organisms had been established in the simple base medium (1 per cent Bacto-peptone and 1 per cent Bacto-lactose) by a series of daily transfers, a transfer was made and examined at half-hour intervals until a distinct turbidity was noted. A loopful of this culture was then placed on a hemocytometer under a thin cover slip and examined and counted under oil immersion. If the average number of bacteria present was between 1 and 5 per small square (between 4 million and 20 million per c.c.), the culture was diluted with sterile water to which had been added 1 per cent of the base medium so that the final concentration was approximately 200 per c.c. One-half c.c., or approximately 100 organisms, was then used as the inoculum for each tube.

Test—Proper quantities of the dye to give the concentrations desired in 10 c.c. were added to the tubes by means of sterile pipettes. Each tube was then thoroughly shaken. Dilutions of the culture were prepared in the manner described above, and ½ c.c. of the final dilution, approximately 100 bacteria, was added to each tube. The volume of each tube was then made up to 10 c.c. by the addition of sterile water by means of a sterile pipette. The tubes were again shaken (in a mechanical shaker) placed in the incubator, and examined for growth at the end of 24 hours and each day thereafter for 5 days. It is recommended that each test be set up in duplicate. Results are recorded as growth or no growth.

NOTES—The simple peptone broth chosen as the base medium supported excellent growth of all the organisms used.

The above method of estimating organisms is one commonly used for counting vaccines. Slight deviations in the size of the inoculum, 50 to 150 organisms rather than 100, did not materially alter the bacteriostatic titer.

The object of using the young culture was to place the organisms in the dye medium at a time when they were in the logarithmic growth phase at which stage they should be most active. The organisms were diluted in a weak culture medium to protect them against the bactericidal effect of distilled water.

The bacteriostatic action of the dye affects the growth of bacteria during the lag phase. It has been found that after this phase immediate and very heavy growth is apparent so that there is no difficulty in determining which tubes show growth by simple examination.

Materials — The Dye Commission furnished samples of several lots of brilliant green including all of those now on the market and Dunham a sample of the original dye (# 190) that he used in the studies of bile brilliant green media on which the present standard medium is based.

Five different organisms were used, *Escherichia coli, Aerobacter aerogenes, Citrobacter sp., Bacillus subtilis* (Marburg type) and *Bacillus cereus.*

Range of Bacteriostasis—Using the above method high dilutions of the dyes proved to be bacteriostatic. Members of the colon group were usually inhibited between 1:200,000 and 1:400,000, while the spore forming organisms were usually inhibited from 1:10,000,000 to 1:50,000,000. However, test

sets could not always be duplicated in regard to the concentration of dyes which would inhibit or permit growth. For instance, using *Aerobacter aerogenes* and NBg 5, growth was obtained in some series in as low a dilution as 1:25,000 and in other series no growth appeared in dilutions as high as 1:125,000.

Variations within a Test Set—Duplicate tubes within the set usually checked each other very closely but, as would be expected in a biological test of this nature, in a few instances a variation of two or more dilutions occurred.

Aftergrowth effects were frequently encountered wherein tubes negative in 24 or 48 hours would show vigorous growth at later periods. This undoubtedly represented lag phases delayed for periods of 24 hours to a week. It was not surprising to find this as it has frequently been possible to train bacteria to become dye resistant. Working with brilliant green and *Escherichia coli*, Mary V. Reed and Elizabeth Genung have recently reported a multiplication of 250 times in tolerance of the organism for the dye through 13 transfers to stronger and stronger dye media in 56 days.[5]

Comparative Test Sets—When two dyes, CBg 4 and NBg 5, were set up in parallel series CBg 4, exhibited a markedly stronger bacteriostatic effect, whatever organism was used, as can be seen from Table I.

The 8 available dyes were set up against *Aerobacter aerogenes*. Growth extending over a week of incubation appeared very similar to that already shown. The results at the end of 24 and 48 hours incubation are shown in Table II a.

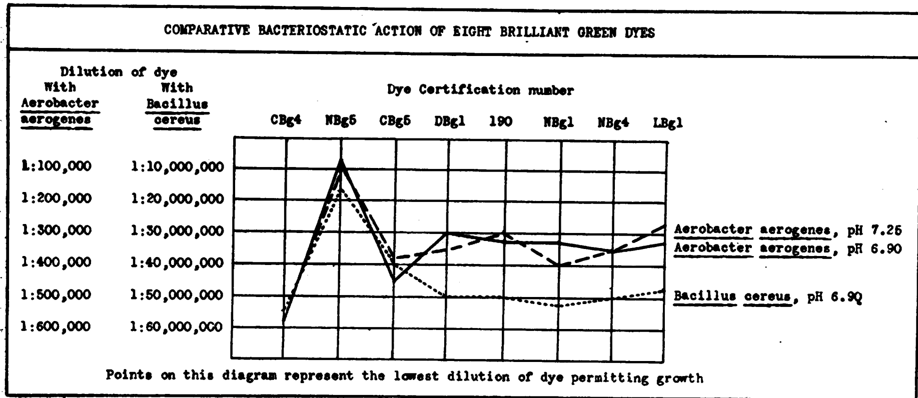
This experiment was repeated using *Bacillus cereus* with results shown in Table II b.

TABLE II
COMPARATIVE BACTERIOSTATIC ACTION OF EIGHT BRILLIANT GREEN DYES

Dye Certification Number	CBg4		NBg5		CBg5		DBg1		190		NBg1		NBg4		LBg1	
Hours of incubation	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
Dilution of dye																
a. Aerobacter aerogenes pH 6.9																
1:50,000	++	++	+	-	++	++	++	++	++	++	++	++	++	++	++	++
1:100,000	++	++	-	-	++	++	++	++	++	++	++	++	++	++	++	++
1:200,000	++	++	-	-	++	++	++	++	++	++	++	++	++	++	++	+-
1:300,000	++	++	-	-	++	++	--	--	+-	--	+-	--	++	--	++	--
1:400,000	++	++	-	-	++	--	--	--	--	--	--	--	--	--	--	--
1:500,000	++	+-	-	-	--	--	--	--	--	--	--	--	--	--	--	--
1:600,000	--	--	-	-	--	--	--	--	--	--	--	--	--	--	--	--
1:700,000	--	--	-	-	--	--	--	--	--	--	--	--	--	--	--	--
b. Bacillus cereus pH 6.9																
1:5,000,000	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:10,000,000	++	++	++	+-	++	++	++	++	++	++	++	++	++	++	++	++
1:20,000,000	++	++	--	--	++	+-	++	++	++	++	++	++	++	++	++	++
1:30,000,000	++	++	--	--	++	++	++	++	++	++	++	++	++	++	++	++
1:40,000,000	++	++	--	--	+-	+-	++	++	++	++	++	++	++	++	++	+-
1:50,000,000	+-	+-	--	--	--	--	--	--	--	--	+-	--	--	--	--	--
1:60,000,000	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1:70,000,000	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
c. Aerobacter aerogenes pH 7.25																
1:50,000	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:100,000	++	++	--	--	++	++	++	++	++	--	++	++	++	++	++	+-
1:200,000	++	++	--	--	++	+-	++	--	++	++	++	++	++	--	+-	--
1:300,000	++	++	--	--	++	--	++	+-	++	++	++	--	+-	--	++	--
1:400,000	++	++	--	--	++	+-	++	--	++	--	++	+-	++	--	--	--
1:500,000	++	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1:600,000	+-	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
1:700,000	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

+ Signs indicate bacteriostasis (no growth of bacteria). - Signs indicate growth. Double columns indicate duplicate tubes.
All tests in lactose-peptone broth at the pH indicated.

CHART I



In view of the fact that brilliant green is commonly used in water analysis in combination with bile at a pH more alkaline than 7.0 it was thought desirable to determine whether the dyes would act the same at a higher pH. Therefore, the above experiment was repeated with a medium of pH 7.25 and essentially the same results obtained, as shown in Table II c.

In order to compare the results given in the last 3 tables the average readings of the tubes shown in those tables are plotted as a curve in Chart I.

DISCUSSION

A method has been presented by which it seems possible to determine the bacteriostatic titer of any given sample of brilliant green. Using this method indications have been obtained that there are distinct differences in the relative toxicity of various batches of this dye. One particular batch, for instance, has been found distinctly more toxic and another distinctly less so than the average run of samples. Data are still too meager, however, to permit definite conclusions at the present time.

It is hoped that other laboratories may be stimulated to check this work and that if possible other methods of measuring bacteriostatic titer can be utilized as a further check. The importance of laboratories doing experimental work recording, in every instance, the lot number of the certified dye which they are using should be again emphasized at this time. The Referees are most anxious to obtain reports of any work which has a bearing on this subject. They in turn will be glad to furnish information on any of the dyes tested to persons interested in bacteriostatic titers.

The dyes which have been used in these experiments include samples of all lots of brilliant green now on the market under certified labels.

The Biological Stain Commission will hereafter refer all samples of brilliant green submitted to them to the Referee, and bacteriostatic titers will be determined and these will be available to any laboratory interested in them.

REFERENCES

1. Churchman, John W. *The Newer Knowledge of Bacteriology and Immunology*, Jordan and Falk, University of Chicago Press, 1928, p. 19.
2. Conn, H. J. Commission on Standardization of Biological Stains (2d ed.), 1929, p. 160.
3. Dunham, H. G., and Schoenlein, H. W. *Stain Technology*, I, 4:129 (Oct.), 1926.
4. Ingraham, Mary A. The Bacteriostatic Action of Gentian Violet and Its Dependence on the Oxidation Reduction Potential. *J. Bact.*, XXVI, 6:573 (Dec.), 1933.
5. Reed, Mary V. The Effect of Certain Tri-Phenyl Methane Dyes on Staphylococcus Aureus and Bacterium Coli Communior. Thesis Smith College, 1933.
Reed, Mary V., and Genung, Elizabeth. *J. Bact.*, XXVII, 29 (Jan.), 1934.