

## Effect of Heat on the Antimicrobial Activity of Brilliant Green Dye

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Antimicrobial activity of brilliant green dye in Trypticase soy broth (BBL) is reduced and ultimately destroyed by prolonged autoclaving at 121 C. Loss of antimicrobial activity is accompanied by decolorization of the dye. This is consistent with other evidence that antimicrobial activity of brilliant green resides in the colored dye ion. The dye is not decolorized when heated in distilled water or peptone, but is decolorized by heating in glucose, glycine, or sodium dodecyl sulfate, showing that decolorization results from reaction with components of the medium. To ensure optimal results, it is recommended that bacteriological media be sterilized by heat prior to addition of brilliant green dye.

The value of certain triphenylmethane dyes as selective agents for isolation of typhoid bacteria was first reported by Drigalski and Conradi (2). These dyes have since been used extensively as aids in the isolation of bacteria of the typhoid and paratyphoid groups (*Salmonella*). Brilliant green (BG) inhibits growth of bacteria at lower concentrations than other dyes (1, 7) and is by far the most widely used dye in selective media, although malachite green and crystal violet are sometimes used (8, 11, 14).

Little consideration has been given to the effects of heat treatment on the inhibitory properties of dyes. In most of the earlier studies (1, 7) the dyes were added after sterilization of the remainder of the medium, thus avoiding the problem. However, a more recent practice (3, 4, 9, 13, 15) has been to sterilize the complete medium with dyes present, and this is the recommended procedure with commercial BG agars (BGA) which contain 12.5 mg of dye/liter. Poelma (13) and Galton et al. (4) recommended autoclaving BGA 12 or 15 min, respectively, at 121 C to obtain optimal selectivity. Lang (8) found that BGA showed good selectivity only when prepared from the ingredients with the dye added after sterilizing the remainder of the medium and cooling it to 50 C.

In the present paper, we report results of a study on the effects of heating BG dye on the inhibitory properties of the dye.

### MATERIALS AND METHODS

BG dye (Matheson, Coleman and Bell) was prepared as stock solutions containing 1.0 and 0.1 mg of dye/ml. These were filter-sterilized using Nalgene 0.2-

$\mu\text{m}$  disposable filters. Trypticase soy broth (TSB; BBL) was prepared in culture tubes. Dye was added before or after sterilizing the TSB as indicated. Volumes of TSB and dye solution were adjusted to give a final volume of 9.9 ml in the tubes prior to inoculation with the test organisms.

**Experimental procedure.** One strain each of *Salmonella anatum*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, and *Streptococcus faecalis* was selected from our stock cultures. These were grown for 24 h at 37 C in TSB, and  $10^{-6}$  dilutions were made in 0.1% peptone-water. They were plated in duplicate on Trypticase soy agar (TSA; BBL) to determine the initial inoculum, and 0.1 ml of this dilution was also inoculated into each of the tubes of test media. A portion of the test media was withdrawn from each tube after 24 and 48 h and plated in duplicate on TSA to determine the number of bacteria present.

**Spectrophotometric measurements.** Optical absorbancies of the test media were measured by using a Beckman model DB-GT double-beam recording spectrophotometer.

### RESULTS AND DISCUSSION

We found in our initial experiments, by adding BG to TSB, that the optical absorbance of the dye in TSB was less than one-half that of the same concentration of dye in distilled water. Furthermore, some turbidity was evident when the dye was added to TSB, especially at higher concentrations. When a BG-TSB mixture was passed through a 0.2- $\mu\text{m}$  Nalgene filter, all the color was removed, indicating that the dye is present as a colloidal complex in TSB. Quantitative measurement of the dye was further hampered by the fact that the dye does not follow Beer's law in water. Furthermore, the optical density of a freshly diluted BG solution

increased slowly for several hours. BG has been reported by Goldacre and Phillips (5) to form an equilibrium between the colored cation and colorless carbinol form at a given pH in aqueous solution with a pK of 7.9. Several hours are required to attain equilibrium. These factors all complicate quantitative measurement, and optical absorbances give only a very rough estimate of the dye present.

Table 1 shows the growth of three types of bacteria in TSB with BG added before and after autoclaving. In the unheated medium, growth of *Salmonella derby* was retarded with as little as 2 mg of BG per liter, and 5 mg of BG per liter, or greater, was lethal. In the autoclaved medium, however, growth was not inhibited by up to 20 mg of BG per liter. Growth of *E. aerogenes* was somewhat retarded when higher concentrations of BG were added after sterilization, but was unaffected when BG was added before sterilization. *P. mirabilis* was killed at all concentrations of BG added after sterilization, but survived and multiplied slightly at up to 10 mg of BG per liter in the autoclaved medium. The dye was partially decolorized by autoclaving as shown by the reduced optical absorbances.

An additional experiment was run to determine if the activity of the dye could be completely abolished by autoclaving until the dye was completely decolorized. *S. aureus* and *S. faecalis* were used because these gram-positive organisms are very sensitive to the dye. The

results with dye unautoclaved and autoclaved for 15 and 60 min at 121 C are shown in Fig. 1 and Table 2. The BG absorption peak at 630 nm was reduced by autoclaving for 15 min and totally eliminated after 60 min. Autoclaving for 60 min completely abolished the inhibitory effects of BG.

We conducted additional experiments to determine the effect of autoclaving BG in other media to determine which components of TSB caused the observed decolorization of the dye (Table 3). An aqueous solution of the dye was

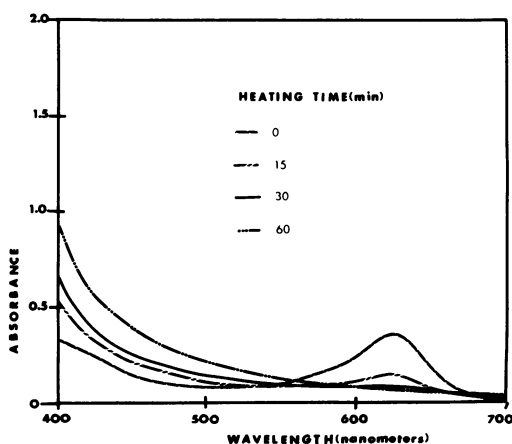


FIG. 1. Changes in the optical absorbance of brilliant green dye (5 mg/liter) in Trypticase soy broth autoclaved at 121 C for various lengths of time.

TABLE 1. Growth of bacteria in Trypticase soy broth containing brilliant green dye added before and after sterilization by autoclaving

Brilliant green concn (mg/liter)	Dye added after sterilization				Dye added before sterilization			
	Optical absorbance at 630 nm	Plate counts/ml			Optical absorbance at 630 nm	Plate counts/ml		
		0 h	24 h	48 h		0 h	24 h	48 h
		<i>Salmonella derby</i>				<i>Salmonella derby</i>		
0		7.2	$5.5 \times 10^8$	$6.8 \times 10^8$	0.080	4.9	$5.0 \times 10^8$	$5.9 \times 10^8$
2	0.236	7.2	$8.1 \times 10^1$	$1.6 \times 10^7$	0.194	4.9	$5.6 \times 10^8$	$5.5 \times 10^8$
5	0.332	7.2	0	0	0.229	4.9	$4.3 \times 10^8$	$3.8 \times 10^8$
10	0.372	7.2	0	0	0.270	4.9	$2.0 \times 10^8$	$4.1 \times 10^8$
20	0.553	7.2	0	0		4.9		$6.5 \times 10^8$
		<i>Enterobacter aerogenes</i>				<i>Enterobacter aerogenes</i>		
0		14	$1.2 \times 10^9$	$1.3 \times 10^9$	0.080	12	$1.0 \times 10^9$	$8.0 \times 10^8$
2	0.236	14	$2.1 \times 10^8$	$1.1 \times 10^8$	0.194	12	$5.0 \times 10^8$	$6.2 \times 10^8$
5	0.332	14	$1.2 \times 10^7$	$9.5 \times 10^8$	0.229	12	$6.8 \times 10^8$	$7.7 \times 10^8$
10	0.372	14		$1.2 \times 10^9$	0.270	12	$6.1 \times 10^8$	$5.6 \times 10^8$
20	0.553	14	$6.9 \times 10^4$	$6.5 \times 10^8$		12	$5.4 \times 10^8$	$6.2 \times 10^8$
		<i>Proteus mirabilis</i>				<i>Proteus mirabilis</i>		
0		13	$6.3 \times 10^8$	$5.0 \times 10^8$	0	6.2	$7.6 \times 10^8$	$9.2 \times 10^8$
2	0.236	13	0	0	0.078	6.2	$4.1 \times 10^8$	$7.7 \times 10^8$
5	0.332	13	0	0	0.122	6.2	$1.7 \times 10^8$	$1.1 \times 10^8$
10	0.372	13	0	0	0.199	6.2	$1.1 \times 10^2$	$1.2 \times 10^2$
20	0.553	13	0	0	0.267	6.2	1	0

TABLE 2. Effect of heating of Trypticase soy broth brilliant green mixture on the inhibitory properties of the dye

Organism	Inoculum (plate count/ml)	Plate count/ml after incubation for 24 h at 37 C in:				
		TSB controls sterilized at 121 C for:		TSB sterilized 15 min at 121 C + BG (10 mg/liter) added after sterilization	TSB + BG (10 mg/liter) added prior to sterilization at 121 C for:	
		15 min	60 min		15 min	60 min
<i>Streptococcus faecalis</i>	4.8	$5.1 \times 10^8$	$4.8 \times 10^8$	0	1	$5.6 \times 10^8$
<i>Staphylococcus aureus</i>	7.1	$7.0 \times 10^8$	$3.6 \times 10^8$	0	7	$6.1 \times 10^8$

TABLE 3. Effect of heating brilliant green dye at 121 C in various media on the optical absorbance of the dyes

Heating time (min)	Optical absorbance at 630 nm of media containing 5 mg of BG per liter heated for various lengths of time				
	Distilled <sup>a</sup> H <sub>2</sub> O	1% Glucose	1% Glycine	1% Peptone	1% Sodium dodecyl sulfate
0	0.936	1.020	0.620	0.960	1.230
15	0.954	0.726	0.628	0.994	0.972
30	1.038	0.689	0.486	1.024	0.774
60	1.038	0.596	0.348	0.996	0.140

<sup>a</sup> All absorbance readings are versus the indicated medium without added BG.

not decolorized by autoclaving, showing that decolorization results from reaction with components of the medium rather than from any sensitivity of the dye to heat. The dye was partially decolorized by heating in 1% solutions of glucose, glycine, and sodium dodecyl sulfate, but not in 1% peptone. The initial optical absorbancies in glycine and sodium dodecyl sulfate were different from those in water, indicating some reaction with these components. We have not determined the nature of the colorless reaction products.

Our results show that the inhibitory properties of BG are readily destroyed by heating in TSB. Loss of antimicrobial activity appears to be closely associated with decolorization of the dye. We have previously observed that BG agar loses its inhibitory properties as the dye is decolorized. Our results are consistent with those of Paetzoldt (12) who found that the colorless leuco and carbinol derivatives of BG were ineffective as inhibitors of *S. aureus*. Krumwiede and Pratt (7) found that inhibition of bacteria by BG was substantially reduced when the dye was decolorized with sulfur dioxide. Kligler (6) found that the quinoid structure of the dye molecule is important for antimicrobial activity.

The optical absorption of the medium containing BG gives some indication of the amount of active dye present. However, some organic

substances, such as bile salts and sodium dodecyl sulfate, reduce the antimicrobial activity of the dye (9, 10, 15) without any reduction in color, so that optical absorption data must be interpreted cautiously.

These results indicate a need for caution in the use of heat to sterilize media containing BG dye as a selective agent. The possibility that the antimicrobial activity of other dyes may be altered by heating should also be considered. Although some workers (4, 13) recommend autoclaving BG agar a specified length of time to attain optimal selectivity, such factors as the geometry and size of containers and variations in the heating cycle of autoclaves can cause variations in the effective heat treatment. These problems can easily be avoided by adding the dye aseptically after sterilization of the remainder of the medium.

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