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# The antibacterial action of crystal violet*

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Crystal violet has an antibacterial action against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Bacillus subtilis*. The effect of the dye, measured as minimum inhibitory concentration or retardation of growth, increases as the pH rises from 6 to 8. Of the four species *E. coli* is most resistant to the dye; the resistances of the other organisms are similar. The mode of action put forward by Stearn & Stearn (1928) that the action of crystal violet is due to the formation of an unionized complex of bacteria with dye, is supported. Gram-negative organisms, such as *E. coli*, have high isoelectric points and contain less acidic components than Gram-positive bacteria which usually have lower isoelectric points, so the former combine with crystal violet less readily and are more resistant to the dye. In extension of this theory, the negative charge on bacteria is increased as the pH of the medium is increased, and the organisms become more sensitive to dye. Evidence is presented which refutes the theory of a poising of the redox potential by crystal violet suggested by Dubos (1929) and Ingraham (1933).

IN 1923, Churchman stated that the growth of Gram-positive bacteria was inhibited by most triphenylmethane dyes but, except at high concentrations, Gram-negative organisms were unaffected. The bacteriostatic activity of gentian violet (an impure form of crystal violet) towards *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus haemolyticus* was modified by pH, being greater at high pH values (Stearn & Stearn, 1926).

I have made experiments to determine the effect of pH on the minimum inhibitory concentration of crystal violet, and the lag or reduction of growth produced by crystal violet in four species of organisms.

## Experimental

### Materials

Crystal violet B.P. re-crystallized from ethanol [$E$ (1% 1 cm) = 2059 at 591 mμ] was used to prepare a 0·01 M aqueous solution.

Nutrient broth was prepared from granules (Oxoid CM1) and 1% potassium dihydrogen phosphate (Analar) added, and the pH adjusted using a freshly prepared solution of potassium hydroxide.

### Organisms

Cultures were prepared from freeze-dried specimens of *E. coli* 1 NCTC 8196, *Staph. aureus* NCTC 7447, *Str. faecalis* NCTC 370, *Bacillus subtilis* NCTC 3610. A suspension was prepared in quarter-strength Ringer solution (Wilson, 1922) from the 24-hr growth on a nutrient agar slope (overnight for *B. subtilis*), followed by filtration through sterile Whatman No. 1 filter paper to remove clumps of bacteria and particles of agar. The extinction of the suspension was checked using

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an EEL nephelometer, and the suspension diluted to give a viable count of about $1 \times 10^7$ organisms/ml, determined by the drop-plate method of Miles & Misra (1938).

### METHODS

10 ml amounts of pH-adjusted broth containing 1 to $6 \times 10^{-7}$ M crystal violet were maintained at 37° for 24 hr to allow equilibrium to be reached between the coloured and the carbinol form of the dye (Goldacre & Phillips, 1949), and bacterial suspension (0·1 ml) prepared as above was then added. The pH-adjusted broth containing no dye was similarly treated to show the effect of pH alone on growth. The dye-broth mixtures were made in triplicate and the controls in duplicate, and average values taken of the extinction. The addition of dye to broth had a negligible effect on extinction both in the presence and absence of growth.

Oxidation-reduction potentials were measured with a platinum electrode against a calomel reference and expressed as $E_h$ (Hewitt, 1950).

## Results

### INHIBITION OF GROWTH AT EXTREMES OF pH

The limiting values of pH for growth in broth at 37° were: *E. coli*, range pH 4·5–9·0, optimum 5·9–7·1; *Staph. aureus*, range 5·6–9·5, optimum 6·7; *Str. faecalis*, range 5·7–9·5, optimum 6·8; *B. subtilis*, range 5·1–9·5, optimum 6·0–6·8.

### INHIBITION OF GROWTH BY pH-ADJUSTED CRYSTAL VIOLET

At pH values above pH 7·5, concentrations of dye below $1 \times 10^{-6}$M prevented growth of *Staph. aureus*, *Str. faecalis* and *B. subtilis* for 48 hr or more; about $1 \times 10^{-5}$M was necessary to prevent growth of *E. coli*. On the other hand, at pH 6·0 nearly $1 \times 10^{-5}$M was required to prevent growth of the first three species, and more than $1 \times 10^{-3}$M for *E. coli*. Fig. 1 shows the effect of pH on the minimum inhibitory concentration of

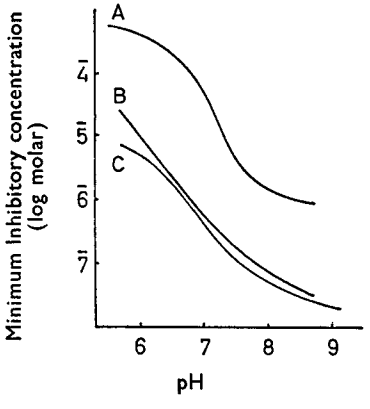


FIG. 1. Effect of pH on minimum inhibitory concentration of crystal violet in nutrient broth at 37°. A. *E. coli*. B. *B. subtilis*. C. *Staph. aureus*.

crystal violet. The curve for *Str. faecalis* (not shown) was similar to those for *Staph. aureus* and *B. subtilis*. At high pH values *Str. faecalis* was more resistant to the dye than *Staph. aureus* or *B. subtilis*.

EFFECT OF pH-ADJUSTED CRYSTAL VIOLET ON GROWTH

The effect of pH on the antibacterial action of crystal violet was determined by plotting extinction against log time in the presence of dye (test) and in the absence of dye (control) at different pH values. Fig. 2 shows the graphs for *E. coli* at pH 6·7.

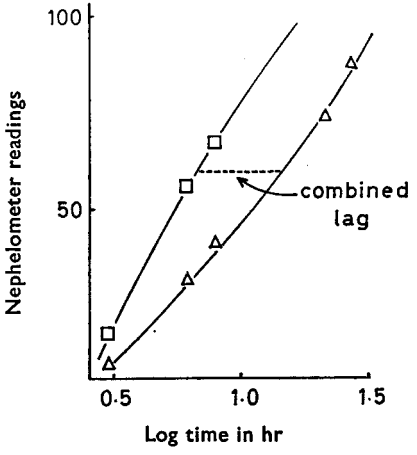


FIG. 2. Effect of $5{\cdot}7 \times 10^{-7}$M crystal violet on the growth of *E. coli* (△) in broth at pH 6·7. □ Control, in absence of dye.

When very low concentrations of dye were used there was little change in the antibacterial effect with pH at pH values below 7, but the effect was much greater at high pH values. For instance, *B. subtilis* in the presence of $1{\cdot}03 \times 10^{-7}$M crystal violet below pH 7 grew as quickly as in the control, but with $1{\cdot}29 \times 10^{-7}$M dye, growth was prevented at pH 8 and above.

Somewhat higher concentrations of crystal violet (2–5×) caused a prolonged lag phase followed by normal growth with *Str. faecalis* and *B. subtilis* at low pH values, while *E. coli* and *Staph. aureus* showed both a prolonged lag phase and an increased generation time. At high pH values growth was prevented.

A method of evaluating the antibacterial effect of crystal violet to include both prolonged lag and reduced growth was introduced. Extinction was plotted against log time for the test and control, and the difference in time between the two at a given arbitrary density I have termed "combined lag".

The Berry & Parkinson method (1955) required a rapidly growing culture giving an opacity in 3 or 4 hr even in the presence of a bacteriostat. The "combined lag" method is not confined to a stated time; it has the advantage that evaluation of bacteriostasis may be assessed in more

severe conditions, in this case at higher pH values and with higher concentrations of dye. A nephelometer reading of 60 was chosen as indicative of appreciable growth, but similar results were obtained if other readings were chosen for comparison of the test and control media.

The combined lag was plotted against pH for each organism (Fig. 3). The increase in combined lag with rise in pH corroborates the results shown in Fig. 1. The curve for *Staph. aureus* was similar to that for *Str. faecalis*.

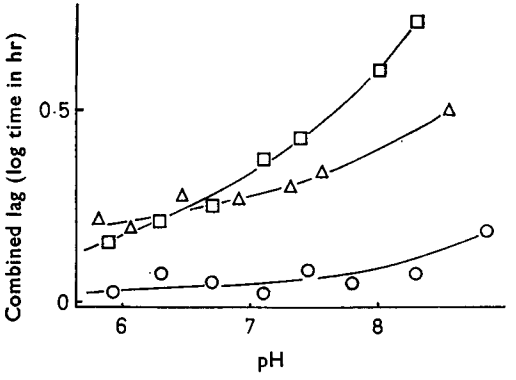


FIG. 3. Effect of pH of broth-dye medium on combined lag produced by crystal violet. △ *Str. faecalis* with $0{\cdot}80 \times 10^{-7}$M dye, □ *E. coli* with $3{\cdot}8 \times 10^{-7}$M dye, ○ *B. subtilis* with $1{\cdot}03 \times 10^{-7}$M dye.

Although buffer was present, during growth of the organisms the pH of the system, particularly when at high initial values, tended towards neutrality. This change was as great as a fall of 0·7 unit from pH 9 with *Str. faecalis*. McCalla (1941) found that crystal violet displaced hydrogen ions from bacteria, which would cause a slight lowering of pH. A reduction in pH would partially antagonize inhibition by crystal violet, and this would necessitate a higher concentration to cause the same inhibitory effect (Fig. 1).

*B. subtilis* spores germinated normally in nutrient broth containing crystal violet, but the vegetative cells formed from the spores were then inhibited or killed.

### ANTAGONISM OF ANTIBACTERIAL ACTION OF CRYSTAL VIOLET BY OXIDIZING AND REDUCING AGENTS

Small amounts of hydrogen peroxide, thioglycollic acid, or methylene blue were added to nutrient broth containing $3{\cdot}5 \times 10^{-5}$M crystal violet. The tubes were inoculated with *E. coli* and observed for growth at 37°. Table 1 shows that no growth occurred in the presence of the dye alone, but when hydrogen peroxide, thioglycollic acid or methylene blue were added the organisms were able to grow. Larger concentrations of hydrogen peroxide or thioglycollic acid prevented growth. Similar results were found with *B. subtilis* using a lower concentration of dye.

TABLE 1. THE EFFECT OF OXIDIZING AND REDUCING AGENTS ON THE ANTIBACTERIAL ACTION OF CRYSTAL VIOLET TOWARDS *E. coli*, AND ON THE REDOX POTENTIALS

| Medium | Growth* | Redox potential (V) |
|---|---|---|
| Nutrient broth .. .. .. .. | + | 0·320 |
| Broth + $3·5 \times 10^{-5}$M crystal violet .. | − | 0·310 |
| Broth + $3·2 \times 10^{-4}$ $H_2O_2$ .. .. | + | 0·400 |
| Broth + dye + $H_2O_2$ .. .. .. | + | 0·405 |
| Broth + $5·7 \times 10^{-4}$M thioglycollic acid | + | 0·120 |
| Broth + dye + thioglycollic acid .. | + | 0·125 |
| Broth + $9·4 \times 10^{-6}$M methylene blue | + | 0·305 |
| Broth + dye + methylene blue .. | + | 0·315 |

* After incubation at 37°, 48 hr: + growth, − no growth.

## Discussion

The extreme susceptibility of the antibacterial activity of crystal violet to pH change made evaluation difficult. At low pH values growth was virtually unaffected, while at high values growth was prevented, e.g. the concentration required to prevent growth of *B. subtilis* at pH 5·6 was 800 times that required at pH 8·7.

The pH of the medium affects the degree of ionization of crystal violet, but because it is more than 90% ionized ($pK_a = 9·3$) over the range 5·6–8·3 (Albert, 1965b) the increase in inhibitory activity with rise in pH is not due to the increase in the number of dye cations.

*E. coli* was the most resistant organism examined. The other species were similar in their susceptibility, but *Staph. aureus* was the least resistant at the highest and lowest pH values used (Fig. 1). Gram-positive organisms such as staphylococci possess more acidic components than Gram-negative bacteria such as *E. coli*, so that the former would combine more readily than the latter with basic dyes. This gave rise to the hypothesis of Stearn & Stearn (1928) that the antibacterial action of the dye was due to the formation of an unionized complex of a cell constituent with the dye.

Those bacteria with a higher isoelectric point, e.g. *E. coli* (5·5), should therefore be more resistant than organisms with lower isoelectric points, e.g. the other three species (1·8–3·0), which is so. This theory can be extended to include the effect of pH. As the pH of the medium is increased the negative charge on bacteria is increased, and this would result in an increase in sensitivity towards crystal violet. Albert (1965a) supposed that *E. coli* possessed anionic groups on its surface with a $pK_a$ of 9 or higher. At pH 6 the anionic groups would be so weakly ionized that combination with crystal violet cations would be negligible, but at pH 8 the anionic groups would be 10% ionized and combination with dye cations would readily occur. This would explain the relative antibacterial effect of crystal violet at these two pH values shown above.

Bacterial growth in broth results in a lowering of the oxidation-reduction potential; this occurred at all pH values, the potential falling by as much as 0·21 V. Dubos (1929) and Ingraham (1933) supposed that dyes

produced bacteriostasis by poising the medium at a potential unsuitable for growth. This is unlikely because crystal violet is bacteriostatic at very low concentrations, and when small amounts of thioglycollic acid or hydrogen peroxide are present in broth the addition of bacteriostatic concentrations of crystal violet causes very little change in potential (Table 1). According to Dubos the potential caused by hydrogen peroxide (0·400 V) should be reduced in the presence of dye, but it was 0·405 V; the potential caused by thioglycollic acid (0·120 V) should be raised in the presence of dye, but it was only 0·125 V.

The antagonistic action of methylene blue towards crystal violet may be explained by supposing that crystal violet interferes with the uptake of hydrogen ions by a coenzyme in the bacterial cell which normally accepts hydrogen ions from a substrate. Methylene blue acts as an alternative hydrogen acceptor and so permits oxidation of the substrate and growth of the bacteria. Fischer & Munoz (1947) suggested that crystal violet might block important biological mechanisms, possibly connected with oxidation processes.

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