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Effect of organic matter and increased inoculum size on the minimum inhibitory concentration of crystal violet towards *Staphylococcus aureus* at various pH values

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The minimum inhibitory concentration towards *Staphylococcus aureus* of crystal violet at pH values 6, 7 and 8, increased when the inoculum size was increased or if egg albumen was added to the medium. When allowance was made for dye uptake, the minimum inhibitory concentration still exceeded that in the absence of organic matter. The increased minimum inhibitory concentration in the presence of muscle could in most cases be attributed to the removal of dye from solution.

It is well known that the presence of organic matter reduces the antibacterial activity of many substances, including that of basic dyes (Graham-Smith, 1919). The higher the concentration of peptone for example in the growth medium, the less effective are dyes against bacteria (Kligler, 1918).

It has also been shown by Churchman & Kahn (1921) that, although single cells of *Escherichia coli* do not grow on crystal violet agar, groups of 30 cells are able so to do —a phenomenon they called "communal activity" of bacteria.

Small pieces of animal tissue reduce the bactericidal action of serum-dye mixtures (Wels, 1922), while serum, yeast or large inocula diminish the effect of crystal violet, presumably due to combination with the dye (Stearn & Stearn, 1924).

I have carried out experiments to determine whether or not the increased minimum inhibitory concentration of crystal violet towards *Staph. aureus*, in the presence of organic matter, was due to absorption by the organic matter.

EXPERIMENTAL

Materials

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

Nutrient broth was prepared from granules (Oxoid CM1) and 1% potassium dihydrogen phosphate (Analar) added, and the pH adjusted to the required value (6, 7 or 8) using a freshly prepared solution of potassium hydroxide.

Cultures were prepared from freeze-dried *Staphylococcus aureus* (NCTC 7447).

Meat muscle was prepared by macerating granules of cooked meat medium (Oxoid CM 81) in water, replacing the water several times until the supernatant liquid was colourless, and drying the separated muscle particles at 60°. 10 mg and 40 mg quantities were autoclaved in test-tubes containing 2 ml water, which was then decanted and the muscle used.

Albumen was removed aseptically from eggs and mixed with sterile nutrient broth.

Estimation of sorption of crystal violet

Bacteria. Organisms were grown on nutrient agar, removed with quarter-strength Ringer solution, washed and centrifuged and a weighed amount of moist bacteria mixed with broth. A total cell count was performed, and a measured volume of suspension added to tubes containing varying amounts of crystal violet solution; broth (at pH 6) was added to produce 5 ml. The mixture was equilibrated at 37° (30 min), centrifuged (10 min at 2300 *g*) and the extinction of the supernatant liquid determined at 591 nm. A second series of tubes was prepared without organisms to act as dye controls, and the extinction determined. The sorption of dye by the organisms was thus calculated, and the experiments were repeated using different volumes of suspension, and at pH 7 and at pH 8.

Muscle. Amounts of broth (10 ml, pH 6) were transferred to test-tubes containing muscle (10 mg) and varying amounts of dye added. After 48 h incubation at 37° the extinction of the supernatant liquid was determined. Another series of tubes was prepared without muscle to act as dye controls, and the extinction again determined. The sorption of dye by the muscle was calculated, and the experiments were repeated with 40 mg muscle, and also at pH 7 and at pH 8.

Albumen. Dye binding was determined by the equilibrium dialysis method (Adams, 1968). Quantities of broth (8 ml; pH 6) were transferred to glass bottles, and bags made from Visking dialysis tubing placed therein. Volumes of 2% w/v albumen solution (0·7 ml) were placed in each bag, varying amounts of dye solution added, and the volume adjusted to 8 ml with broth. The bottles were placed in a reciprocating water bath (24 h at 37°), and the extinction of the liquid in the bottles (outside the bags) determined. Identical dialysis experiments were prepared without albumen to act as dye controls, and the extinction determined. The binding of dye to albumen was calculated, and the experiments were repeated with 2 ml albumen solution, and at pH 7 and at pH 8.

Determination of minimum inhibitory concentration of crystal violet

Different amounts of crystal violet solution were added to 10 ml volumes of broth at pH 6, and inoculated with one drop (about 1/35 ml containing 2–3 $\times$ 10^6 organisms) of an overnight culture of *Staph. aureus*. The tubes were incubated (48 h at 37°) and examined for growth. The experiment was repeated with the addition of 10 mg and 40 mg muscle, 0·5 ml and 1·5 ml albumen solution (containing 10 and 30 mg dry weight albumen respectively), and with different inoculum sizes. The series of experiments was repeated at pH 7 and at pH 8.

RESULTS

The concentrations of crystal violet preventing growth (48 h at 37°) are given in Table 1. Table 2 shows the effect of inoculum size on the minimum inhibitory concentration of crystal violet.

Fig. 1 shows the equilibrium concentrations of dye at pH 6, resulting from different initial concentrations, in the presence of muscle and albumen. The relation between reduction in concentration of dye and equilibrium concentration is shown in Fig. 2. The extent of reduction in concentration of dye by organic matter, under the conditions of the determination of the minimum inhibitory concentration, was calculated as follows. The initial concentration of dye in solution is known from the amount

Table 1. *Effect of organic matter on minimum inhibitory concentration of crystal violet towards* Staph. aureus (*incubated* 48 *h at* 37°).

	With organic matter added to 10 ml broth								
		Conc. of dye × 10^{-6}M				Conc. of dye × 10^{-6}M			
pH	Muscle (mg)	Initial	Uptake	Free dye	Albumen (mg)	Initial	Uptake	Free dye	Without added organic matter
6	10	0·65	0·30	0·35	10	0·59	0·08	0·51	0·40
	40	2·01	1·45	0·56	30	1·40	0·33	1·07	
7	10	0·40	0·29	0·11	10	0·41	0·02	0·39	0·25
	40	1·62	1·35	0·27	30	1·06	0·28	0·78	
8	10	0·056	0·035	0·021	10	0·141	0·015	0·126	0·035
	40	0·147	0·115	0·032	30	0·81	0·23	0·58	

Table 2. *Concentrations of crystal violet* (× 10^{-6}M) *preventing growth of different inoculum sizes of* Staph. aureus (washed) *for* 48 *h at* 37°

	Crystal violet concentrations (× 10^{-6}M) for inoculum sizes of: (× 10^{6}/ml)			
pH	15	1·5	0·15	0·015
6	1·44	0·45	0·29	0·25
7	0·75	0·17	0·13	0·083
8	0·67	0·061	0·041	0·023

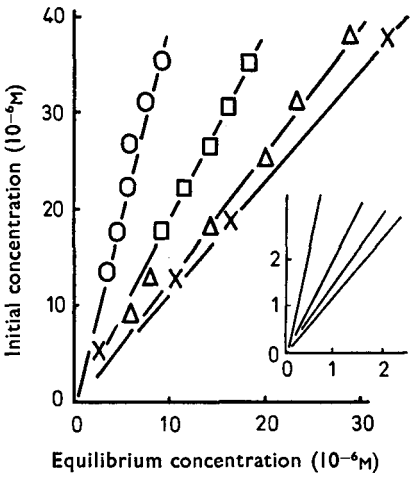

FIG. 1. Equilibrium concentrations of crystal violet produced by different initial concentrations in presence of muscle and albumen at pH 6. ○, 40 mg muscle; □, 10 mg muscle; △, 30 mg albumen; × 10 mg albumen. Inset: enlarged lower portion of graph.

added, and the corresponding equilibrium concentration is found from Fig. 1. The reduction in concentration of dye is obtained from Fig. 2, and this value is subtracted from the initial concentration to give the residual or free dye in solution (Table 1), e.g. at pH 6, in the presence of 40 mg muscle, the minimum inhibitory concentration was 2·01 × 10^{-6}M. From Fig. 1 the equilibrium concentration is 0·47 × 10^{-6}M, corresponding to a reduction in concentration of 1·45 × 10^{-6}M (Fig. 2), leaving a concentration of free dye of 0·56 × 10^{-6}M.

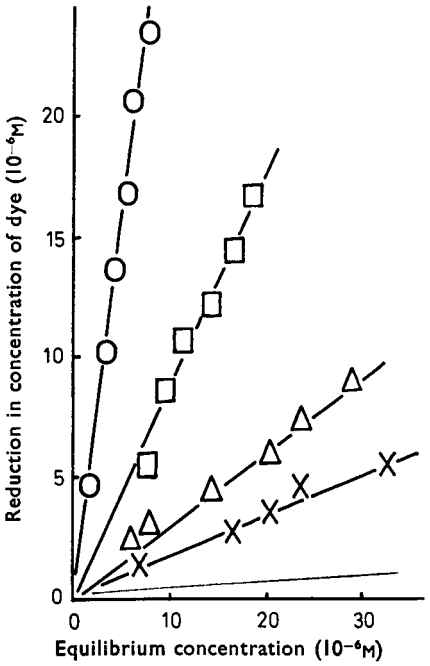

FIG. 2. Reduction in concentration of crystal violet produced by muscle, albumen and bacteria at different equilibrium concentrations at pH 6. ○, 40 mg muscle; □, 10 mg muscle; △, 30 mg albumen; × 10 mg albumen, plain line bacteria.

The graphs for dye sorption by muscle and by albumen at pH 7 and pH 8 were similar to those at pH 6, except that uptake by muscle was greatest at pH 7, slightly less at pH 8, and was least at pH 6; the amount of dye bound by albumen was greatest at pH 8 and least at pH 6.

Dye sorption by *Staph. aureus* itself was difficult to measure since the inoculum size used was so small as to be negligible. Sorption of crystal violet by mg amounts of bacteria is considerable (Adams, 1967), but in the present work it was necessary to extrapolate the results to the smaller inocula used. Fig. 2 includes an approximate indication of the dye uptake at pH 6 (which is similar to that at pH 7 and 8).

DISCUSSION

The addition of 10 mg muscle to 10 ml broth increases the minimum inhibitory concentration *ca* 1·5 ×, and 40 mg 4–6·5 ×, and the effect is similar at the three pH values. Muscle removes crystal violet from aqueous solution slowly but continuously, probably both by absorption by muscle fibres and by reduction by unsaturated compounds such as linoleic acid, which explains its use in anaerobic media. Up to 75% of the dye is removed from solution within 48 h at 37°, e.g. when 10 mg is incubated in 10 ml broth at pH 7 containing $0{\cdot}40 \times 10^{-6}$M dye the concentration of free dye is reduced to $0{\cdot}11 \times 10^{-6}$M, which is below the minimum inhibitory concentration in the absence of muscle, viz. $0{\cdot}25 \times 10$—^{6}M. The concentration of free dye is not always below the minimum inhibitory concentration, but it is never much higher. Except at pH 6 with 40 mg muscle, the increased minimum inhibitory concentration in the presence of muscle can be attributed to the removal of dye from solution.

Under the conditions used, 10 mg albumen binds 5–13% of the dye present, and 30 mg binds 23–28%. Albumen reduces the effect of pH on the minimum inhibitory concentration, e.g. the ratio of the minimum inhibitory concentrations at pH 6 and pH 8 is 11 in the absence, but less than 2 in the presence, of 30 mg albumen. The concentration of free dye is usually much greater than that in the absence of albumen, so that in some way albumen must protect the organisms, in addition to combining with a portion of the dye. Indeed Sykes (1965) has already suggested that the reduction in efficiency of disinfectants in the presence of organic matter was "not so much through the inactivation of disinfectant as through protection of the bacteria". It is possible that the increased viscosity caused by the albumen assists growth of the organism, e.g. by facilitating build-up of CO_2 or by lowering the redox potential.

Increased inoculum size raises considerably the minimum inhibitory concentration of crystal violet. This applies especially at pH 8, the pH at which the organisms are most sensitive. Thus at pH 6 the minimum inhibitory concentration is raised about 6 × by a thousand-fold increase in inoculum size, while at pH 8 the figure is 30 ×. A large inoculum reduces the pH effect of crystal violet; thus the minimum inhibitory concentration at pH 6 is only double that at pH 8 using the largest inoculum, while 10 × the concentration of dye is required to inhibit growth at pH 6 compared with that at pH 8 using the smallest inoculum. This is similar to the effect of albumen discussed above. Churchman and Kahn (1921) observed that a large inoculum of *E. coli* could grow in the presence of crystal violet for some reason other than that of a lowered concentration due to uptake of dye by the bacteria. In the present work the uptake of dye was negligible, and it seems that growth in the presence of dye occurred by some "communal activity" of the organisms.

REFERENCES

ADAMS, E. (1967). *J. Pharm. Pharmac.*, **19**, *Suppl.*, 203S–208S.
ADAMS, E. (1968). *Ibid.*, **20**, *Suppl.*, 18S–22S.
CHURCHMAN, J. W. & KAHN, M. C. (1921). *J. exp. Med.*, **33**, 583–591.
GRAHAM SMITH, G. S. (1919). *J. Hyg., Camb.*, **18**, 1–32.
KLIGLER, I. J. (1918). *J. exp. Med.*, **27**, 463–478.
STEARN, A. E. & STEARN, E. W. (1924). *J. Bact.*, **9**, 491–510.
SYKES, G. (1965). *Disinfection and Sterilisation*, London: Spon.
WELS, P. (1922). Through Stearn & Stearn (1924).