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Binding of crystal violet by nucleic acids of *Escherichia coli*

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The absorption isotherms of DNA and RNA from *E. coli* for crystal violet are sigmoid. Binding of dye was determined i) by difference in its absorbance before and after addition of nucleic acids and ii) by measuring the amount of dye dialysing through a membrane containing dye and nucleic acid. pH change caused no significant change in the degree of binding.

IN the presence of nucleic acids, basic dyes have been observed to undergo changes in their ultraviolet visible spectrum (Michaelis, 1947).

A non-linear relation between the extent of binding by DNA and the equilibrium concentration of rosaniline has been reported by Cavalieri & Angelos (1950), the process being reversible. Peacocke & Skerrett (1956) observed strong interaction between amino-acridine dyes and nucleic acids from herring sperm and *Aerobacter aerogenes*, the binding being independent of pH over the range 3·7 to 7·4.

The present paper reports work on the binding of crystal violet by nucleic acids of *Escherichia coli*.

Experimental

REAGENTS

Saline–EDTA, 0·15M sodium chloride and 0·1M EDTA, adjusted to pH 7 with sodium hydroxide. Acetate–EDTA, 3M sodium acetate and 0·001M EDTA. Concentrated saline-citrate, 1·5M sodium chloride and 0·15M sodium citrate. For dilute saline-citrate this was diluted 1 in 100. All chemicals were of laboratory reagent quality. Crystal violet B.P. was recrystallized from ethanol and a 0·01M aqueous solution prepared.

EXTRACTION OF NUCLEIC ACIDS FROM *E. coli*

Several methods have been described for the extraction of nucleic acids from bacteria (Marmur, 1961; Smith & Burton, 1965; Kirby, Fox-Carter & Guest, 1967). The method of Marmur, primarily devised for the extraction of DNA, was modified as follows.

E. coli 1 was grown in bottles (37°; 24 hr) on the surface of a nutrient agar layer (Oxoid blood agar base, No. 2, CM 271, with additional agar, Oxoid No. 1 L 11). The growth was washed off with quarter-strength Ringer solution, the organisms were centrifuged off, then washed with saline–EDTA to remove metal ions (Mg) which activate nucleases. They were suspended in saline–EDTA to form a suspension free from clumps.

Sodium lauryl sulphate (25% in water; 1 ml/g moist weight organisms) was added and the mixture heated (10 min) at 60° to cause lysis. The suspension increased in viscosity and showed partial clearing; it was

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cooled to room temperature, and one fifth volume of 2·5M sodium chloride solution added. The mixture was shaken (30 min) with an equal volume of chloroform–amyl alcohol (24 : 1), and centrifuged (5 min) at 5,000 *g* when three layers were formed. The uppermost (aqueous) layer was carefully withdrawn and an equal volume of absolute ethanol added with stirring, when the nucleo-proteins "spooled" on to the glass rod.

The nucleoproteins were lightly pressed against the side of the beaker to remove excess ethanol, and quickly dissolved in dilute saline–citrate (1 ml/g moist weight organisms). One tenth volume of concentrated saline–citrate was added to the solution, which was shaken (15 min) with an equal volume of chloroform–amyl alcohol to remove protein. The mixture was centrifuged (5 min at 5,000 *g*) and the upper aqueous layer carefully removed, shaken with further aliquots of chloroform–amyl alcohol until no protein was visible as a whitish precipitate at the interface. The upper layer was carefully removed and solid sodium chloride added to produce a 4M solution to precipitate the RNA. This was removed by centrifuging (10 min at 5,000 *g*), washed with 4M sodium chloride solution, and dissolved in dilute saline–citrate solution (0·2 ml/g moist weight organisms). The RNA was precipitated therefrom with an equal volume of ethanol, as a light flocculent precipitate and then re-dissolved in dilute saline–citrate solution (0·2 ml/g moist weight organisms). All the above 4M sodium chloride solutions which contained the DNA were reserved and treated as follows.

Addition of absolute ethanol gave a DNA precipitate, which was dissolved in dilute saline–citrate solution (0·5 ml/g moist weight organisms). One tenth volume of acetate–EDTA solution was added, a small glass mechanical stirrer fitted and 0·6 volume isopropanol added slowly to the vortex, when DNA spooled on to the stirrer. This was dissolved in dilute saline–citrate and re-precipitated as above. The precipitate was washed with 70% aqueous ethanol, then with 90% aqueous ethanol, and dissolved in dilute saline–citrate, and adjusted to pH 8·0 with sodium hydroxide solution.

A few drops of chloroform were added to the nucleic acid solutions and these were stored at 4°, or the solutions were freeze-dried. Nitrogen and phosphorus determinations* were made. A sample of the DNA solution was heated to 60° and the absorbance at 260 mμ noted, to detect the presence, if any, of single-stranded DNA or of RNA.

DETERMINATION OF BINDING OF DYE BY NUCLEIC ACIDS

Difference-in-absorbance method. The absorption spectrum of an aqueous crystal violet solution was determined on a spectrophotometer between 450 and 650 mμ. This was repeated with the addition of varying amounts of DNA and RNA separately.

The absorbance of crystal violet solution at 591 mμ was determined

* By Mr. G. S. Crouch, School of Pharmacy, University of London, Brunswick Square, E.C.1. Nitrogen by a modified Schöniger method, phosphorus by perchloric acid digestion and molybdenum blue colorimetric finish.

at different pH values over the range 4 to 9. This was repeated after the addition of the nucleic acids.

Equilibrium dialysis method. Pieces of Visking dialysis tubing (4 in × $\frac{5}{8}$ inch diam.) were soaked in distilled water and tied at one end with thread to form a bag. A plastic ring was inserted into the open end, and they were allowed to dry. Glass bottles (3 in × 1 in diam.) were filled with dilute saline-citrate solution (8 ml) and the dialysis bags placed therein. The bags were then filled with known amounts of crystal violet and nucleic acid solutions, the volume being made up to 8 ml with dilute saline-citrate. The bottles were placed in an incubator (2 days; 25°) with occasional agitation. The concentration of crystal violet in the bottles was then determined by measurement of the absorbance at 591 mμ. The amount of dye bound by the nucleic acids was then calculated, allowance being made for the uptake of dye by the dialysis tubing and the bottles. The solution in the bottles was then replaced by an equal volume of fresh saline–citrate and the bags replaced. Dialysis was repeated, the concentration of dye in the bottles again determined, and the release of dye from combination with the nucleic acids calculated.

The experiment was repeated at different pH values using a constant dye concentration and buffer solutions. Dialysis tubing is permeable to crystal violet but not to nucleic acids.

Results

The yields of nucleic acids obtained by extraction of *E. coli* were as follows: DNA 4·1 mg dry weight/g moist weight organisms, $e(_p)_{260} = 9350$. Phosphorus 6·7%, nitrogen 12·7% (both based on dry weight). RNA 2·9 mg dry weight/g moist weight organisms, $e(_p)_{260} = 8040$. Phosphorus 6·8%, nitrogen 12·1%. $e(_p)_{260}$ is the molar absorptivity based on 1 g atom of P per litre.

DNA samples showed a slight increase in absorbance when heated at 60°, indicating the presence of a small amount of RNA or single-stranded DNA (Kirby & others, 1967).

Fig. 1 shows the absorption spectrum of crystal violet alone and in the presence of different amounts of DNA; an essentially similar picture was presented by RNA. Small amounts of nucleic acids reduce the absorbance of crystal violet, large amounts raise the absorbance, and the absorption peak is displaced from 591 to 598 mμ, except for small amounts of RNA.

Fig. 2 shows the absorbance at 591 mμ of crystal violet alone and with the addition of DNA or RNA at various pH values. The difference reflects the binding of the dye with nucleic acid.

Using the equilibrium dialysis method at different pH values the amount of dye bound showed little change with change in pH over the range 4 to 9·2.

Fig. 3 shows the amount of dye bound by the nucleic acids against the equilibrium concentration of dye. A specimen calculation is given in Table 1. A precipitate was produced by equilibrium concentrations above 50×10^{-6}M crystal violet. Retention curves followed approximately the binding curves.

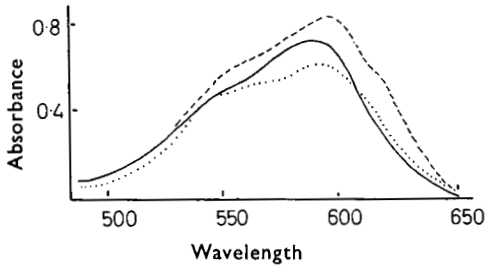

FIG. 1. Absorption spectrum of crystal violet alone, and in the presence of DNA. Full line, 2×10^{-5}M crystal violet; dotted line, dye with 8×10^{-5}M DNA; broken line, dye with $2{\cdot}7 \times 10^{-3}$M DNA.

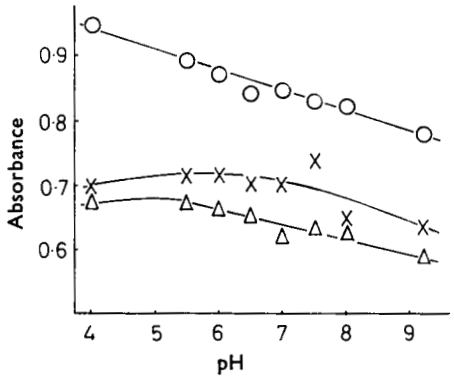

FIG. 2. Effect of pH on absorbance at 591 mμ of crystal violet alone and in the presence of nucleic acids of *E. coli*. ○ dye alone; × dye with DNA; △ dye with RNA shortly after mixing.

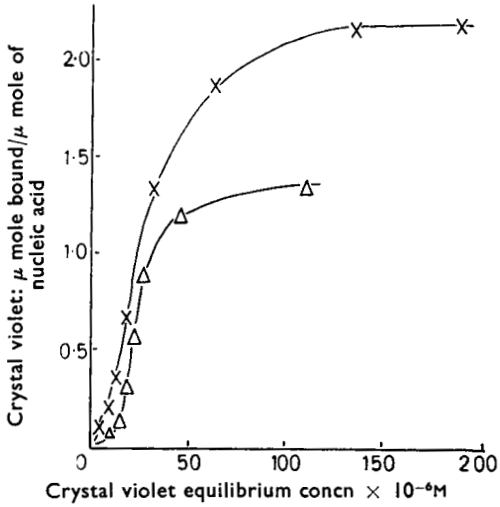

FIG. 3. Amount of crystal violet bound by nucleic acids plotted against equilibrium concentration of dye. × DNA, Δ RNA.

TABLE 1. SPECIMEN CALCULATION OF BINDING OF CRYSTAL VIOLET BY NUCLEIC ACIDS

a	b	c	d	e	f	g	h
Tube	Theoretical concentration of dye if no binding occurred ($\times 10^{-6}$M)	Amount DNA (μmole)	Equilibrium concentration of dye ($\times 10^{-6}$M)	Difference between theoretical and equilibrium concentration of dye (b − d)	Amount of dye taken up by bag and bottle (μmole)*	Amount of dye bound by DNA (μmole)	Amount of dye bound per μmole DNA (μmole) (g/c)
A	12·5	2·65	3·4	9·1	0·0025	$\frac{(9{\cdot}1 \times 16)}{1000} - 0{\cdot}0025$	0·054
D	125	2·65	19·6	105·4	0·0110	$\frac{(105{\cdot}4 \times 16)}{1000} - 0{\cdot}0110$	0·632

* Separately calibrated.
Note. 1 ml of 10^{-6}M contains $1/1000 \times 10^{-6}$ mole.

Discussion

The displacement of the absorption peak of crystal violet to longer wavelengths in the presence of nucleic acids indicates a more polar environment, and suggests complexing between crystal violet and the nucleic acids. A similar phenomenon has been observed with proflavine and herring sperm DNA (Peacocke & Skerrett, 1956), and with crystal violet and bovine serum albumin (Blei, 1957). Like the sorption of crystal violet by whole *E. coli* organisms (Adams, 1967), the absorption isotherms of crystal violet for the nucleic acids were sigmoid, suggesting that Langmuir's law did not apply. Similar results are reported by Peacocke & Skerrett (1956) for proflavine.

Binding of crystal violet approaches a maximum at *ca* 2 mmole dye/mmole DNA, and at *ca* 1·2 mmole/mmole RNA, and precipitates are formed. Experiments showed the binding to be largely irreversible, and to be unaffected by changes of pH over the range 4 to 9.

Peacocke & Skerrett (1956) found little effect of pH over the range 4·7 to 7·4 in the binding of proflavine by the nucleic acids of *Aerobacter aerogenes*.

Since no change in the binding of dye to nucleic acid at pH values between 4 and 9 can be demonstrated, the increase in antibacterial activity at pH 8 and above must be ascribed to other causes.

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