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The absorption by *Escherichia coli* of phenols and their bactericidal activity

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The uptake of several phenols by *E. coli* may be represented by absorption isotherms. The uptake pattern for dilute solutions suggests partitioning between the cells and aqueous solution but at higher concentrations a change in the uptake pattern suggests protein precipitation. An inflexion in the absorption isotherms corresponds with concentrations producing high levels of activity and can be used to forecast concentrations needed to produce rapid rates of kill.

EARLY studies of the kinetics of disinfection established a correlation between the concentration of a bactericide in aqueous solution and the rate of disinfection of a test culture (Chick, 1908) which suggested that the bactericidal reaction is either a true monomolecular reaction or that it simulates a monomolecular reaction. It is known (Nernst, 1917) that reactions in heterogeneous systems are regulated by diffusion and that the kinetics of such reactions always resemble those of a monomolecular reaction. Thus, greater understanding of the bactericidal reaction depends upon studies at the cellular rather than the bulk phase level and this has been the trend in recent years. Salton (1951) has related the bactericidal activity of cetrimide against *Staphylococcus aureus* to its uptake by the cells, whilst Newton (1954) found that cell walls prepared from sensitive organisms absorbed several times as much polymyxin as those prepared from resistant organisms. Few & Schulman (1953) found marked differences in the absorption isotherms for polymyxin-sensitive and polymyxin-resistant organisms. The present communication represents a further attempt to relate the shape of isotherms describing the uptake of phenols by *Escherichia coli* to the bactericidal activity.

Experimental

MATERIALS

Bactericides. The phenolic bactericides were of Analar or Laboratory Reagent quality but were recrystallised or redistilled to the following characteristics. Phenol, m.p. 42·5°; *o*-chlorophenol, b.p. 175°; *m*-cresol b.p. 202°; chlorocresol, m.p. 65°; thymol, m.p. 49·5°; resorcinol, m.p. 109°; hexylresorcinol, b.p. 176°; benzylchlorophenol (5-chloro-2-hydroxydiphenylmethane), m.p. 47°.

Test organism. Escherichia coli (NCTC No 5933), maintained by freeze-drying from peptone solution. *Nutrient agar* for viable counts contained 1% peptone (Oxoid) and 0·5% NaCl gelled by 2% Kobé No 1 Agar, pH 7·4.

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METHODS

Evaluation of bactericidal activity. Freeze-dried *E. coli* were resuspended in distilled water, the suspension was passed through 3 successive 24-hr slope cultures, washed in sterile water and resuspended to a total count of 40×10^9 organisms per ml.

The reaction mixtures, containing $1{\cdot}4 \times 10^8$ *E. coli* per ml, were maintained at 25°. Samples were removed at intervals and diluted with ¼-strength Ringer solution; an appropriate number of standard drops of the dilution were added to 2·0 ml molten nutrient agar at 45°, rolled until solidified and incubated (48 hr) at 37°.

Estimation of uptake of phenols by E. coli. The method used was that described by Beckett, Patki & Robinson (1959a).

Preliminary experiments determined that a suspension of 45×10^9 *E. coli* per ml in 1% solution of phenol absorbed about 10% of the initial phenol. Experiments also revealed a negligible difference between the uptake by resting cells and the same number of freeze-dried cells resuspended in the phenol solutions; because of advantages in handling freeze-dried cells, they were used for the uptake studies.

A phenol solution of known concentration (2 ml) was added at 25° to 45×10^9 *E. coli* freeze-dried cells and equilibrated (10 min) at 25°. The cells were then centrifuged (10 min) at 8,500 *g* after which 1 ml supernatant was removed by means of a clean, dry Agla syringe. 0·5 ml was run into a 100 ml flask and made up to volume with phosphate buffer at pH 6·0. The solution was assayed spectrophotometrically at 270 mμ, using 4 cm cells. The reading (A) represented phenol + exudate.

A further 0·5 ml supernatant was shaken in a separator with 5 ml Analar chloroform and 5 ml phosphate buffer at pH 6·0, extraction being completed with 8×5 ml chloroform into which the phenol partitioned. The combined (40 ml) chloroform volumes were washed with 2×3 ml distilled water, the aqueous washings being added to the aqueous phase remaining in the separator.

The aqueous phase was then heated in a beaker to remove traces of chloroform, cooled, adjusted to 100 ml with buffer and assayed at 270 mμ. This reading (B) represented exudate.

A—B is then the actual phenol remaining (C) in the supernatant after contact with cells.

Chloroform used in the extractions was checked for zero absorbance at 270 mμ against water. Replicate assays of a given supernatant solution showed that estimates of residual phenol were reproducible within ±1·5% of the mean value obtained.

With the higher molecular weight compounds a smaller number of organisms per ml gave a measurable uptake, the actual density of cells used being recorded on the uptake isotherms.

Results

VIABLE COUNTS

An estimate of the overall accuracy of the viable counting technique was made by counting 20 roll tubes prepared from a suspension of *E.*

coli in $\frac{1}{4}$-strength Ringer solution. This gave a value of $\chi^2 = 12{\cdot}35$ which corresponds with P = 0·8–0·9. This was considered satisfactory.

The same test applied to *E. coli*/bactericide reaction mixtures indicated an increase in χ^2 values as the reaction proceeded. Mortalities in excess of 90% tended to produce high values of χ^2 which corresponded with low probabilities. High values of χ^2 may have been due to sampling reaction mixtures containing small numbers of randomly distributed organisms or to a tendency for survivors to aggregate in clumps. Errors in extinction time methods of evaluating bactericidal activity have in fact been attributed to sampling mixtures containing clumped organisms (Berry & Bean, 1954). For this reason, the activities of different bactericides were compared at a mortality level below 90%.

The probit mortality-log time regressions for the death of *E. coli* in each of the bactericides (not illustrated) had distinct "breaks" or inflexions between probits 4·5 and 5·5 as a result of which the regressions were bilinear; *t*-tests showed the slopes of the two parts of the regressions to be significantly different.

Analyses of variance showed the probit-mortality-log time regressions for 10 replicate experiments with one concentration of a phenol to be both linear and parallel for mortalities in excess of that at which the "break" occurred, but not parallel nor necessarily linear for mortalities below the "break." The phenols were therefore compared at a mortality level between 50 and 90%, the level ultimately selected being the mortality corresponding to probit 6·0, i.e. 84·13% (LT84).

COMPARATIVE BACTERICIDAL ACTIVITY OF DIFFERENT PHENOLS

The comparative bactericidal activities of the eight phenols are represented in Fig. 1 where log concentration is plotted against LT84. The regressions in Fig. 1 were extrapolated to intercept the abscissa at

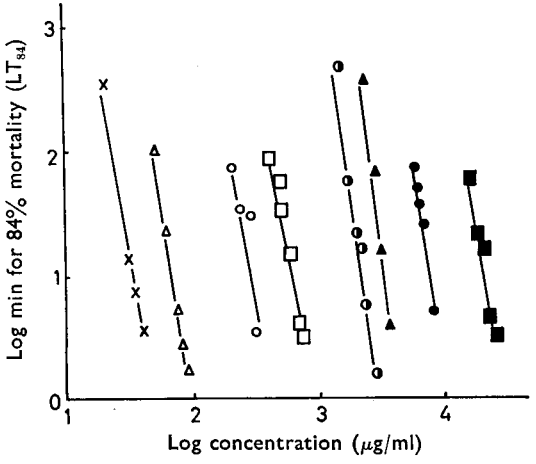

FIG. 1. Bactericidal activity of phenols against *E. coli*. ■, Resorcinol. ●, Phenol. ▲, *m*-Cresol. ◑, *o*-Chlorophenol. □, Chlorocresol. ○, Thymol. △, Hexylresorcinol. ×, Benzylchlorophenol.

a concentration equivalent log LT84 = 0. This represents a mortality of 84% in 1 min and we have called this the *maximum effective concentration* since more rapid reactions are difficult to assay with reasonable certainty. It was used as the basis for comparing the different bactericides.

UPTAKE OF PHENOLS BY *E. coli*

The uptake of phenols by the bacterial cells is represented by absorption isotherms which are shown in Fig. 2. Seven of the eight isotherms are similar in shape and appear to resemble most closely the type S isotherm of Giles, MacEwan, Nakhwa & Smith (1960). However, instead of being continuously curvilinear like the type S isotherms they are bilinear, with the proximal portion being almost perfectly linear. The initial concentration of the bulk solution (Table 1) which corresponded with the "break" or inflexion on the isotherm is calculated by summing the equilibrium concentration (abscissae in Fig. 2) at the inflexion and the amount that had been taken up by the cells (ordinates in Fig. 2). The shape of the absorption isotherm for resorcinol was quite different and resembled the Giles isotherm type L. No inflexion occurred in the resorcinol isotherm.

TABLE 1. RELATIONSHIP BETWEEN "MAXIMUM EFFECTIVE CONCENTRATION"* OF PHENOLS AND CONCENTRATION AT INFLEXION IN ABSORPTION ISOTHERM

Compound	Maximum effective concentration* μg/ml (A)	Concentration at inflexion in absorption isotherm μg/ml (B)	Ratio $\frac{A}{B}$
Resorcinol	$3{\cdot}44 \times 10^4$	—	
Phenol	$1{\cdot}18 \times 10^4$	$1{\cdot}20 \times 10^4$	0·98
m-Cresol	$4{\cdot}28 \times 10^3$	$3{\cdot}25 \times 10^3$	1·32
o-Chlorophenol	$3{\cdot}40 \times 10^3$	$3{\cdot}10 \times 10^3$	1·10
Chlorocresol	$1{\cdot}12 \times 10^3$	$1{\cdot}00 \times 10^3$	1·12
Thymol	$4{\cdot}33 \times 10^2$	$6{\cdot}00 \times 10^2$	0·72
Hexylresorcinol	$1{\cdot}25 \times 10^2$	$1{\cdot}06 \times 10^2$	1·18
Benzylchlorophenol	$5{\cdot}60 \times 10$	$5{\cdot}90 \times 10$	0·95
Mean			1·05

* Defined as "A mortality of 84% in 1 min."

Discussion

A linear probit-mortality-log time regression representing the death of bacteria in a bactericide indicates that the death-rate of the organisms is a function of their resistance. Departure from linearity may indicate a mixed bacterial population or that death is the result of more than one cause or mechanism. For example, in the early stages of the reaction between phenols and *E. coli*, cell exudates appear in the supernatant liquors (Bean & Walters, 1955) and at a later stage the opacity of the cells increases (Beckett, Patki & Robinson, 1959b).

Acceptance of the vitalistic explanation of the course of the bactericidal reaction ignores the observation that when the reaction between a phenol and *E. coli* causes a mortality of more than 99%, the survivors may multiply in what was initially a bactericidal system devoid of intentionally added nutrients (Bean & Walters, 1955). The nutrient material consists of

exudates which are produced during the normal metabolism of the cells: they appear in greater quantity however because of the action of the bactericide.

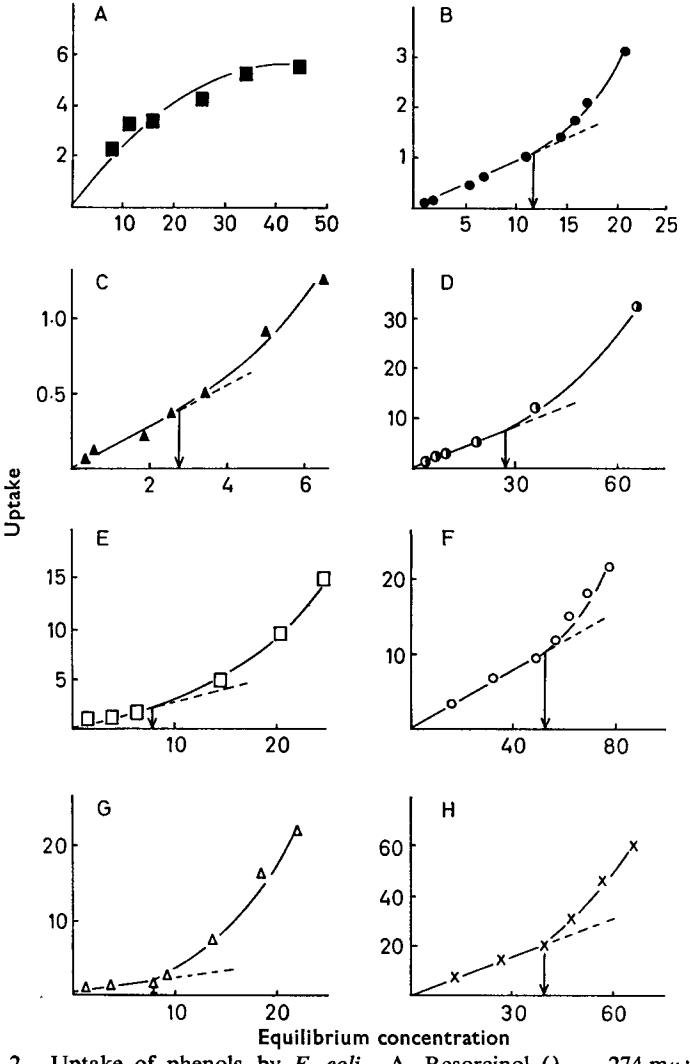

FIG. 2. Uptake of phenols by *E. coli*. A. Resorcinol (λ_{max} 274 mμ; ϵ 926·3). Equilibrium concentration and uptake both $\times 10^{-3}$ μg/ml, by 13×10^{11} *E. coli*. B. Phenol (λ_{max} 270 mμ; ϵ 1496·2). Equilibrium concentration and uptake $\times 10^{-3}$ μg/ml, by 45×10^{9} *E. coli*. C. *m*-Cresol (λ_{max} 271 mμ; ϵ 1445·3). Equilibrium concentration and uptake $\times 10^{-3}$ μg/ml, by 40×19^{9} *E. coli*. D. *o*-Chlorophenol (λ_{max} 273 mμ; ϵ 779·8). Equilibrium concentration and uptake $\times 10^{-2}$ μg/ml, by 65×10^{9} *E. coli*. E. Chlorocresol (λ_{max} 280 mμ; ϵ 1540·1). Equilibrium concentration and uptake $\times 10^{-2}$ μg/ml, by 20×10^{9} *E. coli*. F. Thymol (λ_{max} 274 mμ; ϵ 1830·6). Equilibrium concentration and uptake $\times 10^{-1}$ μg/ml, by 20×10^{9} *E. coli*. G. Hexylresorcinol (λ_{max} 279·5mμ; ϵ 2564·2). Equilibrium concentration and uptake $\times 10^{-1}$ μg/ml, by 4×10^{9} *E. coli*. H. Benzylchlorophenol (λ_{max} 282 mμ; ϵ 2230·5). Equilibrium concentration and uptake, μg/ml, by 2×10^{9} *E. coli*.

For each of the phenols employed in the present experiments, a plot of LT84 against logarithm of the concentration yielded a regression line which was approximately linear (Fig. 1), the slope of the line being the concentration exponent of the phenol. A good fit to linearity was held by earlier workers to indicate that the bactericidal reaction was a monomolecular reaction.

With the exception of that for resorcinol, the isotherms for the uptake of phenols by *E. coli* all resemble the type S isotherm of Giles & others (1960). The linear proximal sections indicate that the amount taken up is proportional to the initial concentration of the bulk aqueous phase and suggest an uptake mechanism which behaves as though the phenol partitioned between two phases, e.g. the external phase and biophase.

At a concentration which is a characteristic of each of the seven phenols, the absorption isotherms indicate a sudden marked increase in the uptake of phenol by the cells, and this continues to rise with increasing bulk concentration. With phenol itself this concentration is the same as the "protein precipitating concentration" recorded by Cooper (1912) and Cooper & Woodhouse (1923). Certainly, at this concentration an increase was noted in the opacity of the bacterial suspension, as indicating a precipitation or coagulation of bacterial proteins.

Giles & MacEwan (1957) describe an adsorption isotherm in which the initial portion is linear, and suggest it represents a condition in which the number of sites available for adsorption remains constant, even though the amount of solute adsorbed increases. They also suggest that this type of uptake occurs when the substrate structure is opened up by the solute, and liken the process of adsorption to the opening of a zip-fastener. Presumably when phenol enters a bacterial cell the hydrogen bonds of the cellular proteins are broken, permitting the unfolding of the chains and the uptake of a greater quantity of phenol. Evidence is offered by Putnam & Neurath (1944) of the unfolding of protein side-chains on the interaction of sodium dodecyl sulphate and serum albumin. In the bactericidal system this mechanism presumably continues up to the concentration represented by the point of inflexion in the absorption isotherm. Above this concentration phase separation takes place or a non-stoichiometric complex is formed (Putnam & Neurath, 1944).

With resorcinol the uptake isotherm resembles the type L isotherm of Giles & others (1960) and indicates a different mechanism or a different type of affinity for the bacterial cell. Undoubtedly the resorcinol isotherm is a reflection of its extreme water-solubility and therefore a different cell-water partition coefficient. Saturation of the bacterial cell is easily attained with resorcinol but not with the remaining seven phenols and this suggests a certain lack of affinity of the cellular lipoproteins for resorcinol.

COMPARISON OF UPTAKE DATA AND MAXIMUM EFFECTIVE CONCENTRATION

For each of the phenols—except resorcinol—the initial bulk concentration at which the inflexion was observed in the uptake isotherm is compared with the *maximum effective concentration* (Table 1). The approxi-

mate constant ratio of the *maximum effective concentration* and concentration of the point of inflexion on the uptake isotherm is in support of the observations of Cooper (1912; 1913) and Cooper & Woodhouse (1923) who record a relation between the bactericidal activities of several phenols and the concentrations at which they precipitate proteins. Cooper & Woodhouse also observed a change in the protein/water partition coefficient at the concentration at which they precipitate the protein, which again appears to be in line with the marked increase in the uptake of the phenols by the cells at the maximum effective concentration.

At approximately the concentration at which the inflexion appears on the phenol absorption isotherm, Loveday & James (1957) observed a change in the electrophoretic mobility of *Aerobacter aerogenes*. They interpreted this as indicating surface saturation of the cells by phenol. In view of the several independent observations on the relation between bactericidal activity and uptake by the cell of bactericide molecules, the point of inflexion on the uptake isotherms may be regarded as an iso-active point and a standard state for comparing bactericidal activity with drug uptake.

Loveday & James (1957) have shown that toxicity of phenols can be correlated directly with surface saturation of the cell and, from measurements of electrophoretic mobility, they have predicted the phenol coefficient of several phenols. Their conclusions that there is a correlation between uptake and bactericidal activity, together with the conclusions reached in this paper, suggest that the course of the bactericidal action may ultimately be explainable without recourse to either the classical monomolecular or vitalistic theories, both of which are open to serious objections.

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