THE INTERACTION OF PHENOLIC COMPOUNDS WITH BACTERIA

PART I. HEXYLRESORCINOL AND *ESCHERICHIA COLI*

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Various quantitative aspects of the interaction of hexylresorcinol with *E. coli* suspensions are described. The amounts of hexylresorcinol bound by the bacteria from solutions of varying concentrations were determined and the speed of this reaction and the influence of temperature on the extent of binding examined. The release of cellular constituents from the bacteria and the changes in the light-scattering properties of *E. coli* suspensions on addition of hexylresorcinol were also investigated. These results are discussed in relation to the site of antibacterial action of hexylresorcinol.

THE influence of various factors on the antibacterial activity of phenolic compounds is a well documented subject, whereas, little data are available concerning the actual interaction of such drugs with bacteria. Some attempts have been made to relate antibacterial activity to some chemical or physico-chemical change on addition of phenol to bacteria but no real success has yet been reported. Various quantitative aspects of the interaction of hexylresorcinol with *Escherichia coli* will be described in this paper including drug binding to, and release of cellular constituents from, the organism and the consequent effects on the light scattering properties of the bacteria. Later publications will be concerned with the influence of other substances, including sodium chloride, butanol and a non-ionic surface-active agent on the interaction of hexylresorcinol with *E. coli* and evaluation of the antibacterial activity of the phenol under conditions similar to those used for drug-binding experiments. Preliminary observations have already been published[1,2].

EXPERIMENTAL

Hexylresorcinol.—Commercial *p-n*-hexylresorcinol was recrystallised from light petroleum (b.p. 40–60°) as colourless needles, m.p. 69° (uncorrected), (Cox[3] gave 68–70°) and log ϵ 3·42 at λ max 280 mλ in distilled water. *Spectrophotometer.*—A Hilger H 700 spectrophotometer was used in conjunction with matched, fused silica cuvettes (1 cm. unless otherwise stated). *Organism.—Escherichia coli* (originally N.C.T.C. 5933) was maintained on nutrient agar slopes. Cultures were incubated for 18–24 hours at 37°.

Preparation of bacterial suspensions. The bacteria were harvested, washed once by centrifuging at 8000 *g* for 10 minutes with distilled water and finally resuspended in 0·13 M phosphate buffer (pH 7·3). The final volume of the suspension was adjusted so that on dilution 1 in

10 (on addition to drug solutions, etc.) the suspension would contain the required number of organisms. Routine standardisation was carried out nephelometrically.

Preparation of suspensions of isolated cell walls. Suspensions of isolated cell walls of *E. coli* were prepared following the method of Salton and Horne[4].

General Technique for Drug-Bacteria Contact

A suitable volume (usually 45 ml.) of an aqueous solution of hexylresorcinol was introduced into a glass centrifuge tube contained in a water bath maintained at 25° (± 1·0°). A known volume (usually 5 ml.) of the bacterial suspension in 0·13 M phosphate buffer was added to the drug solution and the product mixed thoroughly. The final concentration of phosphate buffer was always 0·013 M. After a timed interval of 10 minutes unless otherwise stated, the bacteria were removed by centrifuging at 8000 *g* for 10 minutes and the supernatant solution further clarified by re-centrifuging before examination for the hexylresorcinol content.

Turbidity changes in suspensions of *E. coli* after the addition of hexylresorcinol were determined at 500 mμ. The drug-bacteria suspension was transferred immediately after mixing to a cuvette maintained at 25° in the spectrophotometer. The reference cuvette contained a similar suspension without hexylresorcinol. The optical density of the test suspension was measured at timed intervals after addition of the drug.

Analysis of Solutions derived from Drug-Bacteria Contact

The hexylresorcinol was separated from the cell exudate by extraction with an organic solvent: 50 ml. of drug-containing solution was extracted with chloroform (reagent grade*, 4 × 50 ml portions) and a total of 20 ml. of water was used for washing. The combined solvent extracts, containing the hexylresorcinol, were evaporated to dryness under reduced pressure and the residue dissolved in water and diluted to 50·0 ml. The hexylresorcinol content of this solution was determined spectrophotometrically. The combined aqueous layers, after extraction, were boiled to remove dissolved chloroform, cooled and diluted to 50·0 ml. before spectrophotometric examination for the presence of cell exudate. All results were corrected for the slight background absorption derived from chloroform[5].

RESULTS

The Rate of Interaction of Hexylresorcinol with E. coli

Figure 1 shows the results obtained for the uptake of hexylresorcinol by and the release of cell exudate from *E. coli* with increasing time of contact. The test suspension contained 350 μg./ml. hexylresorcinol and 3×10^9 organisms/ml. The contact times ranged from 2 to 60 minutes.

* Not all commercial samples are suitable.

The Uptake of Hexylresorcinol by E. coli

Duplicate results obtained for the uptake of hexylresorcinol by *E. coli* (10^9 organisms/ml.) from solutions initially containing 20–440 μg./ml. of the drug are shown in Table I (see also curve 1 of Fig. 3). The liberation of cell exudate accompanying hexylresorcinol binding is shown in Figure 3, curve 2, as a function of the drug concentration remaining in the supernatant solution.

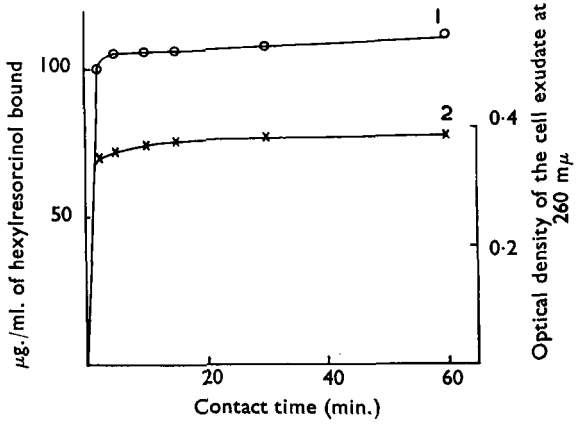

FIG. 1. Uptake of hexylresorcinol by *E. coli* (3×10^9 organisms/ml.) and the release of cell exudate from the organisms with increasing time of contact at 25°. 1. The amount of hexylresorcinol bound (initial concentration 350 μg./ml.). 2. The amount of cell exudate released (optical density at 260 mμ).

Only about 6 per cent of the drug bound by suspensions of intact bacteria was bound by isolated cell wall preparations of the same organism (initial hexylresorcinol concentration 300 μg./ml. and bacteria or isolated cell walls equivalent to $3{\cdot}3 \times 10^9$ organisms/ml.). This figure is of necessity only approximate owing to circumstances previously discussed[6].

TABLE I

THE RELATION BETWEEN THE UPTAKE OF HEXYLRESORCINOL BY *E. coli* (10^9 ORGANISMS/ML.) AND THE INITIAL CONCENTRATION OF HEXYLRESORCINOL

The results were obtained using a single bacterial suspension

Initial concentration of hexylresorcinol μg./ml.	Mean uptake μg./ml.
23·3	2·8
46·5	5·1
69·4	7·7
91·4	10·7
128·6	16·3
174·8	24·0
209·2	34·0
254·4	42·8
289·0	50·8
363·0	74·6
407·4	87·8
436·1	96·3

Influence of Temperature on the Uptake of Hexylresorcinol by E. coli

The uptake of hexylresorcinol by *E. coli* (10^9 organisms/ml.) from solutions initially containing 100 μg./ml. was determined at 25, 30 and 40°: the results are presented in Table II. The amount of cell exudate released under these conditions is also shown in this Table.

Turbidity Changes in Suspensions of E. coli *on Addition of Hexylresorcinol*

The changes in the optical density of suspensions of *E. coli* (final concentration 5×10^8 organisms/ml.) after addition of varying concentrations of hexylresorcinol are shown in Figure 2. The results shown indicate the changes at timed intervals after mixing. Stoppered cuvettes, 1 cm., thermostatically maintained at 25°, were used for these measurements. Microscopic examination of these suspensions showed that no clumping of the bacteria had occurred.

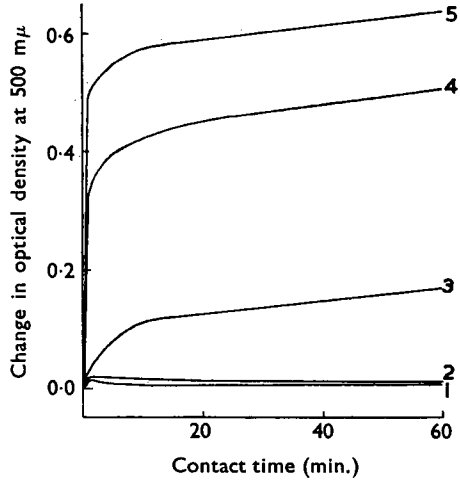

FIG. 2. Change of the turbidity of suspensions of *E. coli* (5×10^8 organisms/ml.) at 25° after the addition of hexylresorcinol. 1. 20 μg./ml. 2. 100 μg./ml. 3. 200 μg./ml. 4. 400 μg./ml. 5. 500 μg./ml.

DISCUSSION

Analytical Methods

The ultra-violet absorption curves of the solutions obtained after contact of *E. coli* with hexylresorcinol showed maxima near 280 mμ; the actual position of the peak varied slightly with the initial concentration, presumably due to the relative concentrations of unchanged hexylresorcinol (λ max 280 mμ) and the cell exudate (λ max 260 mμ). Satisfactory analytical resolution of these mixtures was attained using the extraction procedure described previously[5].

The Rate of Interaction of E. coli *with Hexylresorcinol*

The uptake of hexylresorcinol by *E. coli* was rapid and almost complete after 5 minutes contact time (see Fig. 1); the release of cell exudate during contact showed a similar course. Ten minute contact times were, therefore, considered suitable for all subsequent work with this system.

The Uptake of Hexylresorcinol by E. coli

Curve 1 of Figure 3 shows that the amount of hexylresorcinol bound by *E. coli* is dependent upon the initial concentration of the phenol in solution. There was no difference between the amount of hexylresorcinol bound from aqueous solutions in the presence and absence of phosphate buffer (0·013 M and pH 7·3). The point of no further increase in the

uptake of hexylresorcinol by the bacteria on increasing the phenol concentration in the medium was not attained even with the most concentrated initial solutions (almost saturated). Although the number of bacteria in contact with the hexylresorcinol solutions could have been reduced to

TABLE II

THE EFFECT OF TEMPERATURE ON THE UPTAKE OF HEXYLRESORCINOL BY *E. coli* (10^9 ORGANISMS/ML.)

The initial concentration of hexylresorcinol in contact with the bacteria was 100 μg./ml. Mean results for duplicate determinations

Temperature in ° C.	Concentration in the supernatant solution μg./ml.	Amount of hexylresorcinol bound μg./ml.	Optical density of cell exudate released in the presence of the drug	Optical density of cell exudate released in the absence of the drug
25	87·4	12·6	0·133	0·029
30	86·8	13·2	0·152	0·034
40	86·7	13·3	0·272	0·056

achieve saturation, the precision of the analytical results would have been adversely affected.

The shape of the uptake curve (Fig. 3, curve 1) indicates that the attractive forces exerted by the initially bound molecules facilitate binding of a further quantity of hexylresorcinol. This is also suggested by the changes in the electrophoretic mobilities of bacteria upon progressive addition of phenolic compounds[7]. Alternatively, the molecules bound

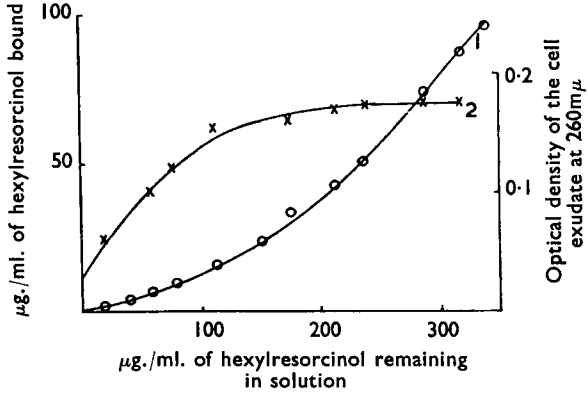

FIG. 3. Uptake of hexylresorcinol by *E. coli* and the release of cell exudate from the organisms (10^9 organisms/ml.). 1. The amount of hexylresorcinol bound from solutions containing 0·013 M phosphate buffer, pH 7·3 at 25°. 2. Release of cell exudate (optical density at 260 mμ) upon increasing the hexylresorcinol concentration.

initially to the cytoplasmic membrane could cause partial disorganisation of the osmotic barrier with resultant penetration by some phenolic molecules.

The amount of cell exudate liberated from *E. coli* suspensions in 10 minutes, as indicated by the optical density at 260 mμ, was influenced by the initial hexylresorcinol concentration (and also by the amount of

hexylresorcinol bound) up to 220 μg./ml. (for 10^9 organisms/ml.). Above this concentration, a constant and limiting value was reached shown in curve 2 of Figure 3. Initially, localised damage to the cytoplasmic membrane of the bacteria is envisaged with consequent release of a limited amount of cell exudate; the components of the cell exudate may possibly be present in the "free" state or may be formed by breakdown of the cytoplasmic constituents. Progressive addition of hexylresorcinol would cause more extensive damage and the release of more cellular constituents until the drug molecules exerted their maximum effect upon the cytoplasmic membrane. Similar interdependence of the concentration of an antibacterial substance (cetyltrimethylammonium bromide, polymyxin and circulin) and the amount of cellular constituents released from bacteria has been demonstrated by other workers[8–11]; however, the amount of cell exudate liberated from the same organisms by different substances will not necessarily be the same.

The relatively small proportion (6 per cent) of hexylresorcinol bound by isolated cell walls of *E. coli* compared with the amount bound by intact cells under identical conditions provides further evidence that the cytoplasmic membrane and possibly the cytoplasm is involved in the latter. The cytoplasmic membrane has also been implicated as the site of interaction of various antibacterial substances, for example phenol[12,14], polymixin and other peptidic antibiotics[15] and some ionic surface-active agents (for a discussion see Salton[16]), with bacteria.

The uptake of hexylresorcinol by bacteria is not a reversible reaction. Therefore, although changes in the contact temperature had little effect on the amount of hexylresorcinol bound (see Table II) it does not follow that values for the heat of reaction are small and only weak binding forces are involved. Either increased breakdown of cytoplasmic constituents or changes in the permeability of the bacteria could effect the considerable increase in the amount of cell exudate liberated on raising the temperature. These results are of particular interest in relation to the temperature coefficients of bactericidal reactions associated with phenolic compounds. Nevertheless, the number of molecules exerting the biological effect may constitute only a very small fraction of those bound by the bacteria and, therefore, any correlation of the changes observed on drug binding and antibacterial activity may ultimately prove impossible.

Turbidity Changes in Suspensions of E. coli *on Interaction with Hexylresorcinol*

Some profound alteration in the light scattering properties of suspensions of *E. coli* (see Fig. 2) occurs after the addition of hexylresorcinol, the effect being dependent on the concentration of the drug and the time of contact. The authors have observed similar turbidity changes occur in bacterial suspensions containing many phenolic substances (for example phenol and chlorocresol) but not all (for example chloroxylenol and thymol). The optical density of bacterial suspensions between 300–600 mμ is attributed solely to the scattering of light by the organisms[17]. An increase in the optical density of a bacterial suspension

may be caused by an increase in the effective reflecting surface area of the cell or to a change in the refractive index of the cell membranes or cytoplasm or to a combination of these effects. The turbidity changes reported in the present paper will be discussed in conjunction with the biological results in Part III[18].

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