THE INTERACTION OF PHENOLIC COMPOUNDS WITH BACTERIA

PART II. THE EFFECTS OF VARIOUS SUBSTANCES ON THE INTERACTION OF HEXYLRESORCINOL WITH *ESCHERICHIA COLI*

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The uptake of hexylresorcinol by suspensions of *E. coli* and the associated release of cellular constituents were examined using heat-killed and butanol-treated organisms. The effect of the addition of cetomacrogol to the system was evaluated in terms of the reduction in the extent of drug binding and the prevention of the hexylresorcinol-dependent light-scattering changes of *E. coli* suspensions. Sodium chloride potentiation of the latter effect and of the release of cell exudate from the bacteria on treatment with hexylresorcinol was also investigated.

IN this paper, the effects of sodium chloride and butanol on the interaction of hexylresorcinol and *Escherichia coli* are examined because of their effects upon the osmotic pressure of the drug-bacteria system. Cetomacrogol was also included since nonionic surface-active agents are employed as emulsifying and solubilising agents in pharmaceutical and cosmetic preparations. Preservation of these products is difficult since the conventional phenolic compounds (among many others) have been shown to be ineffective in the presence of nonionic substances, although the cause of this inactivation has not been established unequivocally[1,2].

The effects of sodium chloride and cetomacrogol on the antibacterial activity of solutions of hexylresorcinol against *E. coli* under analogous conditions to those herein described will be presented and discussed in Part III[3].

EXPERIMENTAL

The experimental techniques were described in Part I[4].

Cetomacrogol (polyethylene glycol 1000 monocetyl ether). This material was obtained from Glovers (Chemicals) Ltd., may be represented by the formula:

$$CH_3(CH_2)_mOCH_2(CH_2OCH_2)_nCH_2OH$$

where m may be 15 or 17 and n may be 19 to 23. The molecular weight is approximately 1300 and the critical micelle concentration in aqueous solution is about 10^{-6} to 10^{-7} M[5].

Preparation of suspensions of heat-killed bacteria. Washed suspensions of *E. coli* (about 10^{10} organisms/ml.) in phosphate buffer were maintained at 100° for 10 minutes. The bacteria were centrifuged, washed once and resuspended in the same medium (2×10^{10} organisms/ml.).

Preparation of suspensions of butanol-treated bacteria. *n*-Butanol was added to suspensions of *E. coli* (about 10^{10} organisms/ml.) in phosphate

buffer to yield a final concentration of 5·0 per cent w/v (0·67 M). After 25 minutes at 20°, the bacteria were centrifuged, washed twice and re-suspended in phosphate buffer (2×10^{10} organisms/ml.).

Colorimetric determination of hexylresorcinol. The method was as described previously[6]. Calibration curves were prepared for solutions containing hexylresorcinol (2–20 μg./ml.) alone and in combination with 0·001 per cent w/v cetomacrogol.

Turbidity changes in bacterial suspensions. The effect of sodium chloride on the turbidity changes of suspensions of *E. coli* treated with

TABLE I

THE UPTAKE OF HEXYLRESORCINOL AND THE RELEASE OF CELL EXUDATE BY HEAT-KILLED AND BUTANOL-TREATED SUSPENSIONS OF *E. coli* (2×10^9 ORGANISMS/ML.)

The initial concentration of hexylresorcinol in contact with the bacteria was 260 μg./ml.

	Preliminary treatment		
	none (control)	heat-killing	butanol
Amount of hexylresorcinol bound in μg./ml. ..	76·0	114	70·0
Optical density at 260 mμ of the cell exudate released during preliminary treatment ..	0·033	0·791	0·133
Optical density at 260 mμ of the cell exudate released during hexylresorcinol treatment ..	0·199	0·049	0·670

hexylresorcinol were determined by the method described in Part I[4], i.e., the reference cuvette contained an equivalent bacterial suspension without added drug.

Observations on suspensions of *E. coli* after the addition of either hexylresorcinol or cetomacrogol, or both, were made using water in the reference cuvette (0·2 cm. cuvettes).

Analysis of solutions derived from drug-bacteria content. In the absence of the surface-active agent, the extraction procedure (cf. Part I[4] was used, see discussion). The colorimetric precedure was applied only to those solutions which contained cetomacrogol.

RESULTS

Influence of Various Factors on the Interaction of Hexylresorcinol with E. coli

Heat-killed and butanol-treated organisms. The results obtained using heat-killed and butanol-treated suspensions of *E. coli* (2×10^9 organisms/ml.) and an initial drug concentration of 260 μg./ml. are shown in Table I.

Sodium chloride. The effect of sodium chloride in various concentrations upon the uptake of hexylresorcinol by, and the release or cell exudate from, *E. coli* (10^9 organisms/ml.) for a solution initially containing 230 μg./ml. of the drug is indicated by the results presented in Figure 3.

Cetomacrogol. Figure 1, curve 1, shows the results obtained for the uptake of hexylresorcinol by *E. coli* (3×10^9 organisms/ml.) from a solution initially containing 350 μg./ml. of hexylresorcinol and 990 μg./ml. of cetomacrogol (the solution also contained phosphate buffer,

pH 7·3, final concentration 0·013 M). The contact times ranged from 2 to 60 minutes and the molar ratio of cetomacrogol to hexylresorcinol was about 4 to 1. Curve 2 of the same Figure shows the results of a comparable experiment in which cetomacrogol was omitted (cf. Part I[4]).

The results shown in Figure 2 demonstrate the effect of increasing concentrations of cetomacrogol on the binding of hexylresorcinol by

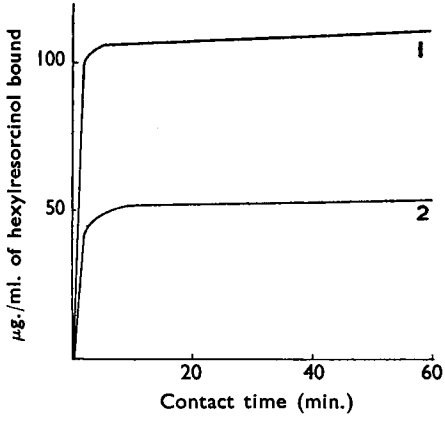

FIG. 1. The rate of uptake of hexylresorcinol by *E. coli* (3×10^9 organism/ml.) at 25° in the presence and absence of cetomacrogol. The initial concentration of hexylresorcinol was 350 μg./ml. or $1{\cdot}8 \times 10^{-3}$ M. 1. In the absence of cetomacrogol. 2. In the presence of 1000 μg./ml or $7{\cdot}7 \times 10^{-3}$ M cetomacrogol.

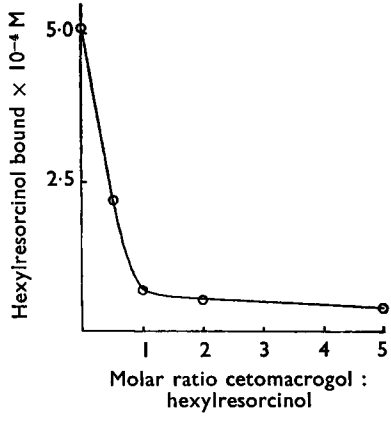

FIG. 2. Uptake of hexylresorcinol by *E. coli* (3×10^9 organisms/ml.) at 25° in the presence of varying proportions of cetomacrogol. The initial hexylresorcinol concentration was $1{\cdot}8 \times 10^{-3}$ M.

suspensions of *E. coli* (3×10^9) organisms/ml. The molar ratios of cetomacrogol to hexylresorcinol ranged from 0·5–5·0 to 1.

For a solution initially containing 500 μg./ml. of hexylresorcinol and cetomacrogol (molar ratio of cetomacrogol to hexylresorcinol of 0·5 : 1), the amount of the phenol bound was 24 μg./5×10^8 organisms/ml.

TABLE II

THE TURBIDITY OF SUSPENSIONS OF *E. coli* (10^9 ORGANISMS/ML.) 10 MINUTES AFTER THE ADDITION OF HEXYLRESORCINOL SOLUTIONS CONTAINING VARIOUS CONCENTRATIONS OF SODIUM CHLORIDE. (0·5 CM. CUVETTES)

Concentration of hexylresorcinol μg./ml.	Optical density at 500 mμ in presence of NaCl			
	0·0 M	0·05 M	0·1 M	0·2 M
50	0·477	0·495	0·503	0·511
100	0·490	0·514	0·512	0·531
200	0·554	0·591	0·603	0·642
300	0·725	0·767	0·799	0·895
350	0·834	0·944	0·968	1·020
400	0·974	1·039	1·068	1·112
500	1·131	1·201	1·234	1·232

Influence of Various Substances on the Turbidity of Suspensions of E. coli

Sodium chloride. Table II shows the effect of the presence of sodium chloride on the turbidity changes of suspensions of *E. coli* observed on

addition of hexylresorcinol. The maximum percentage change in optical density was that observed for suspensions containing 350 μg./ml. of the phenol and 0·2 M sodium chloride. At each concentration of the electrolyte studied, the maximum increase in optical density of the bacterial suspensions also occurred at this concentration of the drug; higher and lower hexylresorcinol concentrations caused a smaller increase. These results were obtained for the simultaneous addition of the phenol and the salt; a greater increase in turbidity was observed when the bacterial suspension was pre-treated with sodium chloride for 10 minutes before addition of hexylresorcinol.

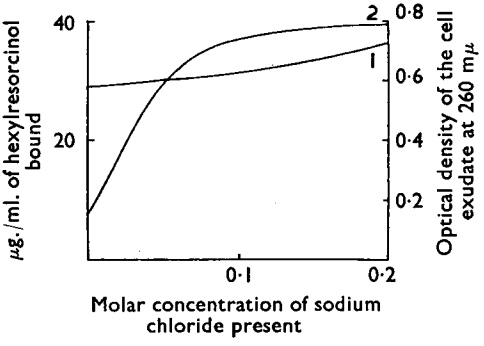

FIG. 3. The effect of sodium chloride on the interaction *E. coli* (10^9 organisms/ml.) with hexylresorcinol (230 μg./ml.) at 25°. 1. The amount of hexylresorcinol bound. 2. Release of cell exudate (optical density at 260 mμ) upon increasing the sodium chloride concentration.

Cetomacrogol. The effect of cetomacrogol on the turbidity changes of suspensions of *E. coli* caused by the addition of hexylresorcinol is demonstrated by the results presented in Figure 4. The results were obtained using solutions containing cetomacrogol in addition to the phenol and a final bacterial concentration of 10^9 organisms/ml. The optical densities of the contact suspensions at 500 mμ (in 0·2 cm. cuvettes) were measured 60 minutes after mixing. Irrespective of the hexylresorcinol concentrations, increases in the concentration of cetomacrogol above $3{\cdot}85 \times 10^{-3}$ M caused a slight decrease in the optical density of the contact suspensions, i.e., less than 0·03 optical density units under the above conditions.

DISCUSSION

Analytical Methods

The extraction procedure described in a previous paper[6] was used to separate unchanged hexylresorcinol from the solutions derived from drug-bacteria suspensions wherever possible. However, this separation was not suitable for solutions containing cetomacrogol and it was necessary to determine the phenol colorimetrically. It was impracticable to determine both the cell exudate and the unchanged hexylresorcinol in solutions containing the nonionic because of foaming.

The straight line calibration curve, obtained on plotting the optical density at 510 mμ against the hexylresorcinol concentration (up to 20 μg./ml. or $0{\cdot}1 \times 10^3$ M) in the final solution after colour development, was virtually unaffected by the presence of cell exudate and cetomacrogol (final concentration 0·001 per cent w/v or $0{\cdot}77 \times 10^{-3}$ M).

Solutions Containing Hexylresorcinol and Cetomacrogol

The molar ratio of cetomacrogol to hexylresorcinol in the solutions used during the present work ranged from 0·5–5·0 to 1. At lower molar ratios, a water insoluble complex separated from the solution. Observations on the probable molecular species present in solutions of hexylresorcinol and cetomacrogol and on the nature of the water insoluble complex have recently been published[1].

Since the critical micelle concentration of aqueous solutions of cetomacrogol is about 10^{-6} to 10^{-7} M, it has not been possible to examine non-micellar solutions of this material. However, the effects of other nonionic surface-active agents of *known* chain lengths at non-micellar concentrations, on the interaction of various phenols with bacteria will be reported at a later date.

Influence of Various Factors on the Uptake of Hexylresorcinol by E. coli

Heat-killed bacteria. The amount of cell exudate liberated during heat treatment of a suspension of *E. coli* is greater than during subsequent treatment with hexylresorcinol or during the interaction of a control suspension with the phenol (Table II). This increase is presumably caused by the destruction of the osmotic barrier of the bacteria, and the escape of fragments of the cytoplasmic components (cell exudate) formed by an "uncoupling reaction" of heat on the cytoplasm, since heat fixation of the cytoplasmic[7] and "protoplast"*[7,8] membranes of bacteria has been demonstrated. The term "uncoupling reaction" is used here to imply the breakdown of proteins, polypeptides and nucleic acids into their simpler components, e.g., peptides, amino acids, sugars. Thus, once the permeability of the osmotic barrier or the cytoplasmic membrane, or both, has been destroyed, the amount of cell exudate released should reflect the uncoupling efficiency of the added agent on the remaining cytoplasmic components. The small amount of cell exudate liberated during the reaction of hexylresorcinol

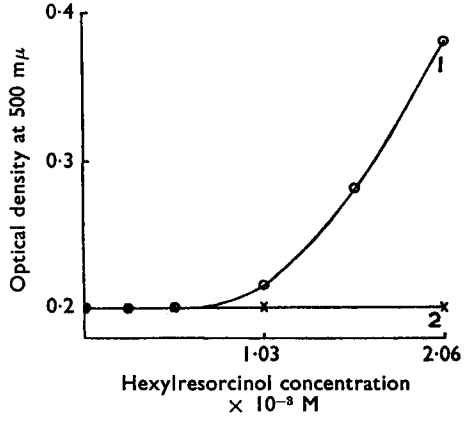

FIG. 4. The effect of cetomacrogol on the turbidity changes of suspensions of *E. coli* (10^9 organisms/ml.) exposed to hexylresorcinol at 25°. The readings were taken at 500 mμ in 0·2 cm. cuvettes, 60 minutes after mixing and using water in the reference cuvette. 1. In the absence of cetomacrogol. 2. With cetomacrogol ($0·77 \times 10^{-3}$ M) added to the bacterial suspension at the same time as the hexylresorcinol.

* Doubt has been expressed whether the bodies formed by growing *E. coli.* in the presence of penicillin in a hypertonic medium are protoplasts in the strict sense of the definition of that term[9].

with heat-killed organisms indicates that the limit of cytoplasmic uncoupling has been reached in the heat treatment.

Butanol-treated bacteria. The concentration of butanol used in these experiments (0·67 M) is in excess of that required to attain a surface pressure of 34 dynes/cm. (0·4 M). Leakage of low molecular weight constituents from bacteria, as well as haemolysis, has been shown to occur above this critical concentration of butanol; it seems reasonable to attribute the former to irreversible denaturation of the cytoplasmic membrane of the bacteria[10]. The amount of cell exudate liberated from *E. coli* by butanol alone may arise either from destruction of the osmotic barrier of the cells and the subsequent escape of naturally "free" cellular constituents, or alternatively, from destruction of the osmotic barrier and a limited uncoupling effect on the cytoplasm. Subsequent addition of hexylresorcinol may cause extensive intracellular uncoupling and release of cytoplasmic components through the previously disrupted osmotic barrier. The relatively small amount of cell exudate obtained on hexylresorcinol treatment of control suspensions of *E. coli* is explicable if the phenol is considered to penetrate the bacterial cell without grossly affecting the permeability of the osmotic barrier.

The data presented in Table II show that the amount of cell exudate released from bacteria depends on the treatment used and that not all of the "potential cell exudate" is necessarily released under one particular set of conditions (see also[11,12]).

Sodium chloride. Sodium chloride, *per se* (at concentration levels up to 0·2 M), has a negligible effect on the amount of cell exudate released from *E. coli* suspensions or on their turbidity. The increase in the amount of cell exudate obtained on adding hexylresorcinol solutions containing sodium chloride to suspensions of *E. coli* may be attributed to the enhanced efficiency of the phenol in the uncoupling effect on cellular constituents and liberation of these substances as cell exudate. The increased turbidity produced by hexylresorcinol in the presence of sodium chloride also indicates the enhanced activity of the phenol, since the presence of the salt has little effect on the amount of the phenol which is bound (Fig. 3). An even greater increase in optical density at 500 mμ of the bacterial suspensions on addition of hexylresorcinol observed when the organisms were pretreated with sodium chloride (0·2 M for 10 minutes) probably has significance in relation to the potentiating effect of the electrolyte on the antibacterial activity of this phenol (see Part III[3]).

The above results seem to indicate that the cell exudate liberated by low concentrations of the phenol alone is derived from a limited "uncoupling reaction" on the cytoplasm, rather than an alteration of the permeability of the osmotic barrier. This explanation accounts for the negligible turbidity changes at these concentrations of hexylresorcinol alone.

Cetomacrogol. The speed of uptake of hexylresorcinol was hardly affected by the presence of the nonionic substance (Fig. 1) whereas, the amount of hexylresorcinol bound was reduced in proportion to the relative molar concentration of cetomacrogol (Fig. 2). The light

scattering changes of the bacterial suspensions associated with addition of hexylresorcinol were abolished in the presence of cetomacrogol (Fig. 4) even when a sufficient concentration of the phenol was taken up to cause such changes in the absence of the nonionic substance.

Complex formation occurs in solutions containing hexylresorcinol and cetomacrogol; steric factors could, therefore, account for the observed reduction in uptake of the phenol by *E. coli* suspensions. The complexing probably prevents cell wall penetration by the phenol, since changes in the light scattering properties of bacterial suspensions upon contact with hexylresorcinol are abolished in the presence of cetomacrogol. Alternatively, it may be postulated that light scattering changes are normally caused by hexylresorcinol molecules penetrating the bacterial surface at specific sites; cetomacrogol could then block hexylresorcinol binding at these sites without interfering with binding over the remainder of the bacterial surface.

Figure 2 shows that the presence of one molecule of cetomacrogol to every two of hexylresorcinol reduces the amount of the phenol bound by approximately 56 per cent. The presence of cetomacrogol in excess of a 2:1 ratio of cetomacrogol to hexylresorcinol has little additional effect on the amount of hexylresorcinol bound by the bacteria: the amount of hexylresorcinol bound per organism under the latter conditions is less than that which would be theoretically required to form a close-packed monomolecular layer round the organism *in the absence of the nonionic substance* (cf. Part III[3]).

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