THE INTERACTION OF PHENOLIC COMPOUNDS WITH BACTERIA

PART III. EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF HEXYLRESORCINOL AGAINST *ESCHERICHIA COLI*

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Received January 5, 1959

Evaluation of the antibacterial activity of solutions containing hexylresorcinol in the presence and absence of cetomacrogol and sodium chloride is described. The relation between the extent of drug binding, light-scattering changes and the release of cell exudate in *E. coli* suspensions on addition of hexylresorcinol with the bactericidal activity is examined. Potentiation of the bactericidal activity of hexylresorcinol by sodium chloride and the inactivation by cetomacrogol is discussed with respect to drug binding, light-scattering changes in the bacterial suspensions and the release of bacterial cell exudate in the presence of these agents.

THE work described in previous papers in this series[1,2] has now been extended to include the antibacterial evaluation of solutions of hexylresorcinol against suspensions of *Escherichia coli*. The choice of method was influenced by a desire to determine whether any relation exists between antibacterial activity and a change in some physical property occurring during the interaction of the phenol and bacteria. The correlation of drug binding, light-scattering changes and cell exudate release in suspensions of *E. coli* observed on addition of hexylresorcinol with the antibacterial activity of the phenol under similar conditions is discussed in the present paper.

EXPERIMENTAL

The experimental techniques described previously[1,2] were used with slight modification; aseptic precautions were observed throughout to prevent chance contamination. Suspensions of *E. coli* were prepared in sterile distilled water instead of phosphate buffer. Solutions of hexylresorcinol were filtered through sintered glass (5/3) and the concentration in the filtrate was checked spectrophotometrically before use. All other solutions were sterilised by autoclaving.

The culture medium employed contained 1·0 per cent Oxoid peptone and 0·5 per cent sodium chloride at pH 7·3.

Bacteriostatic Evaluation

5 ml. volumes of filtered hexylresorcinol solutions (120–500 μg./ml.) were added to 5·0 ml. portions of sterile broth. Each solution was inoculated with one drop of a 24 hour broth culture of *E. coli* delivered from a standard dropping pipette. Ten tubes were set up at each concentration of the drug. The tubes were incubated at 37° for 48 hours and examined for the presence or absence of visible growth. The

minimum inhibitory concentration was that concentration of hexylresorcinol which just inhibited visible growth.

Solutions of hexylresorcinol (50, 125 and 250 μg./ml.) containing cetomacrogol (at molar ratios of 0·5, 1·0 and 2·0 of cetomacrogol to hexylresorcinol) were tested similarly.

Bactericidal Evaluation

The mean single survivor times of suspensions of *E. coli* in solutions of hexylresorcinol were determined under the same conditions as those used for the uptake measurements[1].

General method. Calibrated standard dropping pipettes[3] were used to to transfer 5 drops of a suspension of *E. coli* in water, containing 55×10^9 organisms/ml., to 10 ml. portions of hexylresorcinol solutions; the final bacterial concentration was 5×10^8 organisms/ml. The temperature of the contact suspension was maintained at $25 \pm 1°$ until the addition of 5 ml. of sterile broth after suitable time intervals. The tubes were immediately transferred to an incubator at 37° for 24 hours. The concentration of hexylresorcinol present after dilution was less than the bacteriostatic concentration.

Hexylresorcinol solutions. The death time of *E. coli* in solutions of hexylresorcinol was determined using solutions containing 100–400 μg./ml. of the phenol and 5 tubes at each concentration level. One aliquot at each concentration was quenched at 10 minute intervals from 20–80 minutes after inoculation.

The mean single survivor time of *E. coli* in solutions containing 250 μg./ml. of hexylresorcinol was determined using 20 contact suspensions. One tube of each series was quenched at 7 minute intervals from 29–70 minutes after inoculation. The results were analysed by the method given by Cook and Wills[4].

Hexylresorcinol solutions containing cetomacrogol. Determination of the death time of *E. coli* in a solution containing 500 μg./ml. of hexylresorcinol and 1660 μg./ml. of cetomacrogol (molar ratio of cetomacrogol to hexylresorcinol of 0·5 : 1·0) was as described above for solutions containing hexylresorcinol.

Hexylresorcinol solutions and sodium chloride. 10 ml. portions of the bacterial suspensions, containing 2×10^8 organisms/ml. in distilled water, were added to 10 ml. of 0·4 M sodium chloride solutions and allowed to stand 10 minutes at 25°; 5 ml. of this suspension was then added to 5 ml. of hexylresorcinol solution and the test completed as in the general method. This furnished a test suspension containing 0·1 M sodium chloride and 5×10^8 organisms/ml. together with hexylresorcinol at any pre-determined concentration.

The death time of sodium chloride-treated suspensions of *E. coli* in solutions of hexylresorcinol (100 and 250 μg./ml.) were determined as above for hexylresorcinol alone.

The mean single survivor time of *E. coli*, pre-treated with sodium chloride, in solutions of hexylresorcinol containing 250 μg./ml. of the latter was determined using 20 contact suspensions. One aliquot of

each mixture was quenched at 7 minute intervals from 14 to 56 minutes after inoculation.

RESULTS

Bacteriostatic Evaluation

The minimum inhibitory concentration of hexylresorcinol against *E. coli* in nutrient broth was found to be 120 μg./ml. after 48 hours at 37°. These results established that the quantity of hexylresorcinol carried over into the growth medium in the bactericidal tests was insufficient to inhibit the growth of intact bacteria. The inhibition of the bacteriostatic activity of hexylresorcinol against *E. coli* by cetomacrogol was also demonstrated even for solutions containing the minimum proportion of the nonionic substance and the highest practicable concentration of the phenol (250 μg./ml.).

Bactericidal Evaluation

All results quoted are for reactions at 25°.

Hexylresorcinol solutions. At 100 μg./ml. of hexylresorcinol, surviving organisms were still present after 80 minutes contact time. The approximate death time of *E. coli* in a solution containing 250 μg./ml. hexylresorcinol was 50 minutes. No survivors were apparent after 20 minutes in solutions containing 300 and 400 μg./ml. of the phenol.

The results for the determination of the mean single survivor time of *E. coli* in a hexylresorcinol solution containing 250 μg./ml. are represented by curve 1 of Figure 1. The mean single survivor time was 47 minutes.

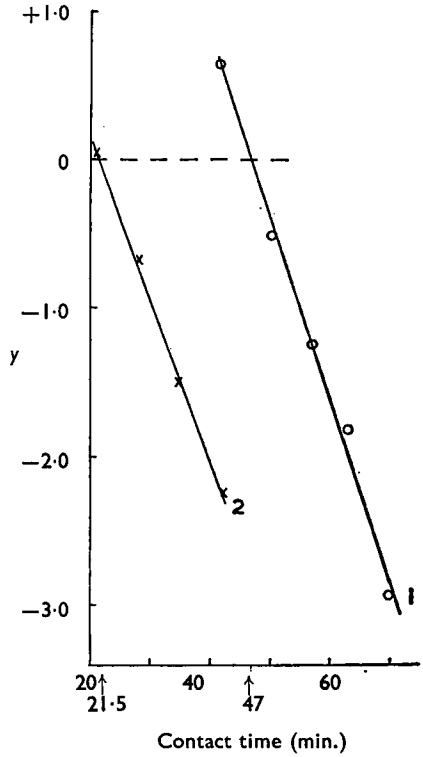

FIG. 1. The relation between y (log $[-\log p]$, where p = the proportion of sterile tubes after incubation) and contact times for the exposure of *E. coli* to a solution of hexylresorcinol containing 250 μg./ml. (curve 1) and for the exposure of sodium chloride-treated *E. coli* to a solution containing the same concentration of hexylresorcinol (curve 2). The reaction temperature was 25° and the initial bacterial concentration was 5×10^8 organisms/ml.

Hexylresorcinol solutions containing cetomacrogol. Viable organisms were still present after 80 minutes contact time.

Hexylresorcinol solutions and sodium chloride. The approximate death times of sodium chloride-treated *E. coli* in solutions containing 100 and 250 μg./ml. of hexylresorcinol were 75 and 35 minutes respectively. The

results for the determination of the mean single survivor time in the latter solution are represented by curve 2 of Figure 1. The mean single survivor time was 21·5 minutes.

Discussion

The potentiating effect of sodium chloride and the inhibitory effect of cetomacrogol on the bactericidal activity of solutions of hexylresorcinol against *E. coli* will now be considered in conjunction with the information presented in previous papers[1,2].

Bactericidal Activity of Solutions of Hexylresorcinol

The amount of hexylresorcinol bound by *E. coli* suspensions from solutions initially containing 250 μg./ml. of hexylresorcinol of mean single survivor time of 47 minutes was 20 μg./5 $\times$ 10^8 organisms/ml., in the presence and absence of phosphate buffer. In Figure 2, this

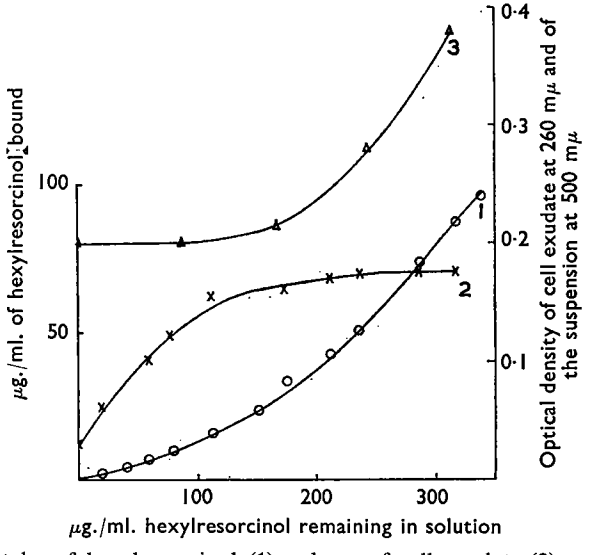

FIG. 2. Uptake of hexylresorcinol (1), release of cell exudate (2) and change of turbidity (3) of suspensions of *E. coli* (10^9 organisms/ml.) on treatment with hexylresorcinol.

hexylresorcinol concentration corresponds to approximately 210 μg./ml. on the abscissa, since the results are presented for a suspension containing 10^9 organisms/ml. At this level, the limiting value of cell exudate released (curve 2) has been achieved and there is a marked increase in the turbidity of the bacterial suspensions (curve 3). Possibly the bactericidal effects become apparent when the drug concentration is sufficient to initiate turbidity changes and, at the same time, effect maximum release of cell exudate. Thus, it is postulated that the bactericidal effects are linked with the interaction and consequent disorganisation of the cytoplasm by the phenol rather than reactions involving the cytoplasmic membrane and its associated osmotic barrier.

Since no turbidity changes could be detected until the hexylresorcinol concentration was sufficient to release the maximum amount, for hexylresorcinol, of cell exudate, no major physical change in the cytoplasmic contents is envisaged during this reaction. But, on addition of more hexylresorcinol to the system, the phenolic molecules probably penetrate into the cytoplasm with consequent effect on turbidity. This is supported by the observation that the addition of a solution of hexylresorcinol to a preparation containing the cytoplasmic constituents of *E. coli* (a disrupted suspension from which the whole cells and the cell walls had been removed) caused turbidity to develop in that preparation. Changes in the refractive index of the cytoplasm are indicated which could be associated with interference with the hydrogen bonding characteristics of nucleic acids, and particularly of deoxyribonucleic acid.

Approximately 10^8 molecules of hexylresorcinol are bound per bacterium under the bactericidal conditions defined above. Assuming that each organism is a smooth cylinder 3 μ in length and 0·6 μ in diameter, its apparent surface area, if equivalent to its geometrical surface area, is $5{\cdot}6 \times 10^8$ Å^2. The minimum surface area occupied by 10^8 molecules of hexylresorcinol, not allowing for intermolecular packing space, would be 26×10^8 Å^2 if the molecules were packed with the phenyl ring perpendicular to the surface, 38×10^8 Å^2 if the molecules were packed with the phenyl ring parallel to the surface and the *n*-hexyl chain protruding from it or 66×10^8 Å^2 if the phenyl ring and the alkyl chain were in the plane of the surface. Thus, accommodation of all the bound molecules as a uni- or bi-molecular layer is obviously impossible and partial penetration is again indicated.

Inactivation of Hexylresorcinol by Cetomacrogol

The simplest explanation of this effect is that the amount of hexylresorcinol bound by the organism is insufficient to cause the biological effect. However, by increasing the concentration of hexylresorcinol present and keeping the cetomacrogol concentration to the lowest practicable level, the amount of hexylresorcinol bound by the organisms (24 μg./5×10^8 organisms/ml. which is equivalent to $1{\cdot}5 \times 10^8$ molecules of hexylresorcinol/organism) may exceed that required to produce the bactericidal effect and light-scattering changes in suspensions of *E. coli* in the absence of the nonionic substance (20 μg./5×10^8 organisms/ml. which is equivalent to $1{\cdot}25 \times 10^8$ molecules of hexylresorcinol/organism, cf. Part II[2]). At this level, the entire amount of the phenol bound obviously cannot be accommodated on the surface of the bacterial cell especially since some cetomacrogol molecules must also be associated with it. Possibly, hexylresorcinol may become bound to the bacteria in the presence of excess cetomacrogol as a monomolecular layer of the phenol-nonionic complex with the nonionic associated with the other surface of the film, thus blocking the building of multilayers of the phenol and also preventing the phenolic molecules from penetrating the bacteria and causing the turbidity changes. If, however, the hexylresorcinol molecules, penetrate the bacteria in association with cetomacrogol molecules

the tendency to hydrogen bond to the oxygen atoms of the ether chains of the nonionic may be greater than the tendency to disrupt and replace the natural hydrogen bonding structure of the nucleic acids.

Potentiation of Hexylresorcinol Activity by Sodium Chloride

It was noted previously[2] that sodium chloride enhanced the effect of hexylresorcinol in producing turbidity changes in bacterial suspensions and the amount of cell exudate released whilst having a negligible effect on the extent of drug binding, but had no effect on these properties in the absence of this phenol. The potentiating effect of sodium chloride on the bactericidal action of hexylresorcinol against *E. coli* is attributed, therefore, to facilitated disorganisation of the cytoplasm and leakage of vital cellular constituents from the organisms.

References

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2. Beckett, Patki and Robinson, *ibid.*, 1959, **11**, 367.
3. Cook and Yousef, *ibid.*, 1953, **5**, 141.
4. Cook and Wills, *ibid.*, 1954, **6**, 638.