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The uptake by *Escherichia coli* and growth inhibitory properties of benzyl alcohol and phenethyl alcohol

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The effects of benzyl alcohol (0–4 mg/ml) and phenethyl alcohol (0–2·5 mg/ml) on the rate of mass increase in cultures of *E. coli* were studied by absorbance measurements. Growth rate constants in the partially inhibited cultures were calculated. With both agents the growth rate was reduced on increasing the alcohol concentration, the extent of this reduction becoming progressively larger for equal increments in concentration. The inhibition of growth was shown to be reversible and to result from a uniform inhibition of the individual cells. The overall cellular uptake of benzyl alcohol was approximately the same as that of phenethyl alcohol. With both agents the uptake was proportional to the concentration of alcohol remaining in the medium and was independent of contact time within the range of 10–90 min. Phenethyl alcohol is, however, more effective than benzyl alcohol in inhibiting growth. This is attributed to a greater uptake of the former by the cellular membrane.

Studies of the kinetics of growth in bacterial cultures treated with subinhibitory concentrations of antibiotics have proved useful in elucidating their mechanisms of action. This paper describes the results of applying this method to benzyl alcohol and to β-phenethyl alcohol, two structurally similar simple aromatic alcohols with bactericidal properties. The former is used extensively as a preservative, the latter is recommended in preparing selective media for Gram-positive organisms. The cellular uptake of these alcohols is compared with their growth inhibitory properties.

MATERIALS AND METHODS

The organism used was *Escherichia coli* (NCTC 1093). The media, conditions of culture and methods for measuring absorbance have been described previously (Rye & Wiseman, 1966). Total cell counts and cell size measurements were obtained using a Model B Coulter electronic particle counter (Rye & Wiseman, 1967). Viable counts were made by the pour plate method using tryptone soya broth in the dilutions and tryptone soya agar in the plates. Inactivation of the alcohols was obtained by dilution. Colonies were counted after 24 h incubation at 37°.

β-Phenethyl alcohol (carbinol-^{14}C) was prepared by the reduction of phenylacetic acid (carboxyl-^{14}C) using lithium aluminium hydride (Nystrom & Brown, 1947) and was purified by distillation under reduced pressure at 15 mm mercury. Benzyl alcohol (carbinol-^{14}C) and other labelled compounds were obtained from the Radiochemical Centre, Amersham.

Studies of growth inhibition

Method A. The absorbance of exponentially growing cultures of *E. coli* was measured at intervals and when this reached a value of 0·1, 10 ml volumes were mixed

with equal volumes of solutions of the alcohols at 37°. Incubation of these partially inhibited cultures together with control cultures diluted with growth medium alone was continued with shaking; growth was followed by absorbance measurements. In some experiments the absorbance measurements were supplemented by determining the [^{14}C] content of the cells. Glucose in the growth medium was replaced by [^{14}C] glucose (specific activity 0·01 μCi/mg) and the changes in cell carbon content during growth in the presence and absence of the alcohols determined (Rye & Wiseman, 1968a).

Method B. Exponentially growing cells of *E. coli* were harvested by filtration when the absorbance had reached 0·1. They were then washed and suspended in glucose-free medium at 37°. The absorbance was adjusted to 0·1 and 10 ml volumes were mixed with equal volumes of solutions of the alcohols in glucose-free medium at 37°. After incubating these mixtures for 45 min, glucose (1 mg/ml) was added and the subsequent growth followed by means of absorbance measurements.

Uniformity of cellular inhibition

This was studied using a method similar to that described by Rye & Wiseman (1968b). Control, benzyl and phenethyl alcohol-treated cultures of *E. coli* were incubated in the presence of 2 μg/ml of ampicillin for periods of time which were sufficient to allow a doubling in the total cell mass to occur. This concentration of ampicillin is sufficient to suppress cellular division without affecting the rate of growth. Measurements were made of the coefficients of variation of the cell size distributions before and after the experimental growth periods.

Uptake of benzyl alcohol (BA) and phenethyl alcohol (PEA) by E. coli

Concentrated cell suspensions in glucose-free medium containing approximately $1{\cdot}5 \times 10^{11}$/ml were prepared by harvesting by centrifugation cells from 5 litre volumes of exponentially growing cultures when the absorbance had reached 0·6. 0·5 ml of various dilutions of the labelled alcohols in glucose-free medium were added to 1·5 ml of the concentrated cell suspensions and also to controls containing 1·5 ml of glucose-free medium. These mixtures were allowed to stand at 20° with occasional shaking for periods of up to 90 min. After centrifuging at 5000 rev/min for 10 min, the radioactivity in duplicate 0·2 ml samples of the supernatant liquids was determined using a Packard Tricarb liquid scintillation counter. The alcohol uptake in each suspension was obtained by calculating K, the ratio of the alcohol concentration within the cells to the concentration in the suspending medium.

This was carried out using the equation:

$$K = 1 + \frac{2}{V}\left\{\frac{C - C^1}{C^1}\right\}$$

which takes account of the relatively large volume V occupied by the cells in the 2 ml suspension. C^1 and C are the alcohol concentrations in the supernatant liquids from the treated cell suspension and control mixture respectively. V was obtained by multiplying the total cell count by the mean cell volume and was assumed to remain constant throughout the determination.

Measurement of partition coefficients

Volumes of 10 ml of known dilutions of BA and of PEA in glucose-free medium were mixed with equal volumes of chloroform, n-heptane and octanol and shaken for

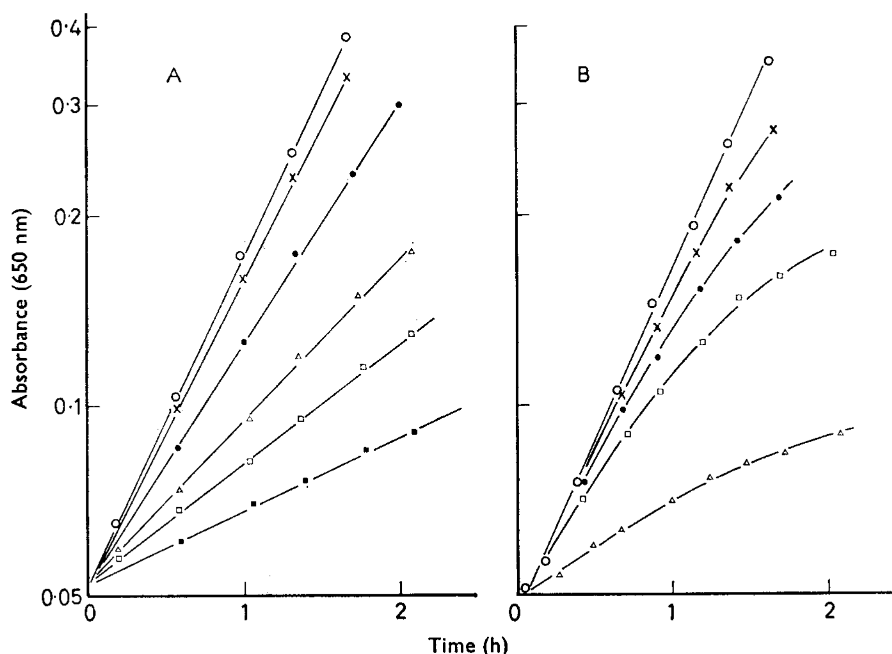

FIG. 1. The effects of (A) benzyl alcohol and (B) phenethyl alcohol on the absorbance of cultures of *E. coli* partially inhibited using method A. Benzyl alcohol concentations ○ 0, × 1, ● 2, △ 3, □ 3·5, ■ 4 mg/ml. Phenethyl alcohol concentrations ○ 0, × 1, ● 1·5, □ 2, △ 2·5 mg/ml.

6 h at 20°. In each case, after separating the two phases, the concentration of alcohol remaining in the aqueous layer was determined by measuring the absorbance at 260 nm. From these measurements the partition coefficients of the alcohols between these organic solvents and glucose-free medium were calculated.

RESULTS

The changes in absorbance occurring over a period of 120 min in cultures partially inhibited by BA and PEA using method A are shown in Fig. 1. In the presence of BA growth occurred exponentially at a rate dependent on its concentration. With PEA growth occurred at a gradually decreasing rate throughout the period of incubation. In similar experiments using media containing [^{14}C] glucose, the ratio of absorbance to cell [^{14}C] content remained constant throughout the growth period, indicating that absorbance measurements are a valid measure of total cell mass even in cultures partially inhibited by BA or PEA.

The reversibility of the growth inhibitory properties of BA and PEA was investigated by diluting cultures partially inhibited by these agents with an equal volume of growth medium. The results given in Fig. 2 show that BA-inhibited cultures on dilution immediately resume growth at a rate characteristic of the new alcohol concentration whereas the growth rate in PEA-inhibited cultures only increases gradually.

For PEA, the results of growth inhibitory studies using method B are shown in Fig. 3. With both agents growth commenced immediately after the addition of glucose and was observed to occur at an exponential rate. The growth rate constants in these cultures and in the exponentially growing cultures of Fig. 1(A) were calculated by the method of least squares and are shown in Fig. 4 plotted with alcohol concentration.

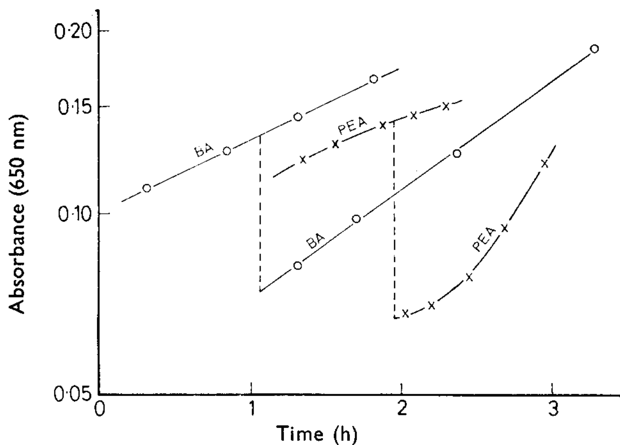

FIG. 2. The effects of dilution on the absorbance of cultures of *E. coli* partially inhibited by benzyl alcohol ○——○ and phenethyl alcohol ×——×. Benzyl alcohol concentration before dilution 2·5 mg/ml, after dilution 1·25 mg/ml. Phenethyl alcohol concentration before dilution 2 mg/ml, after dilution 1 mg/ml.

Each point on the graph is the mean of at least five determinations and is given together with its standard deviation. Increasing the concentrations of either BA or PEA causes a reduction in the growth rate, the extent of the reduction produced by equal increments in alcohol concentration becoming progressively larger at high concentrations. The inhibitory pattern for BA obtained by method B is identical to that obtained using method A. At all concentrations, PEA is more effective in inhibiting growth than equivalent concentrations of BA.

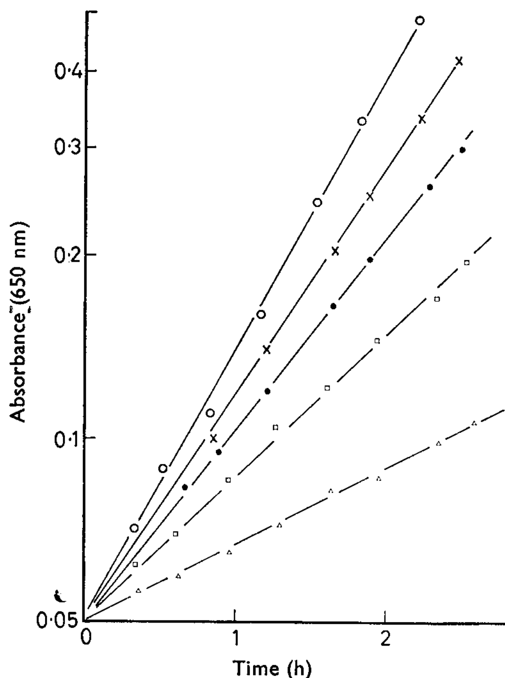

FIG. 3. The changes in absorbance after the addition of glucose to glucose-starved suspensions of *E. coli* in the presence of ○ 0, × 1, ● 1·5, □ 2, △ 2·5 mg/ml of phenethyl alcohol (method B).

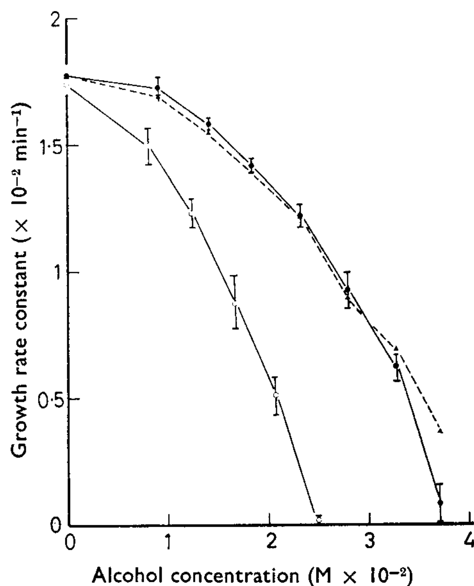

FIG. 4. The exponential growth rate constants of *E. coli* in cultures partially inhibited by benzy alcohol ●——● and phenethyl alcohol ○——○ using method B. The broken line ▲---▲ gives the results for benzyl alcohol using method A. Each result is the mean of at least five determinations and is shown with its standard deviation.

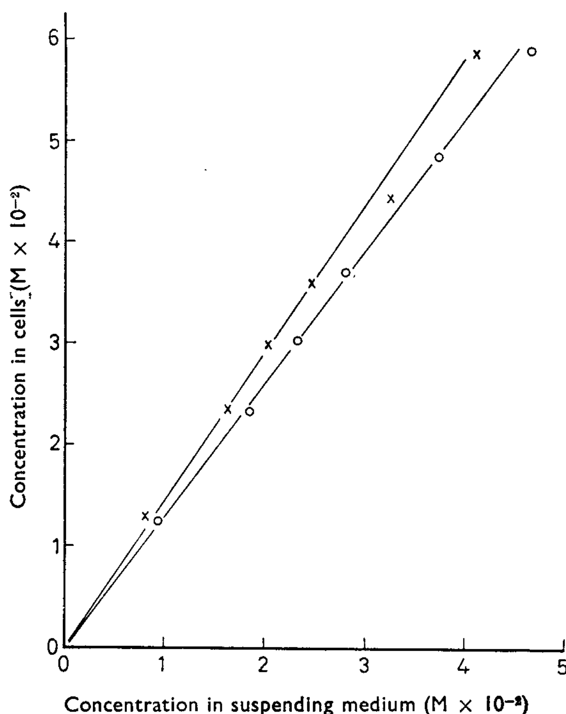

FIG. 5. The uptake of benzyl alcohol ○——○ and phenethyl alcohol ×——× by suspensions of *E. coli* in glucose-free medium. The results for benzyl alcohol and phenethyl alcohol lie on straight lines with slopes of 1·30 and 1·45 respectively.

The uptake of BA after 30 min and of PEA after 60 min by cultures of *E. coli* is shown in Fig. 5. Throughout the concentration range studied the cellular uptake was directly proportional to the concentration of alcohol remaining in the suspending medium. The value of K for PEA (1·45) was only slightly greater than that for BA (1·3) and uptake measurements made after contact times of between 10 and 90 min showed these values to be independent of time. The partition coefficients of BA and PEA between chloroform, octanol and n-heptane and glucose-free medium at 20° are: BA: chloroform 7·0, PEA: chloroform 15·2; BA: octanol 11·7, PEA: octanol 21·5; BA: heptane 0·21, PEA: heptane 0·58. PEA enters the organic phase more readily than does BA with each solvent.

The viability of suspensions of *E. coli* in glucose-free medium incubated in the presence of 4 mg/ml of BA and 3 mg/ml of PEA was determined at 20 min intervals for 2 h. No significant loss in viability was observed.

In experiments made in the presence of ampicillin, the coefficients of variation of the cell size distributions, after a doubling in the total cell mass had occurred, were 0·352, 0·368 and 0·315 in the control culture, BA (2 mg/ml) and PEA (2 mg/ml) treated cultures respectively. These values are not significantly different from that of 0·346 observed at the commencement of the growth period. These results show therefore that the decreased growth rate in partially inhibited cultures is due to a general and uniform inhibition of growth rather than to a loss in viability of some of the cells.

DISCUSSION

Studies of microbial growth in cultures partially inhibited by antibacterial agents have been used to investigate the mechanisms of interaction between molecules of inhibitor and cell receptor sites (Garrett, Miller & Brown, 1966). Evidence has also been obtained which suggests that different enzyme systems control the rate of growth over different ranges of inhibitor concentration (Harris & Morrison, 1961). Growth in the partially inhibited cultures can be studied either by means of absorbance measurements or by following the changes in total or viable cell count. For the successful interpretation of results obtained using counting techniques it is however necessary that the number of receptor sites in the individual cells remains constant and independent of cell mass (Garrett & others, 1966). This is unlikely to be the case if changes in cell size occur in the presence of inhibitor and since PEA causes the formation of long filaments in cultures of *E. coli* (Berrah & Konetzka, 1962), absorbance measurements were selected in these investigations for studying growth.

Rate constants can be calculated only if growth occurs exponentially in the presence of the antibacterial agent. The decrease in growth rate resulting from the addition of inhibitors to exponentially growing cultures may occur gradually, an exponential rate being attained only after prolonged periods of incubation (Mielck & Garrett, 1970). Using method A, exponential growth was not achieved on treating cultures with PEA within the 2 h experimental period. Furthermore the response of cells during recovery from PEA treatment occurred gradually. These observations could be explained if the transfer of PEA across the cellular membrane occurred slowly, but no evidence for this was found in the uptake measurements made after short contact times. Nevertheless the results in Fig. 3 for cultures inhibited by method B suggest that during the period of glucose starvation PEA is able to equilibrate between the medium and its intracellular sites of action thus enabling an immediate and complete manifestation of its inhibitory

properties to be observed after the addition of glucose. With BA no significant difference between using method A and method B could be detected.

The inhibitory patterns shown in Fig. 4 are unlike those which have been reported previously when other agents were used and cannot readily be interpreted using the models elaborated either by Garrett & others (1966) or by Harris & Morrison (1961). Garrett & others (1966) suggested that the inhibitor in the biophase equilibrates with receptor sites, growth occurring at a rate proportional to the fraction of sites remaining uncombined. Similarly Harris & Morrison (1961) postulate that the inhibitor combines competitively or non-competitively with cellular enzymes, the velocity of the enzyme catalysed reaction in the presence of inhibitor being proportional to the concentration of uncombined enzyme. Both models predict that the growth rate will decrease on increasing the inhibitor concentration and that on plotting growth rate with concentration the rate will approach zero asymptotically at high concentrations. The inhibitory patterns obtained with sulphonamides (Garrett & Wright, 1967), lincomycin (Mielck & Garrett, 1969) and erythromycin (Garrett, Heman-Ackah & Perry, 1970) are in approximate agreement with this prediction. With other agents, however, modifications to the basic theory were necessary in order to explain the experimental evidence. Thus with chloramphenicol and tetracycline (Garrett & others, 1966) and spectinomycin (Mielck & Garrett, 1970), where a linear relation between growth rate and inhibitor concentration was observed, it was postulated that only a small fraction of the receptor sites required blocking for total inhibition of bacetrial growth. With tetracycline and oxytetracycline (Jones & Morrison, 1962) the complex inhibitory patterns were attributed to multiple enzyme inhibition, different enzymes being inhibited to differing extents after different threshold concentrations of inhibitor had been reached.

There have previously been no quantitative studies reported of the growth inhibitory properties of either BA or PEA and the results of investigations of the mode of action of PEA have been conflicting. Berrah & Konetzka (1962) reported that in the presence of PEA, DNA synthesis was inhibited whereas RNA and protein synthesis were unaffected. Treick & Konetzka (1964) found that when added to stationary phase cells this agent immediately inhibited DNA synthesis but that an increase in DNA content of between 40–60% occurred when exponentially growing cells were used. These results indicated that PEA prevents the initiation of DNA replication and it was suggested that the site of action of PEA might be the bacterial cell membrane. Lark & Lark (1966) reached a similar conclusion.

Other reports however claim the chief effects of PEA to be an interference in the function or biosynthesis of mRNA (Rosenkranz, Carr & Rose 1964), an inhibition of enzyme induction (Rosenkranz, Carr & Rose, 1965) or a general inhibition of RNA synthesis (Prevost & Moses, 1966). Silver & Wendt (1967) showed that PEA altered the cellular permeability barrier and concluded that its effects resulted from a non-specific reversible attack on the cell membrane, the absorption of PEA by the membrane producing a breakdown of its structural integrity. This could cause an inhibition of such cellular processes as DNA synthesis either indirectly by allowing leakage of small molecules from the cells, or directly if the process were coupled to the membrane.

The results reported here are not inconsistent with this proposed mode of action. Both BA and PEA are likely to be absorbed by cellular membranes, the hydrocarbon moiety penetrating and being reversibly bound by hydrophobic interactions within the phospholipid region, the relatively polar hydroxyl groups remaining associated with

the protein layer. Absorption of these molecules would then interfere with membrane bound enzymes by inducing conformational changes in the membrane structure. An orientation of the molecules perpendicular to the plane of the membrane would allow cooperative absorption, each membrane bound molecule facilitating the uptake of further molecules. The molecular structure of these alcohols, each being monofunctional and possessing a hydrocarbon residue, favours such an arrangement and would be expected to produce an uptake pattern corresponding closely to the S1 isotherm described by Giles, MacEwan & others (1960). The inhibitory patterns given in Fig. 4 are then readily explained if the extent of inhibition is assumed to be proportional to the amount of membrane bound inhibitor. The uptake of BA by wool has been shown to follow an S1 isotherm (Giles & others, 1960) and Bean & Das (1966) reported that the uptake by *E. coli* of six phenolic agents approximately followed an S1 mechanism after a short initial linear portion.

The results shown in Fig. 5 do not conflict with this hypothesis. The values of K reported here are a reflection of the total alcohol concentration within the cell and not just of the alcohol bound at the cell membrane. Thus the presence of alcohol in simple solution in the aqueous biophase of the cell would readily mask an S1 uptake at the cell membranes and would produce a linear uptake isotherm as shown in Fig. 5. It is evident from Fig. 4 that PEA is more effective than BA in inhibiting growth. Since however, the overall uptake of these alcohols is essentially similar, it seems probable that within the cell the proportion of PEA which is bound by the membrane is greater than that of BA. The partition data presented support this conclusion since in all three solvents PEA enters the organic phase more readily than BA.

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