Bacterial Survival in Suspension in Polyethylene Glycol Solutions

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SUMMARY

The % survivals of *Escherichia coli* (B, Jepp, commune), *Serratia marcescens* (UK8) and *Aerobacter aerogenes* (Hinshelwood), in the presence of polyethylene glycol were studied as a function of molecular weight and % polyethylene glycol. Polyethylene glycol is shown to cause a loss of viability; experiments are described which suggest loci for its action.

INTRODUCTION

During an investigation of the protective action of solutes on the survival of bacteria as aerosols it was observed that polyethylene glycol (PEG) was toxic (Cox, 1965). The present paper reports *in vitro* experiments which were designed to elucidate this toxic action on *Escherichia coli* (B, Jepp, commune), *Serratia marcescens* (UK8) and *Aerobacter aerogenes* (Hinshelwood).

METHODS

Organisms. Cultures of *Escherichia coli* (B, Jepp, commune), *Serratia marcescens* (UK8) and *Aerobacter aerogenes* (Hinshelwood), were grown in a 2 % tryptone medium (pH 7.6) for 16 hr at 37° by a shake flask technique. Each day suspensions of organisms were prepared by centrifugation from the culture fluid and resuspension in phosphate buffer (pH 7.6), unless stated otherwise.

Procedure. The bacterial suspensions were serially diluted in phosphate buffer (pH 7.6) to give the chosen concentration, usually 10^5 colony-forming units/ml.; 0.5 ml. of this suspension was added to 4.5 ml. of buffer + polyethylene glycol (PEG) (supplied by L. Light and Co. Ltd., Colnbrook) mixtures of differing PEG compositions, % (w/v), and were allowed to equilibrate. After a standard time, 1 ml. samples were removed and added to 9 ml. buffer. The degree of survival in each sample was determined by surface drop plates (Miles & Misra, 1938) on 1 % tryptone agar or 1 % peptone agar in Petri dishes. After overnight incubation at 37° the colonies were counted on six plates/sample.

So that more information could be obtained, this procedure was modified to allow a study of the ability of *Escherichia coli* B to reproduce phage T7, when these *E. coli* organisms were treated with PEG. The technique with phage in aerosol studies was reported previously (Cox & Baldwin, 1964, 1966). The bacteria, after centrifugation, were resuspended to give a concentration of 3×10^9 /ml. in tryptic meat broth at 37°

containing 0.004 M-potassium cyanide added to prevent phage development. After 2 min, to allow the cyanide to take effect, phage T7 was added and the mixture maintained at 37° for a further period of 10 min. to allow adsorption to occur. After centrifugation, which left unadsorbed phage in the supernatant fluid, the deposited bacteria were resuspended at room temperature in broth containing 0.004 M-cyanide, and the centrifugation and resuspension steps were repeated. Dilution in phosphate buffer gave 10^5 phage T7 infected *E. coli* B organisms/ml. suspension. The procedure then followed that detailed above for non-infected bacteria except that the ability to reproduce phage was determined by plaque formation in 0.5 % soft agar in tryptic meat broth layered on 1 % tryptone agar in Petri dishes. As a check on the handling procedure the experiments with infected bacteria were conducted in the presence of *Bacillus subtilis* var. *niger* spores, at a concentration similar to that of the *E. coli* B. In this manner the % plaque formation (and the % colony formation for a control of non-infected *E. coli* B) could be related to the initial bacterial concentrations allowing for dilution, or to the number of *B. subtilis* var. *niger* colonies, for the sample under consideration.

The estimates of colony formation of *Escherichia coli* B, and for plaque formation by phage T7, initially were conducted with tryptone plates containing crystal violet which inhibited the growth of *Bacillus subtilis*. This procedure, however, also partially inhibited the growth of *E. coli* B so crystal violet was omitted. The two bacteria were readily differentiated on the control plates, since *E. coli* B formed white colonies and *B. subtilis* var. *niger* formed orange colonies; the presence of *B. subtilis* var. *niger* did not interfere with phage plaque formation.

RESULTS

Time of contact between polyethylene glycol (PEG) and bacteria

Results with each of the five organisms in the presence of PEG of molecular weights of 200 and 15,000 at 50 % PEG and 40 % PEG in phosphate buffer (pH 7.6) respectively, were obtained. The curves approximated to a two-stage loss of viability in that an initial rapid decay (0–1 min.) occurred, followed by a much slower decay (1–60 min.). A convenient standard equilibration time of 30 min. was adopted, unless stated otherwise.

Age of bacterial culture

The influence of age of the bacterial culture was investigated in detail for only the Jepp strain of *Escherichia coli*. It was found that there was no significant difference in the behaviour of cultures of various ages from 5 hr to 8 days, timed from the end of the 16-hr growth period. This enabled cultures to be prepared weekly. Less detailed studies suggested that the same was true for the other four organisms.

Temperature for equilibration

The % survival of a suspension of *Escherichia coli* Jepp in PEG (1540) and PEG (9000) was determined at 4°, 10°, 22°, 30° and 37° as a function of % PEG made up in phosphate buffer (pH 7.6). The effects were least at 4° and greatest at 22°. As a consequence the experimentally convenient temperature of the room (21°) was used for later experiments.

Bacterial survival as a function of molecular weight and % PEG

The % survival (mean of two determinations) of each of the five organisms was studied as a function of % PEG of molecular weights 200, 300, 400, 600, 1540, 9000, 15,000 made up in phosphate buffer (pH 7.6) Figures 1-4 illustrate the survival observed for PEG 200, 600, 1540, 15,000, respectively. The data were plotted as a function of % PEG, rather than moles/l., because the molecular weight dependence

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Figs. 1-4. The survival of bacteria as a function of polyethylene glycol (of different molecular weights)+phosphate buffer mixtures at 21°. Fig. 1. Molecular weight 200. Fig. 2. Molecular weight 600. Fig. 3. Molecular weight 1540. Fig. 4. Molecular weight 15,000. ○, *Escherichia coli* B; △, *E. coli* Jepp; □, *E. coli* commune; ■, *Serratia marcescens*; ●, *Aerobacter aerogenes*.

was less apparent and virtually disappeared with *Escherichia coli* Jepp. Results obtained with PEG (300) were similar to those obtained with PEG (200), while with PEG (400) the survivals were intermediate between those using PEG 200 and 600. For the polymer fractions of 9000 and 15,000, the pattern for the five organisms was similar in that the survival curves all showed a break at about 25% PEG, followed by a marked change of slope at about 30% PEG. For lower molecular weight fractions the patterns were less consistent.

Effects of bacterial concentration

Escherichia coli (Jepp, B) was studied for the effect of the bacterial concentration. The results for concentrations of 1×10^9 and 1×10^4 bacteria/ml. were similar and showed no marked effect of concentration over this range.

Clumping behaviour

Suspensions at a bacterial concentration of 1×10^9 bacteria/ml. were studied to determine whether the clumping behaviour of the bacteria was modified in the presence of PEG (15,000). With *Escherichia coli* B a stable dispersion formed at 30 % PEG, corresponding to the minimum in the survival curve for PEG (15,000) (Fig. 4); at PEG concentrations higher and lower than 30 %, flocculation or aggregation (LaMer & Healy, 1963) occurred, i.e. the state of clumping and % survival appeared to be related. In PEG (200) a similar relationship was observed between clumping behaviour and % survival.

Fig. 5

Fig. 6

Fig. 5. The survival of *Escherichia coli* B in polyethylene glycol (molecular weight 15,000) at 21°. ○, Polyethylene glycol in phosphate buffer; organisms collected in phosphate buffer. ■, Polyethylene glycol in tryptic meat broth; organisms collected in tryptic meat broth. △, Polyethylene glycol in distilled water; organisms collected in distilled water. ●, Polyethylene glycol in distilled water; organisms collected in phosphate buffer. □, Polyethylene glycol in distilled water; organisms collected in tryptic meat broth.

Fig. 6. Survival of *Escherichia coli* B in polyethylene glycol + phosphate buffer mixtures as a function of polyethylene glycol (15,000) concentration and pH value. —, 0 % PEG; ○, 10 % PEG; □, 20 % PEG; ●, 25 % PEG; ■, 30 % PEG; △, 40 % PEG.

Competitive action of other solutes with PEG

Protection of *Escherichia coli* Jepp by sucrose in the presence of PEG (1540) at 30 % PEG, but not at 60 % PEG, was reported previously (Cox, 1965). As an extension of these observations, but with *E. coli* B, solutions of PEG (15,000) in distilled water, phosphate buffer and tryptic meat broth, were studied (Fig. 5). The concentration at which PEG became toxic was dependent upon the presence of other

solutes. The substantial protection afforded by tryptic meat broth was also markedly apparent for 0.8 % tryptone (or greater), provided that the tryptone was present during contact of the bacteria with PEG (15,000). Contact of bacteria with tryptic meat broth after PEG treatment did not completely prevent the PEG stress. The degree of survival was dependent upon the diluting fluid used for the assay of survival (Fig. 5). The protective action of tryptone, even at 2 %, was not marked for PEG (200).

Influence of pH value

One of the differences between distilled water, phosphate buffer and tryptic meat broth was the pH value of these liquids. Figure 6 gives % survival of *Escherichia coli* B as a function of pH value over the range pH 4–11 and of % PEG (15,000); it shows that the toxic action of PEG (15,000) was least in the region of pH 8. The distilled water used for the data of Fig. 5 showed a value of pH 5; the curve at pH 5 in the presence of buffer closely follows the curve for distilled water. The addition to distilled water of a small quantity of buffer salt (0.001 M) to give pH 8 resulted in behaviour close to that at pH 8 in Fig. 6, i.e. the apparently greater toxicity of PEG (15,000) in distilled water was due to the pH value rather than to the low ionic strength.

Table 1. *Survival in polyethylene glycol of Escherichia coli B and Escherichia coli B + phage T7 complex*

	No. of deter- minations	Arithmetic mean	Standard deviation
% survival of control	7	26.4	3.4
% lysis*	5	94.2	8.5
% lysis†	4	56.7	3.8

* On non-dyed agar plates.

† On agar plates + crystal violet.

The Escherichia coli B + phage T7 complex

Escherichia coli B infected with phage T7 in an undeveloped state were prepared by the method described. The results of subjecting these bacteria and the non-infected controls to 30 % PEG (15,000) for 10 min. are given in Table 1. The data included results for culture ages of from 16 hr to 7 days, and for phage:bacteria ratios of 1:2, 1:5, 3, 7 and 30:1. It is concluded that the action of PEG (15,000) upon *E. coli* B, did not affect those reactions involved in phage reproduction; however, the combined action of PEG (15,000) and crystal violet partially inhibited phage growth and lysis. In the terms used previously (Cox & Baldwin, 1964, 1966) the action of PEG (15,000) upon *E. coli* B is to prevent *E. coli* B reproduction.

The result of subjecting the *Escherichia coli* B infected with phage T7 and the non-infected controls to PEG (200) at 60 % for 10 min. was that lysis and survival values of the order of 12 % were obtained. This suggests that PEG (200) influenced the *E. coli* B phage productive capacity (Cox & Baldwin, 1964, 1966). i.e. the action of high and low molecular weight PEG would seem to be at different loci of action.

DISCUSSION

The use of phage showed that the reproductive capacity of *Escherichia coli* B was impaired by polyethylene glycol (PEG; 15,000). Since the latter penetrates the cell wall very slowly, if at all, and does not penetrate the cytoplasm, both the cell wall and cell division seem to be implicated as at least one locus of action of PEG (15,000). PEG of molecular weight 200, being able to pass through the cell wall but not the cytoplasmic membrane, may be able to operate at a second locus of action concerned with *E. coli* B phage productive capacity, but which cannot be reached and preserved by tryptone. The clumping behaviour of bacteria in PEG (15,000 and 200) also suggests that changes in the cell surface occur, since clumping behaviour depends upon the interactions of cell-wall surfaces. It is perhaps a coincidence that the stable suspension forms at the % PEG, which gives a minimum in the survival curve (Fig. 1, 4), although the action of tryptic meat broth is both to prevent the action of PEG (15,000) and also to prevent dispersion.

Since the data exhibit a more regular pattern when related to a % rather than a molar concentration scale, the concentration of the repeat unit of the PEG may be the important factor. Percentage PEG values are approximately converted to moles repeat unit/l. by multiplying the % value by a factor of 0.22. Of the five organisms, *Escherichia coli* Jepp showed the least dependence upon molecular weight, although any such relationship may be fortuitous, since it was shown that PEG of low and high molecular weight had different actions. Other substances (from the Dow Chemical Co. Michigan) such as polypropylene glycol (400); Ambiflo H7, H52, H438 (300, 1000, 3500 respectively) a dialkoxyether of polyoxyalkylene glycol; Hyprin GP30 (266) a hydroxypropyl glycerol; polyglycol 15-200 (2600) $\text{CH}_2\text{OR}_n\text{H}.\text{CHOR}_n\text{H}.\text{CH}_2\text{OR}_n\text{H}$, where R is $\text{C}_2\text{H}_4\text{O}-\text{C}_3\text{H}_6\text{O}$, behaved somewhat like PEG. Thus the phenomenon does not appear to be specific with regard to the nature of the agent.

The action of PEG may involve its adsorption; it was suggested (Cox, 1965) that the wetting properties of the bacterial surface might be effected. However, since phage T7 reproduction was not impaired by PEG (15,000) a change of wetting properties is not a likely explanation. Polymer phase separations in which a mixture of polymers forms a single homogeneous phase or separates into phases depending upon concentration, etc., may be pertinent. Such systems have been studied by Dervichian (1954), by Hyde, Langbridge & Lawrence (1954), by Albertsson (1960) and by Polson *et al.* (1964). Polson *et al.* found that with mixtures of protein + polyvinyl alcohol type, precipitation and denaturation of protein occurred, whereas mixtures of protein + dextran or + PEG resulted in phase separation without denaturation. The protein precipitation was dependent upon protein concentration, temperature, pH value and PEG concentration; this behaviour is similar to that described in the present paper.

The protective action of sucrose and perhaps protein is consistent with phase separation since multiplicity of hydroxyl groups produces marked increases in solubility (Hyde *et al.* 1954). The effect of pH value is consistent also with phase separation of an ionic polymer, since the solubility of such a polymer is related to its charge and this will change with the pH value. An understanding of the influence of temperature in terms of phase separation would require knowledge of the phase

diagram, since temperature changes can cause dramatic effects (Albertsson, 1960), while the marked recovery in viability for PEG of high molecular weight (Fig. 4) is not contrary to phase separation (Hyde *et al.* 1954). It therefore seems possible that the action of PEG might be explained through the phase separation of groups which normally would be in solution and attached to the cell membranes. Such an action would then have to cause a detrimental change in the structure of the cell membranes.

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