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Effect of Polysorbate (Tween) 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation

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Log phase cultures of *Pseudomonas aeruginosa* in nutrient broth containing polysorbate 80 were much less resistant to the action of benzalkonium chloride, chlorhexidine diacetate and polymyxin B sulphate than cells grown in plain broth.

THE present work was started in an attempt to investigate the resistance of *Pseudomonas aeruginosa* to chemical agents. The method used was based on the principles proposed by Brown & Garrett (1964) and modified by Brown & Richards (1964) for use with this organism.

The increase in numbers of exponentially dividing cells can be described by

$$X = X_0 e^{kt} \qquad (1)$$

where X is the number of cells per unit volume at time t, and X_0 is the concentration at time $t = t_0$: the apparent first order rate constant k describes the rate of growth and may be derived from the slope of the line described by the logarithmic transformation of equation (1)

$$\log X = {}^{kt}/_{2{\cdot}303} + \log X_0 \qquad (2)$$

When a chemical agent is added to a culture of log phase cells, any change in the constant k may be attributed to the action of the chemical.

Benzalkonium, chlorhexidine and polymyxin B sulphate were initially examined because *Ps. aeruginosa* is less resistant to these agents than to others. (Kohn, Gershenfeld & Barr, 1963a,b; Riegelman, Vaughan & Okumoto, 1956; Anderson & Stock, 1958).

Experimental

The test organism used was *Ps. aeruginosa* strain NCTC 8203; the basic medium was Oxoid nutrient broth No. 1 whilst the incubation and reaction temperature was 37·5°. Growth rates were followed by measuring the absorbance at 420 mμ with a Unicam 600 spectrophotometer. Antibacterial agents used were benzalkonium chloride B.P.C., chlorhexidine diacetate (I.C.I.) and polymyxin B sulphate kindly supplied by Messrs Burroughs Wellcome & Co. Details of methods and materials have been described previously (Brown & Richards, 1964).

EFFECT OF CHEMICAL AGENTS ON GROWTH RATE

Replicate inocula of log phase cells in nutrient broth were added separately to pre-warmed broth containing graded concentrations of Tween 80. Each concentration was duplicated. Every culture was allowed to grow until it was at a suitable stage in the log phase when one of each pair was inoculated with 0·5 ml of an aqueous solution of the antibacterial agent. The other one of each pair was simultaneously

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inoculated with water. The final volumes were about 100 ml. Spectrophotometric readings were taken at intervals and representative results with benzalkonium (35 μg/ml), chlorhexidine (10 μg/ml) and polymyxin (3·3 units/ml) are shown in Figs 1–3 respectively.

EFFECT OF TWEEN 80 ON SURVIVAL TIME WITH CHLORHEXIDINE

An end-point experiment was made to determine whether the enhancing effects of Tween 80 on the action of chlorhexidine against exponentially dividing cells could also be shown with washed log phase cells.

Preparation of inoculum. An overnight culture was used to inoculate nutrient broth to give a final volume of 100 ml: this was then incubated. Replicate samples of log phase cells were then used to inoculate separately, plain broth and broth with 0·5% Tween 80. These two cultures were allowed to grow into the log phase when 8 ml of each were removed, the cells washed once by centrifugation and resuspended in 80 ml water to give two suspensions, P (from plain broth) and T (from broth with Tween).

Preparation of reaction mixtures. Two series of 15 tubes were made, each consisting of 3 replicates of 5 concentrations of chlorhexidine in water (10, 20, 50, 100 and 200 μg/ml).

One series was inoculated with 0·5 ml samples from P and the other with 0·5 ml samples from T. These were designated reaction mixtures and were maintained at 37·5°.

Determination of survival time. Samples of 0·5 ml were taken from each reaction mixture after 0·25, 0·5 and 2 hr; they were added to 5 ml recovery medium and incubated one week. The recovery medium was that of Riegelman, Vaughan & Okumoto (1956) without agar. Colony counts showed that the reaction mixtures initially contained about 4×10^3/ml viable cells. Positive controls to test the efficiency of recovery media consisted of 0·5 ml samples of P and T separately added to 9·5 ml distilled water at 37·5°. Samples of 0·5 ml were then withdrawn and added to 5 ml of recovery media containing 0·5 ml of the 200 μg/ml chlorhexidine solution. Negative controls consisted of chlorhexidine solutions of each concentration used. These were incubated at 37·5° and 0·5 ml samples withdrawn and used to inoculate 5 ml recovery media.

Results and discussion

EFFECT OF CHEMICAL AGENTS ON GROWTH RATE

There was no appreciable effect when benzalkonium was added to log phase cells in nutrient broth to produce a concentration of 35 μg/ml (Fig. 1A). The same concentration immediately reduced to about zero the growth rate in broth containing 0·02% Tween (Fig. 1B). This enhancing effect is particularly remarkable because Tween 80 is used as an antagonist of benzalkonium (Kohn, Gershenfeld & Barr, 1963a). Such antagonism is shown by Figs 1B and 1C, a comparison of which shows that a 10 fold increase in Tween 80 almost eliminated the inhibitory effect of 35 μg/ml benzalkonium. It was not possible to use concentrations of

benzalkonium greater than about 35 μg/ml in plain broth because of precipitation effects.

A similar phenomenon was observed with 10 μg/ml chlorhexidine which

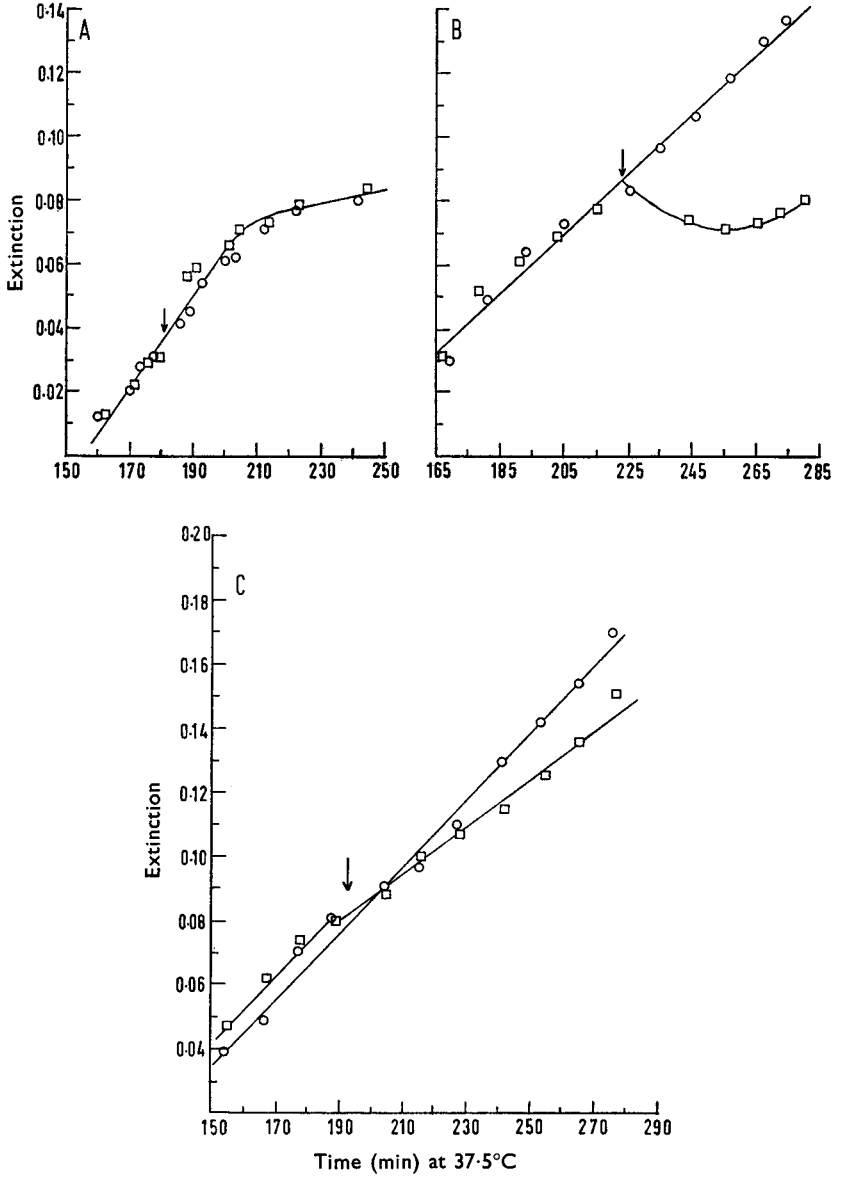

FIG. 1. Effect of Tween 80 on the action of 35 μg/ml benzalkonium chloride against log phase cultures of *Ps. aeruginosa*. ○, Control culture. □, Test culture. A, nutrient broth. Benzalkonium added after 180 min. B, nutrient broth + Tween 80, 0·02%. Benzalkonium added after 223 min. C, nutrient broth + Tween 80, 0·2%. Benzalkonium added after 193 min.

produced lysis in plain broth (Fig. 2A). The rate of lysis was increased in the presence of 0·02% Tween 80, but 0·5% eliminated any observable effect of the chlorhexidine (Fig. 2B). The enhancing of chlorhexidine activity by Tween 80 was much less marked than with benzalkonium and polymyxin.

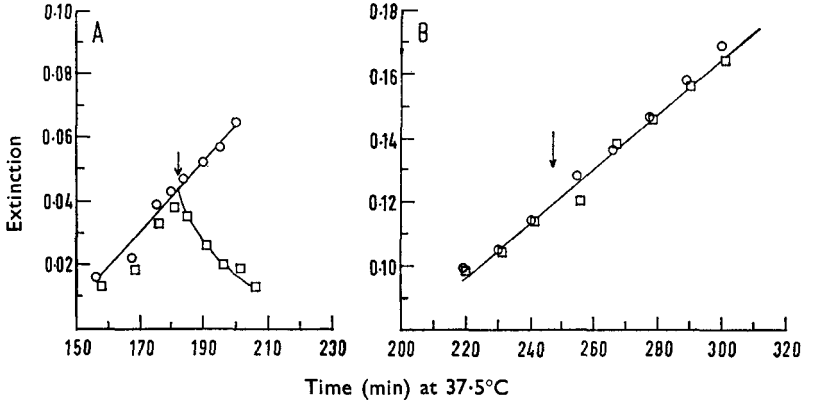

FIG. 2. Effect of Tween 80 on the action of 10 μg/ml chlorhexidine diacetate against log phase cultures of *Ps. aeruginosa*. ○, Control culture. □, Test culture. A, nutrient broth. Chlorhexidine added after 182 min. B, nutrient broth + Tween 80, 0·5%. Chlorhexidine added after 247 min.

The inhibitory effect of 3·3 units/ml polymixin B sulphate on the growth rate of *Ps. aeruginosa* was apparently enhanced in the presence of each of the concentrations (up to 0·5%) of Tween 80 tested. The effect increased with increasing concentration of Tween 80 (Fig. 3A and B).

Antibacterial agents were added at different stages in the log phase and with one exception the results were similar to those shown in Figs 1–3. When benzalkonium was added to plain broth cultures sufficiently early in the log phase it did not affect the rate of growth but did affect the onset of the final stationary phase which occurred sooner than with the control. This phenomenon did not occur in the presence of Tween 80.

These experiments were repeated using another strain of *Ps. aeruginosa* NCTC 7244, isolated from an eye infection. Tween 80 gave substantially the same effect with these chemical agents.

EFFECT OF TWEEN 80 ON SURVIVAL TIME WITH CHLORHEXIDINE

There is a correlation between the effects of Tween 80 on growth rate and lysis of *Ps. aeruginosa* in the presence of chlorhexidine observed spectrophotometrically, and its effects on survival time of washed cells. Log phase cells grown in the presence of Tween 80 and then washed showed no recovery after any of the contact periods with any concentration of chlorhexidine. Log phase cells grown in plain broth and washed were more resistant to the chlorhexidine and showed growth after 120 min contact with 20 μg/ml and up to 15 min contact with 50 μg/ml. In both

systems cells grown in the presence of Tween 80 were less resistant than cells grown in its absence.

The resultant concentration of Tween 80 in the survival time reaction mixtures, where the cells were washed with water, was much less than that previously shown to have an effect on the reactions measured optically with log phase cells. However, *Ps. aeruginosa* produces much slime

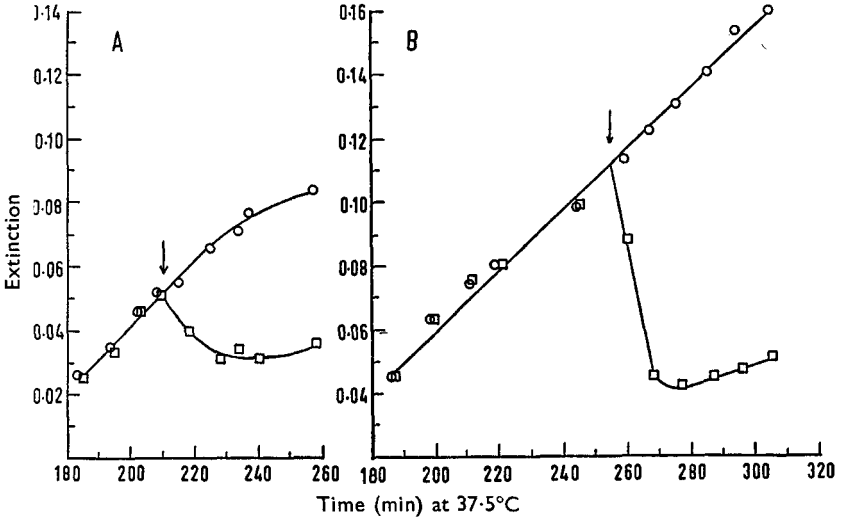

FIG. 3. Effect of Tween 80 on the action of 3·3 units/ml polymyxin B sulphate against log phase cultures of *Ps. aeruginosa*. ○, Control culture. □, Test culture. A, nutrient broth. Polymyxin added after 210 min. B, nutrient broth + Tween 80, 0·5%. Polymyxin added after 254 min.

(Rhodes, 1959) and we have verified that strains NCTC 8203 and 7244 are not exceptions. It seems likely that in all instances the presence of Tween 80 increased the rate at which the slime dispersed from the surface of the actively dividing cells and this may have rendered the organism more sensitive to chemical attack, both during growth and after washing.

Another possibility is that Tween 80 is affecting cell membrane permeability and allowing the penetration of chemicals in low concentrations which would not enter the cell in the absence of Tween. We are testing both these hypotheses.

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