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The effect of Polysorbate (Tween) 80 on the growth rate of *Pseudomonas aeruginosa*

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Problems associated with growth rate measurement for *Pseudomonas aeruginosa* have been investigated. There is an apparent change in the rate of growth at an early stage when determined spectrophotometrically. This change is associated with cell clumping. The presence of polysorbate 80 in the culture medium eliminated this effect. The reproducibility of the growth rate measurement has been established.

THE resistance of *Pseudomonas aeruginosa* to chemical antibacterial agents causes serious difficulties in ophthalmology (Brown, Foster, Norton & Richards, 1964) and in the control of cross infection (Rogers, 1960). Brown & Garrett (1964) investigated the activity of chemical agents by measuring their effect on the growth rate of exponentially dividing cells of *Escherichia coli*. The advantages of this method were discussed by them.

The present work was undertaken to find out if the method is suitable for use with *Ps. aeruginosa*. There are difficulties associated with determining the growth rate of this organism because of its tendency to clump and also to form a pellicle. This paper reports results of adding polyoxyethylene sorbitan mono-oleate [polysorbate (Tween) 80] to the culture media in an attempt to overcome these difficulties.

Experimental

Ps. aeruginosa (NCTC 8203) was the test organism and Oxoid nutrient broth No. 1 was used as the basic medium. Incubation was at 37·5°. A broth culture from an isolated colony was used to inoculate replicate agar slants which were stored frozen. A fresh slant was used for each experiment.

GROWTH RATE DETERMINATIONS

Preliminary experiments showed that aeration of log phase cultures stimulated pellicle formation to the extent that rate determination was not possible. Without artificial aeration the formation of a pellicle was delayed for the minimum time necessary to establish the rate (about 40 min). Growth rates of log phase cells were measured using a Unicam 600 spectrophotometer at 420 mμ (Fig. 1 plain broth); in addition direct microscopic observation was made. The procedure of Brown & Garrett (1964) was used to reduce any lag phase to a minimum. An overnight broth culture was used to inoculate pre-warmed broth and the absorbance was then measured at intervals. At a suitable value, such that the cells were known to be dividing exponentially, a sample was further diluted with broth in an aluminium capped 250 ml conical flask maintained at

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37·5°, the final volume being 100 ml. This final culture was incubated until the cell concentration was once more sufficiently high to allow absorbance readings (about 130 min). Measurements were then made at intervals to determine the growth rate. Growth rates of the initial log phase for five replicate cultures were determined on each of three days (Table 1). The reproducibility of these measurements was determined by an analysis of variance (Table 2).

TABLE 1. GROWTH RATE CONSTANTS $\times 10^4$ FOR REPLICATE *Ps. aeruginosa* CULTURES ON DIFFERENT DAYS IN PRESENCE AND ABSENCE OF TWEEN 80

	Day 1	Day 2	Day 3
Nutrient broth	12·22	12·55	12·89
	12·31	12·50	12·37
	12·41	12·50	12·22
	12·38	12·59	12·55
	12·85	12·63	12·86
Nutrient broth + Tween 80, 0·02%	9·87	9·44	9·60
	9·52	9·60	9·44
	9·02	10·13	9·65
	9·52	9·65	10·16

TABLE 2. ANALYSIS OF VARIANCE OF GROWTH RATE CONSTANTS IN TABLE 1.

	Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio (F)
Nutrient broth	Between days	$5·95 \times 10^{-10}$	2	$2·97 \times 10^{-10}$	1·7
	Within days	$6·0 \times 10^{-9}$	12	$5·0 \times 10^{-10}$	
	Totals	$6·6 \times 10^{-9}$	14		
	s.d. : % of mean = 1·7				
	Tabulated value of (F) at 0·05 significance level = 19·4				
Nutrient broth + Tween 80, 0·02%	Between days	$1·4 \times 10^{-9}$	2	$7·0 \times 10^{-10}$	1·4
	Within days	$9·2 \times 10^{-9}$	9	$1·0 \times 10^{-9}$	
	Totals	$10·6 \times 10^{-9}$	11		
	s.d. : % of mean = 3·2				
	Tabulated value of (F) at 0·05 significance level = 19·3				

EFFECT OF TWEEN 80 ON GROWTH CURVE

The shortness of the initial log phase (Fig. 1 plain broth) caused some difficulty in making a sufficient number of measurements to establish the growth rates of several cultures growing simultaneously. Microscopic examination showed that the end of the initial log phase occurred at the same time as clumping of cells. *Ps. aeruginosa* produces considerable amounts of slime (Rhodes, 1959) and it seemed possible that this contributed to the clumping.

Tween 80 was added to the broth in an attempt to disperse the cells and thus prolong the initial phase. Replicate inocula were added to nutrient broth containing eight concentrations of Tween 80 graded between 0·004 and 0·5%. Fig. 1 illustrates representative growth curves obtained with and without Tween 80 in unaerated broth. Growth rates were determined for four replicate cultures on each of three days using nutrient broth containing 0·02% Tween 80 (Table 1). An analysis of variance is given in Table 2. The effect of aeration on cultures growing in the presence of 0·1% Tween 80 is illustrated in Fig. 2. Aeration was effected by shaking the flask at a rate of 120 throws/min.

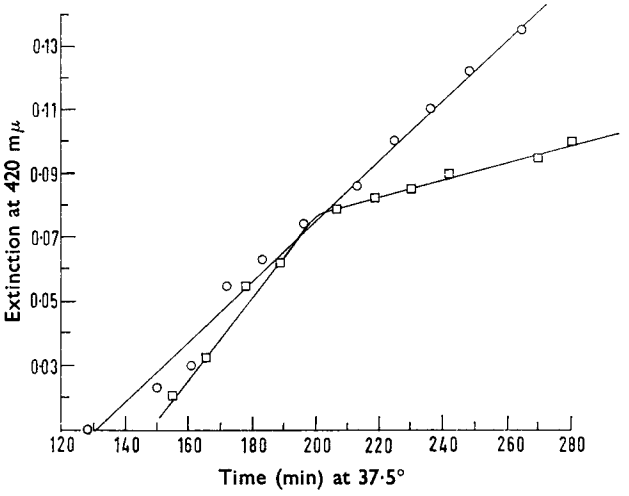

FIG. 1. Effect of Tween 80 on the growth curve of *Ps. aeruginosa* in nutrient broth. ○ Nutrient broth + Tween 80, 0·02%. □ Nutrient broth.

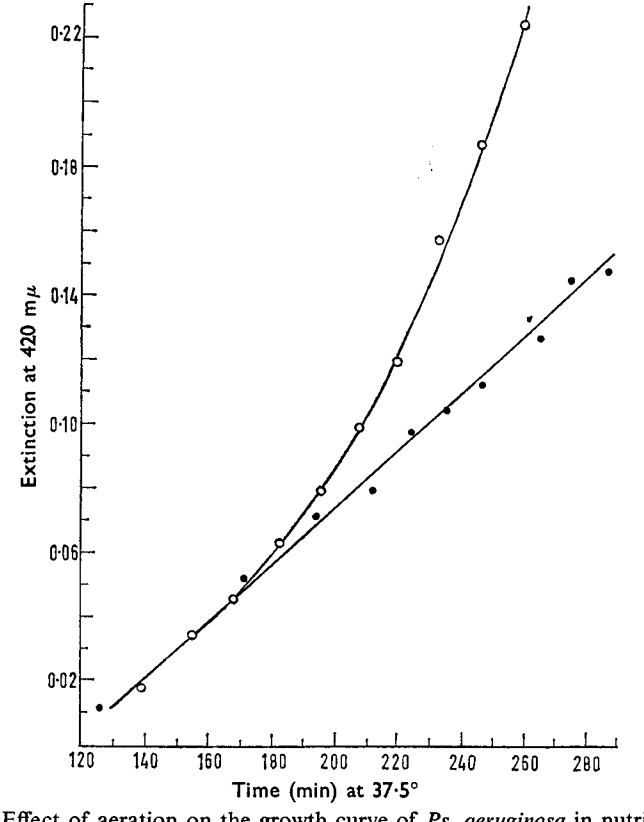

FIG. 2. Effect of aeration on the growth curve of *Ps. aeruginosa* in nutrient broth + Tween 80, 0·1%. ○ Culture shaken at 120 throws/min. ● Control, not aerated.

EFFECT OF ADDING WATER TO LOG PHASE CELLS

Initial work showed that the addition of 1 ml of distilled water to 100 ml (approx.) cultures of log phase cells had an effect greatly in excess of that expected from dilution alone. This phenomenon is important because it is intended to investigate the activity of chemical antibacterial agents by measuring alterations in growth rate after the addition of small volumes of an aqueous solution of the agent. Replicate log phase cultures were incubated in a water bath at 37·5°. Sterile distilled water was boiled and cooled to 37·5° immediately before use and added to one or more of the cultures as follows:

(*a*) 1 ml added to the culture using a 1 ml pipette and the culture afterwards briefly shaken by hand to mix.

(*b*) As in (*a*) except that the culture is briefly shaken by hand during addition of water.

These experiments were repeated using 0·5 ml water. Representative results are illustrated in Fig. 3.

Results and discussion

Fig. 1 illustrates the characteristic shoulder to the growth curve for *Ps. aeruginosa* in nutrient broth. The presence of 0·02% or more Tween 80 in the medium eliminated this shoulder. Microscopic examination made concurrently showed that clumping was absent in the presence of the Tween. This would suggest that Tween 80 has the effect of dispersing cell aggregates which contributed to the observed apparent alteration in rate. The growth rate in nutrient broth in the presence of concentrations of Tween 80 within the range 0·02–0·5% showed no difference. In cultures which were not aerated, pellicle formation occurred after 250 min in plain broth but took progressively longer in the Tween broth as the concentration of the agent increased: 0·05% Tween 80 delayed formation until after 290 min. The presence of Tween 80 in the aerated cultures also delayed but did not prevent pellicle formation. Increasing the concentration of Tween 80 to 0·1% failed to delay the pellicle formation in aerated cultures after 220 min (Fig. 2).

Tables 1 and 2 show that the reproducibility of the growth rate determinations in the presence and absence of Tween 80 is satisfactory. The presence of Tween 80 satisfactorily prolonged the log phase and made it feasible to investigate chemical antibacterial activity against *Ps. aeruginosa* by measuring the effect on growth rate (Brown & Garrett, 1964).

The addition of 0·5 ml water to cultures of log phase cells while being gently shaken by hand (for mixing without aeration) produced no measurable effect on the growth rate compared with that of a control culture (Fig. 3A). When 1 ml water was added to log phase cultures not shaken during the addition it had the effect of reducing the growth rate to zero for a significant time after which the initial rate of growth was resumed (Fig. 3B). *Ps. aeruginosa* is known to be very sensitive to osmotic effects (Bernheim, 1963) and this may account for the phenomenon.

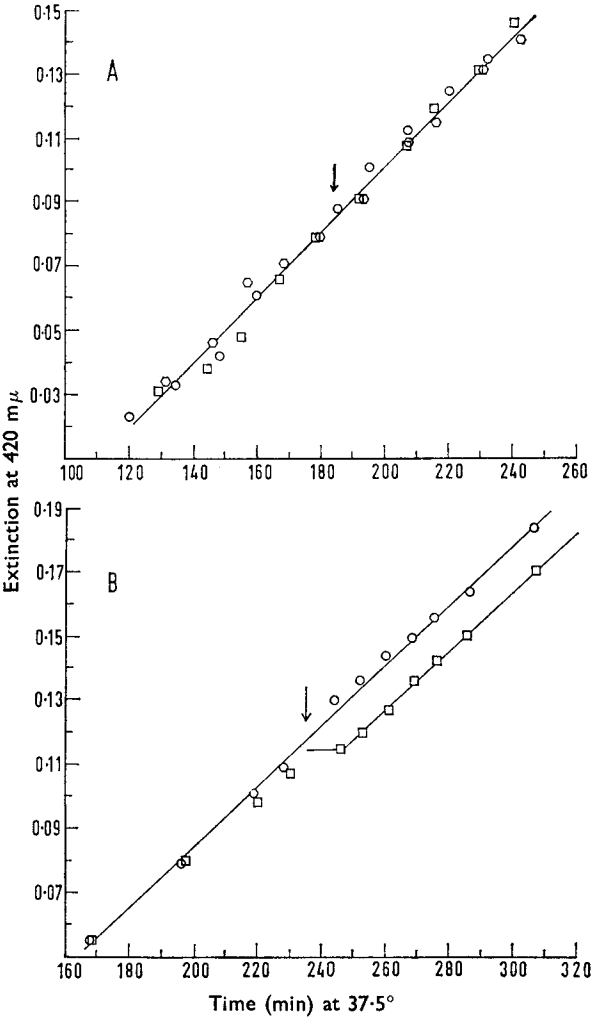

FIG. 3. Effect on growth rate of method of adding water to cultures of *Ps. aeruginosa*. A. Shaken during addition. ○ Nutrient broth + Tween 80, 0·02%. □, ◇ Replicate cultures containing nutrient broth + Tween 80, 0·02% + 0·5 ml water added after 184 min. B. Not shaken during addition. ○ Nutrient broth + Tween 80, 0·02%. ■ Nutrient broth + Tween 80, 0·02% + 1·0 ml water added after 235 min.

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