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Preservation of fluorescein solutions against contamination with *Pseudomonas aeruginosa*

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A combination of phenylmercuric nitrate and phenylethyl alcohol was more effective in preserving fluorescein solutions against contamination with *P. aeruginosa* than phenylmercuric nitrate alone. *P. aeruginosa* cells, grown in the presence of phenylethyl alcohol and then washed were rendered sensitive to chemical inactivation.

Fluorescein solutions are difficult to preserve effectively against contamination by *Pseudomonas aeruginosa* (Dale, Nook & Barbiers, 1959). Phenylmercuric nitrate (PMN) is generally acknowledged to be the most suitable preservative for these solutions and is recommended by the B.P.C. 1968. Kohn, Gershenfeld & Barr (1963) considered that the rate of kill of aqueous solutions of PMN against *P. aeruginosa* was too slow for PMN to be recommended as a preservative for ophthalmic solutions.

Disodium ethylenediamine tetraacetate (EDTA) enhances the activity of benzalkonium, chlorhexidine and polymyxin against logarithmic phase cells of *P. aeruginosa* (Brown & Richards, 1965). This effect was considered to be the result of EDTA chelating divalent cations from the bacterial cells and thus affecting the permeability properties of the cells to the chemical antibacterials under test. EDTA, however, did not enhance the activity of solutions of PMN against *P. aeruginosa* cells, rather it caused a reduction in the activity of the organic mercurial (Brown, 1968). In this situation it was thought that the EDTA chelated mercury ions and thus rendered the PMN less effective against the *P. aeruginosa* cells.

Phenylethyl alcohol (PEA) has been recommended as a preservative for use in ophthalmic solutions (Brewer, Goldstein & McLaughlin, 1953), but Kohn & others (1963) found PEA, like PMN, to have too slow an action against *P. aeruginosa* cells for use in ophthalmic solutions.

Silver & Wendt (1967) concluded that PEA exerted its antibacterial effect by modifying the permeability properties of the bacterial cell. Therefore PEA would seem to offer similar possibilities of EDTA of being used in combination with other antibacterial agents to enhance their antibacterial activity.

The purpose of this present work was to investigate the effectiveness of combinations of PEA and PMN in the preservation of fluorescein solutions against contamination with *P. aeruginosa*.

EXPERIMENTAL

The test organism used was *P. aeruginosa* strain NCTC 8203, and the growth medium for liquid cultures was Difco nutrient broth, Difco bacto-agar 2% was added to obtain the solid culture medium. Incubation was at 37°. The fluorescein sodium, PMN and EDTA were all BDH laboratory chemicals and the PEA (N.F.) was obtained from S. B. Penick & Company, New York. Cell numbers were estimated by

colony counts. The inactivating broth of Riegelman, Vaughan & Okumoto (1956) without agar, was used for the first dilution and nutrient broth was used for subsequent dilutions. The maintenance of stock cultures has already been described (Brown & Richards, 1964).

Effect of PEA *and* PMN *on survival time*

An end point experiment was made to determine the effect of PEA on the action of PMN in the preservation of fluorescein solutions against contamination with *P. aeruginosa*.

Preparation of inoculum. An overnight culture was used to inoculate nutrient broth at 37° to give a final volume of 100 ml; after incubation for 2·5 h this culture formed the source of inoculum.

Preparation of reaction mixtures. Five formulations (A–E) of fluorescein sodium 2% were prepared. Solutions A–E were preserved with PMN 0·002%; PEA 0·6%; PMN 0·002% with PEA 0·6%; PMN 0·002% with PEA 0·4% and PMN 0·002% with PEA 0·2% respectively. 9·5 ml quantities were filled into glass tubes having plastic screw caps and sterilized at 121° for 20 min. Duplicate tubes of each solution were inoculated with 0·5 ml samples of the prepared culture to give a final bacterial concentration of approximately 3×10^5 cells per ml. These inoculated solutions were designated reaction mixtures and were maintained at 21–22°.

Determination of survival time. Samples of 0·5 ml were taken from each reaction mixture after 10, 20, 30, 60 and 180 min; they were added to 9·5 ml recovery medium and incubated for 7 days. The recovery medium was that of Riegelman & others (1956) without agar. Positive controls to test the efficiency of the recovery medium consisted of 0·5 ml samples of prepared culture separately added to recovery medium

Table 1. *Survival times for* P. aeruginosa *in 2·0% fluorescein solutions*

Preservative	Replicate[a]	Contact time (min) at 21° 10	20	30	60	180
PMN 0·002% (A)	1	+	+	+	+	−
	2	+	+	+	+	−
PEA 0·6% (B)	1	+	+	+	+	−
	2	+	+	+	−	−
PMN 0·002% PEA 0·6% (C)	1	+	+	+	−	−
	2	+	−	−	−	−
PMN 0·002% PEA 0·4% (D)	1	+	+	−	−	−
	2	+	+	+	−	−
PMN 0·002% PEA 0·2% (E)	1	+	+	+	−	−
	2	+	+	+	+	−

Controls: Duplicate controls separately containing 0·5 ml of "C" plus 9·5 ml inactivating recovery broth inoculated to a final concentration of approximately 3×10^5 cells/ml log phase *P. aeruginosa*. All gave growth.

a, +, −, visible growth or not after 7 days incubation at 37° in inactivating recovery broth.

N.B. Inocula consisted of 0·5 ml log phase *P. aeruginosa* in nutrient broth to give a final concentration of approximately 3×10^5 cells/ml.

containing 0·5 ml of the fluorescein solution preserved with PMN 0·002% plus PEA 0·6%. The additional control procedure of Brown (1968) was also followed. Samples of all positive tubes were incubated on a milk agar medium consisting of 10% dried defatted milk (Carnation nonfat dry milk) and 2% agar to observe for pigment production and clearing of the casein. The results are in Table 1.

Effect of growth media on survival time

An end point experiment was made to determine whether *P. aeruginosa* cells grown on nutrient agar plus various additives produced cells having different resistance to fluorescein solutions preserved with either PMN 0·002% or the PMN/PEA combination.

Preparation of inoculum. An overnight culture was used to inoculate duplicate 900 ml flat culture bottles containing 200 ml solid media. There were 7 different media consisting of nutrient agar; nutrient agar plus PMN 0·001%; nutrient agar plus

Table 2. *Survival times for* P. aeruginosa *in fluorescein solutions*

Media used to obtain source of inocula[1]	Formulation[2]	Replicate[3]	Contact time (min) at 22°							
			30	60	90	120	150	180	300	24 h
Nutrient agar	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	+	−
	D	1	+	+	+	+	+	+	−	−
		2	+	+	+	−	−	−	−	−
Nutrient agar + PMN 0·001%	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	+	−
	D	1	+	+	+	+	−	−	−	−
		2	+	+	+	+	−	−	−	−
Nutrient agar + EDTA 0·1%	A	1	+	−	−	−	−	−	−	−
		2	+	+	+	−	−	−	−	−
	D	1	+	−	−	−	−	−	−	−
		2	+	−	−	−	−	−	−	−
Nutrient agar + calcium chloride 0·1%	A	1	+	+	+	+	+	+	−	−
		2	+	+	+	+	+	+	+	−
	D	1	+	+	+	+	−	+	−	−
		2	+	+	+	+	−	−	−	−
Nutrient agar + dextrose 1·0%	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	−	−
	D	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	−	−
Nutrient agar + PEA 0·25%	A	1	+	+	−	−	−	−	−	−
		2	+	+	+	+	−	−	−	−
	D	1	+	−	−	−	−	−	−	−
		2	+	−	−	−	−	−	−	−
Nutrient agar + 0·5% polysorbate 80	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	−	−
	D	1	+	+	+	+	+	−	−	−
		2	+	+	+	+	−	−	−	−

Controls: Duplicate controls separately containing 1·0 ml of each of "A" and "D" plus 9·5 ml inactivating recovery broth were inoculated to a final concentration of approximately 2×10^5 cells/ml of the cells grown on nutrient agar + EDTA 0·1%. All gave growth.

[1] Inoculum gave a final concentration of approximately 2×10^6 cells/ml.

[2] Formulation "A" was fluorescein sodium 2·0% with PMN 0·002%. Formulation "D" was fluorescein sodium 2·0% with PMN 0·002% and PEA 0·4%.

[3] +, −, Visible growth or not after 7 days incubation at 37° in inactivating recovery broth.

EDTA 0·1%; nutrient agar plus anhydrous calcium chloride 0·1%; nutrient agar plus dextrose 1%; nutrient agar plus PEA 0·25% and nutrient agar plus polysorbate 80 0·5%. The inoculated media were then incubated for 48 h after which the surface growth was harvested by gently washing the surface of each culture with 10 ml sterile distilled water. The suspensions thus obtained from the duplicate bottles were bulked. The bulked suspensions were centrifuged, the supernatants removed and the cells resuspended in an equal volume of nutrient broth. A viable count was then made on each of the 7 suspensions. These suspensions formed the source of inocula and were refrigerated at 2–4° until needed.

Preparation of reaction mixtures. 9·5 ml quantities of formulations A and D were prepared as before and duplicate tubes of both solutions were inoculated with 1·0 ml of the prepared suspensions to give a final concentration of approximately 2×10^6 cells per ml. (A further count was made to check the actual inoculum in each case.) These inoculated solutions were designated reaction mixtures and were maintained at 21–22°.

Determination of survival times. Survival times were determined as previously described except that the contact times were 30, 60, 90, 120, 150, 180, 300 min and 24 h. The results are given in Table 2.

Effect of PEA *on growth rate*

Replicate inocula of overnight *P. aeruginosa* cells were used to inoculate 4 × 250 ml flasks of prewarmed nutrient broth (W–Z) to give a final volume of 100 ml. Two of the flasks (W and Y) contained plain nutrient broth and the other 2 flasks (X and Z) contained nutrient broth plus PMN 0·001%. The growth rates of cultures W–Z were followed by means of colony counts. At time 145 min after inoculation, 0·25 ml sterile distilled water prewarmed to 37° was separately added to W and X and 0·25 ml prewarmed PEA was separately added to Y and Z to give a final concentration of 0·26% PEA. The results are shown in Fig 1.

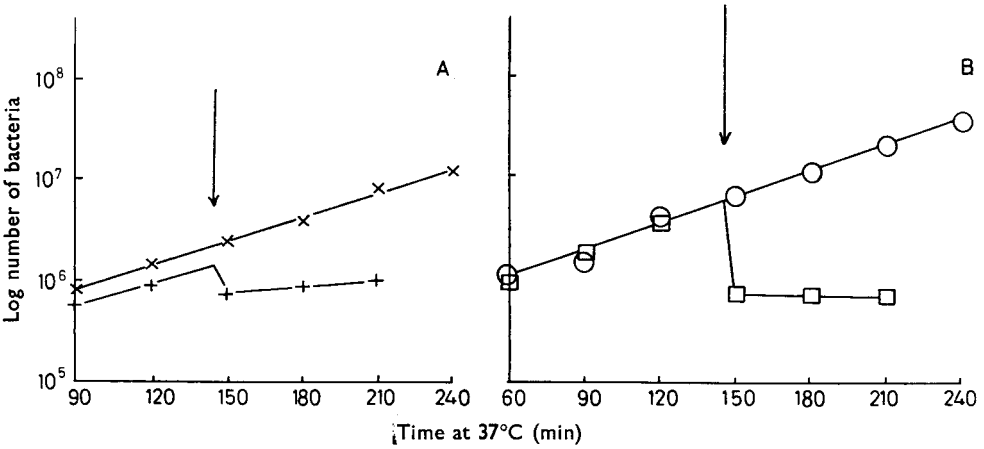

FIG. 1. The effect of PEA 0·26% against growing cultures of *P. aeruginosa* in nutrient broth alone (A) and plus PMN 0·001% (B). × Nutrient broth (W). + Nutrient broth plus PEA 0·26% at 145 min (Y). ○ Nutrient broth plus PMN 0·001% (X). □ Nutrient broth plus PMN 0·001% plus PEA 0·26% at 145 min (Z).

RESULTS AND DISCUSSION

Effect of PEA *and* PMN *on survival time*

Formations C and D both achieved sterility within 60 min of being contaminated with *P. aeruginosa* cells to a final concentration of approximately 3×10^5 cells per ml (Table 1). This was a faster sterilization time than was obtained with the official B.P.C. formulation A. The formulations containing PEA 0·6% both showed a marked precipitate after autoclaving.

Effect of growth media on survival time

From Table 2 it is seen that formulation D has a shorter sterilization time than formulation A against 6 of the 7 cell suspensions. In the seventh case, further time intervals between 3 and 24 h would be needed to determine which formulation has the more rapid action against the cells of this suspension.

These results again indicate that the formulation containing the combination of PMN 0·002% with PEA 0·4% would seem to be an improvement on the existing B.P.C. formulation which contains PMN 0·002% alone.

The cells grown on nutrient agar in the presence of EDTA 0·1% and the cells grown on nutrient agar in the presence of PEA 0·25% were both more sensitive to the action of PMN and the PMN/PEA combination than the cells grown on nutrient agar alone. EDTA is known to affect the permeability of *P. aeruginosa* cells making them more sensitive to several chemical agents (Brown & Richards, 1965). These results show that PEA also has an effect on the cell that enhances the activity of the chemical agents tested.

The lowered resistance of the cells grown on the EDTA and the PEA media is considered to result from these agents having an action on the cells during the growth period. It is not considered to be caused by chemical carried over in the inocula. The washing of the bacterial suspensions and their subsequent thousand-fold dilution, when used as inocula, was thought to ensure that any agent carried over would be present in such high dilution as to be unable to influence the results. Both EDTA and PEA have to be present in fairly high concentration to influence antibacterial action. Furthermore, Brown (1968) showed that PMN in the presence of EDTA was less effective against *P. aeruginosa* cells than PMN alone. Our own results with EDTA and PMN confirmed those of Brown.

Cells grown in the presence of polysorbate 80 or dextrose appeared to have a similar resistance to the cells grown on plain nutrient agar. Excess calcium did not seem to affect the resistance of the cells. It is concluded that the significant concentration of calcium is that concentration that is sufficient for normal cell synthesis. Concentrations higher than this do not appear to affect the resistance of the pseudomonas cells. Growth in the presence of PMN did not seem to affect the resistance of the cells to subsequent chemical inactivation.

Effect of PEA *on growth rate*

From Fig. 1A it can be seen that PEA 0·26% effected a 45% kill of *P. aeruginosa* cells growing in nutrient broth at 37°. The same concentration of PEA, however, caused an 80% kill of cells growing in nutrient broth plus PMN 0·001% (Fig 1B). It is considered that the PEA enabled the PMN, which at this concentration is not effective against the organism, to kill a significant number of cells. We believe the PEA to be

acting on the permeability of the *P. aeruginosa* cells thus enabling concentrations of PMN which could not on their own effect penetration into the cell to pass into the cell and exert an antibacterial effect. Therefore, under the conditions of this experiment, the PMN and PEA have a synergistic action against growing cells of *P. aeruginosa*. PEA therefore might be used with advantage in combination with PMN and possibly in combination with other antibacterial agents in the preservation of pharmaceuticals against contamination with *P. aeruginosa*.

It is suggested that the effectiveness of PEA, in combination with another chemical antibacterial agent, against *P. aeruginosa* may be related to the permeability of the pseudomonas cell to the agent. If the antipseudomonal activity of the agent is limited because it cannot penetrate into the pseudomonas cell then it may be expected that combining it with PEA will result in increased antipseudomonal activity. If the agent, however, is already able to penetrate the pseudomonas cell, then a combination with PEA is unlikely to increase its activity.

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