tetrazol activity, 100 mg./Kg. of pentylenetetrazol was injected subcutaneously into each mouse. This dose is considered to be approximately twice the CD_{50} in untreated mice (25). In both tests, an animal was considered protected if the tonic extensor component of the seizure was abolished. The control animals, which were injected with 7% gum acacia or distilled water, showed 0% protection against electroshock and subcutaneous pentylenetetrazol.

All of the test compounds were evaluated at a dose level of 200 mg./Kg. with the exception of compound 4 (Table II, 100 mg./Kg.). However, none of the derivatives of III were shown to be effective in preventing the tonic hind leg extension produced by pentylenetetrazol. It was not possible to test higher doses of the experimental compounds since amounts greater than 200 mg./Kg. produced toxic symptoms.

The results from the electroshock testing demonstrated that several of the analogs were more protective against electroshock than was the parent compound (III). At a dose level of 200 mg./Kg., III protected 20% of the test animals against electroshock. The electroshock data appear in Tables I and II.

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Keyphrases

N-Methyl-2-phenylsuccinimide derivatives–synthesis
Mannich reaction
Lithium amide–higher homolog synthesis
IR spectrophotometry–structure
Anticonvulsive activity against electroshock, pentylenetetrazol

Survival of *Pseudomonas aeruginosa* in Fluorescein Solution

Preservative Action of PMN and EDTA

By MICHAEL R. W. BROWN

Colony counts were made for a year on washed and unwashed cells of *Ps. aeruginosa* inoculated into fluorescein sodium solution, 2 percent in water (pH 8.6), into nutrient broth, into tris buffer (pH 8.6), and into water. Broth exerted a protective effect in all storage liquids (22°) and also supported growth, depending on the amount of broth. Water-washed inocula lost viability in less than a day in fluorescein solution. Viability of broth inocula was maintained for more than a year in all vehicles. Fluorescein solution preserved with PMN-sterilized inocula of about 10^6/ml. *Ps. aeruginosa* cells within 5 hr. EDTA in combination with PMN was less effective.

PSEUDOMONAS AERUGINOSA causes general problems of cross infection (1, 2) and is a particular hazard in ophthalmology (3, 4). Dale *et al.* (5) have reviewed many reports of *Ps. aeruginosa* as a contaminant of fluorescein eye drops.

The B.P.C. recommendation of phenylmercuric nitrate (PMN) as an ophthalmic preservative is supported by many studies, nearly all of which

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show it to be a relatively slow but effective sterilizing agent (6–9). Other workers have found PMN to be less than effective in the case of fluorescein. Anderson *et al.* (10) found that 0.002% PMN failed to sterilize fluorescein drops after several days contact, but 0.004% PMN achieved sterility within a day. These workers did not use contact times of less than a day and the recovery media did not contain inactivators.

EDTA enhanced the activity of chlorhexidine, benzalkonium, and polymyxin against *Ps. aeruginosa* growing both in serum and broth (11). Furthermore, acquired resistance by this organism to a quaternary ammonium compound was eliminated using EDTA (12, 13).

The present work was done to obtain quantitative information about survival of *Ps. aeruginosa* in fluorescein and other aqueous solutions and to study the preservative properties of 0.1% EDTA (13) used in combination with PMN.

EXPERIMENTAL

Ps. aeruginosa NCTC[1] 6750, NCTC 8203, and NCTC 7244 were used as test bacteria. The growth medium was oxoid[2] nutrient broth No. 1 and incubation was at 37°. B.D.H.[3] fluorescein sodium (microscopical quality) was used. Other chemicals were Analar quality (B.D.H.). Cell numbers in broth cultures were estimated by measuring the absorbance at 420 mμ with a Unicam 600 spectrophotometer and by colony counts using broth as a diluent. Experimental details have been described previously (14).

Survival in Solutions Without Preservative

Survival experiments were made using screw-capped 20-ml. and 100-ml. glass bottles in a water bath at 22°. Inocula were added to fluorescein sodium solution 2% in distilled water (Fig. 1). Colony counts were made at intervals during the first day, then daily, then weekly, until a consistent pattern of results occurred. Representative suspensions were then counted at intervals for about a year. Samples were examined microscopically for cell clumping. An identical control procedure was carried out, adding inocula to distilled water (Fig. 2), to nutrient broth (Fig. 2), and to tris buffer pH 8.6 (pH of fluorescein solution) (Fig. 3). A total of about 50 suspensions was stored.

Preparation of Inocula—A technique similar to that described previously (14) was used to obtain a reproducible growth cycle in nutrient broth. Twenty-four hour cultures were used. The original culture was diluted with fresh broth (culture OR) so that the storage liquids contained initially about 10^3 cells/ml. and duplicates contained 10^6/ml. In addition, the contribution of the original growth medium to survival was assessed. This was done by using inocula washed by centrifugation (3 times) with either fresh broth (culture WB) or 0.2 *M* NaCl then water (culture WW). Preliminary experiments showed that the volume of broth added as part of the cell inoculum contributed to the survival of *Ps. aeruginosa* in the various storage liquids. Preliminary work also suggested that the size of container was significant. Consequently, inocula

[1] National Collection of Type Cultures, Colindale, London, England.
[2] Oxo Ltd., London, England.
[3] B.D.H., Poole, Dorset, England.

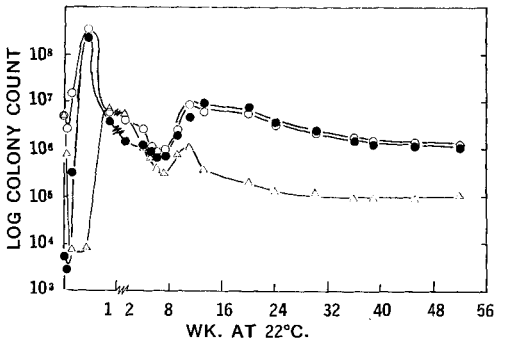

Fig. 1—Survival of Ps. aeruginosa *in fluorescein solution. Key: ●, 10 ml. broth inoculum in 90 ml. fluorescein solution (10^3/ml.); ○, 10 ml. broth inoculum in 90 ml. fluorescein solution (10^6/ml.); △, 0.5 ml. broth inoculum in 99.5 ml. fluorescein solution (10^6/ml.).*

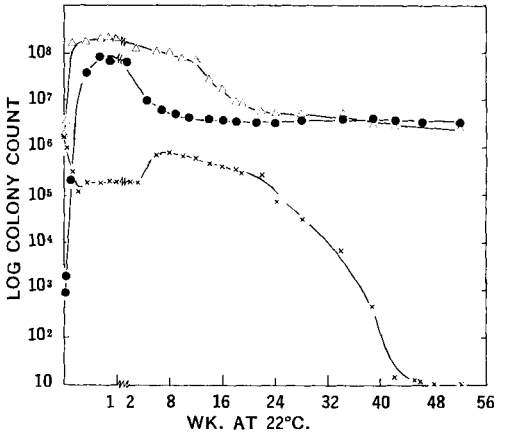

Fig. 2—Survival of Ps. aeruginosa *in water and broth. Key: ×, 0.5 ml. broth inoculum in 99.5 ml. water, (10^6/ml.); ●, 10 ml. inoculum washed in fresh broth and added to 90 ml. water (10^3/ml.); △, 0.5 ml. broth inoculum in 99.5 ml. broth (10^6/ml.).*

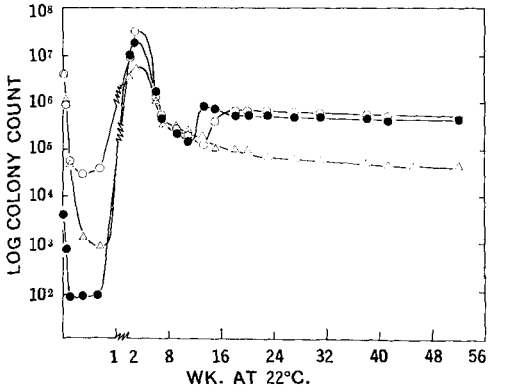

Fig. 3—Survival of Ps. aeruginosa *in tris buffer (pH 8.6). Key: ●, 10 ml. broth inoculum in 90 ml. tris buffer (10^3/ml.); ○, 10 ml. broth inoculum in 90 ml. tris buffer (10^6/ml.); △, 0.5 ml. broth inoculum in 99.5 ml. tris buffer (10^6/ml.).*

TABLE I—SURVIVAL TIMES FOR *Ps. aeruginosa* IN SOLUTIONS PRESERVED WITH PMN AND EDTA

Soln.	Organism Strain (NCTC)	Replicate[a]	Contact Time: 45 min.	90 min.	3 hr.	5 hr.	1 Day	2 Days	7 Days
Fluorescein + 0.002% PMN	6750	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	8203	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	7244	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
Fluorescein + 0.002% PMN + 0.1% EDTA	6750	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
Fluorescein + 0.004% PMN	6750	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	+	−	−	−	−
		2	+	+	−	−	−	−	−
Fluorescein + 0.004% PMN + 0.1% EDTA	6750	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	−	−	−	−	−
		2	+	+	−	−	−	−	−
0.002% PMN	6750	1	+	+	+	+	−	−	−
		2	+	+	+	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	+	−	−	−
	7244	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
0.002% PMN + 0.1% EDTA	6750	1	+	+	+	+	−	−	−
		2	+	+	+	+	+	−	−
	8203	1	+	+	+	+	−	−	−
		2	+	+	+	+	+	−	−
	7244	1	+	+	+	+	+	+	−
		2	+	+	+	+	+	−	−
0.004% PMN	6750	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	8203	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
	7244	1	+	+	+	−	−	−	−
		2	+	+	+	−	−	−	−
0.004% PMN + 0.1% EDTA	6750	1	+	+	+	+	+	−	−
		2	+	+	+	+	+	−	−
	8203	1	+	+	+	+	+	−	−
		2	+	+	+	+	+	−	−
	7244	1	+	+	+	+	−	−	−
		2	+	+	+	+	+	−	−

[a] +, −, visible growth or not after 1-week incubation in recovery broth. Duplicate controls separately containing 2% fluorescein, 0.1% EDTA, and recovery broth alone were also inoculated and all gave growth.

consisted of 1 ml. added to 99 ml. storage liquid (*A*); 1 ml. to 9 ml. (*B*), in 20-ml. bottles; 10 ml. to 90 ml. (*C*); 0.5 ml. to 99.5 ml. (*D*). Inoculation was always made to give both 10^3 cells/ml. and 10^6/ml. approximately.

Storage Liquids—*Sodium Fluorescein Solution, 2%*—Inoculum OR was used to give suspensions *B*, *C*, and *D*; WB to give *B*, *C*, and *D*; WW to give *A* and *B* (Fig. 1).

Distilled Water—Inoculum OR gave suspensions *B* and *D*; WB gave *B*, *C*, and *D* (Fig. 2).

Nutrient Broth—Inoculum OR gave suspensions *A* and *D* (Fig. 2).

Tris Buffer—Inoculum OR gave suspensions *B*, *C*, and *D* (Fig. 3). Storage was at 22°.

Survival in Preserved Solutions

One-half milliliter broth inocula of all three test strains to a final concentration of about 10^6 cells/ml. were added to various combinations of fluorescein, EDTA, and PMN in 20-ml. solutions in a water bath at 22° (Table I). One-half milliliter samples were taken at various time intervals, added to 10 ml. recovery broth, and incubated at 37° for 7 days. As a control procedure, samples from all positive tubes were incubated on a milk agar medium (10% dried, defatted milk,[4] and 1.5% agar) and examined for pigment and clearing of the casein.

Recovery Broth—The recovery broth of Kohn *et al.* (9) for *Ps. aeruginosa* after contact with PMN was used, with *M*/372 $CaCl_2 \cdot 6H_2O$ added to inactivate EDTA (0.1% equivalent to *M*/372). The broth was composed of: 0.3 Gm. lecithin, 3 Gm. polysorbate 80,[5] 2.93 Gm. dehydrated thioglycollate medium USP, 59 mg. $CaCl_2 \cdot 6H_2O$ (autoclaved separately), water to 100 ml. This medium was selected after preliminary experiments using colony counts with the basic lecithin–polysorbate–thiogly-

[4] Marvel milk granules, Cadbury Bros. Ltd., Bournville, England.

[5] Tween 80. Atlas Chemical Industries, Inc., Wilmington, Del.

collate medium in the presence and absence of $M/372$, $M/37$, and $M/3.7$ $CaCl_2 \cdot 6H_2O$.

RESULTS AND DISCUSSION

Solutions Without Preservative

Fluorescein Solution—Unwashed broth inocula of *Ps. aeruginosa* added to fluorescein solution rapidly dropped in count when diluted 100-fold or more (Fig. 1, open triangle). The count rose again after this initial drop and remained relatively constant. This pattern was the same for inocula of 10^6/ml. and 10^3/ml. The initial inhibitory effect of fluorescein solution was eliminated when the inoculum was in a sufficiently large volume of broth (Fig. 1, open and closed circles). The larger volume of broth protected the cells from the initial drop in colony count and also allowed the count to rise and be maintained at a higher level for inocula of both 10^6/ml. and 10^3/ml. The results from inocula washed in fresh broth (not illustrated) were similar to those with unwashed cells and showed that the washing process with broth had no significant effect upon subsequent survival in fluorescein solution. Inocula of cells washed in water showed rapid death and were sterile in less than a day.

Water, Broth, and Tris Buffer—The pattern of survival of *Ps. aeruginosa* in water was similar to that in fluorescein and representative results are given in Fig. 2. Inocula with the greater amounts of broth were protected in comparison to those with less, and viability was maintained at higher levels. The effect was similar for both 10^6/ml. and 10^3/ml. inocula. Figure 2, times sign, shows that 0.5 ml. broth inoculum added to 99.5 ml. water retained a relatively constant count for about 6 months; this dropped until the storage liquid was sterile after about 1 year. An inoculum of 10 ml. broth added to 90 ml. water initially showed a rise in count to about 10^8/ml. This dropped for about 2 months when a count of about 4×10^6/ml. was maintained for the rest of the experimental period. A similar pattern occurred with cells washed in fresh broth (Fig. 2, closed circle). The pattern for broth as a storage liquid (Fig. 2, open triangle) was similar to that of water with the high broth inoculum. All inocula into tris buffer showed an initial drop in count (Fig. 3). Inocula with the greater amounts of broth (Fig. 3, open and closed circles) were protected against initial death and ultimately viability was maintained at higher levels when compared to results with small broth inocula.

Broth exerted a protective effect in all the storage liquids and also supported growth. The protection and growth were apparently related to the amount of broth present. The size of cell inoculum and size of storage container apparently had no influence on survival. All three strains gave similar results.

The magnitude of the initial drop in count (about 99.9%) when broth inocula was diluted 100-fold in fluorescein solution, tris buffer (pH 8.6), and water was unexpected. This result, together with that using water-washed inocula (sterilized within a day) shows that the kind of inoculum used in studies with *Ps. aeruginosa* is crucial. It would seem that washing in water has a damaging effect upon *Ps. aeruginosa* and that the use of water-washed cell inocula may give misleading results in antibacterial studies.

In practice it is likely that contamination from *Ps. aeruginosa* would be from cells present with nutrient material. The results from this study show that aqueous vehicles will support viability of large populations of this organism for periods of a year or more. Inocula maintained viability to about the same extent in fluorescein solution, tris buffer, and water.

Preserved Solutions

PMN alone was consistently effective in sterilizing inocula of about 10^6 cells/ml. in fluorescein solution (Table I). There was little difference between the effects of 0.004% and 0.002% PMN, and sterility was achieved with all strains between 1.5 and 5 hr. Kohn *et al.* (9) obtained closely similar results. In the absence of fluorescein, sterility was also achieved within several hours using PMN alone in water. It might be anticipated that a high molecular weight anion such as fluorescein might react with a cation such as the phenylmercuric ion. This possibility is not excluded by the results which, nevertheless, show PMN to be effective within the experimental limits.

In every instance except one (0.004% PMN) solutions with EDTA took longer to achieve sterility than those without it. This apparent antagonism may be due to EDTA chelating the mercury, making it less available for antibacterial action. These results confirm the efficacy of PMN as a relatively slow acting preservative againt *Ps. aeruginosa* and suggest that EDTA exerts a slight antagonistic action in combination with PMN in the test circumstances.

It is questionable whether this preservative effect of PMN is a sufficient protection, especially for drops used with damaged eyes. The difficulty of preserving eye drops against *Ps. aeruginosa* is well known (4) and the present work shows the dangers of long-term contamination of inadequately preserved drops including fluorescein. The use of sterile, single-dose units for damaged eyes or, in the case of fluorescein, sterile, impregnated paper strips would seem to reduce this hazard.

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Keyphrases

Fluorescein solution
Pseudomonas aeruginosa–fluorescein solution
Cells, *P. aeruginosa*–water, broth washed
Phenylmercuric nitrate–preservative activity
EDTA effect–phenylmercuric nitrate activity