studied. The method provides rapid and effective separation of iodide and iodate ions and should serve as a suitable technique for the quality control of radiopharmaceuticals containing I^{131}.

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8-Hydroxyquinoline Sulfate as a Preservative for Tuberculin PPD (Mantoux)

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The preservative 8-hydroxyquinoline sulfate (8-HQS) has been tested for its antimicrobial activity in solutions of tuberculin purified protein derivative (PPD). All tests were carried out at room temperature (18–22°). Two yeast and three mold species were destroyed readily by 0.01 per cent 8-HQS. The preservative also was effective against small inocula (approximately 10–100 cells) of *Pseudomonas aeruginosa,* whereas large inocula (1000 cells) usually grew out. Four strains of *Staphylococcus aureus* died quickly in the solution with or without the presence of 8-hydroxyquinoline sulfate.

TUBERCULIN PURIFIED protein derivative (PPD) is a specific protein isolated from the culture filtrate of *Mycobacterium tuberculosis.* Introduced intracutaneously or percutaneously, it produces localized induration and swelling in a person who has had an infection or is currently infected with *M. tuberculosis.* Intracutaneous testing with tuberculin solutions of specified strength is used widely as part of tuberculosis prevention programs.

PPD is prepared as a concentrated stock solution preserved with phenol. For intracutaneous use (Mantoux test), the stock solution is diluted to contain graded amounts, usually 0.2, 1, 2, 20, and 50 mcg. of PPD per milliliter. Phenol, however, reduces the potency of diluted PPD solutions (1, 2); for this reason, Magnusson *et al.* (3) devised a diluent for PPD which contains 8-hydroxyquinoline sulfate[1] (8-HQS) as a preservative.

Although 8-HQS has been used for many years in the diluent for PPD, there have been no reports concerning its efficacy as a preservative for this material. However, when 8-HQS was used as a preservative for antimeasles serum, the serum became contaminated with bacteria; two children who received the contaminated serum died, while a third became ill and recovered (4). There is no information regarding the concentration of 8-HQS used. In the same report, Olin and Lithander described the results of injecting a contaminated solution of tuberculin which contained no preservative. Of the 11 children given Mantoux tests with the contaminated material, all showed symptoms of toxicity, and one died. In both of these incidents, *Staphylococcus aureus* was the contaminant.

The reported failure of 8-HQS to prevent the growth of contaminants in antimeasles serum and the ability of contaminants to grow in tuberculin in the absence of a preservative suggested that an investigation of the efficacy of 8-HQS in PPD should be made. This report gives the results of such an investigation.

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[1] Marketed as Chinosol.

MATERIALS AND METHODS

PPD.—PPD prepared as a concentrated stock solution (5) was diluted to contain 50 tuberculin units (TU) per milliliter; 1 TU = 0.00002 mg. protein.

WHO Diluent.—The diluent devised by Magnusson *et al.* (3) was used for all preparations. This diluent is a phosphate buffered saline (1.45 Gm. KH_2PO_4, 7.6 Gm. $Na_2HPO_4 \cdot 2H_2O$, 4.8 Gm. NaCl in 1.05 L. of distilled water) containing 0.005% polysorbate 80[2] and 0.01% 8-hydroxyqinoline sulfate (8-HQS). For some experiments, diluent was made without 8-HQS.

Cultures.—The source of the cultures, methods for preparation of inoculum, and inoculation of preserved solutions with challenge organisms have been described previously (7). The only deviation from these methods was a triple washing of inoculum and the resuspension with Magnusson's (WHO) diluent, from which the 8-HQS was omitted.

Testing of Preservative Activity.—Inoculated solutions were incubated at room temperature (18–22°) and subcultured with a standard loop to Difco nutrient broth for bacteria or Difco Sabouraud agar for yeasts and molds. When quantitative results were desired, 0.1-ml. quantities of the preserved solutions or dilutions thereof were spread on suitable agar media in Petri dishes.

8-Hydroxyquinoline Sulfate.—8-Hydroxyquinoline sulfate (Eastman Organic Chemicals, 1776) was used. The method for analysis was developed in these laboratories. Because it has not been published previously in detail, it is described as follows.

Spectrophotometric Determination of 8-Hydroxyquinoline Sulfate

Principle.—The method is based upon the characteristic light absorption which 8-HQS shows in the ultraviolet region with an absorption maximum at 240 mμ. At this wavelength and in the

[2] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

TABLE I.—EFFECT OF 8-HQS ON YEASTS AND MOLDS

Culture	Cells Inoculated/ ml. of PPD	Viable Cells/ml. — Diluent without Chinosol[a] 1 Day	4 Days	Diluent with Chinosol[b] 1 Day	4 Days	Diluent with Chinosol[c] and PPD[d] 1 Day	4 Days
C-27 *S. ellipsoideus*	5,000	1,300	1,500	20	<10	110	<10
C-28 *D. kloeckeri*	1,000	400	9,000	<10	<10	30	<10
C-30 *R. glutinis*	26,000	4,500	56,000	5,700	<10	390	<10
C-32 *R. oryzae*	400	750	20,000	130	<10	230	<10
C-35 *C. spinosa*	400	100	300	70	<10	90	140

[a, b, c] Chemical analysis of these solutions found 0, 0.010, and 0.0095% 8-HQS, respectively. [d] This solution contained 50 TU/ml.; the others contained none.

TABLE II.—EFFECT OF 0.01% 8-HQS ON BACTERIA

Culture	Viable Cells/ml. — Diluent without Chinosol Zero Time	8 Days	PPD with Chinosol 1 Day	4 Days	8 Days
C-4 *P. aeruginosa*	600	4,000,000	160	50,000	200,000
C-6 *P. aeruginosa*	600	8,000,000	5,000	90,000	860,000
C-16 *P. aeruginosa*	600	5,000,000	<10	20,000	200,000
C-9 Proteus sp.	300	90,000	4,000	1,600	1,500
C-10 *S. aureus*	60,000	...	<10	<10	...
C-11 *S. aureus*	60,000	...	7,000	<10	...

concentration range of 0.0001 to 0.0006%, the 8-HQS concentration is inversely proportional to the logarithm of the per cent transmittance.

Standard Graph.—A stock solution of 0.02% 8-HQS in WHO diluent (3) is diluted with WHO diluent to give four points between 0.0001 and 0.0006% 8-HQS, recording the per cent transmittance at 240 mμ in a Beckman DB spectrophotometer. The per cent transmittance (log scale) is plotted against the 8-HQS percentage (arithmetic scale). WHO diluent is used as a blank.

Procedure.—The sample of tuberculin PPD solution of unknown 8-HQS content is diluted 1:50 or 1:100 with WHO diluent, and its per cent transmittance is determined between 300 and 220 mμ in the Beckman DB spectrophotometer using as blank WHO diluent with the same PPD content as the sample tested. The shape of the absorption curve serves for identification of the 8-HQS, while the per cent transmittance at the absorption peak (240 mμ) on the same curve permits reading of the 8-HQS concentration by reference to the standard graph.

Example.—A sample of a tuberculin PPD-Mantoux solution, diluted 1:50 with WHO diluent, shows a 34.5% transmittance at 240 mμ, corresponding to 0.000335% 8-HQS. Therefore, the undiluted sample contains 50 × 0.000335% = 0.0167% 8-HQS.

RESULTS

Table I shows the effect of 8-HQS on yeasts and molds. For this experiment, the authors inoculated diluent, diluent with 8-HQS, and diluent with both 8-HQS and PPD. Three of the fungi grew well in the diluent without 8-HQS, even when the inocula were one-tenth of those shown in Table I. However, 0.01% 8-HQS destroyed all fungi. The presence of the PPD did not influence the activity of the 8-HQS.

Bacteria were inoculated into similar solutions, as described in Table I. Two sets of tubes were inoculated—one to obtain approximately 60,000 cells per milliliter, the other to obtain approximately 600 cells. Table II shows that even 600 cells of the three strains of *Pseudomonas aeruginosa* and one *Proteus* species were able to initiate growth in PPD solution containing 0.01% 8-HQS, but 60,000 cells of *S. aureus* were destroyed quickly. Two additional strains of *S. aureus* also were investigated, and the results were similar. In diluent with 8-HQS but without PPD, results were similar to those shown in Table II. Diluent which did not contain 8-HQS was also lethal to *S. aureus*, although it supported the growth of the other bacteria, as shown in Table II.

Table II shows the necessity of prolonging the incubation for several days when testing the efficacy of a preservative. Although the bacterial populations of *P. aeruginosa* sometimes decreased by 99.9% during the first day after inoculation, the survivors grew well.

When the concentration of 8-HQS in a PPD solution containing 50 TU/ml. was increased to 0.02 and 0.04%, 0.02% was generally more inhibitory to *P. aeruginosa* than 0.01% (Table III), but

TABLE III.—EFFECT OF CONCENTRATION OF 8-HQS ON SURVIVAL OF VARYING NUMBERS OF *P. aeruginosa* INOCULATED INTO PPD

Culture	Cells[a] Inoculated/ ml. of PPD	No. Tubes with Viable Cells / No. Tubes Inoculated with *P. aeruginosa*[b] — 0	0.01%[c]	0.02%	0.04%
C-4	10^1	8/9	0/9	0/9	0/9
	10^2	9/9	1/9	1/9	0/9
	10^3	9/9	5/9	2/9	2/9
	10^4	9/9	6/9	4/9	1/9
C-6	10^1	9/9	0/9	1/9	0/9
	10^2	9/9	2/9	2/9	0/9
	10^3	9/9	6/9	0/9	2/9
	10^4	9/9	5/9	3/9	3/9
C-16	10^1	9/9	0/9	1/9	0/9
	10^2	9/9	0/9	1/9	2/9
	10^3	9/9	6/9	3/9	3/9
	10^4	9/9	4/9	1/9	1/9

[a] 10^1—average of ten viable cells per milliliter of PPD (range 4 to 30 cells); 10^2, 10^3, 10^4 indicate average inocula of 100, 1000, and 10,000 cells/ml. [b] Aggregate results of six separate experiments. Subcultured 8–10 days after inoculation. [c] Per cent 8-HQS.

Table IV.—Stability of 8-HQS at 4° in Solutions Containing 50 TU of PPD per Milliliter

Percentage of Chinosol	
Added	Found[a]
0	0
0.010	0.012
0.020	0.021
0.040	0.042

[a] Stored 4 months at 4°C.

Table V.—Effect of Elevated Temperatures on Stability of 8-HQS in Solutions Containing 50 TU of PPD per Milliliter

Code No.	Percentage of Chinosol				
	Added	Found	After 1 Wk.		
		4°	20°[c]	30°	37°
366-1[a]	0.010	0.012	0.010	0.098	0.090
366-2[b]	0.010	0.012	0.011	0.012	0.010
366-3[b]	0.000	0.000	0.000	0.000	0.000

[a] Magnusson's diluent with PPD. [b] Magnusson's diluent without PPD. [c] Room temperature varied from 18–22°C.

even 0.04% of 8-HQS did not always destroy all bacteria. Against the smallest inoculum (10^1 organisms) of all three strains of *P. aeruginosa*, 0.01% 8-HQS gave consistent protection, although 0.02% failed in some instances to kill all organisms. Such inconsistencies were observed (Table III) when reviewing the results of six separate experiments in which a total of nine inoculated tubes were utilized for each variable. The reason for these inconsistencies is unknown, but numerous studies to identify the organisms which grew showed that they were *P. aeruginosa*.

8-HQS is stable in PPD solution. Solutions of 50 TU/ml. in Magnusson's diluent were prepared with varying concentrations of 8-HQS and stored in upright flasks at 4° for 4 months. Table IV shows that the 8-HQS was stable at this temperature. Table V shows that, even at elevated temperatures, 8-HQS had adequate stability in solutions containing 50 TU/ml. which were stored in screw-capped test tubes.

However, 8-HQS may be absorbed by the stoppers used for sealing vials (6). The authors believe that this has happened under practical conditions of storage because of the following experience.

Vials of PPD-Mantoux, which had been filled 17 and 5 months previously, stoppered, and subsequently stored at 4°, were inoculated with the three cultures of *P. aeruginosa*. The inoculum per milliliter of PPD solution was 25, 250, 2,500, and 25,000 cells. Subcultures made after 4 days at room temperature showed that viable cells were present in all tubes. Identical results were obtained when control tubes containing Magnusson's diluent without 8-HQS were inoculated and subcultured. Chemical analysis showed that none of the 0.01% of 8-HQS added to the PPD at the time of preparation was present at the time of inoculation.

DISCUSSION

Biologicals packaged in multiple-dose containers must contain a preservative. Unfortunately, some widely used preservatives are inadequate, especially against *P. aeruginosa*. Also, some, *e.g.*, benzethonium chloride, may be inactivated by the biological product (7); some, *e.g.*, phenol, may inactivate the biological. Numerous workers, *e.g.*, Lachman (8), have shown that preservatives may be absorbed by the rubber closure of the vial.

For these reasons, it is essential that the preservative activity in biologicals be tested after the products have been packaged in the final containers for several months. Both bacteriological and chemical tests appear desirable when introducing a new preservative or new packaging material. Thereafter, chemical tests should suffice.

The experiments demonstrate that, under the conditions of the tests (20–22°), 0.01% 8-HQS added to PPD solutions is an effective preservative against contamination with certain yeasts and molds. The use of four strains of *S. aureus* as test organisms gave only limited information, since at room temperature the inocula died even in the absence of 8-HQS. However, according to the report of Olin and Lithander (4), a strain of *S. aureus*, which was able to grow in a tuberculin solution at 4°, 22°, and 37°, was inhibited effectively by 0.01% 8-HQS. This observation agrees with earlier reports which indicate that low concentrations (0.001–0.02%) of 8-HQS inhibit the growth of staphylococci and of streptococci (9).

Of special interest is that under the conditions of the tests 8-HQS has a bactericidal action against *P. aeruginosa*, provided the inoculum used is not in excess of 100 cells per milliliter. Effectiveness of a preservative against Pseudomonas strains is important, as these organisms are ubiquitous and are encountered frequently as contaminants in biological products. They are able to grow well, even at refrigerator temperatures, in solutions containing few nutrients and can cause fatal generalized infections (10, 11). For these reasons, *Pseudomonas* species deserve special attention as challenge organisms in the critical appraisal of the antimicrobial activity of preservatives used for biologicals.

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