# Interaction of 8-Hydroxyquinoline Sulfate with Components Present in a Tuberculin PPD Solution II

## Effect of 8-Hydroxyquinoline on Metallic Impurities

By S. LANDI and H. R. HELD

**The presence of 8-hydroxyquinoline sulfate (8-HQS) as a preservative in tuberculin purified protein derivative (PPD) solutions leads to the formation of a dark crystalline deposit. In this investigation it has been shown that the deposit was due to chelates resulting from the reaction of metals with 8-hydroxyquinoline (8-HQ) liberated from its sulfate. The metal impurities in tuberculin solution originate mainly from the buffer salts and the PPD. The removal of these metal impurities from the tuberculin PPD did not affect the potency of the preparation. Previous workers have shown the need to maintain the metal content within fairly narrow limits in order to render 8-HQ effective as a preservative. The necessity to keep the metal content within narrow limits in order to render the preservative effective, and to avoid the development of organo-metallic deposits, was considered an important drawback to its usefulness as a preservative.**

8-HYDROXYQUINOLINE SULFATE (8-HQS)[1] is added to tuberculin solutions as an antimicrobial agent. These solutions are prepared by adding PPD (10, 50, or 1,000 TU[2]/ml.), polysorbate 80[3] (0.005%), and 8-HQS (0.01%) to a buffered solution of pH 7.38 (1). These solutions are perfectly clear when freshly prepared. However, upon storage at 5° in a 0.5 L. bulk, these solutions showed some turbidity which was accompanied by a dark deposit. Furthermore, after dispensing 1- and 5-ml. volumes of the original clear solutions in vials or ampuls, there was no turbidity, but on careful observation, a few tiny dark particles were present after approximately 6 months' storage at 5° or at 37°.

The authors wished to establish the reason for the appearance of this undesirable deposit and, therefore, attempted to determine which substances took part in its formation, and if the presence of such deposit would affect the biological potency of the tuberculin preparation.

### MATERIALS AND METHODS

**Reagents**—8-Hydroxyquinoline sulfate (8-HQS) (Eastman Organic Chemicals, 1776); 8-hydroxyquinoline (8-HQ) (Fisher Scientific Co., 0-261); 8-hydroxyquinoline zinc salt (8-HQZn) (Eastman Organic Chemicals, 7884); polysorbate 80 (polyoxyethylene 20 sorbitan monooleate); chloroform (Fisher Scientific Co., C-298).

**8-Hydroxyquinoline Iron Salt (8-HQFe)**—8-HQFe was prepared by dissolving 4.35 Gm. of 8-HQ in 14 L. of distilled water and adding dropwise, while stirring, a solution of 2.70 Gm. $FeCl_3 \cdot 6H_2O$ in 50 ml. $H_2O$. The black precipitate was filtered on Whatman No. 1 paper and dried in a vacuum desiccator over $CaCl_2$.

**8-Hydroxyquinoline Aluminum Salt (8-HQAl) and 8-Hydroxyquinoline Copper Salt (8-HQCu)**—These metal chelates were prepared by adding an excess of 8-HQS (10% solution) to 1 L. of a millimolar aqueous solution of $AlCl_3$, respectively, $CuSO_4$ (reagent grade chemicals) and neutralization of the mixture at pH 7–8 by the dropwise addition of concentrated ammonia. The yellowish-green precipitates were filtered on a Millipore filter, washed thoroughly with distilled water, and vacuum dried over $CaCl_2$.

**Tuberculin PPD (Trichloroacetic Acid Precipitate) and Tuberculin PPD (Ammonium Sulfate Precipitate)**—Both methods of preparation and analysis of the tuberculin PPD preparations were previously carried out (2).

**Buffer Solution (pH 7.38)**—Isotonic phosphate-buffered solution, pH 7.38 (1.45 Gm. of $KH_2PO_4$, 7.60 Gm. of $Na_2HPO_4$, and 4.80 Gm. of NaCl in 1050 ml.). This buffer is also used as diluent for the preparation of tuberculin PPD solution (Mantoux) (1). Distilled water from a metal Barnstead still was used to prepare the buffer solution.

**Isolation of a Dark Deposit from Tuberculin PPD Solution (0.01% 8-HQS; 0.005% polysorbate 80)**—Five-hundred milliliters of a tuberculin PPD solution (1,000 TU/ml.) which had developed a dark deposit during 9 months' storage at 5°, was filtered through a Millipore TH filter[4] (pore size 0.45 $\mu$).

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[1] Marketed as Chinosol.

[2] The biological potency of tuberculin is expressed in tuberculin units (TU). One TU is biologically equivalent to 0.00002 mg. of the international standard tuberculin PPD.

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

[4] Obtained from Millipore Filter Corporation, Bedford, Mass.

The crystalline greenish black deposit was washed with distilled water and dried together with the filter in a vacuum desiccator over $CaCl_2$.

Twelve milliliters of a tuberculin PPD solution (2 mg. PPD/ml.) was prepared and sterilized by filtration. An aliquot of the solution was transferred into a glass-stoppered Pyrex tube and 8-HQS (0.01% final concentration) was added to the remainder of the solution. Both solutions were stored at 5° for 3 weeks. During this time a black deposit (0.3 mg.) had formed in the solution containing 8-HQS. The deposit was removed by filtration of the solution through a Gelman GA-6 filter[5] (0.45 $\mu$) and the filtrate as well as the original solution without 8-HQS were tested for biological potency.

One-milliliter ampuls (capacity 1.5 ml.) filled with 1ml. tuberculin PPD solution (50 TU/ml.) and stored at 5° over a period of 3.5 years contained tiny black specks. The contents of 350 ampuls were filtered through a Gelman GA-6 filter, and the black specks retained on the filter were washed thoroughly with water containing 0.005% polysorbate 80. Filter and deposit were dried in a vacuum desiccator over $CaCl_2$. The weight of the greenish black deposit was 11 mg.

One-milliliter vials (capacity 1.7 ml.) filled with 1 ml. tuberculin PPD solution (50 TU/ml.) and closed with equilibrated[6] white rubber stoppers[7] and sealed with three-piece aluminum caps[8] were stored for 2.5 years at 10°. During storage a greenish dark deposit developed that was isolated by filtering the contents of 770 vials as described above for ampuls. The weight of the greenish black deposit was 11.4 mg.

**Ultraviolet Spectrophotometry**—The samples were dissolved in chloroform (approximately 4 to 8 mcg./ml.), and the ultraviolet absorption spectrum was determined with a Beckman model DU spectrophotometer using chloroform as a blank.

**Infrared Spectrophotometry**—KBr pellets of the samples were prepared and the infrared absorption spectra were recorded from 650 to 5,000 $cm.^{-1}$ using a Beckman model IR-4 spectrophotometer fitted with NaCl optics.

**Neutron Activation Analysis**—The Millipore filter with the dark deposit from tuberculin PPD solution (1,000 TU/ml.) and a Millipore filter used as a blank were sealed in quartz tubes and irradiated with neutrons for 2 days in an atomic reactor. After activation, the filters were taken out of the tubing and examined with a multichannel pulse height $\gamma$-ray spectrometer. $\gamma$-Ray spectra were taken at intervals, as the activated sample decayed during a 3-month period.

**Biological Testing**—The tuberculin preparations to be compared were diluted in a buffer solution (pH 7.38, containing 0.005% polysorbate 80) to give the following two concentrations, 0.004 and 0.001 mg./ml. The dose injected intradermally into 20 BCG-sensitized guinea pigs was 0.1 ml. at each of these concentrations as previously described by Landi (1). At 24 hr. after injection the diameter of each reaction zone was measured, and the result was recorded as the sum of two perpendicular diameters in millimeters. The estimate of potency was made by the method of Long, Miles, and Perry (4).

The dark deposit obtained from ampuls and vials was also tested for potency. A solution of the deposit was prepared by dissolving 1 mg. of the deposit in 1 ml. 0.02 *N* HCl and further diluting it to the following two concentrations, 0.004 mg./ml. and 0.001 mg./ml. by the addition of buffer (pH 7.38; 0.005% polysorbate 80); 0.1 ml. of these concentrations and 0.1 ml. of the buffer used to dilute the dark deposit were injected intradermally and at random into 16 BCG-sensitized guinea pigs.

## RESULTS AND DISCUSSION

The deposit isolated from tuberculin PPD solutions containing 8-HQS (0.01%) was found to be soluble in chloroform and mineral acids. Microscopic examination of this deposit revealed that it consisted of tiny crystals and crystal fragments (Fig. 1). These crystals gave a bright yellow fluorescence under ultraviolet light (wavelength 360 m$\mu$). In a tuberculin solution some of these crystals were invisible in daylight but their presence was easily revealed under ultraviolet light.

Figure 2 shows the ultraviolet spectra in chloroform of a deposit from tuberculin PPD solution (1,000 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80) and for 8-HQFe, 8-HQZn, and 8-HQ. The steep absorption maximum at 260 m$\mu$ (Fig. 2, curve A) obtained with the deposit is in close agreement with the maximum at 256 m$\mu$ (8-HQFe), 258 m$\mu$ (8-HQZn) (Fig. 2, curves B and C, respectively), 260 m$\mu$ (8-HQAl), and 263 m$\mu$ (8-HQCu), while 8-HQ has a maximum in chloroform at 243 m$\mu$ (Fig. 2, curve D). The 8-HQ metal complexes served as controls. Other UV light absorbing substances such as tuberculoprotein with an absorption maximum at approximately 270 m$\mu$ and nucleic acid at approximately 260 m$\mu$ present in the tuberculin solution could not be considered as part of the deposit. These substances are insoluble in chloroform while the ingredients in the deposit were completely soluble in this organic solvent. The only other UV-absorbing and chloroform-soluble ingredient of tuberculin solution, which could be present in the deposit, was polysorbate 80; however, this nonionic surfactant has a very flat UV absorption maximum at a wavelength of 230 m$\mu$. Therefore, polysorbate 80 certainly could not account for the steep absorption maximum of 260 m$\mu$ given by the deposit.

To verify these findings the infrared spectra (Fig. 3) of the following substances were compared: (A) the deposit which formed in a tuberculin PPD solution (1,000 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80); (B) 8-HQFe; (C) 8-HQZn; (D) 8-HQ; (E) 8-HQS, (F) polysorbate 80; (G), nucleic acid from filtrate of *M. tuberculosis* var. *hominis*, (H) tuberculin PPD (trichloroacetic acid precipitation); (I) tuberculin PPD (ammonium sulfate precipitation). Figure 3 shows that only the IR spectra of 8-HQFe and 8-HQZn are similar to the IR-spectrum of the deposit. The substances D to I cannot constitute a major component of the deposit because their following distinct peaks are missing in the spectrum of the deposit (wave numbers in $cm.^{-1}$ Fig. 3): 897, 974, 1,900 for 8-HQ; 888, 993, 1,192, 1,400, 1,595, 3,000 for 8-HQS; 945, 1,000–1,200,

[5] Obtained from Gelman Instrument Company, Ann Arbor, Mich.
[6] Equilibrated against buffer solution of pH 7.38 (0.01% 8-HQS final concentration) (3).
[7] Designation V-32-86, obtained from The West Company, Phoenixville, Pa.
[8] Designation 13-31, obtained from The West Company, Phoenixville, Pa.

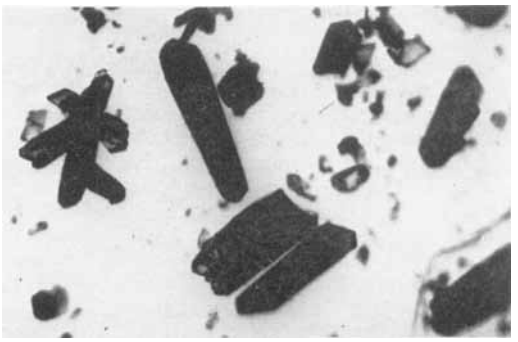

*Fig. 1—Greenish black crystals and crystal fragments from ampuls (50 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80). Magnification approx. 200×.*

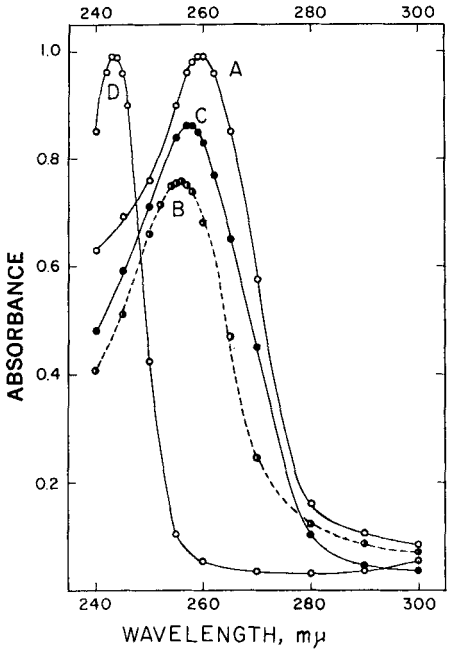


*Fig. 2—Ultraviolet absorption spectra in chloroform. Key:* A, *dark deposit from tuberculin PPD solution;* B, *8-HQFe;* C, *8-HQZn;* D, *8-HQ.*

1,726, 2,920 for polysorbate 80; 1,000–1,150, 1,200–1,280, 1,580–1,730 for nucleic acid; 1,010–1,130, 1,200–1,260, 1,600–1,730 for tuberculin PPD (trichloroacetic acid precipitation); and 1,520–1,570, 1,600–1,680 for tuberculin PPD (ammonium sulfate precipitation).

The IR spectra (Fig. 3) do not give any clue as to which metals have taken part in the formation of the deposit, since the IR spectra of 8-quinolinolates of different metals are very similar to each other according to Charles *et al.* (5) and Stone (6). Due to the extremely small amount of residue collected on a Millipore filter the deposit was tested for metals on the filter itself by the neutron activation analysis technique. Among the metals which can be determined by this technique only iron, zinc, and sodium were found in appreciable amounts (7.0% Fe, 1.6% Zn, and 0.6% Na), whereas other metals (antimony, cobalt, copper, chromium, gold, lanthanum, manganese) were present in trace amounts only (Table I).

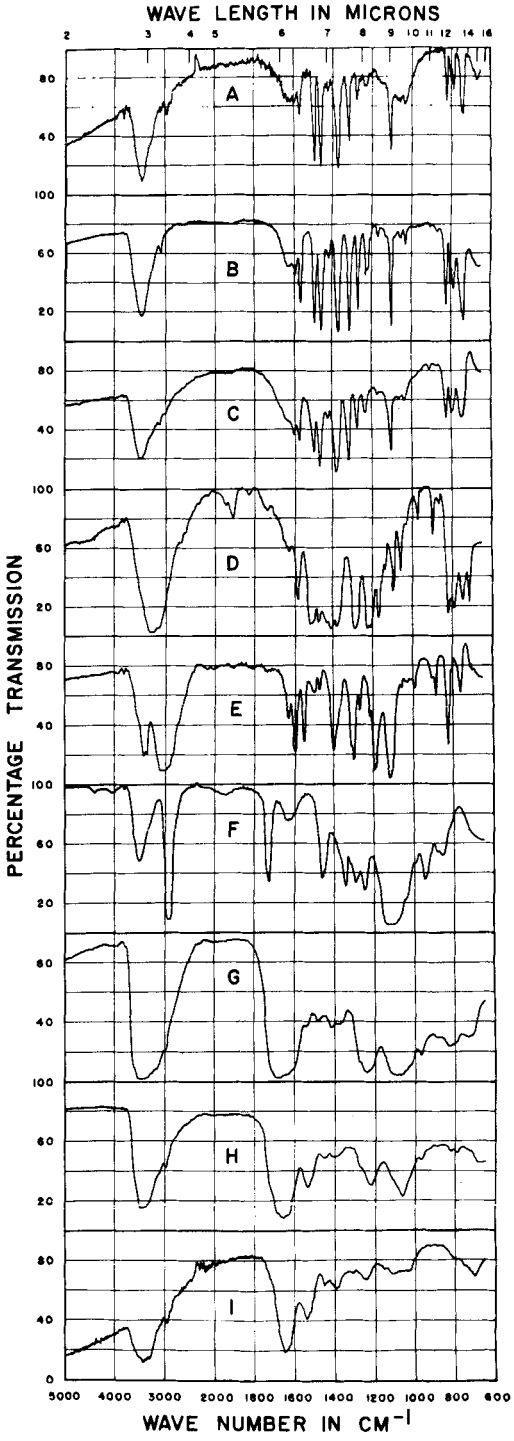


*Fig. 3—Infrared absorption spectra of components from tuberculin PPD solution. Key:* A, *dark deposit from tuberculin PPD solution;* B, *8-HQFe;* C, *8-HQZn;* D, *8-HQ;* E, *8-HQS;* F, *polysorbate 80;* G, *nucleic acid, prepared from filtrate of* M. tuberculosis *var.* hominis, H, *tuberculin PPD (trichloroacetic acid precipitation);* I, *tuberculin PPD (ammonium sulphate precipitation).*

TABLE I—ACTIVATION ANALYSIS ON A DARK DEPOSIT FROM TUBERCULIN PPD SOLUTION[a]

| Metal[b] | Amount, % |
|---|---|
| Iron | 7.03 |
| Zinc | 1.60 |
| Sodium | 0.62 |
| Copper | 0.36 |
| Chromium | $5.13 \times 10^{-3}$ |
| Antimony | $3.36 \times 10^{-4}$ |
| Gold | $2.97 \times 10^{-4}$ |
| Cobalt | $1.70 \times 10^{-4}$ |
| Scandium | $0.58 \times 10^{-4}$ |
| Manganese | Trace |
| Lanthanum | Trace |
| Bromine | Trace |

[a] The deposit had formed from 500 ml. tuberculin PPD solution (1,000 TU/ml.; 0.01% 8-HQS; 0.005% polysorbate 80) during 9 months' storage at 5°. [b] Looked for and not detected: Rb, Cs, Hg, Pt metals (all judged to be less than 1 p.p.m.); Cd, Mo, As, Ag, Ge (all judged to be less than 5 p.p.m.). The activation technique is not sensitive for the detection of Ca, Al, Pb, Si, S, P, I, Ni, Mg, and other light elements.

The above findings strongly suggest that chelation between 8-HQ and the metals present in tuberculin PPD solutions has taken place. In fact it is well known that traces of heavy metals are present in reagent grade chemicals (7, 8) and in the tuberculoprotein (2) used to prepare tuberculin PPD solutions. It is also known that when 8-HQS is added to a buffered solution (pH 7.38) it is dissociated into 8-HQ and $H_2SO_4$ (3, 9). 8-HQ is one of the best analytical reagents for metals (10–12) since it readily forms chelates which are of extremely low solubility. It is therefore not surprising that chelation could take place in tuberculin PPD solutions, when 8-HQS is employed as a preservative. Therefore, when tuberculin solutions, at pH 7.38, are stored in the bulk form (0.5 L. or more), chelation will at first render the solution slightly turbid and upon further storage a deposit will be formed which will settle at the bottom of the container. Magnusson and Bentzon (13) and Jensen *et al.* (14) reported a similar dark deposit which had formed in tuberculin PPD stock solution containing 8-HQS.

In order to verify to what extent the ingredients present in tuberculin PPD solutions contribute to the formation of the dark deposit, the following experiments were carried out. Solutions of phosphate-buffered saline (pH 7.38) containing 0.005% polysorbate 80 and 0.01% 8-HQS, and other solutions containing PPD in concentrations of 10, 50, and 1,000 TU/ml., respectively, in addition to polysorbate 80 and 8-HQS, were prepared. One liter of each solution was stored at 5° in glass-stoppered volumetric flasks. During a period of 9 months' storage, a deposit appeared in all the solutions. However, in those containing PPD the amount of deposit increased with increasing quantities of PPD. The distilled water used to prepare the buffered solution was only a minor contributor of metals. The distilled water from a metal Barnstead still may contain up to a total of 0.1 p.p.m. heavy metals according to Healy *et al.* (15). To determine if polysorbate contributed to the formation of the deposit, 0.01% 8-HQS was added to a buffered solution (pH 7.38) with 0.005% polysorbate 80 and without polysorbate 80. These two solutions were stored for 9 months at 37°. In both solutions an equal amount of dark deposit was formed indicating that the addition of polysorbate 80 had no noticeable influence on the amount of deposit present in these solutions.

Besides the metals present in the tuberculin PPD solution, a minor metal contribution could be made by the glass of the container and in the case of vials, by the rubber stopper and the metal seal. For instance when tuberculin PPD solutions are dispensed in multiple-dose vials sealed with equilibrated rubber stoppers (equilibrated against 100 p.p.m. 8-HQS) (3), these closures will influence the amount of deposit formed in these tuberculin solutions according to a "push-pull" mechanism engendered by the partition coefficients of the metal chelates between the tuberculin solutions and the rubber closures. As described previously (3), during equilibration of a rubber stopper against a buffered solution (pH 7.38) containing 100 p.p.m. 8-HQS, its base 8-HQ will distribute itself between rubber and aqueous solutions according to its partition coefficient. When 8-HQ enters the rubber, it reacts chemically with the heavy metal ions present in the rubber whereby the metals chelate with 8-HQ. These metal chelates have their own partition coefficients between the organic phase (rubber stopper) and the aqueous phase. Consequently, when rubber stoppers are used the chelates in the stopper and in the solution distribute themselves according to their partition coefficients until an equilibrium is reached. This may contribute to the formation of a deposit which may not necessarily be dark as the color of 8-HQ chelates from different metals varies from nearly colorless to black.

In order to find out if the removal of metals from tuberculin PPD would affect the potency of the preparation, the potency of a tuberculin solution (2 mg. PPD/ml. at pH 7.38) was compared with the potency of the same solution after removal of metal chelates which formed after addition of 8-HQS. It was found that the relative potency of the tuberculin solution after removal of metals and compared with the same tuberculin solution before removal of metals was 1.05 (0.89–1.24).[9] This relative potency is not significantly different from 1.00 and therefore there is no evidence of a loss or of an increase in potency when metal chelates are removed.

Skin tests were also carried out in BCG-sensitized guinea pigs using the dark greenish deposit isolated from vials and ampuls. No tuberculin skin reaction could be detected in the animals indicating that there was no tuberculin PPD present in either deposit.

Formation of an insoluble deposit is undesirable in pharmaceutical solutions and therefore represents a considerable drawback to the use of the preservative 8-HQS in tuberculin PPD solutions. The formation of such deposit could be avoided by (*a*) pretreating a stronger buffer solution (pH 7.38), for instance 10 times its usual strength with 0.01% 8-HQS, then filtering the metal chelates out and diluting the clear filtrate with distilled water containing 0.01% 8-HQS to the required buffer strength to be used to prepare tuberculin PPD solutions; the same treatment could be carried out on a more concentrated (2 mg./ml.) tuberculin PPD solution; (*b*) using closures with low metal content when dispensing tuberculin solutions into vials.

On the other hand, the complete exclusion of metals would not be desirable because 8-HQ is markedly antimicrobial only in the presence of

[9] Figures in brackets represent 95% confidence limits.

traces of certain heavy metals, *e.g.*, copper ($Cu^{2+}$), iron ($Fe^{3+}$ or $Fe^{2+}$) while other metals such as cadmium, zinc, nickel, and especially cobalt antagonize the antimicrobial properties of 8-HQ (7, 16, 17).

These incompatibilities render this preservative (8-HQS) cumbersome to use in tuberculin PPD solutions.

## SUMMARY

1. It has been shown that tuberculin PPD solutions to which 0.01% 8-HQS had been added as preservative, developed a crystalline deposit when stored for several months.

2. The deposit was found to consist of metal chelates of 8-HQ in which the metals involved were iron, zinc, sodium, and traces of some other metals.

3. The main sources of metals causing precipitation were found to be the reagent grade chemicals constituting the buffer and the PPD used to prepare the tuberculin solutions; a minor but still significant source was the distilled water.

4. The removal of metals from tuberculin PPD caused by the presence of the preservative 8-HQS did not affect the biological potency of the preparation and tuberculin PPD was not present in the deposit.

5. The tendency of the preservative, 8-HQS, to give rise to an organo-metallic deposit in tuberculin PPD solutions, is a considerable drawback. Especially so, since a trace of iron or copper has to be present in order to render this preservative effective.

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*Keyphrases*

Tuberculin solution—stability
Preservative—tuberculin solution
8-Hydroxyquinoline $SO_4$—preservative
Metal–8-hydroxyquinoline chelates—activity effect
Biological assay—sensitivity
UV spectrophotometry—identity, chelate
IR spectrophotometry—identity, chelate
Neutron activation analysis—metal identity

# Microbiological Evaluation of PCMX Complexes

By M. D. RAY*, K. E. AVIS, and C. C. FLANIGAN, JR.

**The minimum inhibitory concentration of *p*-chloro-*m*-xylenol (PCMX) was established against *Bacillus subtilis, Pseudomonas aeruginosa,* and *Aspergillus niger,* using a serial dilution and a pour plate procedure. The inoculum size was standardized by absorbance of light at 450 mμ wavelength. The drug was subsequently complexed with methylcellulose, polyethylene glycol 6000, and polysorbate 80. A minimum inhibitory concentration for the drug against each of the microorganisms was established in the presence of varying concentrations of the macromolecules. The results demonstrated that in most cases the antimicrobial activity of PCMX was reduced in the presence of the macromolecules studied. Since it had been previously established that PCMX interacted with these molecules, it was concluded that the reduction in biological activity was a direct result of the molecular interaction.**

PHENOL AND ITS DERIVATIVES have served a major function in antisepsis and disinfection since the introduction of phenol by Lister (1) in aseptic surgery. Although the value of these agents is widely acknowledged, there has been an extensive search in recent years for phenolic derivatives which have an increased spectrum of microbicidal activity with less toxicity to tissue. Considerable interest has been generated in this country and in Europe in the use of *p*-chloro-*m*-

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