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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xylenol (PCMX) as an antiseptic in external pharmaceutical products (2–14), as a disinfectant in cleaning solutions (15), and as a preservative for pharmaceutical and nonpharmaceutical commodities (16). Reports have also indicated that PCMX has a very low toxicity to tissue (17–22).

A recent investigation has shown, however, that PCMX reacts with various nonionic macromolecules often present in formulations to form complexes (23). The present investigation was undertaken to explore the effect of such interactions on the biological activity of the agent.

## EXPERIMENTAL

**Reagents**—Recrystallized PCMX,[1] m.p. 115–115.5°; polyethylene glycol 6000[2]; methylcellulose 15 cps., USP[3]; polysorbate 80,[4] and Sabouraud medium.[5]

**Microorganisms**—*Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* ATCC 10145, and *Aspergillus niger* ATCC 6275.

**Apparatus**—Beckman spectrophotometer model DB, Bausch and Lomb spectronic 20, Thomas-Hoover melting point apparatus, Precision Scientific Company Thelco oven, constant-temperature water bath.

### Procedures

**Determination of Cell Count**—The microorganisms used in this investigation—*B. subtilis*, *Ps. aeruginosa*, and *A. niger*—were selected because each had a previously reported sensitivity to PCMX and because they represented three general groups of microorganisms, Gram-positive bacteria, Gram-negative bacteria, and fungi, respectively. Sabouraud liquid medium and Sabouraud agar medium were employed as culture media throughout the investigation. The authors felt it advisable to use the same culture media for each organism so that any binding between the PCMX and culture media would be uniform throughout the study. Although, admittedly, bacteria do grow better in other media, Sabouraud media were selected in deference to the more fastidious *A. niger*. It was shown by controls that the bacteria used in this study did grow quite well in Sabouraud liquid medium and Sabouraud agar medium.

A rapid and accurate method for determining cell counts was adopted similar to the light absorption method reported by Guidry and Maier (24). In the author's investigation a loopful of each of the test organisms was transferred at zero time to 50 ml. of sterile liquid medium. Cultures of *B. subtilis* and *Ps. aeruginosa* were incubated at 37°, and the *A. niger* at 27°. After 48 hr. of growth, the suspensions were transferred to a sterile Waring blender bowl and subjected to a dispersion treatment for 30 sec. The dispersed cultures were diluted with a measured volume of nutrient medium, and percent transmittance readings were taken, employing a Bausch and Lomb spectronic 20, at wavelengths of 450 mμ and 500 mμ. Immediately thereafter, a measured aliquot from each dispersed culture was transferred to freshly prepared Sabouraud agar plates and incubated. After 48 hr. the plates were removed and the colonies counted. Several replicate tests were performed in this manner, and a relationship was established between percent transmittance and cell count (see Table I).

**Determination of Standard Minimum Inhibitory Concentration (MIC)**—An approximate MIC for PCMX against each of the test organisms was determined by inspecting for visible growth of the organisms in a series of concentrations of PCMX in nutrient medium. A standard MIC was subsequently determined by quantitating the growth of each organism in a series of solutions of PCMX having a range which extended above and below the approximate MIC. Replicate tubes of each concentration were inoculated with a measured volume of the standardized microbial suspensions. After incubation for 48 hr., aliquots from each tube were transferred to Sabouraud agar plates. Colony counts were made after a 48-hr. incubation period, comparing this count with the theoretical number that would have been present if all the cells initially introduced into the medium had survived. The effect of PCMX was then evaluated as lethal, inhibitory, or noninhibitory based upon whether the plates showed no growth, growth of fewer colonies than the theoretical cell count, or growth of considerably more colonies than were representative of the cell count of the initial inoculum.

**Determination of MIC of Complexation Mixtures**—The PCMX and macromolecule were weighed by difference into a dry volumetric flask. Sufficient liquid nutrient medium was added to attain a prescribed volume. The mixtures were transferred to conical flasks, capped with aluminum foil, and sterilized by autoclaving at 121° for 15 min. After cooling, the solutions were placed in a 30° chamber for 48 hr. to permit complexation to occur, after the manner described by Breuninger and Goettsch (23).

An exception was required for the methylcellulose solutions, because the heat of the sterilization process caused the methylcellulose to be partially dehydrated and precipitated from solution. To correct this, the samples containing methylcellulose were refrigerated for 8 hr. to rehydrate the methylcellulose prior to being placed in the 30° chamber.

A series of concentrations of each macromolecule was prepared, and the antimicrobial activity of PCMX at each of these concentrations was determined. The concentrations employed were: polyethylene glycol 6000–0.22, 0.33, 0.44, 0.88, and

TABLE I—CORRELATION BETWEEN PERCENT TRANSMITTANCE AND VIABLE CELL COUNT

| Organism | Wavelength, mμ | Percent, T | Viable Cell Count[a] |
|---|---|---|---|
| *Aspergillus niger* | 450 | 75 | $2 \times 10^6$ |
| *Aspergillus niger* | 500 | 87 | $2 \times 10^6$ |
| *Pseudomonas aeruginosa* | 450 | 35 | $2 \times 10^7$ |
| *Pseudomonas aeruginosa* | 500 | 42 | $2 \times 10^7$ |
| *Bacillus subtilis* | 450 | 78 | $1 \times 10^8$ |
| *Bacillus subtilis* | 500 | 86 | $1 \times 10^8$ |

[a] Determined by multiplying actual plate colony counts by appropriate dilution factors.

[1] Ottasept Extra. Supplied through the courtesy of Ottawa Chemical Company, Toledo, Ohio.
[2] Fisher Scientific Company, Fair Lawn, N. J.
[3] Methocel 15 cps. Supplied through the courtesy of Dow Chemical Company, Midland, Mich.
[4] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.
[5] Baltimore Biological Laboratory, Inc., Baltimore, Md.

1.32%; methylcellulose—0.5, 1.0, and 1.5%; and polysorbate 80—0.5, 1.0, and 1.5%. A known number of cells, as determined by optical density measurement, was added to each complexation mixture and incubated at the appropriate temperature for 48 hr. At the end of the incubation period an aliquot from each sample was transferred to Sabouraud agar plates. The inoculated plates were examined and the colonies counted after 48 hr. of incubation.

Controls were employed to establish the viability of the organisms, the ability of the nutrient to support the growth of each organism, and the ability of PCMX to inhibit growth of each organism in the absence of the macromolecule.

## RESULTS AND DISCUSSION

**Methylcellulose Complex**—In the presence of methylcellulose, PCMX had less inhibitory effect on the growth of *B. subtilis* and *Ps. aeruginosa*, as reflected in an increase in the MIC (Figs. 1 and 2). The MIC for PCMX against *B. subtilis*, in all the concentrations of methylcellulose studied, was found to be 1:15,000 (0.007%). Against *Ps. aeruginosa* the intensity of the effect of methylcellulose varied with the concentration of the complexing agent. When PCMX was equilibrated with 0.5% methyl-

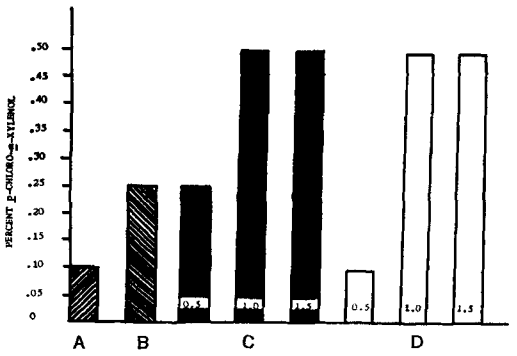


*Fig. 1—Effect of complexation on the activity of* p-*chloro*-m-*xylenol*—pseudomonas aeruginosa. *Key: A, Minimum inhibitory concentration of unreacted* p-*chloro*-m-*xylenol; B, Minimum inhibitory concentration of* p-*chloro*-m-*xylenol in presence of polyethylene glycol 6000 (0.22-1.32%); C, Minimum inhibitory concentration of* p-*chloro*-m-*xylenol in presence of three concentrations of methylcellulose; D, Minimum inhibitory concentration of* p-*chloro*-m-*xylenol in presence of three concentrations of polysorbate 80.*

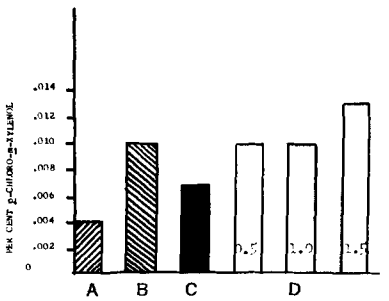


*Fig. 2—Effect of complexation on the activity of* p-*chloro*-m-*xylenol*—bacillus subtilis. *See Fig. 1 for key.*

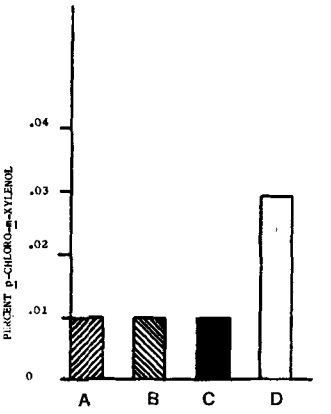


*Fig. 3—Effect of complexation on the activity of* p-*chloro*-m-*xylenol*—aspergillus niger. *See Fig. 1 for key.*

cellulose, a MIC of 1:400 (0.25%) was observed, as compared with an MIC of 1:1000 (0.1%) in the absence of a complexing agent. With a concentration of 1.0% and 1.5% an MIC of 1:200 (0.5%) was observed.

The activity of PCMX against *A. niger* was apparently unaffected by complexation with methylcellulose (Fig. 3). In all concentrations of methylcellulose studied, an MIC of 1:10,000 (0.01%) was noted, which was the same as the MIC of uncomplexed PCMX against *A. niger*. There was no apparent explanation for this lack of effect, since complexation had occurred in the work of Breuninger and Goettsch (23), although the metabolic effects of the organism on the complex may be a factor.

Through the use of controls it was shown that methylcellulose alone had no stimulatory or inhibitory effect, in the concentration range studied, on any one of the three organisms. Solutions of 0.5, 1.0, and 1.5% methylcellulose in water failed to support the growth of any of the organisms, but did not inhibit the growth in Sabouraud liquid medium. It was concluded therefore that any diminution in the activity of PCMX was a direct result of interaction with methylcellulose.

**Polysorbate 80 Complex**—The activity of PCMX against all three test organisms was considerably diminished in the presence of polysorbate 80, in the range of concentrations studied. Against *B. subtilis* the MIC of PCMX when complexed with 0.5% and 1.0% polysorbate 80 was found to be 1:10,000 (0.01%), and with 1.5% polysorbate 80 was found to be 1:7,500 (0.015%) (Fig. 2). The MIC for unreacted PCMX against *B. subtilis* was 1:26,000 (0.004%).

There was a uniform reduction in the activity of PCMX against *A. niger* (Fig. 3) at all three concentration levels of polysorbate 80 employed, to an MIC of 1:3300 (0.03%). The MIC of uncomplexed PCMX was 1:10,000 (0.01%).

Complexation of PCMX with 0.5% polysorbate 80 had no effect on the activity of the drug against *Ps. aeruginosa* (Fig. 1), showing an MIC of 1:1000 (0.1%). When equilibrated with 1.0% and 1.5% polysorbate 80, however, PCMX showed a marked reduction in activity, with an MIC of 1:200 (0.5%).

Through the use of suitable controls, it was demonstrated that polysorbate 80 contributed no

stimulatory or inhibitory activity of its own against the three test organisms. Therefore, where polysorbate 80 caused a reduction in the activity of PCMX, it was thought that the reduction was directly attributable to complexation between the agent and the macromolecule, thereby lessening the availability of the drug to exert its antimicrobial activity. Where no inhibition occurred there may have been insufficient polysorbate 80 present.

**Polyethylene Glycol 6000 Complex**—The activity of PCMX against both *B. subtilis* and *Ps. aeruginosa* was reduced when complexed with polyethylene glycol 6000 (Figs. 1 and 2). The MIC against *Ps. aeruginosa* was found to be 1:400 (0.25%), and against *B. subtilis*, 1:10,000 (0.01%). The MIC for unreacted PCMX against the two organisms was 1:1000 (0.1%) and 1:26,000 (0.004%), respectively.

Polyethylene glycol 6000 did not inhibit the activity of PCMX against *A. niger* in the range of concentrations studied (Fig. 3). This observation, coupled with the fact that methylcellulose had a like effect, suggested the possibility that *A. niger* was capable of disrupting the complex formed with these two macromolecules, thereby restoring the availability of PCMX to exert is antifungal activity, but not the complex formed with polysorbate 80.

Controls similar to those described above for methylcellulose were used to establish the fact that polyethylene glycol 6000 alone had no contributing effect on the growth of the organisms. It was assumed, therefore, that any reduction in the activity of PCMX when complexed with polyethylene glycol 6000 was a direct result of that interaction.

The studies of Breuninger and Goettsch (23) showed that the solubility of PCMX was decreased upon the addition of polyethylene glycol 6000 up to about 0.5%, and that a subsequent increase in the concentration of polyethylene glycol 6000 caused an increase in the solubility of PCMX. In our study, contrary to what might be expected, colony counts of *B. subtilis* and *Ps. aeruginosa* at a polymer concentration of 0.22% were lower than counts at higher concentrations. Polyethylene glycol 6000 had no apparent effect on the activity of PCMX against *A. niger*. Table II shows the effect of polyethylene glycol 6000 on the MIC of PCMX.

**Comparison with Equilibrium Dialysis Studies**—In the equilibrium dialysis studies of Breuninger and Goettsch, moles of bound PCMX were plotted against moles of unbound PCMX in the presence of 2% methylcellulose and 2% polysorbate 80. The slope for the plot in the presence of methylcellulose was found to be 0.14 and in the presence of polysorbate 80, it was 9.3. This comparison showed that polysorbate 80 had a much greater binding affinity for PCMX than did methylcellulose.

From our data the increase in the required MIC in the presence of the various concentrations of polysorbate 80 and methylcellulose as compared with PCMX alone was calculated. These percentages are shown in Tables III and IV.

A comparison of percent increase in MIC for PCMX–polysorbate 80 complex with PCMX–methylcellulose complex does not reveal a striking parallel with the greater binding affinity that Breuninger and Goettsch found for PCMX-polysorbate 80 complex as compared with PCMX-methylcellulose complex. However, there is a degree of correlation in that the percent increase for PCMX-polysorbate 80 against *B. subtilis* was 150 but only 81 with PCMX-methylcellulose, and against *A. niger* was 200 and 0, respectively. Thus it would appear that with these two organisms the greater binding effect between PCMX and polysorbate 80 resulted in a greater inhibition of activity than with PCMX and methylcellulose.

TABLE II—EFFECT OF POLYETHYLENE GLYCOL 6000 ON THE MINIMUM INHIBITORY CONCENTRATION OF *p*-CHLORO-*m*-XYLENOL

| Organism | Percent Polyethylene Glycol 6000 | Percent Increase in MIC[a] |
|---|---|---|
| *Bacillus subtilis* | 0.22 | 60 |
| | 0.33 | 60 |
| | 0.44 | 60 |
| | 0.88 | 60 |
| | 1.32 | 60 |
| *Pseudomonas aeruginosa* | 0.22 | 60 |
| | 0.33 | 60 |
| | 0.44 | 60 |
| | 0.88 | 60 |
| | 1.32 | 60 |
| *Aspergillus niger* | 0.22 | 0 |
| | 0.33 | 0 |
| | 0.44 | 0 |
| | 0.88 | 0 |
| | 1.32 | 0 |

[a] MIC = Minimum inhibitory concentration.

TABLE III—EFFECT OF METHYLCELLULOSE ON THE MINIMUM INHIBITORY CONCENTRATION OF *p*-CHLORO-*m*-XYLENOL

| Organism | Percent Methylcellulose | Percent Increase in MIC[a] |
|---|---|---|
| *Bacillus subtilis* | 0.5 | 81 |
| | 1.0 | 81 |
| | 1.5 | 81 |
| *Pseudomonas aeruginosa* | 0.5 | 150 |
| | 1.0 | 400 |
| | 1.5 | 400 |
| *Aspergillus niger* | 0.5 | 0 |
| | 1.0 | 0 |
| | 1.5 | 0 |

[a] MIC = Minimum inhibitory concentration.

TABLE IV—EFFECT OF POLYSORBATE 80 ON THE MINIMUM INHIBITORY CONCENTRATION OF *p*-CHLORO-*m*-XYLENOL

| Organism | Percent Polysorbate 80 | Percent Increase in MIC[a] |
|---|---|---|
| *Bacillus subtilis* | 0.5 | 150 |
| | 1.0 | 150 |
| | 1.5 | 225 |
| *Pseudomonas aeruginosa* | 0.5 | 0 |
| | 1.0 | 400 |
| | 1.5 | 400 |
| *Aspergillus niger* | 0.5 | 200 |
| | 1.0 | 200 |
| | 1.5 | 200 |

[a] MIC = Minimum inhibitory concentration.

## SUMMARY AND CONCLUSIONS

In almost all cases the activity of PCMX against three microorganisms, *B. subtilis*, *Ps. aeruginosa*, and *A. niger* was shown to be diminished when allowed to interact with the three macromolecules, polyethylene glycol 6000, methylcellulose, and polysorbate 80, in the concentration ranges studied. Exceptions to this general observation were obtained

with *A. niger* when evaluating the effect of methylcellulose and polyethylene glycol 6000 on PCMX; and the result obtained with *Ps. aeruginosa* in evaluating the effect of polysorbate 80 at a concentration of 0.5% on PCMX. In these instances, no increase was observed. In all cases where a reduction in the antimicrobial activity of PCMX was demonstrated, the reduction was thought to be a direct result of the molecular interaction with the nonionic macromolecule, thereby diminishing the availability of the drug to exert its antimicrobial activity.

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*Keyphrases*

*p*-Chloro-*m*-xylenol (PCMX)—microbiological evaluation
Minimum inhibitory concentration—PCMX
Complex formation—PCMX activity
Equilibrium dialysis—PCMX, methylcellulose, polysorbate 80
Spectrophotometry—cell count determinations


# Studies on the Effects of Reserpine in Mice as Influenced by its Diluent

By W. VELDKAMP, G. A. JOHNSON, and H. H. KEASLING

**The effects of dextroamphetamine sulfate and benzphetamine hydrochloride on locomotor activity in mice pretreated with reserpine varied depending on the reserpine preparations used. Pretreatment with reserpine suspended in aqueous 0.25 percent methylcellulose (pH 5.8) had little effect on the response to dextroamphetamine sulfate but decreased the response to benzphetamine hydrochloride. Pretreatment with a reserpine solution (pH 3.5) significantly enhanced the locomotor response to both compounds at the higher doses tested. The difference in effect produced by the reserpine preparations could not be explained on the basis of differences in brain amine levels at the time locomotor activity was measured since both preparations had depleted brain amines to the same extent. Further studies revealed that variations in pH of the reserpine diluent had a marked effect on brain reserpine levels, on brain amine levels when measured 1 hr. after reserpine administration, and on the rate of onset and intensity of ptosis produced in the mouse.**

VAN ROSSUM *et al.* (1) and van Rossum and Hurkmans (2) have reported that reserpine pretreatment in mice has no effect on the locomotor activity response to dextroamphetamine sulfate while the effects of derivatives of amphetamine which have large substituents on the nitrogen atom, such as benzylamphetamine and benzphetamine hydrochloride,[1] are antagonized by pretreatment with reserpine. On the other hand, Smith (3) has reported an enhancement of the effects of dextroamphetamine sulfate on the locomotor activity of mice when pretreated with reserpine.

The present study shows that the effect obtained with dextroamphetamine sulfate and benzphetamine hydrochloride in reserpinized mice will vary depending upon the reserpine preparation used in pretreating the mice. A number of acids such as ascorbic, Burn and Rand, (4), acetic, Fleming and Trendelenberg, (5), and citric, Fleming, (6) have been used to dissolve reser-

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[1] Trademarked as Didrex by the Upjohn Co., Kalamazoo, Mich.