Inject 2 μl. of the benzene solution using a syringe fitted with an adapter that allows the needle to penetrate only $1^1/_4$ inches beyond the septum. The adapter is unnecessary for trichloroethanol or its acetate. Inject 2 μl. of a 0.5 μg. per ml. standard before and after each set of samples in order to determine the exact retention time and provide for quantitative measurement of each sample.

Calculations. Since 2 μl. of sample extract represents 0.010 gram of original sample, the standard corresponds to 0.1 p.p.m. The response for chloral hydrate is linear up to 6 nanograms, and for trichlorfon, to 12 nanograms. Trichloroethanol and its acetate give a linear response for amounts up to 30 nanograms. Therefore, the method is satisfactory for a range of 0.1 to 1.2 p.p.m. for trichlorfon and 0.1 to 3.0 p.p.m. for trichloroethanol. For higher levels, dilutions have to be made. The parts per million of any sample is calculated as follows:

$$\text{P.p.m.} = \frac{(\text{area of sample})(0.1)(\text{dilution factor})}{(\text{average area of standards})}$$

Discussion

Recovery Experiments. Recovery experiments were conducted on all of the tissues and crops listed in Table I by adding known amounts of the compound at the blending step, and processing the sample by the appropriate procedure. The results of these experiments are listed in Table I. All recoveries were conducted at a 0.1 p.p.m. level. Chloral hydrate, and especially trichloroethanol, are quite volatile. Recovery of these compounds is, therefore, not as good as for trichlorfon. Typical control and recovery chromatograms for trichlorfon and trichloroethanol analyses at 0.1 p.p.m. are shown in Figure 1.

The conversion of trichloroethanol to the acetate by the acetylation procedure in the presence of plant tissue extracts is quantitative through 1.0 p.p.m. Since there was no need to go beyond this level, experiments at still higher levels were not attempted.

Control Values. Untreated samples were analyzed for apparent trichlorfon and trichloroethanol peaks. The number of control samples analyzed ranged from three for each animal tissue to ten for each moist crop. In all cases, control values were far less than 0.1 p.p.m. for both compounds. Control values were not calculated for chloral hydrate because it has the same retention time as trichlorfon. Chlorinated hydrocarbon insecticides do not interfere in the method because of their nonpolar nature.

Sensitivity. The method is capable of measuring 0.1-p.p.m. residues of all three compounds with good precision. At this level, relatively little interference from control peaks is encountered. The procedure is inherently capable of considerably greater sensitivity, if required.

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TOXICITY STUDIES

Toxicity of Metal Complexes of Octamethylpyrophosphoramide in Water and Dimethylsulfoxide

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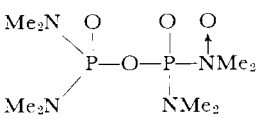
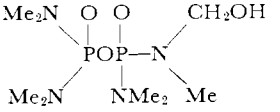
The LD_{50} values for metal complexes of octamethylpyrophosphoramide (OMPA) toward mice have been determined. When water is the solvent, all OMPA complexes except that of Co(II) are more toxic than the combined toxicity of the metal salt and OMPA. When dimethylsulfoxide (DMSO) is the solvent, the toxicity of $[Fe(OMPA)_3][FeCl_4]_3$ is extremely high (LD_{50} = 4.8). The toxicity of the other OMPA complexes in DMSO is either the same as or less than the combined toxicity of the metal salt and OMPA.

Octamethylpyrophosphoramide (OMPA) acts as a typical organophosphorus poison by inhibiting the action of acetylcholinesterases (*2*). The active metabolite of OMPA is thought to be either



or



A mechanism has been proposed that

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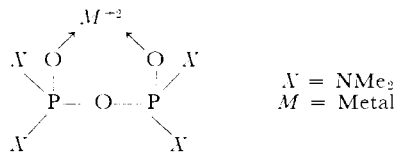
includes both species, with the more stable alcohol represented as the likely active metabolite. In either case there is evidence that OMPA is oxidized in vivo before it has any effect on the function of acetylcholinesterase.

Previous work in this laboratory (*3*, *6*) has resulted in the isolation of stable complexes of octamethylpyrophosphoramide with a wide variety of metal ions. These complexes are believed to involve coordination through the phosphoryl oxygens.



X = NMe_2
M = Metal

The present investigation is concerned with measuring the toxicity of metal complexes of OMPA in an effort to determine whether the metal ion has any effect on the toxicity of OMPA. Both water and dimethylsulfoxide were used as solvents.

Experimental

Random-bred Ha/ICR Swiss Webster white male mice weighing 25 to 30 grams were obtained from Cumberland View Farms in Tennessee. The animals were housed five to a cage and fed pellet Chow (Ralston Purina Co., St. Louis) with water *ad libitum* for three days to allow sufficient time for recovery from the rigors of shipping.

Before testing, the animals were grouped four or six per cage. Dosages were spaced in geometric progression, so that one dosage group would yield a 10 to 20% mortality, another group an 80 to 90% mortality, and at least one other group was in an intermediate dosage range. For each compound tested, the minimum number of groups was three, and in most cases four groups of six animals were used.

Each mouse was injected intraperitoneally with 0.2 ml. of test solution via disposable 1-ml. tuberculin syringes. The animals were observed for 10 days to 2 weeks.

Distilled water and reagent grade dimethylsulfoxide (Matheson, Coleman, and Bell) were used as solvents. The metal perchlorate salts were obtained from G. Frederick Smith Chemical Co. Anhydrous iron (III) chloride was obtained from Matheson, Coleman, and Bell. Pennsalt Chemical supplied a 91% solution of octamethylpyrophosphoramide which was purified by fractional distillation under vacuum (*3*).

Solutions of the complexes were prepared immediately prior to injection. Stock solutions were prepared by weighing the metal salts, complexes, or OMPA in appropriate volumetric flasks and filling to the mark with distilled water or dimethylsulfoxide. A Mettler H-6 analytical balance was used for weighings.

The data were treated by the statistical method of Litchfield and Wilcoxon (*5*). In Tables I and II the LD_{50} values are reported at the 95% confidence level. The slopes of the dosage mortality curves are also indicated. The slopes of mortality curves for most compounds used in this study were high enough to give fairly accurate LD_{50} values.

Results and Discussion

The LD_{50} values for the metal salts, OMPA, and complexes of OMPA are presented in Tables I and II. The predicted LD_{50} values are calculated by

Table I. LD_{50} Values for Metal Salts

Compound	Solvent	Animals Tested	Onset Symptoms, Minutes	Time of Death after Injection	LD_{50}, Mg./Kg.	Slope
$LiClO_4 \cdot 3H_2O$	H_2O	24	<1	7 min.–12 hours	1160 ± 110	1.1
$NaClO_4 \cdot H_2O$	H_2O	38	<1	6 min.–26 hours	1150 ± 200	1.3
$NaClO_4 \cdot H_2O$	DMSO	24	<1	8 min.–2 days	1178 ± 141	1.2
$Pb(ClO_4)_2 \cdot 6H_2O$	H_2O	28	1	11 min.–5 days	275 ± 33	1.1
$Mn(ClO_4)_2 \cdot 6H_2O$	H_2O	34	1	2 min.–8 hours	410 ± 143	1.5
$FeCl_3 \cdot 6H_2O$	H_2O	18	1	2 hours–3 days	370 ± 185	1.4
$FeCl_3 \cdot 6H_2O$	DMSO	12	<1	2.5 hours–2 days	260 ± 52	1.2
$FeCl_3$	DMSO	12	2	24 hours–8 days	18 ± 11	1.8
$Co(ClO_4)_2 \cdot 6H_2O$	H_2O	24	2	3 min.–3 days	160 ± 56	1.5
$Ni(ClO_4)_2 \cdot 6H_2O$	H_2O	30	1	4 min.–12 hours	100 ± 45	1.6
$Cu(ClO_4)_2 \cdot 2H_2O$	H_2O	32	1	20 min.–3 days	29 ± 4	1.2
$Zn(ClO_4)_2 \cdot 6H_2O$	H_2O	26	2	19 min.–48 hours	76 ± 46	1.5
$Mg(ClO_4)_2$	H_2O	30	1	1 min.–36 hours	1500 ± 150	1.2

Table II. LD_{50} Values for OMPA Complexes in H_2O and DMSO

Compound	Solvent	Animals Tested	Onset Symptoms, Minutes	Time of Death after Injection	Slope	Exptl. LD_{50}, Mg./Kg.	Predicted LD_{50}	% Diff.
OMPA	H_2O	30	5	6 min.–1 hour	1.1	14.9 ± 1.3	...	...
OMPA	DMSO	24	1	24 min.–35 hours	1.1	9.7 ± 0.9	...	...
$LiClO_4 \cdot 2OMPA$	H_2O	26	13	15 min.–45 min.	1.4	15.0 ± 4.9	17.6	+14.8
$NaClO_4 \cdot OMPA$	H_2O	26	1	15 min.– 4 hours	1.3	15.1 ± 3.0	21.3	+29.1
	DMSO	30	1	14 min.–36 hours	1.2	12.2 ± 1.6	13.9	+12.2
$Mg(ClO_4)_2 \cdot 3OMPA$	H_2O	30	3	20 min.–12 hours	1.4	12.0 ± 3.2	19.0	+36.9
	DMSO	30	1	2 min.–36 hours	1.9	12.0 ± 5.3	12.1	0
$Pb(ClO_4)_2 \cdot 3OMPA$	H_2O	16	3	19 min.–41 min.	1.1	18.0 ± 5.4	22.0	+18.2
$Mn(ClO_4)_2 \cdot 3OMPA$	H_2O	20	9	20 min.–36 min.	1.2	14.0 ± 2.0	19.3	+27.4
	DMSO	24	2	28 min.–36 hours	2.0	18.0 ± 9.9	12.6	−30.0
$Fe(ClO_4)_3 \cdot 3OMPA$	DMSO	12	1	2 hours–6 days	1.3	13.8 ± 6.2	13.7	0
$[Fe(OMPA)_3]\ [FeCl_4]_3$	H_2O	22	8	20 min.–30 min.	1.2	21.0 ± 2.5	26.0	+19.2
	DMSO	18	28	33 min.– 4 days	1.3	4.8 ± 1.4	17.0	+76.5
$Co(ClO_4)_2 \cdot 3OMPA$	H_2O	22	9	14 min.–39 min.	1.3	19.0 ± 4.2	19.0	0
	DMSO	18	4	7 min.– 2.5 hours	1.3	10.5 ± 4.2	12.6	+16.7
$Ni(ClO_4)_2 \cdot 3OMPA$	H_2O	24	3	18 min.–30 min.	1.3	14.6 ± 3.7	19.3	+24.3
$Cu(ClO_4)_2 \cdot 3OMPA$	H_2O	30	2	20 min.–49 min.	1.1	17.4 ± 2.1	21.2	+21.8
$Zn(ClO_4)_2 \cdot 3OMPA$	H_2O	24	3	21 min.–38 min.	1.2	17.6 ± 2.3	19.5	+9.8
	DMSO	24	1	42 min.–26 hours	1.4	12.5 ± 3.8	12.7	0
$2SbCl_5 \cdot OMPA$	DMSO	24	2	58 min.– 4 days	1.5	35.0 ± 17.5	30	−16.7
DMSO	...	41	1	1 min.–38 min.	1.1	11,700 ± 105	...	...

assuming that the toxicity of the metal complex is dependent only on the amount of OMPA present. For example, $LiClO_4 \cdot 2OMPA$ is 84.5% OMPA by weight. The predicted LD_{50} equals the LD_{50} of OMPA in water, 14.9, divided by 0.845 to give 17.6 mg. per kg. The per cent difference is calculated by

$$\frac{LD_{50} \text{ predicted} - LD_{50} \text{ measured}}{LD_{50} \text{ predicted}} \times 100$$

The predicted LD_{50} values for the complexes dissolved in DMSO were calculated in the same way by using the LD_{50} value of 9.7 mg. per kg. for OMPA in dimethylsulfoxide.

In Table II, the predicted LD_{50} values for the complexes are not generally in agreement with experimental values. When water is the solvent, all OMPA complexes except those of Co(II) are more toxic than expected. The toxicity of the metal salts in water is negligible when compared with OMPA in all cases except Cu(II) and Zn(II) (Table I).

There is no apparent correlation between the per cent difference in toxicity (Table II) and the toxicity of the original metal salts. For example, Cu(II) and Zn(II) metal salts are the most toxic in water, but the complexes of OMPA with Cu(II) and Zn(II) are less toxic than the other complexes. An indication of the strength of the metal interaction with the phosphoryl oxygens of OMPA can be obtained by comparing the decrease in the P=O stretching frequency. For the metal complexes the decrease in the P=O stretching frequency is as follows: Na (13 $cm.^{-1}$), Li (20 $cm.^{-1}$), Mg (20 $cm.^{-1}$), Mn (27 $cm.^{-1}$), Zn (31 $cm.^{-1}$), Co (34 $cm.^{-1}$), Ni (39 $cm.^{-1}$), Cu (50 $cm.^{-1}$), Fe (51 $cm.^{-1}$), Pb (60 $cm.^{-1}$). The effect of the metal ion on the toxicity of OMPA in water is indicated by the per cent difference between experimental and predicted LD_{50} values. The increasing order is Co(II), Zn(II), Li(I), Pb(II), Fe(III), Cu(II), Ni(II), Mn(II), Na(I), Mg(II). A comparison of these two orders reveals no general trend.

When DMSO is the solvent, $[Fe(OMPA)_3][FeCl_4]_3$ is much more toxic than expected. The DMSO data for other complexes show small differences from the predicted value with the exception of $2SbCl_5 \cdot OMPA$ which is less toxic than expected. However, the large error limits in the LD_{50} for this complex do not justify any further conclusion.

The toxicities of both $FeCl_3 \cdot 6H_2O$ and anhydrous $FeCl_3$ were examined in DMSO in an attempt to determine the cause of the high toxicity of $[Fe(OMPA)_3][FeCl_4]_3$ in DMSO. Anhydrous iron(III) chloride is more toxic than $FeCl_3 \cdot 6H_2O$ (Table I). The toxicity of $Fe(OMPA)_3(ClO_4)_3$ is only one third that of $[Fe(OMPA)_3][FeCl_4]_3$ in DMSO. These results indicate that $[FeCl_4]^-$ is the apparent cause of the high toxicity of $[Fe(OMPA)_3][FeCl_4]_3$ in DMSO.

The ability of DMSO to adsorb rapidly and completely through animal tissues may be responsible for the change in toxicity of OMPA in water and DMSO. Dimethylsulfoxide is not toxic (LD_{50} = 11,700), but its ability to penetrate animal tissues will bring more of the toxic material to the surface of acetylcholinesterase in a shorter time. Since the active metabolite of OMPA is thought to be an oxidized form (*2*), the oxidizing character of DMSO may increase the ease with which the active metabolite is formed which may account for the higher toxicity of OMPA in DMSO.

Although DMSO penetrates animal tissues very quickly, the time required for all deaths to occur (Table II) was much longer for DMSO solutions of complexes. The time required for the first death to occur was not much different in either solvent, and the onset of symptoms was actually faster in DMSO than in water (Table II). The cause of these differences is not clear, since DMSO is quickly reduced to dimethylsulfide and eliminated (*1*).

The symptoms were very similar for studies in both water and DMSO: lacrimation, salivation, irregular respiration, muscle tremors, gasping, and convulsions causing death. Therefore, the effects are assumed to be due to the OMPA present, and that the major function is to inhibit acetylcholinesterase.

Acetylcholinesterase is thought to have an imidazole ring, a serine hydroxyl, a phenolic hydroxyl, and an anionic site as active centers (*4*). The inhibition of acetylcholinesterase can involve phosphorylation of the enzyme or blocking of its active centers. In either case, the ability of acetylcholine to acetylate acetylcholinesterase will be reduced.

The ability of the metal ions in complexes of OMPA to increase the toxicity of OMPA in water may be due to a combination of two effects. When looking at these effects, one must consider all species present in the aqueous solutions. The complexes of OMPA are not stable in water and are highly dissociated. This result in the presence of three species: complex ion, metal ion, and free OMPA

$$6\ H_2O + [M(OMPA)_3]^{+2} \rightleftharpoons [M(H_2O)_6]^{+2} + 3OMPA$$

The bonding of the metal ions to the phosphoryl oxygens in OMPA will ower the electron density on the oxygens which in turn will cause the phosphorus atoms to become more electrophilic. This will make phosphorus more susceptible to nucleophilic attack by acetylcholinesterase. Metal ions or the charged complex ion also can block anionic sites. The aquated metal ions also may be coordinating to other basic donor sites at the same time as OMPA is phosphorylating the enzyme. This may account for the synergistic effect observed for metal complexes of OMPA in water.

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