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METABOLITES OF PINACOLYL METHYLPHOSPHONOFLUORIDATE (SOMAN) AFTER ENZYMATIC HYDROLYSIS *IN VITRO*

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Abstract—Analytical procedures for the characterization and quantitative estimation of phosphonic acids derived from Soman, based upon paper chromatography and partition between isobutanol–benzene and an aqueous phase, were developed.

Utilization of 32P-labeled phosphonic acids permitted the determination of as little as 10^{-2} μmole of acid.

Application of these methods to the enzymatic hydrolysis products of Soman suggests that the principal, if not the only, metabolite of Soman incubated with rat plasma or liver tissue *in vitro* is pinacolyl methylphosphonic acid.

INTRODUCTION

IT has been shown that organophosphorus cholinesterase inhibitors undergo enzymatic hydrolysis when incubated with mammalian tissue.[1–3] In the case of diisopropyl phosphorofluoridate (DFP), $[(CH_3)_2CHO]_2P(O)F$, Mounter and Dien[4] showed that approximately 1 mole of F^- was produced for each 2 moles of CO_2 evolved during enzymatic hydrolysis in bicarbonate buffer, suggesting that hydrolysis was exclusively at the P—F bond. By means of paper chromatography Cohen and Warringa[5] characterized the phosphorus-containing metabolite following administration 32P-DFP *in vivo* as diisopropyl phosphoric acid. This substance is nontoxic, except at very high dosage levels, and produces none of the symptoms characteristic of DFP poisoning.[6]

The rapid detoxification of Soman, a potent anticholinesterase,[7] in the presence of liver tissue was described by us recently.[8] The metabolites of Soman analogous to those obtained from DFP[5] would be HF and pinacolyl methylphosphonic acid (PMPA), $(CH_3)_3C \cdot CH(CH_3)O \cdot P(CH_3)(O)OH$. However, secondary cleavage leading to a dealkylated product has been reported in the reaction of DFP with pseudocholinesterase *in vitro*,[9] resulting in the formation of monoisopropyl phosphoric acid. The corresponding metabolite of Soman would be methylphosphonic acid (MPA), $CH_3P(O)(OH)_2$.

We were interested in studying the metabolism of Soman by rat plasma and liver tissue *in vitro*, since no direct identification of the metabolites of Soman has been previously reported. For this purpose both PMPA and MPA were first sythesized as

reference compounds. This report is concerned with the analytical procedures developed for characterization and estimation of these substances. The methods were then applied to the identification of phosphorus-containing metabolites after incubation of Soman with rat plasma and liver tissue *in vitro*.

MATERIALS AND METHODS

Soman of 96% purity was obtained from the Chemical Process Division of these laboratories.

^{32}P-Labeled Soman was supplied by the Defense Research Board of Canada; its specific activity at the time of receipt was 10 mc/mmole. Upon arrival, the radioactive Soman was diluted 1 : 100 with benzene. The benzene solution was then extracted with 0·1 M Tris buffer at pH 7·4 to remove water-soluble impurities. The purity of the resulting ^{32}P-Soman solution in benzene approximated 95% on the basis of stoichiomatric reaction with chymotrypsin.* It was stored over anhydrous sodium sulfate in the deep freeze.

Isobutanol–benzene solvent was prepared by mixing one volume of benzene with one volume of secondary butyl alcohol. The solvent was saturated with the appropriate aqueous medium prior to use.

Synthesis of PMPA. Fifty mmoles of Soman was added to a threefold excess of 0·1 M NaOH and stirred for 96 hr at room temperature. The pH was lowered to 9·0 with HCl and the volume of the solution reduced to 100 ml by removal of H_2O under vacuum. The pH was then lowered to 1·5 with HCl and the mixture extracted with ten 100-ml portions of chloroform. The chloroform was evaporated from the extract, leaving a light yellow oily liquid, which was dried over P_2O_5. The compound gave a negative test for F^- ion and a neutralization equivalent (pK_a) of 2·54.

Analysis: $C_7H_{17}PO_3$; calculated: C, 46·6; H, 9·5; P, 17·2 found: C, 46·0; H, 9·5; P, 17·1

Synthesis of MPA. Ten g of methylphosphonyl dichloride was added slowly to 100 ml of H_2O. The mixture was evaporated over a steam bath *in vacuo*, giving a white oil which solidified upon cooling. The white powder was dried over P_2O_5 and recrystallized from chloroform solution (m.p. 104° to 106° compared with 105° reported by Hoffman[10]). Titration with NaOH yielded a pK_1 of 2·2 and pK_2 of 7·7 compared with corresponding values of 2·2 and 7·8 reported by Nylen.[11]

Analysis: CH_5PO_3; calculated: C, 12·5; H, 5·2; P, 32·2 found: C, 12·4; H, 5·1; P, 31·9

^{32}P-PMPA. The purified ^{32}P-Soman was hydrolyzed in excess 0·1 N NaOH for 96 hr followed by adjustment of the pH to approximate neutrality with HCl.

^{32}P MPA. The purified ^{32}P-Soman was hydrolyzed in 12 N HCl at room temperature overnight followed by digestion at 100° for 3 hr. The solution was diluted and adjusted to approximate neutrality with NaOH.

Borate and Tris buffers. Molarity was 0·01 at pH 7·4.

Acid borate or 'acid tris' solutions. Nine volumes of 0·01 M buffer plus 1 volume of 50% trichloracetic acid and sufficient 10 N H_2SO_4 (10 N HCl in the case of acid Tris) to yield a pH approximating 0·5.

* The authors wish to thank Dr. H. O. Michel for carrying out this estimate of purity.

EXPERIMENTAL AND RESULTS

A. Estimation of phosphonic acids as orthophosphate ion

PMPA and MPA were oxidized by potassium persulfate[12] and then estimated as orthophosphate by a method based upon that of Boltz and Mellon[13] For this, 1-ml aliquots of the phosphonic acid were added to 2 ml of deionized water. Each sample received 0·5 ml of 2% $K_2S_2O_8$ and was heated for 10 min at 100°; 2 ml of 7·5% Na_2SO_3 was added and the samples heated for 5 min at 100°. The samples were cooled, and 2 ml of 2% ammonium molybdate in 30% H_2SO_4 and 0·5 ml of 0·3% hydrazine sulfate were added. The samples were again heated for 15 min, cooled to room temperature, and diluted to a final volume of 10ml; their color intensities were measured in a Klett–Summerson colorimeter fitted with a 66 filter.

The relation between phosphonic acid concentration and colorimeter reading was linear in the range of 0·5 to 5 × 10^{-1} μmole, permitting estimation of the amount of phosphonic acid present in samples derived from the partition procedure presented below.

B. *Distribution of PMPA and MPA from isobutanol–benzene and aqueous solutions*

In order to facilitate solubility in the organic solvent used, the pH of the system was lowered well below the pK_a of the phosphonic acids[14] namely to 0·5. A 9·9 ml volume of acidified borate solution was placed in a 30-ml separatory funnel. Ten μmoles of phosphonic acid in 0·1 ml was added, followed by extraction with 10 ml of isobutanol benzene previously saturated with the acidified borate solution. An aliquot of the upper solvent phase was taken to dryness at 80°–90° under a jet of dry air. Five ml of water was added to the residue; the sample was then heated in a waterbath at 100°for 10 min to ensure solution. In the case of the aqueous phase, the sample was again evaporated at 80°–90°/under an air jet to roughly 0·5-ml volume. This was diluted and was then adjusted to a pH of 4·0–5·0 with NaOH.

TABLE 1. DISTRIBUTION OF PINACOLYL METHYLPHOSPHONIC (PMPA) AND METHYLPHOSPHONIC (MPA) ACIDS BETWEEN ISOBUTANOL BENZENE AND ACIDIFIED BORATE SOLUTION

Compound	Amounts added (μmoles)	Amount recovered	
		Organic solvent (μmoles)	Aqueous phase (μmoles)
PMPA	10	9·8 (9·7–9·9)*	0·6 (0·5–0·7)
MPA	10	0·6 (0·5–0·7)	9·3 (8·8–9·8)

* Confidence limits, 95% in parentheses.

The solutions of samples derived from both the solvent and aqueous phases were further diluted, if necessary to yield concentrations lying within the linear portion of the calibration curve relating concentration of the phosphonic acid to Klett reading. Results from six separate extractions with each acid are shown in Table 1. It may be seen that PMPA is almost completely extracted into the isobutanol–benzene phase from the aqueous phase, whereas MPA remains very largely in the aqueous phase under these conditions.

C. Partition of P-labeled ^{32}PMPA and MPA

The utilization of ^{32}P-labeled phosphonic acids of high specific activity enabled a study of the distribution of the compounds in the isobutanol–benzene–aqueous buffer system with 1% or less of the amount required for colorimetric analysis of the unlabeled acids.

Phosphonic acid (8·2 μg), equivalent to 23,700 and 19,270 counts per minute for PMPA and MPA, respectively, was added to acid borate solution and shaken with acid-borate-saturated isobutanol–benzene. After 20 min, a 3-ml aliquot from each phase was centrifuged at 3,000 rev/min for 10 min. One-ml aliquots of the centrifuged solutions were transferred to planchets containing 2 drops of 1 N NaOH in ethanol, dried, and counted in a Geiger-Mueller end-window counter. Known amounts of ^{32}P-labeled MPA and PMPA were added to planchets containing 1 ml of acid borate buffer or isobutanol–benzene, respectively, plus 2 drops of 1 N NaOH in ethanol, and were dried and counted as standards. After correction for background, the per cent recovery in each phase could be calculated. The results from six trials with each radioactive acid, shown in Table 2, agree with the distribution of the phosphonic acids found by the colorimetric method (Table 1).

TABLE 2. DISTRIBUTION OF ^{32}P-LABELED PMPA AND MPA ACIDS BETWEEN ISOBUTANOL BENZENE AND ACIDIFIED BORATE SOLUTION

Compounds	Amounts added (μmoles × 10^2)	Amounts recovered	
		Organic solvent (μmoles × 10^2)	Aqueous phase (μmoles × 10^2)
PMPA	4·50	4·46 (4·27–4·65)*	0·125 (0·123–0·127)
MPA	4·50	0·215 (0·153–0·277)	4·29 (4·14–4·44)

Confidence limits, *95%, in parentheses.

D. Chromatographic characterization of PMPA and MPA

The chromatographic homogeneity of PMPA and MPA was determined by ascending, single-phase paper chromatography. Strips of Whatman 1 paper, 2·2 cm on the top, 1·2 cm on the bottom, and 17·8 cm long, were used. The solutions were spotted on the paper 2 cm from the bottom, and the chromatograms were developed to a height of 14–15 cm in test tubes (1 × 8 in.) The solvent systems listed in Table 3 were based on previously reported systems[15, 16] used with compounds other than those dealt with in this report. The phosphorus compounds were located on the developed chromatograms with ultraviolet light by the method of Hanes and Isherwood.[17] The results from ten trials of each acid with each of four different solvent systems are given in Table 3. One spot was obtained for each phosphonic acid, whether unlabeled or labeled with ^{32}P in each of the four solvent systems, indicating chromatographic homogeneity in the samples of PMPA and MPA. This observation, along with the previous analytical data regarding melting point and elemental analysis, suggests that the compounds are of high purity. In addition, the R_f values for PMPA and PMA were sufficiently separated to permit recognition of these acids whether alone or together.

E. Chromatography of ^{32}P-labeled PMPA and MPA; application to biological studies

The solvent system, ethanol–H_2O–conc.NH_4OH (85 : 15 : 5 v/v) reported by Cheftel *et al.*,[18] was subsequently adopted for the chromatography of the labeled phosphonic acids because of its short development time (solvent front ascended 14–15 cm in 2 hr) and the greater separation obtained between PMPA and MPA. The R_f values (± S. D.) by this method for PMPA and MPA (six trials, each acid) were 0·94 ± 0·02 and 0·15 ± 0·02 respectively; R_f values when a mixture of PMPA and MPA was chromatographed were identical with those obtained with the separate acids.

TABLE 3. R_f VALUES OF PINACOLYL METHYLPHOSPHONIC (PMPA) AND METHYLPHOSPHONIC (MPA) ACIDS

Compounds	Number of trials	Solvent *(R_f ± S. D.)			
		1	2	3	4
Separate application					
PMPA	10	0·70 ± 0·02	0·70 ± 0·01	0·94 ± 0·01	0·96 ± 0·01
MPA	10	0·05 ± 0·01	0·13 ± 0·01	0·77 ± 0·01	0·78 ± 0·01
Mixture					
PMPA	10	0·72 ± 0·01	0·70 ± 0·01	0·98 ± 0·01	0·96 ± 0·01
MPA	10	0·06 ± 0·01	0·14 ± 0·01	0·77 ± 0·02	0·76 ± 0·01

*Solvent: 1. 2-propanol–conc. N H_4OH (75:25, v/v) [15]
2. 2-propanol–H_2O–conc. N. H_4OH (75:24:1, v/v) [15]
3. acetone–35% chloracetic acid (60:40, v/v) [16]
4. acetone–25% trichloroacetic acid (75:25, v/v) [16]

A chromatogram scanner, (Atomic Accessories, model RSC-160) was used to find the loci of the labeled phosphonic acids, with 40 counts per minute per spot giving a detectable peak on the recorder. Localization of spots by means of ^{32}P counts was then applied to the hydrolysis products of Soman from the biological studies reported below.

An 8% (wet weight) homogenate of rat liver was prepared in 0·01 M Tris buffer (pH 7·4). The homogenate was centrifuged at 20,000 rev/min (40,000 *g*) for 30 min in a refrigerated Spinco (model L) centrifuge. The supernate, representing the finer particulate portion of the homogenate[19] plus the nongranular fraction, was incubated with 2×10^{-5} M unlabeled Soman for 1 hr at 37°. In addition, rat plasma samples diluted 1 : 5 with 0·01 M Tris buffer (pH 7·4), were incubated similarly with 4×10^{-6} M unlabeled Soman. (The pretreatment of the rat preparations with unlabeled Soman was performed to cover binding sites which would otherwise remove part of the subsquently added ^{32}P-Soman by fixation to proteins).[8] One ml of 10^{-5} M ^{32}P-Soman, equivalent to 1×10^{-2} μmole, was then added to 9 ml of each preparation. Controls were also run with ^{32}P-PMPA and ^{32}P-MPA added to each tissue. After 30 min at 37°, trichloracetic acid was added to 5% concentration, followed by 10 N HCl to pH 0·5. The mixtures were centrifuged for 10 min at 3,000 rev/min and the supernates collected. The residues were washed three times with the acid Tris solutions, the washings being separated from the residues by centrifugation and then added to the original supernates. The combined supernates and washings were extracted with equal volumes of isobutanol–benzene. The phases were separated and clarified by

centrifugation. For chromatography, each phase was evaporated to 0·5 ml and then spotted on the paper. Results are given in Table 4.

It may be noted that the R_f values for the spots obtained from ^{32}P-Soman after hydrolysis by rat plasma or liver tissue are virtually identical with one another and with that obtained with co-chromatographed ^{32}P-PMPA. No spot was detectable from ^{32}P-Soman undergoing enzymatic hydrolysis corresponding to that given by ^{32}P-MPA under these conditions.

TABLE 4. R_f VALUES FOR THE HYDROLYSIS PRODUCTS AFTER ADDITION OF ^{32}P-LABELED SOMAN, PMPA, OR MPA TO RAT PLASMA AND RAT LIVER PREPARATIONS

Sample	No. of trials	Aqueous phase (R_f values ± S. D.*)	Isobutanol–benzene phase (R_f values ± S. D.*)
Plasma + PMPA	5	none	0·91 ± 0·01
Plasma + MPA	5	0·15 ± 0·01	none
Plasma + Soman	5	none	0·92 ± 0·01
Liver + PMPA	5	none	0·92 ± 0·01
Liver + MPA	5	0·16 ± 0·00	none
Liver + Soman	5	none	0·93 ± 0·01

*R_f Values were obtained with the system, ethanol–H_2O–N H_4OH (85:15:5) reported by Cheftel *et al.*[18]

F. Estimation of enzymatic hydrolysis products of Soman

^{32}P-Soman was incubated with similar preparations of rat plasma and liver under the same conditions used in the previous section for paper chromatography. The hydrolysis products were extracted as before, clarified by centrifugation, transferred to planchets, dried, and counted as previously described. The thrice-washed residue was solubilized with NaOH and counted as a control for recovery of total counts added. No significant number of counts above background was obtained from the residues.

The values obtained (Table 5) include a small amount of residual Soman in the case of liver tissue as determined by enzymatic assay.[20] The results of concurrent studies for spontaneous hydrolysis of ^{32}P-Soman in the presence of heat-inactivated plasma or liver tissue (boiled 10 min) are also included. It may be noted that spontaneous hydrolysis with the heat-inactivated preparations accounts for 15·7 and 13·2% of the ^{32}P counts recovered from plasma and liver tissue respectively. Table 5 also shows that the relative distribution of counts for ^{32}P-containing metabolites in the organic solvent and aqueous phases following hydrolysis both by the active and heat-inactivated preparations closely approaches the distribution obtained with PMPA alone (Table 2).

DISCUSSION

Esterases other than cholinesterases reacting with organophosphorus compounds have been classified as A esterases when the enzyme could hydrolyze the phosphorus compound and as B esterases when the enzyme was inhibited by low concentration (about 1×10^{-7} M) of Paraoxon or DFP.[21] Inasmuch as the preparations of rat plasma and liver tissue used in this study were pretreated with 4×10^{-6} M and

2×10^{-5} M Soman, respectively, before using this agent as a substrate, the enzymes present in these preparations appear to fit within the category of the A esterases. The observation of Cohen and Warringa[22] that the A esterase for DFP from hog kidney also hydrolyzed Soman provides additional support for this conclusion.

TABLE 5. METABOLITES FROM ^{32}P-SOMAN AFTER INCUBATION WITH RAT PLASMA AND LIVER TISSUE

Conditions	^{32}P-Soman added (μmoles $\times 10^2$)	^{32}P-Containing metabolites recovered	
		Organic solvent (μmoles $\times 10^2$)	Aqueous phase (μmoles $\times 10^2$)
With plasma	1·000	0·932*(0·792–1·070)†	0·026*(0·018–0·034)
With heat-inactivated plasma	1·000‡	0·146 (0·124–0·168)	0·004 (0·003–0·005)
With liver tissue	1·000	0·966 (0·822–1·110)§	0·027 (0·019–0·935)§
With heat-inactivated liver tissue	1·000‡	0·127 (0·108–0·146)	0·004 (0·003–0·005)

* Includes metabolites recovered with heat-inactivated plasma.

† Confidence limits, 95%, in parentheses.

‡ The difference between ^{32}P-GD added and that recovered as metabolites was accounted for by $0{\cdot}85 \times 10^{-2}$ μmoles of residual Soman as estimated by enzymatic assay.[20]

§ Metabolites recovered (11·2%) were derived from residual Soman as determined by enzymatic assay[20] in addition to those hydrolyzed with heat-inactivated liver.

We reported earlier that Soman, incubated with rat liver tissue, undergoes loss of toxicity to mice and concurrent decrease in anticholinesterase potency *in vitro*.[8] These findings plus evidence for the enzymatic hydrolysis of Soman presented above suggest that Soman may undergo rapid metabolism by the liver *in vivo*.

In the case of Paraoxon, an organophosphorus compound which is also rapidly metabolized by liver[21], Westerman[23] showed that passage through the portal circulation as a result of intraperitoneal injection significantly lessened its anticholinesterase action, as compared with subcutaneous injection. In agreement with this principle, we found the LD_{50} of Soman by intraperitoneal and subcutaneous injection was 158 μg/kg and 92·4 μg/kg respectively.* In addition, in the case of isopropyl methylphosphonofluoridate (Sarin), a compound closely related to Soman, Adie[24] observed a clear negative correlation between the capacity of a rabbit liver to hydrolyze Sarin and susceptibility of the animal to poisoning by intraperitoneal injection of the agent. When the anticholinesterase was injected by the subcutaneous route, no such correlation was demonstrable. These findings suggest that other factors than ability of the liver to hydrolyze organophosphorus compounds may be of importance in detoxification processes for organophosphorus anticholinesterases. In this connection, Fleisher *et al.*[20] observed that another mode of detoxification for Sarin, namely fixation to nonspecific tissue sites other than cholinestrase, underwent marked alteration after pretreatment with ethyl *p*-nitrophenyl thionobenzene phosphonate *in vivo*. Using ^{32}P-labeled Sarin, they found a 12-fold increase in the amount of ^{32}P bound to brain protein, consistent with high inhibition of brain cholinesterase activity, and a 3·3-fold increase in toxicity. Preliminary studies indicate that these observations are also applicable to Soman-poisoned rats.[8]

* L. W. Harris and J. H. Fleisher; unpublished data.

No evidence was obtained that MPA is a degradation product of Soman incubated with rat plasma or liver. This is taken to indicate that dealkylation of Soman doesnot occur *in vitro* under the conditions used in this study. This interpretation is in agreement with the finding of Oosterbaan *et al.*[25] that the phosphoryl residue of liver aliesterase inhibited by DFP does not undergo dealkylation. The observation by Berends *et al.*[19] that DFP-inhibited serum cholinesterase undergoes dealkylation is not in conflict with our findings, inasmuch as this was obtained with an enzyme system different from that used in this study.

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