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SPECIFICITY OF SUBSTITUTED PHENYL PHOSPHORUS COMPOUNDS FOR ESTERASE INHIBITION IN MICE

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Abstract—Forty-three substituted phenyl phosphorus compounds were studied in relation to selective esterase inhibition in mice, ability to potentiate the toxicity of malathion, and metabolic conversion to more active antiesterase agents. The malathion potentiation and the degree of *in vivo* inhibition of the aliphatic esterases hydrolyzing malathion were generally related. A similarity appeared among compounds containing the *o*-methyl-, *o*-ethyl- and *p*-ethylphenyl groupings in their activity for producing neurotoxicity in hens (activity as recorded in the literature) and for potentiation of malathion in mice. However, several compounds lacking the *o*-methyl- and *o*-ethyphenyl groupings were quite selective aliphatic esterase inhibitors and were active in malathion potentiation in mice but did not yield a neurotoxicity with hens. The most active potentiator of malathion by the assay method used was di-*o*-tolyl mono-*p*-tolyl phosphate. Metabolic activation to more potent esterase inhibitors was demonstrated for many aryl phosphates containing alkyl substituents in several positions on the phenyl group.

TRI-ARYL PHOSPHATES are widely used as plasticizers, solvents, flame retardants, additives to extreme pressure lubricants and as lead scavengers in gasoline. The activity of tri-*o*-cresyl phosphate (TOCP) and certain other substituted phenyl phosphates in the production of a neurotoxicity characterized by peripheral and central demyelination in certain vertebrates is well established,[1–8] although the mechanism of this action is not clear.[4–6, 9–11] TOCP, presumably through the action of metabolites,[5, 12] is a highly selective *in vivo* inhibitor of certain aliphatic esterases and "pseudo-cholinesterases".[4, 9, 11, 13–17] Formation of active antiesterases from TOCP occurs *in vivo*,[5, 12] and can be effected by liver slices *in vitro*[5] and presumably by a rat liver soluble plus microsome system fortified with diphosphopyridine nucleotide and magnesium ions.[18] Another unusual biological activity displayed by TOCP[19, 20] and its thiono analog[20] is an ability to potentiate the toxicity to rodents of malathion[19, 20] and certain other carboxyester and amide-containing organophosphates.[20] This potentiation presumably depends on the selective inhibition by TOCP metabolites of esterases involved in the detoxification of the potentiated compounds.[19, 20] It has been suggested that agents capable of inhibiting hydrolytic detoxification might render people hypersensitive to the toxic effects of certain ester-type drugs.[21]

The specificity of tri-aryl phosphates and related compounds was further investigated with mice in relation to their conversion to more potent anti-esterase agents, *in vivo* and *in vitro* specificity in esterase inhibition, and ability to potentiate the toxicity of malathion. Since evidence is available that the organophosphate-induced ataxia in chickens may result from an esterase inhibition, the structure–activity relationship for this ataxia as reported in the literature is compared with that for esterase inhibition in mice.

METHODS AND MATERIALS

Compounds

The substituted phenyl phosphorus compounds studied and their sources are indicated in Table 1. All compounds were used as received without further purification and the results should therefore be comparable with other studies[6, 8, 22] where these same materials were used. Malathion (O:O-dimethyl-S-(1:2-dicarboethoxyethyl)-phosphorodithioate) was a sample of 96 per cent purity provided by the American Cyanamid Co., Stamford, Connecticut, U.S.A.

Treatment of mice

Twenty-five-gram female white mice from the Rolfsmeyer Farms (Madison, Wisconsin, U.S.A.) were treated intraperitoneally while under light ether anesthesia. Corn oil solutions of the phosphates were used, and in certain cases it was necessary to warm the corn oil to effect solution of the phosphorus compound. Injection volumes were 0·125 ml per 25-g mouse for the substituted phenyl phosphates and 25 μl per 25-g mouse for malathion.

A standard 100 mg/kg dosage of triaryl phosphate was administered 24 hr prior to either sacrifice of the mice for esterase activity assays or treatment with malathion for toxicity potentiation studies. Control mice were treated with corn oil only. None of the aryl phosphorus compounds produced any mortality or symptoms of poisoning within 3 days after injection of this 100 mg/kg dosage. Twenty or more mice were used in determining the toxicity of malathion administered 24 hr after each of the aryl phosphates. LD_{50} values as mg toxicant per kg mouse were estimated from logarithm–probit mortality plots. The LD_{50} 24 hr after administering malathion was about 1500 mg/kg with control mice and from 59 mg/kg to greater than 1200 mg/kg for mice pretreated with the aryl phosphorus compounds.

Enzyme determinations

Mouse tissues were removed rapidly following sacrifice. Heparinized blood was diluted 1:4 with bicarbonate buffer (0·0357 M $NaHCO_3$ and 0·164 M NaCl) and centrifuged to separate the corpuscles which were discarded. This 1:4 diluted plasma was used at 0·4 ml for assay with malathion as a substrate and at 0·1 ml with propionyl choline as a substrate. Homogenates of whole brain or liver were prepared at 20 per cent w/v in bicarbonate buffer with a Potter–Elvehjem type glass homogenizer and 0·4 ml homogenate was used for assay with each of the substrates.

Esterase activity was determined indirectly by CO_2 evolution from a bicarbonate buffer (0·0357 M $NaHCO_3$ and 0·164 M NaCl) in an atmosphere of 5 per cent CO_2 and 95 per cent N_2 at 38 °C in 10-ml Warburg flasks. The enzyme source in 0·40 ml was usually added to the side arm of the flask, and the substrate in 1·60 ml to the main compartment. The enzyme with or without added inhibitor was incubated for 20 min at 28 °C and 40 min at 38 °C before tipping to mix it with the substrate.

The esters at 0·0125 M were added in 1·60 ml to the main compartment of the flask so that the final concentration after tipping the enzyme was 0·01 M. Solutions of acetyl-β-methylcholine chloride, propionylcholine-*p*-toluene sulfonate (PrCh) and acetylthiamine chloride hydrochloride were prepared directly in the bicarbonate buffer. Emulsions of tributyrin and 96 per cent pure malathion and a suspension of testosterone propionate (test. pr.) were prepared by adding Triton X-100 (Rohm and

TABLE 1. STRUCTURE–ACTIVITY RELATIONSHIPS FOR CERTAIN ARYL PHOSPHORUS COMPOUNDS

Compounds*†	Ataxia in chickens‡ (1 g/kg oral)	Mouse LD_{50} malathion	% inhibition *in vivo* 24 hr post-treatment					Liver microsome activation§	
			Plasma malathion	Liver malathion	Plasma PrCh	Liver toc. ac.	Liver test. pr.	−DPNH	+DPNH
Symmetrical triaryl phosphates									
1. $(PhO)_3P(O)$	No[2, 6, 7]	450	62	50	31	0	0	57	52
2. $(2\text{-}MePhO)_3P(O)$	Yes[1, 2, 5–8]	190	97	85	94	97	73	+3	93
3. $(3\text{-}MePhO)_3P(O)$	No[2, 5–8]	340	92	59	92	17	22	80	88
4. $(4\text{-}MePhO)_3P(O)$	No[2, 5–8]	230	81	61	0	37	23	4	2
5. $(2\text{-}EtPhO)_3P(O)$	No[6, 8]	510	87	69	76	49	20	7	91
6. $(3\text{-}EtPhO)_3P(O)$	No[8]	140	86	76	83	0	0	77	84
7. $(4\text{-}EtPhO)_3P(O)$	Yes[8]	80	91	82	63	53	0	4	43
8. $(2\text{-}n\text{-}PrPhO)_3P)O)$	No[8]	>1200	35	48	0	0	0	3	21
9. $(2{:}3\text{-}Me_2PhO)_3P(O)$	No[22]	200	79	73	87	30	0	2	82
10. $(2{:}4\text{-}Me_2PhO)_3P(O)$	No[6]	1100	0	0	0	0	0	1	60
11. $(2{:}5\text{-}Me_2PhO)_3P(O)$	No[6, 7, 22]	1150	35	60	76	41	0	25	80
12. $(2{:}6\text{-}Me_2PhO)_3P(O)$	No[6, 7, 22]	1100	34	44	18	0	0	10	22
13. $(3{:}4\text{-}Me_2PhO)_3P(O)$	No[7, 22]	370	83	64	40	0	0	33	57
14. $(3{:}5\text{-}Me_2PhO)_3P(O)$	No[6, 7, 22]	760	85	51	30	0	0	41	50
15. $(2\text{-}Me\text{-}4\text{-}EtPhO_3P(O)$	No[8]	850	83	53	16	0	0	3	2
16. $(2{:}4/2{:}5\text{-}Me_2PhO)P(O)$	No[22]	1100	70	75	70	44	25	8	78
Asymmetrical triaryl phosphates									
17. $(PhO)_2P(O)(OPhCl\text{-}2)$	No[6]	200	94	59	40	40	0	88	80
18. $(PhO)_2P(O)(OPhMe\text{-}2)$	Yes[6, 7]	180	46	69	85	83	57	26	91
19. $(2\text{-}MePhO)_2P(O)(OPhMe\text{-}4)$	Yes[6, 7]	54	95	82	92	97	82	6	82
20. $(4\text{-}MePhO)_2P(O)(OPhMe\text{-}2)$	Yes[6–8]	110	93	77	95	86	76	6	80
21. $(4\text{-}MePhO)_2P(O)(OPhEt\text{-}2)$	Yes[8]	80	95	88	93	87	75	8	80
22. $(4\text{-}MePhO)_2P(O)(OPhEt\text{-}4)$	No[8]	160	82	78	12	76	38	+8	12
23. $(2\text{-}EtPhO)_2P(O)(OPhMe\text{-}4)$	Yes[8]	170	91	73	79	70	30	+4	84
24. $(3\text{-}EtPhO)_2P(O)(OPhEt\text{-}4)$	No[8]	120	78	82	83	28	16	47	75
25. $(4\text{-}EtPhO)_2P(O)(OPhMe\text{-}4)$	No[8]	180	90	87	42	89	64	+1	32
26. $(4\text{-}EtPhO)_2P(O)(OPhPr\text{-}n\text{-}2)$	Yes[8]	120	94	85	92	79	49	6	49
27. $(2\text{-}n\text{-}PrPhO)_2P(O)(OPhMe\text{-}4)$	No[8]	440	89	53	0	0	0	+4	55
28. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPh)$	No[22]	>1200	17	12	14	25	0	7	18
29. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhMe\text{-}2)$	Yes[7, 8]	320	89	87	72	0	0	3	77
30. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhEt\text{-}2)$	Yes[8]	>1200	30	0	0	0	0	12	9
31. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhEt\text{-}4)$	No[8]	280	92	45	45	0	0	35	36
32. $(3{:}5\text{-}Me_2PhO)_2P(O)(OPhPr\text{-}n\text{-}2)$	No[8]	320	90	67	71	0	0	24	80

TABLE 1.—*continued*

Compounds*†	Ataxia in chickens‡ (1 g/kg oral)	Mouse LD_{50} malathion	% inhibition *in vivo* 24 hr post-treatment					Liver microsome activation§	
			Plasma malathion	Liver malathion	Plasma PrCh	Liver toc. ac.	Liver test. pr.	−DPNH	+DPNH
Triaryl phosphorothionates									
33. $(2\text{-MePhO})_3P(S)$	Yes[2]	>1200	0	18	15	20	0	+6	13
34. $(4\text{-MePhO})_3P(S)$	—	>1200	0	0	0	0	0	1	2
Triaryl phosphites									
35. $(PhO)_3P$	—	100	92	75	63	78	76	88	88
36. $(4\text{-MePhO})_3P$	—	550	77	57	32	32	17	59	57
Dialkyl-*o*-tolyl phosphates									
37. $(EtO)_2P(O)(OPhMe\text{-}2)$	No[6]	>1200	53	0	65	45	0	+1	12
38. $(n\text{-}PrO)_2P(O)(OPhMe\text{-}2)$	No[22]	>1200	0	20	55	29	0	+6	54
39. $(2\text{-Et-hexyl-O})_2P(O)(OPhMe\text{-}2)$	No[6]	1050	60	0	67	0	0	8	35
Others									
40. $(2\text{-MePhO})_2P(O)OP(O)(OPhMe\text{-}2)_2$	No[22]	840	52	0	63	0	0	27	45
41. $(3{:}5\text{-}Me_2PhO)_2P(O)Cl$	No[6]	660	88	43	83	53	0	27	33
42. Ph_3P	—	>1200	0	0	0	0	0	3	10
43. $Ph_3P(S)$	—	>1200	0	0	0	0	0	5	11

* Abbreviations: Me = methyl; Et = ethyl; Pr = propyl; Ph = phenyl; 2:4/2:5 for compound 16 designates a mixture of these xylenols used in synthesis.

† The compounds were obtained from the following sources: Compounds 3–9, 11–16, 20–32, 38 and 40 from Dr. H. F. Bondy, Coalite and Chemical Co., England; Compounds 1, 10, 18, 19, 37, 39 and 41 from Dr. R. Hansberry, Shell Development Co., Modesto, California, U.S.A.; Compounds 2, 17 and 33–36 from Eastman Organic Chemicals Dept., Eastman Kodak Co., Rochester, New York, U.S.A.; and compounds 42 and 43 from Dr. Helmut Tietz, Biological Laboratories, Farbenfabriken Bayer, Leverkusen-Bayerwerk, Germany.

‡ Superscripts refer to literature cited.

§ Aryl phosphorus compounds at 1×10^{-4} M were incubated with mouse liver microsomal fraction ±DPNH, and the inhibitors formed were assayed with mouse plasma propionylcholinesterase. Results are presented as % cholinesterase inhibition after incubation with 1×10^{-5} M total aryl phosphate equivalent. The difference between the −DPNH and the +DPNH should be used in interpreting the degree of activation.

Haas Co., Philadelphia, Pennsylvania, U.S.A.) in acetone to dissolve the ester, evaporating the solvent and then adding the buffer with thorough mixing to yield a final concentration of 1·1 mg/ml Triton X-100. An emulsion of tocopherol acetate (toc. ac.) was prepared in the same manner except that 5·5 mg/ml Triton X-100 were used. For tissue blanks the substrate was replaced with buffer, and for substrate blanks the tissue was replaced with buffer. The high emulsifier level apparently caused no interference in the assays.

The b_{30} values (μl CO_2 liberated/30 min) under the assay conditions for the brain tissue were 113 with acetyl-β-methylcholine, 239 with tributyrin and 49 with acetyl thiamine; for the plasma were 50 with malathion and 75 with propionylcholine; and for the liver were 64 with malathion, 68 with tocopherol acetate and 53 with testosterone propionate.

For assay of the inhibitors *in vitro*, the aryl phosphates were added in acetone solutions to the side arm of the Warburg flask, the acetone evaporated and the enzyme preparation added. After mixing to aid solution of the inhibitor and enzyme in the side arm, the concentration of the aryl phosphate was from 1×10^{-4} to 1×10^{-7} M. All concentrations stated for inhibitors are those during the inhibition reaction and not during the assay of the esterase.

Metabolism to more potent esterase inhibitors

The ability of suitably fortified liver fractions to metabolize the aryl phosphorus compounds was investigated. Immediately after removing the liver from mice or chickens, 14 per cent (w/v) homogenates in cold 0·05 M potassium phosphate buffer at pH 7·0 were prepared in a chilled glass homogenizer. After preliminary centrifugation at 15,000 *g* for 30 min at 2 °C to remove the cell debris and mitochondria, the supernatant was recentrifuged for 60 min at 105,000 *g* and 2 °C. The supernatant from this 105,000 *g* centrifugation was designated the "soluble" and the precipitate the "microsome" fraction. The microsome fraction was reconstituted with similar phosphate buffer to the original homogenate volume. A single study was made using the chicken spinal cord with a similar fractionation procedure.

The aryl phosphorus compound in acetone was added to a 25 ml Erlenmeyer flask and the solvent evaporated to leave a residue of 0·2 μmoles aryl phosphate on the bottom of the flask. To this flask was then added 0·33 ml, 0·05 M, pH 7·0 potassium phosphate buffer, 0·67 ml water containing no cofactor or 3·6 μmoles di- or tri-phosphopyridine nucleotide (DPN or TPN) or their reduced forms (DPNH or TPNH), and 1·0 ml of the microsome, soluble, or microsome plus soluble preparation. Two hours incubation with shaking in air at 38 °C were then allowed for metabolism of the aryl phosphate. Two milliliters acetone were then added and the resulting precipitate removed by centrifugation. The acetone was removed under reduced pressure and aliquots of the remaining aqueous material added to the esterase source. The activity of esterase inhibitors formed by the liver fractions was assayed as described above. Suitable controls with mouse liver fractions showed no inhibition resulting with this procedure except that due to the organophosphate.

In a few studies the ease of extraction into *n*-hexane was determined for the anti-esterase products from TOCP. The products from *in vitro* activation, after precipitatating the proteins with acetone and removing the precipitate and the acetone, were assayed against mouse plasma propionylcholinesterase. The remaining supernatant

was partitioned with an equal volume of *n*-hexane and the antiesterase agents recovered in each phase were similarly assayed. The hexane was removed at room temperature with a mild air jet prior to esterase assay. Products from the *in vivo* activation of TOCP were also studied 2 and 24 hr after injection of mice with 500 mg/kg TOCP in corn oil. The liver and small intestine were removed, homogenized in acetone, the acetone-soluble fraction recovered and after evaporation of the solvent, the residue partitioned between an equal volume of water and *n*-hexane. Aliquots of each phase were used for assaying the activity in inhibiting mouse plasma propionylcholinesterase.

RESULTS

In vivo *studies*

Many compounds were highly active in potentiating malathion (i.e. potentiated LD_{50} of less than 200 mg/kg) and in effecting ataxia in hens (compounds 2, 7, 18–21, 23 and 26 of Table 1). These compounds contained at least one 2-methyl, 2-ethyl or 4-ethyl substituent on a phenolic group. Only the 2-methylphenyl and 2-ethylphenyl di-(3:5-dimethylphenyl) phosphates (compounds 29 and 30) and the thiono analogue

TABLE 2. CORRELATION OF MALATHION TOXICITY AND INHIBITION OF MALATHION-HYDROLYZING ESTERASES 24 HR AFTER INJECTION OF MICE WITH ARYL PHOSPHORUS COMPOUNDS AT 100 MG/KG

LD_{50} malathion	No. of compounds	Ave. % inhibition malathion hydrolysis *in vivo*	
		Plasma	Liver
54–80	3	94	83
100–140	5	89	79
160–190	5	81	78
200–320	6	88	65
340–450	4	82	57
510–850	6	79	46
1050–1150	5	40	36
>1200	9	15	11

Summary of data presented in Table 1.

of TOCP (compound 33) produce ataxia in hens, but were not highly active in potentiating malathion toxicity to mice. These three compounds are less toxic to chickens than most of the other ataxia-producing materials.[2, 7, 8] Moderate to high activity in malathion potentiation (potentiated LD_{50} of less than 350 mg/kg) without activity for producing ataxia in hens resulted with ten compounds (3, 4, 6, 9, 17, 22, 24, 25, 31 and 32 of Table 1), none of which contained 2-methylphenyl or 2-ethylphenyl groupings with the exception of tri(2:3-dimethylphenyl) phosphate (compound 9).

The activity of the aryl phosphorus compounds in producing *in vivo* inhibition of the malathion-hydrolysing esterases of liver and plasma was generally related to their ability to increase the susceptibility of mice to malathion (Table 2). This correlation was better with the liver than with the more sensitive plasma esterase.

From the specificity of *in vivo* esterase inhibition by the aryl phosphates it is evident that malathion and propionyl choline are largely hydrolyzed by different enzymes of mouse plasma, and that malathion is hydrolyzed by a different liver enzyme than

tocopherol acetate or testosterone propionate, although the latter two substrates may be hydrolyzed at least partially by the same enzyme. The compound specificity for *in vivo* inhibition of the liver and plasma malathion-hydrolyzing enzymes was somewhat similar.

None of the substituted phenyl phosphorus compounds studied effected significant inhibition of the activity of brain homogenates in hydrolyzing acetyl-β-methylcholine or acetyl thiamine when the brains were removed for assay 24 hr after injection of a 100 mg/kg dose. The brain enzyme hydrolyzing tributyrin was more sensitive such that 50–65 per cent inhibition resulted with compounds 18–20, 30–40 per cent with 2 and 23, and 20–30 per cent inhibition with phosphates 6, 9, 21, 24, 25, 27 and 38, while no significant inhibition resulted with the other materials. The specificity for *in vivo* inhibition of the brain tributyrin-hydrolyzing esterase was different than that for inhibition of the plasma and liver enzymes studied. It must be noted that these analyses on *in vivo* inhibition of the brain esterases are based on single and not replicated analyses.

The approximate toxicity to mice was determined for the aryl phosphorus compounds that were most active in increasing the toxicity of malathion (i.e. potentiated LD_{50} of 200 mg/kg or less). LD_{50} values for these phosphates 72 hr after injection in corn oil solutions were mostly greater than 1000 mg/kg (compounds 2, 6, 9, 17, 19, 22 and 24–26). Compound 18 yielded an LD_{50} of 800–1000 mg/kg, compound 7 was 400–600, compounds 21, 23 and 35 were 300–400 and compound 20 was 200–300 mg/kg.

Preliminary studies showed that TOCP injected at 400 mg/kg increased the susceptibility of mice to several chemicals injected 24 hr later. The compounds affected and the approximate factor of decrease in their mg/kg LD_{50} are as follows: malathion, 10×; the insecticide, dimethoate (O:O-dimethyl-S-(N-methylcarbamoylmethyl)-phosphorodithioate), 5×; butyrylcholine, 6×; and 2-dimethylaminoethyl acetate, 3·5×. Less than a two factor change in susceptibility resulted with the following injected compounds: thiamine, acetylthiamine, atropine, veratrine, protoveratrine and the insecticides DDVP (O:O-dimethyl-2:2-dichlorovinyl phosphate) and Dowco 109 (O-4-*tert*-butyl-2-chlorophenyl-O-methyl methylphosphoroamidothioate).

In vitro *studies*

Studies on the sensitivity of certain of the esterases to *in vitro* inhibition by ten aryl phosphorus compounds are presented in Table 3. In no case was the liver esterase which hydrolyzes malathion greatly inhibited by 1×10^{-4} M aryl phosphate. The plasma enzymes hydrolyzing propionyl choline and particularly malathion were more sensitive. The selectivity for inhibiting the plasma malathion-hydrolyzing esterase compared with the cholinesterase (pI_{50} malathion-esterase/pI_{50} PrCh-esterase) was usually greater with the phosphates than with the phosphites studied.

Under suitable conditions, fractions from mouse liver homogenates were effective in converting TOCP to more efficient inhibitors of mouse plasma cholinesterase. The microsome fraction fortified with either DPNH or TPNH was active in this respect, as was the microsome plus soluble fraction fortified with DPN or TPN. The oxidized or reduced forms of the pyridine nucleotides were about equally effective as cofactors for the microsome plus soluble fraction, while the reduced forms were much more effective than the oxidized forms with the microsome fraction. No great

difference was noted in the activity of the di- and tri-phosphopyridine nucleotides but the concentration of cofactor was not varied in these studies.

A comparison was made of the mouse plasma cholinesterase inhibitors formed from TOCP *in vitro* and those present in the liver and small intestine of mice 2 or 24 hr after injection of 500 mg/kg TOCP. The only criterion used in this comparison was the partitioning of the inhibitors between equal volumes of *n*-hexane and water. The inhibitors recovered from the liver *in vivo* partitioned greater than 90 per cent into an equal volume of hexane from water, the inhibitors from small intestine were less

TABLE 3. INHIBITORY ACTIVITY *in vitro* OF CERTAIN ARYL PHOSPHORUS COMPOUNDS ON MOUSE PLASMA CHOLINESTERASE AND THE MOUSE PLASMA AND LIVER ENZYMES HYDROLYZING MALATHION*

Compound	Mouse plasma-pI_{50}	
	PrCh	malathion
$(PhO)_3P(O)$	5·3	6·2
$(3\text{-}MePhO)_3P(O)$	5·7	6·1
$(4\text{-}MePhO)_3P(O)$	<4·0	5·4
$(3\text{-}EtPhO)_3P(O)$	5·0	6·3
$(4\text{-}EtPhO)_3P(O)$	<4·0	5·2
$(4\text{-}MePhO)_2P(O)(OPhEt\text{-}4)$	<4·0	5·4
$(4\text{-}EtPhO)_2P(O)(OPhMe\text{-}4)$	<4·0	5·2
$(PhO)_2P(O)(OPhCl\text{-}2)$	5·6	6·4
$(PhO)_3P$	5·4	6·0
$(4\text{-}MePhO)_3P$	4·7	4·5

* The pI_{50} (negative logarithm of molar concentration producing 50 per cent inhibition) for these compounds with the mouse liver enzyme hydrolyzing malathion was in every case greater than 4·0.

completely recovered in the hexane, and those formed by either fortified microsome plus soluble or the microsome fraction alone partitioned about equally between hexane and water. In studies on the nature of the *in vivo* metabolites of TOCP in rats it has been found[23] that at least three metabolites can be partially to completely extracted into hexane. It appears likely that the most active metabolite formed *in vivo* is the same as that formed by the microsome–DPNH preparation *in vitro* and that it is susceptible to further metabolism by hydrolysis and possibly oxidation.

Certain structural requirements are evident (Table 1) for the conversion of aryl phosphates to more potent mouse plasma propionylcholinesterase inhibitors (activation) by the mouse liver microsome-reduced pyridine nucleotide system. By comparison of the inhibitors present after incubation in the presence and absence of DPNH, the following relationships can be seen: (1) For symmetrical monosubstituted triaryl phosphates, the greatest degree of activation results with 2-methyl or 2-ethyl substituents although compounds with 2-*n*-propyl or 4-ethyl substituents are also activated. (2) For asymmetrical monosubstituted triaryl phosphates the same requirements hold for a 2-methyl or 2-ethyl group for maximum activation and 2-*n*-propyl or 4-ethyl group for activation. (3) This relationship did not hold for the di-(3:5-dimethylphenyl)-mono- (2 or 4-alkylphenyl)-phosphates since the 2-methyl and 2-*n*-propyl derivatives were activated but not the 2- or 4-ethyl phenyl derivatives. (4) With

symmetrical tri(dialkylphenyl)-phosphates the degree of activation varied greatly with the position of the substituents such that activation decreased in the order: 2:3 > 2:4 or 2:5 > 2:6; 3:4 or 3:5. The 2:4-dimethylphenyl phosphate and a mixed 2:4/2:5-dimethylphenyl phosphate were highly activated but not the 2-methyl-4-ethyl analog. (5) Other 2-methylphenyl phosphates activated were the O:O-dialkyl-*o*-cresyl phosphates and tetra-*o*-cresyl pyrophosphate. (6) No activation resulted with two triaryl phosphites, and with triphenyl phosphine and its sulfide, however, this does not rule out the possible activation of other phosphines or phosphites since the analogous phosphates for the derivatives studied were not activated. (7) The high inhibition resulting with tri-*m*-cresyl phosphate and tri-*m*-ethylphenyl phosphate in the absence of DPNH made it impossible to ascertain under these experimental conditions whether or not activation had occurred with these *m*-alkylphenyl phosphates.

The microsomal fraction from chicken liver was also effective in the activation of TOCP and tri-*p*-ethylphenyl phosphate (Table 4). With TOCP the active metabolite

TABLE 4. ACTIVATION OF TOCP AND TRI-*p*-ETHYLPHENYL PHOSPHATE BY CHICKEN LIVER MICROSOMAL FRACTION WITH OR WITHOUT DPNH FORTIFICATION

(Aryl phosphates at 1×10^{-4} M were incubated with microsomal fraction ±DPNH, and the inhibitors formed were assayed at 1×10^{-5} M with cholinesterase; results as b_{30} with 0·01 M PrCh at 38 °C are the average of duplicate runs, each of which showed the same effect of DPNH on the inhibitor formation.)

Esterase source	Esterase control	$(2\text{-MePhO})_3P(O)$		$(4\text{-EtPhO})_3P(O)$	
		−DPNH	+DPNH	−DPNH	+DPNH
Chicken spinal cord (20 mg/ml)	154	113	83	110	95
Chicken plasma (100 μl/ml)	185	147	49	142	145
Mouse plasma (25 μl/ml)	63	35	8	27	18

A similar study was made using a fraction from chicken spinal cord prepared and assayed in an identical manner to the liver microsomal fraction. No inhibition of any of the esterases resulted with any of the treatments.

could be detected with chicken spinal cord cholinesterase or with cholinesterase of mouse or chicken plasma. The metabolite of tri-*p*-ethylphenyl phosphate was apparently inactive when assayed with chicken plasma cholinesterase. No activation of either substrate occurred with a fraction from chicken spinal cord which was separated under the same centrifugation conditions used to obtain the liver microsome fraction.

DISCUSSION

Potentiation of the toxicity to mammals of certain organophosphates in mixtures presumably results from one compound interfering with the detoxification of a second compound. It has been shown by a variety of procedures[19–21, 24–28] that malathion is potentiated by those organophosphates which selectively inhibit an esterase

active in hydrolysis of one or both of the carboethoxy groups of the malathion molecule. This mechanism for malathion potentiation is generally supported by the evidence presented in the present study.

The structure–activity relationships for aryl phosphates in effecting potentiation of malathion toxicity to mice and in producing ataxia in hens are somewhat similar but certainly not identical. If both types of biological activity are related to esterase inhibition, then the effective antiesterases are present in different concentrations in the two organisms or the antiesterases differ in their selectivity for inhibiting the physiologically important esterases involved. There is considerable evidence[11] that true and pseudocholinesterase inhibition is not involved in the production of ataxia in hens. The failure of an ataxia-producing dose of tri-*p*-ethylphenyl phosphate to produce cholinesterase inhbition in hens has been used as evidence[10] for discarding the "pseudocholinesterase inhibition" hypothesis for ataxia production. However, a metabolite of tri-*p*-ethylphenyl phosphate formed *in vitro* by either the mouse or chicken liver microsomal fraction is an effective inhibitor of certain esterases, but apparently not for the chicken plasma cholinesterase. It is also known that certain compounds inactive in producing neurotoxicity in hens are active in effecting paralysis in cats.[2]

Numerous investigators have been intrigued with the great selectivity in esterase inhibition in animals treated with various organophosphorus esters. Many phosphate esters with ammonium or sulfonium groupings are highly selective for cholinesterase inhibition, and others such as certain bis-aryl phosphoramidates are selective for aliphatic esterases.[29, 30] In the present study it was found that a high selectivity for aliphatic esterases resulted with triaryl phosphates containing only *p*-alkyl substituents such as methyl or ethyl.

Many esterases are inhibited by organophosphates. The normal physiological function of many of these enzymes is unknown. Just as the organophosphate cholinesterase inhibitors have proven invaluable in ascertaining the significance of the various reactions mediated by cholinesterase, so selective inhibitors of other carboxylic esterases should help ascertain the role of these esterases in the body. Certain aliphatic esterases are involved in drug metabolism and the potentiation between organophosphates has given a dramatic demonstration of this role. The significance of inhibition of esterases other than acetylcholinesterase in the acute and chronic toxicity of the varied organophosphates, and in the infrequently observed ataxia or neurotoxicity, and anesthetic activity[31] to mammals is not clearly established. Further studies are also needed on the inhibition of these other esterases in relation to the poisoning of insects by organophosphates and the acquired resistance of insects to these compounds, the poisoning of nematodes and other lower animals and the herbicidal action of phosphate esters, and the activity of some compounds of this type as anticarcinogenic agents.

TOCP and many other aryl phosphates are metabolized to more potent esterase inhibitors. The chemical nature of the active metabolites has not been determined. The reaction is probably at least initiated by an oxidation since it can be effected with a liver microsome-reduced pyridine nucleotide system in an oxygen-containing atmosphere. The active products can be readily demonstrated in rats, mice, chickens and cockroaches. The metabolite formed from TOCP *in vivo* by rats has now been

highly purified, and is active at 1×10^{-8} M as an inhibitor of human plasma cholinesterase.[23]

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