Biochemical Pharmacology, 1963, Vol. 12, pp. 73–83. Pergamon Press Ltd., Printed in Great Britain.

POTENTIATION AND NEUROTOXICITY INDUCED BY CERTAIN ORGANOPHOSPHATES

JOHN E. CASIDA, RONALD L. BARON*, MORIFUSA ETO† and JUDITH L. ENGEL

Department of Entomology, University of Wisconsin, Madison, Wis., U.S.A.

(*Received* 30 *July* 1962; *accepted* 2 *October* 1962)

Abstract—The following types of phosphorus compounds were found to be active in potentiating the toxicity of malathion to mice: triphenyl phosphates and phosphonates containing *o*- and *p*-, methyl and ethyl substitutents; certain di-(substituted-phenyl) phenylphosphonates and *N*-methylphosphoramidates; *S,S,S*-trialkyl phosphorotrithioites and phosphorotrithioates; and certain saligenin cyclic phosphorus esters. Some compounds in the latter two groups also produced ataxia in hens. Certain of the saligenin cyclic phosphorus esters were as potent in effecting ataxia as the dialkyl phosphorofluoridates, but required much larger doses to produce parasympathomimetic effects. Also considered are the activity of the 112 phosphorus esters investigated for inhibition *in vitro* of mouse plasma esterases hydrolyzing malathion and propionylcholine, and the stability of the saligenin cyclic phosphorus esters to enzymatic and nonenzymatic hydrolysis.

SELECTIVE organophosphate inhibitors for acetyl- and pseudocholinesterase have been extensively investigated. Limited studies on selective aliphatic esterase inhibitors have shown diaryl *N*-methylphosphoramidates and certain triaryl phosphates to be of particular interest.[1–3] Potentiation of the toxicity of malathion provides an indirect assay *in vivo* for selective aliphatic esterase inhibitors, since malathion is greatly increased in toxicity to mammals on inhibition of the aliphatic esterases acting as detoxifying enzymes.[4–9] By the use of the malathion potentiation assay with mice, 112 compounds‡ were tested for selective aliphatic esterase inhibition *in vivo*. Selected

* Permanent address: Dept. of Pharmacology, U.S. Food and Drug Administration, Washington, D.C.

† Permanent address: Dept. of Agricultural Chemistry, Kyushu University, Fukuoka, Japan.

‡ Sources for the organophosphorus compounds investigated were as follows: Compounds 1, 3, 6, 8–28, 31–36, 43–59, 61, 62, 64, 66, 67, 69, 75, 77, 86, 87 from Drs. R. S. Gordon and E. G. Jaworski of Monsanto Chemical Co., St. Louis, Mo.; 60, 63, 65, 68, 70, 74, 78, 80, 85, Dr. A. D. F. Toy, Victor Chemical Works, Chicago Heights, Ill.; 40, 92–98, 110, Dr. D. R. MacDougall, Chemagro Corp., Kansas City, Mo.; 2, 4, 7, 99, 100, 111, Dr. R. J. Rowlett, Jr., Virginia-Carolina Chemical Corp., Richmond, Va.; 39, 41, 42, 81–84, Dr. J. E. Johnson, Dow Chemical Co., Midland, Mich.; 38, 76, 79, Dr. E. L. Clark, American Cyanamid Co., Stamford, Conn.; 5, 37, 91, Aldrich Chemical Co., Inc., Milwaukee, Wis.; 112, Dr. H. H. Moorefield, Union Carbide Chemicals Co., Clayton, N. C. The remaining chemicals (Cmpds. 29, 30, 71–73, 88–90, 101–109) were prepared by coupling the appropriate phosphorus chloride and phenol.[10–12] Malathion [*O:O*-dimethyl-*S*-(1:2-dicarboethoxyethyl)-phosphorodithioate] was a sample of 96% purity provided by the American Cyanamid Co., Stamford, Conn. Zytron® [(*O*-2,4-dichlorophenyl)-*O*-methyl *iso*propylphosphoramidothioate] was obtained from the Dow Chemical Co., Midland, Mich.

Abbreviations for designating structures: Me = methyl; Et = ethyl; Ph = phenyl; x = position of ring substituent unknown.

compounds were further examined for neurotoxic activity with hens, since many organophosphates display both types of biological activity.[9, 10]

METHODS

Treatment of mice and antiesterase assays in vitro

Female white mice (25 g) from Dan Rolfsmeyer Co. (Madison, Wis.) were treated intraperitoneally while under light ether anesthesia. Corn oil was used as the solvent, and in certain cases it was necessary to warm the corn oil to effect solution. The problem of low solubility necessitated injecting suspensions of several materials, particularly compound 89. Injection volumes were 0·10 ml per 25-g mouse for each compound.

Standard malathion potentiation studies (Table 1) were made in a manner similar

TABLE 1. ACTIVITY OF PHOSPHORUS ESTERS IN MALATHION POTENTIATION AND *in vitro* INHIBITION OF MOUSE PLASMA ESTERASES HYDROLYZING PROPIONYLCHOLINE AND MALATHION

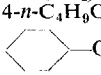
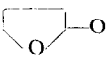
Compounds	Malathion LD_{50} mg/kg*	Mouse plasma-pI_{50}† PrCh	Malathion
Symmetrical triaryl phosphates			
1. $(2\text{-MeOPhO})_3P(O)$	370	6·3	6·2
2. $(2\text{-}CH_2{=}CHCH_2PhO)_3P(O)$	700	(22)	(12)
3. $(2\text{-PhPhO})_3P(O)$	920	(26)	(0)
4. $(4\text{-ClPhO})_3P(O)$	175	(43)	6·5
5. $(4\text{-}NO_2PhO)_3P(O)$	300	5·7	5·7
6. $(4\text{-}Me_3CPhO)_3P(O)$	970	(13)	(0)
7. $(x\text{-}C_8H_{17}PhO)_3P(O)$	900	(10)	(0)
8. $(2,4,5\text{-}Cl_3PhO)_3P(O)$	710	(12)	(0)
9. $(2,4,6\text{-}Cl_3PhO)_3P(O)$	1000	(9)	(0)
Diphenyl phosphates			
10. $MeOP(O)(OPh)_2$	650	(7)	(40)
11. $EtOP(O)(OPh)_2$	750	(20)	(42)
12. $n\text{-}C_4H_9OP(O)(OPh)_2$	560	(36)	5·5
13. $n\text{-}C_8H_{17}OP(O)(OPh)_2$	220	(22)	5·5
14. $n\text{-}C_{10}H_{21}OP(O)(OPh)_2$	260	(7)	5·3
15. $Et_2CHCH_2OP(O)(OPh)_2$	450	(49)	5·8
16. $C_3H_7(Me)CHCH_2OP(O)(OPh)_2$	350	(43)	5·7
17. $Me_2CHC_5H_{11}OP(O)(OPh)_2$	130	(39)	5·6
18. $C_4H_9(Et)CHCH_2OP(O)(OPh)_2$	230	(40)	6·4
19. $Me_2CHCH_2(Me)_2CC_2H_4OP(O)(OPh)_2$	170	(31)	6·0
20. $C_3H_7(HO)CH(Et)CH(HO)CHOP(O)(OPh)_2$	460	5·1	6·2
21. $HOC_2H_4OC_2H_4OP(O)(OPh)_2$	250	5·0	5·9
22. $MeOC_2H_4OP(O)(OPh)_2$	560	(37)	6·2
23. $n\text{-}C_4H_9OC_2H_4OP(O)(OPh)_2$	630	5·3	5·8
24. $PhOC_2H_4OP(O)(OPh)_2$	180	5·1	6·3
25. $2\text{-}ClPhOC_2H_4OP(O)(OPh)_2$	350	5·5	5·7
26. $PhCH_2OC_2H_4OP(O)(OPh)_2$	350	5·7	6·2
27. $n\text{-}C_4H_9OC_5H_{10}OP(O)(OPn)_2$	300	5·0	6·5
28. $PhCH_2OP(O)(OPh)_2$	570	(16)	5·7
29. $2\text{-}EtPhOP(O)(OPh)_2$	90	(24)	5·0
30. $4\text{-}EtPhOP(O)(OPh)_2$	115	5·0	5·8
31. $2\text{-}PhPhOP(O)(OPh)_2$	470	(19)	5·5
32. $4\text{-}Me_3CPhOP(O)(OPh)_2$	320	5·1	6·1
33. $4\text{-}Et(Me_2)CPhOP(O)(OPh)_2$	250	(39)	6·2
34. $4\text{-}n\text{-}C_4H_9OC(O)PhOP(O)(OPh)_2$	180	5·3	5·8
35.  $OP(O)(OPh)_2$	820	(8)	5·7
36.  $OP(O)(OPh)_2$	640	(35)	6·3

TABLE 1—*continued*

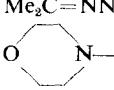
Compounds	Malathion LD_{50} mg/kg*	Mouse plasma-pI_{50}† PrCh	Malathion
37. $SCNP(O)(OPh)_2$	150	5·5	5·5
38. $NH_2C(O)NHC(O)CH_2SP(S)(OPh)_2$	800	(7)	(8)
Other di-and triaryl phosphates			
39. $MeOP(S)(OPhCl_3\text{-}2,4,5)_2$	520	(11)	(22)
40. $EtOP(S)(OPhCl_2\text{-}2,4)_2$	135	(16)	(20)
41. $MeOP(O)(OPhCl\text{-}2\text{-}CMe_3\text{-}4)_2$	350	(19)	6·2
42. $MeOP(S)(OPhCl\text{-}2\text{-}CMe_3\text{-}4)_2$	860	(32)	(47)
43. $ClC_2H_4OP(O)(OPh)(SPhCl_5)$	420	5·4	5·6
44. $C_4H_9(Et)CHCH_2OP(O)(OPhCl\text{-}2)_2$	105	6·9	6·3
45. $C_4H_9(Et)CHCH_2OP(O)(OPhOMe\text{-}2)_2$	500	5·2	(44)
46. $C_4H_9(Et)CHCH_2OP(O)(OPhOEt\text{-}2)_2$	660	(38)	(18)
47. $C_4H_9(Et)CHCH_2OP(O)(O\text{-}2\text{-naphthyl})_2$	350	(45)	6·2
48. $C_4H_9(Et)CHCH_2OP(O)(OPhMe\text{-}3)(OPhMe\text{-}4)$	110	(46)	(35)
49. $C_4H_9(Et)CHCH_2OP(O)(OPhCl\text{-}2)(OPhOMe\text{-}2)$	180	6·8	6·3
50. $Me_2CHC_5H_{11}OP(O)(OPhOMe\text{-}2)_2$	350	5·4	6·0
51. $Me_2CHC_5H_{11}OP(O)(O\text{-}1\text{-naphthyl})_2$	650	(24)	5·3
52. $n\text{-}C_{12}H_{25}SP(S)(OPhCl_2\text{-}2,4)_2$	500	5·1	(41)
53. $n\text{-}C_4H_9OC_2H_4OP(O)(OPhCl\text{-}2)_2$	105	7·2	6·3
54. $n\text{-}C_4H_9OC_2H_4OP(O)(OPh)(OPhCl\text{-}2)$	160	6·7	6·7
55. $PhOP(O)(OPhCl\text{-}2)_2$	180	5·7	6·3
56. $PhOP(O)(OPhCMe_3\text{-}4)_2$	400	(17)	5·7
Di- and triaryl phosphites			
57. $(PhO)_3P$	740	(34)	(29)
58. $(4\text{-}ClPhO)_3P$	150	5·7	5·3
59. $(4\text{-}Me_3CPhO)_3P$	>1200	(33)	5·3
60. $(x\text{-}C_9H_{19}PhO)_3P$	840	5·4	5·9
61. $(3,5\text{-}Me_2PhO)_3P$	600	5·0	6·1
62. $ClC_2H_4OP(OPhCl\text{-}4)_2$	150	5·3	5·9
63. $HOP(OPhMe\text{-}3)_2$	460	5·5	5·8
Derivatives with carbon to phosphorus bond			
64. $ClCH_2P(O)(OPh)_2$	430	(47)	6·7
65. $Cl_3CP(O)(OPh)_2$	500	5·2	5·6
66. $n\text{-}C_7H_{15}P(O)(OPh)_2$	360	5·4	6·5
67. $n\text{-}C_{16}H_{33}P(O)(OPh)_2$	480	(11)	5·3
68. $PhCH{=}CHP(O)(OPh)_2$	440	6·3	6·7
69. $PhP(O)(OPh)_2$	640	(36)	5·4
70. $PhP(S)(OPh)_2$	700	(8)	(15)
71. $PhP(O)(OPhMe\text{-}2)_2$	75	(33)	(9)
72. $PhP(O)(OPhEt\text{-}2)_2$	60	5·5	(16)
73. $PhP(O)(OPhEt\text{-}4)_2$	75	(37)	(15)
74. $PhP(O)(OPhCl\text{-}4)_2$	180	(34)	6·0
75. $PhP(O)(OPhCMe_3\text{-}4)_2$	900	(15)	(6)
76. $PhP(S)(OPhNO_2\text{-}4)_2$	175	(7)	(10)
77. $Ph_2P(S)OPhNO_2\text{-}4$	120	(0)	(12)
78. $Ph_2P(O)OEt$	900	(8)	(19)
79. $Ph_3P(O)$	>1200	(6)	(4)
80. $PhP(OPh)_2$	740	5·0	5·2
Derivatives with nitrogen to phosphorus bond			
81. $MeNHP(O)(OPhCl_3\text{-}2,4,5)_2$	<50	5·8	6·2
82. $MeNHP(O)(OPhCl\text{-}2\text{-}CMe_3\text{-}4)_2$	58	5·6	5·7
83. $MeNHP(S)(OPhCl\text{-}2\text{-}CMe_3\text{-}4)_2$	950	(9)	(14)
84. $n\text{-}C_8H_{17}NHP(O)(OPhCl_2\text{-}2,4)_2$	250	5·7	6·2
85. $Me_2C{=}NNHP(O)(OPh)_2$	200	5·6	6·5
86. 	650	(7)	(0)
87. $(4\text{-}MeOPhNH)_2P(O)Me$	1000	(10)	(0)

TABLE 1—*continued*

Compounds	Malathion LD_{50} mg/kg*	Mouse plasma-pI_{50}† PrCh	Malathion
Others			
88. 2-MePhOP(O)OPh-2-CH_2	75	7·7	7·2
89. PhP(O)OPh-2-CH_2	125	7·5	7·0
90. PhP(S)OPh-2-CH_2	<50	5·5	(37)
91. ClP(S)C(Me_2)CH – CMe	320	(3)	(0)
92. $(MeS)_3P(O)$	250	5·0	(8)
93. $(EtS)_3P(O)$	380	5·5	(30)
94. $(i\text{-}C_3H_7S)_3P(O)$	175	5·0	(13)
95. $(n\text{-}C_4H_9S)_3P(O)$	<50	7·1	5·9
96. $(n\text{-}C_5H_{11}S)_3P(O)$	<50	6·5	5·8
97. $(n\text{-}C_6H_{13}S)_3P(O)$	340	6·1	5·2
98. $(n\text{-}C_8H_{17}S)_3P(O)$	1000	(14)	(3)
99. $(n\text{-}C_3H_7S)_3P$	<50	5·8	(37)
100. $(n\text{-}C_4H_9S)_3P$	<50	6·4	5·7

* Mice treated with 100 mg of the test organophosphorus compound per kg 24 hr prior to treatment with malathion.

† Figures in parentheses are percentage inhibition with 1×10^{-5} M.

to a reported procedure.[9] A 100 mg dose of the test organophosphate per kg was administered and followed 24 hr later by varying doses of malathion to determine the LD_{50} of the malathion. None of the organophosphates indicated in Table 1 produced any mortality or marked signs of poisoning within 3 days after injection of this 100 mg/kg dose. Twenty or more mice were used in determining each LD_{50} value reported;

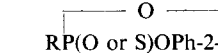
TABLE 2. MALATHION LD_{50} AT VARYING TIME INTERVALS AFTER TREATMENT OF MICE WITH FOUR PHOSPHORUS ESTERS

Compound (20 mg/kg)	Malathion LD_{50} as mg/kg after indicated interval (hours) 0	1	6	24	72	120
95. $(n\text{-}C_4H_9S)_3P(O)$	950	550	58	85	700	1100
82. MeNHP(O)(OPhCl-2-CMe_3-4)$_2$	900	600	190	170	500	500
88. 2-MePhOP(O)OPh-2-CH_2	15	16	95	370	900	900
90. PhP(S)OPh-2-CH_2	18	16	14	90	750	850

values as milligrams of toxicant per kilogram of mouse were estimated from logarithm-probit mortality plots. The LD_{50} of malathion at 24 hr was about 1500 mg/kg with control mice, and ranged from less than 50 to greater than 1200 mg/kg for mice pretreated with the test organophosphorus compounds.

In one study four organophosphorus compounds were administered at 20 mg/kg and the malathion LD_{50} determined at varying intervals thereafter (Table 2). In another study utilizing some of the more active or more toxic compounds (Table 3),

TABLE 3. BIOLOGICAL ACTIVITY OF SALIGENIN CYCLIC PHOSPHORUS ESTERS AND CERTAIN OTHER SELECTED COMPOUNDS

Compounds		Mouse plasma-pI_{50}* PrCh	Mouse plasma-pI_{50}* Malathion	Toxicity to mice (mg/kg) Approx. LD_{50}	LD_{50} of malathion† In equitoxic mixture with compound	LD_{50} of malathion† Mixed with compound (1 kg/mg)	Toxicity to chickens (mg/kg) Approx. LD_{50}	Toxicity to chickens (mg/kg) Minimal ataxic dose
								
R	O or S							
101. MeO	O	7·5	7·0	30	200	370	8–12	n.a.‡
102. PhO	O	7·8	6·8	45	85	850	13–19	1·5–2
88. 2-MePhO	O	7·7	7·2	140	45	145	30–60	2–5
103. 3-MePhO	O	7·5	6·9	26	145	390	25–30	1–2
104. 4-MePhO	O	7·9	6·8	70	65	200	25–30	0·25–0·5
105. 3,5-Me_2PhO	O	7·6	7·0	25	210	800	95	4–8
106. 2-ClPhO	O	7·3	6·6	65	62	210	25–50	12–25
107. Et	O	7·7	6·5	6	260	310	1–2	n.a.
108. $ClCH_2$	O	7·0	6·7	22		540	25	n.a.
89. Ph	O	7·5	7·0	140	40	70	200–400	200
90. Ph	S	5·5	(37)	290	86	960	400–800	50–100
109. Me_2N	O	6·1	5·9	7	660	580	5–10	n.a.
Others								
99. $(n\text{-}C_3H_7S)_3P$		5·8	(37)	300	400	1000		
100. $(n\text{-}C_4H_9S)_3P$		6·4	5·7	1400	350	1000		
110. $(n\text{-}C_3H_7S)_3P(O)$		6·5	5·5	85	350	1050		
95. $(n\text{-}C_4H_9S)_3P(O)$		7·1	5·9	285	370	1100		
96. $(n\text{-}C_5H_{11}S)_3P(O)$		6·5	5·8	3500	700	1000		
111. $PhP(O)(OPhNO_2\text{-}4)_2$		6·2	6·3	67	140	630		
								
112. $ClP(S)OC_6H_{10}\text{-}2\text{-}CHCH_3$		(47)	(35)	39	350	1040		

* Figures in parentheses are percentage inhibition at 1×10^{-5} M.
† Simultaneous administration of test organophosphorus compound and malathion.
‡ n.a. = No ataxia signs evident with any sublethal dosages.

the LD_{50} of the test organophosphate was determined; the compound then was mixed with an equitoxic amount of malathion and the LD_{50} of the simultaneously administered mixture was determined. Thus, the mixture contained the two components in the ratio of their individual LD_{50} values. Mice were also treated simultaneously with 1 mg of the test compound per kg and varying malathion doses (Table 3).

The antiesterase activity *in vitro* of all compounds was assayed with mouse plasma utilizing propionylcholine (PrCh) and malathion substrates as previously described.[9] Results are reported as pI_{50} values (negative logarithm of molar concentration producing 50% inhibition) with a 30-min preincubation of inhibitor and enzyme prior to addition of the substrate to initiate the assay.

Treatment of chickens

White leghorn hens aged 6 to 18 months and 1·4 to 2·2 kg in weight were used. All compounds were administered directly as corn oil solutions except for certain cyclic phosphorus compounds that required small amounts of dimethylformamide initially to effect solution. Noncyclic di- or triaryl phosphates were administered orally as a single treatment per bird, with or without corn oil as a solvent, depending on the dose. A single intraperitoneal injection was used to administer the saligenin cyclic phosphorus compounds, while multiple daily intraperitoneal doses were administered with the *S,S,S*,-trialkyl phosphorus compounds and Zytron®. The values for acute toxicity were obtained without the use of atropine to reduce the cholinergic shock. Usually 10 to 30 birds were used to approximate the acute lethal and minimal ataxia levels for each compound.

Hydrolysis of saligenin cyclic phosphorus compounds

Nonenzymatic hydrolysis studies were made with 0·05 M sodium barbital, pH 9·5, at 27° with 1×10^{-4} M organophosphate. To increase substrate solubility, the organophosphates were initially dissolved in ethanol, and buffer was immediately added to yield a final ethanol concentration of 0·5%. Aliquots were withdrawn for analysis at 0, 3, 5, 10, 20, 40, 80, and 120 min and 24 hr. Enzymatic studies utilized heparinized, undiluted rabbit plasma, and the soluble fraction (15,000 × *g*, 30 min, 5°) from a 20% homogenate of rabbit liver prepared in phosphate buffer (KPO_4, pH 8·0, 0·05 M). To 1·5 ml of 3×10^{-4} M organophosphate substrate in phosphate buffer containing 0·5% ethanol was added 0·2 ml enzyme. In a separate study it was established that this ethanol level was not inhibitory. Boiled enzymatic preparations were used as controls. After incubation times of 0, 5, 10, 20, 40, and 80 min and 24 hr at 27°, tubes were withdrawn and 3·5 ml additional buffer added. With both the nonenzymatic and the enzymatic studies, the extent of hydrolysis was evaluated with the 4-aminoantipyrine reagent.[11] The red dye formed could be completely extracted into chloroform if it was derived by coupling with a free phenol, but the color remained in the aqueous phase if the phenolic derivative contained an ionized phosphate group.[11] Only initial hydrolysis at the cyclic P-O-aryl bond would yield a red dye, on coupling with aminoantipyrine, that would remain in the aqueous phase on extraction with chloroform.

All results were plotted as logarithm per cent organophosphate unhydrolyzed versus minutes of reaction, and the half-life values were read directly from these

linear plots. The initial site of hydrolysis was ascertained by the method indicated above in each case where a red dye resulted with aminoantipyrine.

RESULTS

Malathion potentiation and antiesterase activity

In all mouse-toxicity studies, the time sequence of toxicity signs indicated but did not necessarily establish that malathion was the lethal agent when mixtures of compounds were administered.

Five types of highly active potentiators were found (Table 1). Triphenyl phosphates and phosphonates containing 2-MePhO, 4-MePhO, 2-EtPhO, and 4-EtPhO substituents (Cmpds. 29, 30, 48, 71–73) were highly potent, as were the previously examined series of tri-(substituted-phenyl) phosphates containing these substituents.[9] High activity appeared in di-(substituted-phenyl) phenylphosphonates containing 2-MePhO, 2-EtPhO, 4-ClPhO, 4-NO_2PhO, and 4-EtPhO groups (Cmpds. 71–74, 76, 77, 111). Diaryl *N*-methylphosphoramidates (Cmpds. 81, 82) were quite active as might be anticipated from the specificity as esterase inhibitors of similar compounds as found by Myers *et al.*[1, 2] The *S,S,S,*-trialkyl phosphorotrithioates and their trivalent phosphorus analogs were also found to be very active. Based mainly on the pentavalent series, the optimal activity for malathion potentiation and esterase inhibition *in vitro* appeared from C_3 to C_5 for the alkyl group (Cmpds. 95, 96, 99, 100). The potency of the saligenin cyclic phosphorus esters (Table 3) was anticipated from their formation *in vivo* as metabolites[11, 13] of active *o*-cresyl phosphates[6, 9] and their high reactivity and potency as esterase inhibitors, with the exception of the more stable dimethylphosphoramidate analog (Cmpd. 109). The toxicity of the saligenin cyclic phosphorus compounds was greater with alkyl or alkylamino substituents on the phosphorus (Cmpds. 101, 107–109) than for most aryl analogs. The greatest degree of potentiation with simultaneous administration of equitoxic mixtures of malathion and the test compound appeared with the aryl analogs of this series (Cmpds. 88–90, 102–104, 106).

The time interval between the potentiator and the malathion for maximal potentiation (Table 2) varied from less than 1 hr (Cmpd. 88) to more than 24 hr (Cmpd. 82). By 72 hr, the degree of potentiation with these compounds was greatly reduced. The results on rate and duration of action with *S,S,S*-tributyl phosphorotrithioate (Cmpd. 95) are consistent with a study on cholinesterase inhibition in rats by this compound.[14] In these studies it was found that actions other than cholinesterase inhibition are important in poisoning by the phosphorotrithioate.

Several di- and triaryl phosphates containing 2-ClPhO substituents were quite active (Cmpds. 44, 49, 53–55) compared with analagous compounds with PhO or 2-MeOPhO groups (Cmpds. 18, 27, 45). However, the 2-ClPhO substituent reduced or destroyed the selective inhibition of the plasma esterase hydrolyzing malathion compared with the enzymes hydrolyzing propionylcholine.

Malathion potentiation was similar 24 hr after administration of corresponding tri- and pentavalent phosphorus esters. The activity *in vitro* and selectivity in esterase inhibition (Tables 1 and 3) varied between the tri- and pentavalent derivatives, although the pentavalent analogs were usually more potent (Cmpds. 58, 59, 80, 99,

100, 57, 61, compared respectively with 4, 6, 69, 110, 95 of the present study and 1 and 14 of a previous study[9]). The relatively high activity reported for triphenyl phosphite[9] probably resulted from impurities in the sample. These results indirectly indicate the oxidative conversion *in vivo* of the phosphites to phosphates in mice, but no direct evidence is available for such a conversion.

Substitution of P=S for P=O in the compounds greatly reduced the activity both as esterase inhibitors *in vitro* and as malathion potentiators (Table 1, Cmpds. 42, 70, 76, 83, 90, compared respectively with 41, 69, 111, 82, 89). This probably resulted from the reduced reactivity conferred by the P=S and the relatively slow oxidation rate *in vivo* to the P=O compound compared with the hydrolysis rate of these active antiesterase metabolites.

Chicken toxicity

Certain of the saligenin cyclic phosphorus compounds were found to be highly toxic and some induced ataxia at very low intraperitoneal dosages.[13, 15] Ataxia resulted with the aryl saligenin cyclic phosphates and phosphonates, but not with the generally more acutely toxic alkyl or dimethylamino analogs (Table 3, Cmpds. 101, 107–109). No tests were made with atropine to determine the effect on the LD_{50} and possible ataxia at higher dosages of this group of compounds. The dose differential between the acute lethal and minimal ataxic level was greatest with the 4-MePhO analog (Cmpd. 104). This compound was as active as the dialkyl fluorophosphorus compounds[16] in inducing ataxia, but differed from them in the large differential between the dosage inducing ataxia and that inducing cholinergic shock. The phenylphosphonates (Cmpds. 89, 90) induced ataxia at slightly sublethal doses; however, the low solubility of these compounds made it difficult to obtain precise results.

With triaryl phosphates, only one *o*-cresyl group is required for neurotoxic activity.[17] The present studies with chickens (Table 3) are in general agreement with the available literature on structure-neurotoxicity correlations for *o*-cresyl phosphorus compounds,[9, 10, 17–20] if it is assumed that the action in the body is dependent on a hydroxylation and cyclization mechanism.[11, 13, 15] The phenylphosphonate analog (Cmpd. 89) appeared to be less active or of similar activity in inducing ataxia to two possible precursors, the phenylphosphonothionate (Cmpd. 90) and $PhP(O)(OPhMe\text{-}2)_2$ (Cmpd. 71), which would presumably form compound 89 *in vivo*. Compound 71 induced ataxia at an oral dose of 250 but not at 125 mg/kg. The relatively low activity of the cyclic phenylphosphonate (Cmpd. 89) may be due both to low solubility and high reactivity. This might result in equal or even lower doses of more soluble and more hydrolytically stable precursors (Cmpds. 71, 90) yielding a larger amount of the effective agent (Cmpd. 89) at the site of action. The saligenin cyclic phosphorus compounds with alkyl phosphate and alkyl phosphonate groupings (Cmpds. 101, 107, 108) did not yield ataxia. The following potential precursors of such compounds also failed to yield ataxia: $[C_4H_9(Et)CHCH_2O]_2P(O)OPhMe\text{-}2$ and $(n\text{-}C_3H_7O)_2P\text{-}(O)OPhMe\text{-}2$ at 1000 mg/kg oral[17, 20]; $MeP(O)(OPhMe\text{-}2)_2$ at 100 mg/kg intramuscular;[16] and $EtP(O)(OPhMe\text{-}2)_2$, $ClCH_2P(O)(OPhMe\text{-}2)_2$ and $MeOP(O)(OPhMe\text{-}2)_2$, where no signs of ataxia appeared in the survivors with acute oral LD_{50} values of 25–50, 50–100, and 400–800 mg/kg, respectively. In general, where a saligenin cyclic phosphorus compound induced ataxia, the corresponding probable precursors with *o*-cresyl

groups also induced ataxia, and where ataxia was not evident with the saligenin cyclic phosphorus compound, the precursors were also inactive.

Many *o*- and *p*-ethylphenyl phosphorus compounds effect ataxia in hens, but the contribution of possible metabolic intermediates with antiesterase activity in this response is not clear. The *o*- and *p*-ethylphenyl diphenyl phosphates (Cmpds. 29, 30) effected ataxia at a 1000 mg oral dose per kg, whereas di-(*o*-ethylphenyl) phenylphosphonate (Cmpd. 72) gave ataxia at 250 mg/kg and the *p*-ethyl analog (Cmpd. 73) did not at 2000 mg/kg. The possible α-methylsaligenin cyclic phosphate and phosphonate metabolites of the *o*-ethylphenyl compounds have not been prepared, and their contribution to the biological activity of *o*-ethylphenyl phosphorus compounds is not known.

The toxicity and ataxia-inducing properties of several *S,S,S*-trialkyl phosphorus compounds were examined after multiple intraperitoneal doses of the compounds. The two *S,S,S*-tributyl cotton defoliants were most extensively examined. With the phosphorotrithioate (Cmpd. 95 or DEF®) at 100 mg/kg daily for 10 days, ataxia appeared in 10 and 18 days after treatment was started, while with 7 days of treatment at this dose, ataxia appeared on day 14. With the phosphorotrithioite analog (Cmpd. 100 or Merphos®), seven daily doses of 100 mg/kg gave ataxia on day 33, and ten daily doses of 100 mg/kg gave ataxia on day 25. The *S,S,S*-tripropyl esters were considerably more toxic. The trithioate (Cmpd. 110) yielded ataxia in 10 days when 15 mg/kg was administered daily for 7 days, and in 17 to 21 days after ten daily injections of 5 mg/kg. Higher levels were lethal, but even at these lower levels the hens suffered apparent cholinergic shock prior to the appearance of the ataxia signs. *S,S,S*-tripropyl phosphorotrithioite (Cmpd. 99) was lethal with seven doses of 50 mg/kg and was not further investigated. Other *S,S,S*-trialkyl phosphorotrithioate analogs were tested at 100 mg/kg daily for 10 days. The ethyl, amyl, hexyl, and octyl analogs (Cmpds. 93, 96–98) gave no signs of toxicity or ataxia. Further studies with the amyl analog at 200 mg/kg for 7 days and the hexyl analog at 300 mg/kg daily for 10 days failed to give any indication of ataxia with these compounds.

Since two phosphorus-containing herbicides gave ataxia on repeated administration this study was extended to a third herbicide, Zytron®. A daily intraperitoneal dose of 200 mg/kg for 8 days was lethal, whereas 100 mg/kg for 7 days gave ataxia signs in 21 days. A dose of 50 mg/kg for 10 to 14 days gave ataxia-like signs in 21 days. The signs with Zytron® were not those of tri-*o*-cresyl phosphate ataxia in that cholinergic shock appeared within a few days and extended into the ataxia period, which persisted for at least 3 months. Partial recovery from the poisoning signs appeared slowly after the 3-month ataxia period.

Relative hydrolytic stability of the saligenin cyclic phosphorus compounds

Hydrolytic half-life values in 0·05 M barbital, pH 9·5, varied from less than 5 min for the PhP(O) and $ClCH_2P(O)$ derivatives (Cmpds. 89, 108) to many days for the $Me_2NP(O)$ analog (Cmpd. 109). Half-life values between 22 and 26 min were obtained with the PhP(S), 2-ClPhOP(O), and EtP(O) derivatives (Cmpds. 90, 106, 107); between 40 and 50 min for the 2-MePhOP(O) and 4-MePhOP(O) analogs (Cmpds. 88, 104); about 60 min for the PhOP(O) and 3-MePhOP(O) (Cmpds. 102, 103) and 80 min for the 3,5-Me_2PhOP(O) (Cmpd. 105) derivatives; and 150 min for the MeOP(O) analog

(Cmpd. 101). The general order of increasing stability was phosphonate < aryl phosphate < alkyl phosphate < dimethylphosphoramidate.

Rabbit plasma and liver were most effective in increasing the hydrolysis rates of the alkyl phosphate and phosphonate analogs. Neither liver nor plasma effected as much as a 2× increase in hydrolysis rate for compounds 88, 102, 103, 105, and 106; and liver but not plasma gave a 2× increase with compound 90 and 4× with compound 104. The liver effected an 8× increase with the MeOP(O) (Cmpd. 101) and 15× with the EtP(O) (Cmpd. 107) analogs, whereas the plasma effected more than a 10× increase in hydrolysis rate with both these compounds.

In all cases of enzymatic and nonenzymatic hydrolysis, the initial site of cleavage was the cyclic P-O-aryl bond. The catalytic activity of phosphate ion on the nonenzymatic hydrolysis has been previously noted.[12]

DISCUSSION

Extensive studies on possible potentiation by mixtures of organophosphorus insecticides followed the observation of greater than additive toxicity of a mixture of malathion and *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothionate (EPN).[21, 22] The potentiation appears to result primarily from the interference of one material with the metabolism or detoxification of the other. Selective aliphatic esterase inhibitors thus inhibit the esterases that detoxify malathion through hydrolysis of the carboethoxy groups.[4-9] The present study greatly extends the types of compounds known to effect malathion potentiation. Not only insecticides, but also certain plant defoliants and many organophosphates used for nonagricultural purposes possess this type of biological activity. With the insecticides, impurities may contribute to the potentiation, as has already been shown with *O*,*O*-dimethyl *S*-(*N*-methylcarbamoylmethyl)-phosphorodithioate where an impurity potentiates the oral toxicity of this insecticide to certain mammals.[23] Most aryl phosphate insecticides contain only one aryl group, but the di- and triaryl derivatives appearing as manufacturing impurities may contribute to the biological activity in either a deleterious or beneficial manner.

Organophosphates that are highly potent in inhibiting esterases other than acetylcholinesterase at nonlethal dosages may be important in a number of ways. Such compounds might hypersensitize people to the toxic effects of certain ester-type drugs[5] or might be used advantageously with such drugs to prolong their action. They may help establish the physiological significance of these other esterases and their possible involvement in the action of organophosphates currently used or being tested as insecticides, herbicides, fungicides, nematocides, and antihelminthic agents. Certain of these materials (Cmpds. 95, 100, and triphenyl phosphate) greatly increase the toxicity of malathion to houseflies and mosquitoes, particularly with resistant strains.[24] The aryl saligenin cyclic phosphorus esters also synergize the insecticidal activity of malathion.[25] Their use in this way must be carefully evaluated from the benefit in insect control with these "synergists" compared with the increased toxicity of the mixture to mammals and other possible side effects to mammals resulting from the use of the synergist. The possible neurotoxic activity must be carefully evaluated, since this response varies with experimental animals and only limited evidence is available from accidental human exposures leading to ataxia and central and peripheral neuropathy.

The availability of organophosphates of greater selectivity and potency as aliphatic esterase inhibitors and ataxia-inducing agents should facilitate resolution of these intriguing problems.

Acknowledgments—Publication approved by the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported in part by grants from the U. S.Army Chemical Research and Development Laboratories (Grant DA-CML-18-108-61-G-6) and the National Institutes of Health (Contract RG-5304). The skilled technical assistance of Miss Judith O'Neil, Miss Andrea Woodward and Mrs. Norma Avanzado is gratefully acknowledged. Without the generous assistance of several industrial laboratories in making available many of the esters studied, this investigation would not have been possible.

REFERENCES

1. D. K. Myers, *Studies on Selective Esterase Inhibitors.* Unpublished doctoral thesis, Amsterdam (1954).
2. D. K. Myers, A. Kemp, Jr., J. W. Tol and M. H. T. de Jonge, *Biochem. J.* **65**, 232 (1957).
3. B. Holmstedt, *Pharmacol. Rev.* **11**, 569 (1959).
4. J. W. Cook, J. R. Blake and M. W. Williams, *J. Ass. off. agric. Chem., Wash.* **40**, 664 (1957).
5. K. P. Dubois, *Arch. Industr. Hlth* **18**, 488 (1958).
6. S. D. Murphy R. L. Anderson and K. P. Dubois, *Proc. Soc. exp. Biol., N.Y.* **100**, 483 (1959).
7. F. W. Seume and R. D. O'Brien, *Toxicol. appl. Pharmacol.* **2**, 495 (1960).
8. K. P. Dubois, *Advanc. Pest Control Res.* **4**, 117 (1961).
9. J. E. Casida, *Biochem. Pharmacol.* **5**, 332 (1961).
10. R. L. Baron, *Delayed Neurological Effects of Certain Organophosphate Esters in Chickens.* Unpublished doctoral thesis, Madison, Wis. (1962).
11. M. Eto, J. E. Casida and T. Eto, *Biochem. Pharmacol.* **11**, 337 (1962).
12. M. Eto and Y. Oshima, *Agric. biol. Chem., Tokyo* **26**, 452 (1962).
13. J. E. Casida, M. Eto and R. L. Baron, *Nature, Lond.* **191**, 1396 (1961).
14. S. D. Murphy and K. P. Dubois, *Arch. Industr. Hlth* **20**, 161 (1959).
15. R. L. Baron, D. R. Bennett and J. E. Casida, *Brit. J. Pharmacol.* **18**, 465 (1962).
16. D. R. Davies, P. Holland and M. J. Rumens, *Brit. J. Pharmacol.* **15**, 271 (1960).
17. C. H. Hine, M. K. Dunlap, E. G. Rice, M. M. Coursey, R. M. Gross and H. H. Anderson, *J. Pharmacol. exp. Ther.* **116**, 227 (1956).
18. D. Henschler, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **237**, 459 (1959).
19. H. F. Bondy, E. J. Field, A. N. Worden and J. P. W. Hughes, *Brit. J. industr. Med.* **17**, 190 (1960).
20. W. N. Aldridge and J. M. Barnes, *Biochem. Pharmacol.* **6**, 177 (1961).
21. J. P. Frawley, E. C. Hagan, O. G. Fitzhugh, H. N. Fuyat and W. I. Jones, *J. Pharmacol. exp. Ther.* **119**, 147 (1957).
22. J. P. Frawley, H. N. Fuyat, E. C. Hagan, J. R. Blake and O. G. Fitzhugh, *J. Pharmacol. exp. Ther.* **121**, 96 (1957).
23. J. E. Casida and D. M. Sanderson, *Nature, Lond.* **189**, 507 (1961).
24. F. W. Plapp, Jr. and G. W. Eddy, *Science* **134**, 2043 (1961).
25. M . Eto, Personal communication (1962).