Trialkyl Phosphate and Phosphorothiolate Anticholinesterases. II. Effects of Chain Length on Potency*

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ABSTRACT: The potency of diethyl alkyl phosphates or *O,O*-diethyl *S*-alkyl phosphorothiolates as anticholinesterases increases gradually with the chain length of the alkyl group; with a six-carbon chain it reaches a maximum which is about the same for both classes of compounds. While triethyl phosphate is inactive, its thiolo analog shows some potency. The findings are interpreted in terms of the existence of a hydrophobic patch, of limited size, near the esteratic site of the enzyme. The mechanism of inhibition involves phosphorylation, but the inhibitory excellence is due to high affinity for the enzyme surface near the esteratic site.

We have recently shown (Bracha and O'Brien, 1968) that trialkyl phosphates or phosphorothiolates which are structurally similar to Amiton[1] (*O,O*-diethyl *S*-(2-diethylaminoethyl) phosphorothiolate) but lack its nitrogen are good acetylcholinesterase inhibitors, in spite of the fact that they have no charged substituent and are poor phosphorylators. It is customary to predict the potency of organophosphates by the nature of the substituents on the main side chain, and by the way they labilize the P–O–C bonds (see review by O'Brien, 1960). Thus, simple trialkyl phosphates have not attracted much interest, since they do not possess the properties usually associated with good organophosphorus inhibitors.

Preliminary studies showed that although the lower phosphates had little activity, increasing the length of the side chain beyond C_4 increased potency substantially. This finding was unexpected, because the phosphorylating ability of an organophosphate is not expected to increase with a mere lengthening of an alkyl side chain, but rather to diminish. Clearly, binding to the enzyme surface must be a dominant factor. To explore these mechanisms we therefore prepared a series of diethyl *n*-alkyl phosphates (as well as their thiolo analogs), with side chain extending from C_2 to C_{10} and measured their anticholinesterase activity and toxicity.

Results

The compounds, whose syntheses are described under Experimental Section, are shown with their physical properties in Table I and their anticholinesterase activity and their toxicity to mice and houseflies in Tables II and III. All the compounds prepared were either diethyl *n*-alkyl phosphates or diethyl *n*-alkyl phosphorothiolates, with the alkyl varying in length from C_2 to C_{10}. No phosphorothionates were prepared since previous studies have shown them to be totally inactive against cholinesterase (O'Brien and Hilton, 1964; Bracha and O'Brien, 1968). It was apparent (Figure 1) that after a certain length was achieved, increase in chain length promoted potency. However, an abrupt cutoff point was noted at C_6 after which activity of higher homologs remained constant at the C_6 level. The potency of the sulfur-containing compounds was similar in magnitude to that of the phosphates once the cutoff point was reached, indicating that a similar binding process was responsible for their activity.

The toxicity of the compounds to mice usually paralleled their cholinesterase inhibition. However, their toxicity for houseflies was low, as in Amiton and its carbon analogs.

Discussion

Binding to the Enzyme Surface. It is evident that simple alkyl phosphates and phosphorothiolates can be very effective anticholinesterases if the alkyl group is large. The most active compounds are only eightfold less potent than the well-known anticholinesterase, paraoxon (O'Brien, 1963).

Because *n*-alkyl chains differ little in electronic character, one may conclude that the variations in their potency are not due to electronic effects upon the phosphorus, but must be upon the affinity for the enzyme surface. The reaction may be represented by

$$PX + E \underset{}{\overset{K_a}{\rightleftharpoons}} PXE \xrightarrow{k_2} PE + X$$

where P is the diethyl phosphoryl group, X the *n*-alkyl chain, E the enzyme, K_a the affinity step, and k_2 the phosphorylation step (Main, 1964). Then K_a is the parameter most likely to be affected by carbon chain length. By contrast, most variations in potency of

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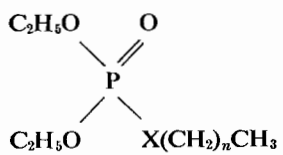
[1] Abbreviations used: Amiton, *O,O*-diethyl *S*-(2-diethylaminoethyl) phosphorothiolate; DPA, 1,1-dimethyl-2-phenylaziridinium.

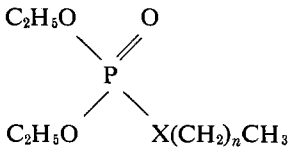
TABLE I: Physical Constants, Yields, and Analyses of Diethyl Alkyl Phosphates or Diethyl Alkyl Phosphorothiolates.



Compd	Carbons in Chain (n + 1)	X	Yield (%)	Bp (mm)	n_D^{20}	MR_e[a]	ΣBR_e[b]	Formula	Calcd (%) C	Calcd (%) H	Calcd (%) P	Found (%) C	Found (%) H	Found (%) P
1	2	O	Commercially available	100 (18)	1.4060	256.12	256.26	$C_6H_{15}O_4P$	36.35					
2	2	S	67	127–129 (12)	1.4604	289.49	290.35	$C_6H_{15}O_3PS$	36.35	7.63	15.63	36.30	7.55	15.48
3	3	O	64	96–97 (6)	1.4090	276.43	276.85	$C_7H_{17}O_4P$	42.86	8.73	15.79	42.71	8.59	15.60
4	3	S	70	135–137 (12)	1.4602	309.98	310.95	$C_7H_{17}O_3PS$	39.61	8.08	14.59	39.40	8.06	14.32
5	4	O	58	28–81 (1.0)	1.4124	296.90	297.46	$C_8H_{19}O_4P$	45.71	9.11	14.73	45.74	9.12	14.54
6	4	S	58	86–85 (0.1)	1.4596	330.27	331.55	$C_8H_{19}O_3PS$	42.46	8.46	13.69	42.35	8.42	13.54
7	5	O	58	89–90 (1.0)	1.4168	317.69	318.06	$C_9H_{21}O_4P$	48.20	9.44	13.81	48.24	9.52	13.60
8	5	S	60	93–94 (0.1)	1.4600	350.84	352.15	$C_9H_{21}O_3PS$	44.98	8.81	12.89	45.19	8.71	12.92
9	6	O	60	97–98 (1.0)	1.4210	338.58	338.66	$C_{10}H_{23}O_4P$	50.41	9.73	13.00	50.47	9.63	12.79
10	6	S	75	152–153 (10)	1.4583	370.90	372.75	$C_{10}H_{23}O_3PS$	47.23	9.12	12.19	47.10	9.03	12.24
11	7	S	78	117–116 (0.1)	1.4577	391.10	393.35	$C_{11}H_{25}O_3PS$	49.23	9.39	11.54	49.10	9.34	11.78
12	8	S	67	156–157 (10)	1.4256	379.66	379.86	$C_{12}H_{27}O_4P$	54.12	10.22	11.63	53.98	10.14	11.50
13	8	S	76	134–136 (0.1)	1.4588	411.95	413.95	$C_{12}H_{27}O_3PS$	51.04	9.67	10.97	50.96	9.62	10.59
14	9	S	70	139–140 (0.1)	1.4602	432.83	434.55	$C_{13}H_{29}O_3PS$	52.68	9.86		52.40	9.72	
15	10	O	54	125–126 (0.05)	1.4282	420.43	421.06	$C_{14}H_{31}O_4P$	57.12	10.62	10.52	57.06	10.51	10.64
16	10	S	70	143–145 (0.05)	1.4617	453.77	455.15	$C_{14}H_{31}O_3PS$	54.16	10.07	9.98	54.19	10.12	9.96

[a] Molar refraction (found). [b] Molar refraction (calcd) (see text).

TABLE II: Inhibitory Power of Diethyl *n*-Alkyl Phosphates and Diethyl *n*-Alkyl Phosphorothiolates.[a]



n	(a) Phosphates, X = O	(b) Phosphorothiolates, X = S	Ratio a:b
1	$>10^{-2}$	1.4×10^{-5}	>710
2	4.5×10^{-5}	7.0×10^{-6}	6.4
3	3.3×10^{-4}	1.0×10^{-5}	33.0
4	8.5×10^{-6}	2.4×10^{-6}	3.4
5	2.1×10^{-6}	7.0×10^{-7}	3.0
6		8.5×10^{-7}	
7	1.65×10^{-6}	7.3×10^{-7}	2.3
8		7.0×10^{-7}	
9	7.7×10^{-7}	5.9×10^{-6}	0.13

[a] Values shown are I_{50}'s; that is, molar concentration giving 50% inhibition following incubation at 25° for 10 min. Note that $n = m - 1$, where m is the value in Figure 1.

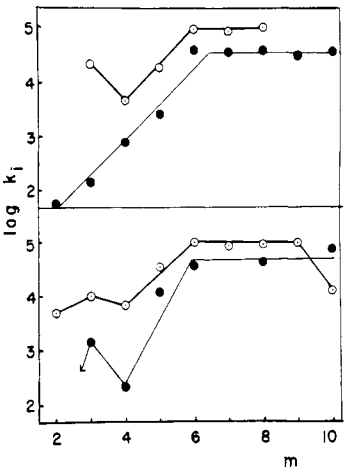


FIGURE 1: Relation between anticholinesterase potency and number of carbons in longest branch of main side chain (m). Lower part: (●) diethyl *n*-alkyl phosphates and (⊙) diethyl alkyl phosphorothiolates. Upper part: (●) *O*-ethyl *S*-*n*-alkyl methylphosphonothiolates and (⊙) *O*-ethyl *S*-ω-(*t*-butylalkyl) methylphosphonothioates (from Brestkin *et al.*, 1964). The intercept of the X axis with the Y axis represents that value of k_i below which measurements of potency could not be determined accurately.

organophosphates have been interpreted in terms of electronic factors operating upon k_2 (Heath, 1961; O'Brien, 1960), although the studies of Becker *et al.* (1963) and Brestkin *et al.* (1964) discussed below, are important exceptions. We have measured here the apparent bimolecular rate constant (k_i) which Main (1964) and Kitz and Wilson (1962) have shown to equal k_2/K_a.

Belleau and Lacasse (1964) distinguished between two types of enzyme–inhibitor interaction: (a) nonspecific accommodative perturbation of the network of nonpolar chains, due mainly to hydrophobic interaction or dissolution of nonpolar alkyl substituents in the enzyme surface, and (b) specific perturbation of the enzyme surface by extremely well-fitting substituents through the action of short-range van der Waals' forces. The nonpolar nature of the esterase surface will supply a substantial driving force for absorption of the nonpolar moiety into the protein. Thus, serum albumin would bind, nonspecifically, certain hydrocarbon-like molecules (Lovrien, 1963) while other proteins have shown nonspecific binding of short alkanes (Wishnia and Pinder, 1964). Naturally, there would be numerous sites for nonspecific hydrophobic bonding which would involve the various nonpolar amino acid residues of the esterase. However, we need concern ourselves only with those sites which are in the immediate vicinity of the active sites on the enzyme.

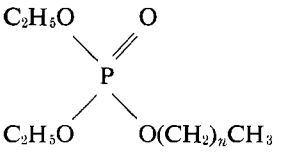
In a previous paper (Bracha and O'Brien, 1968) evidence was provided that in six series of alkyl phosphates, phosphorothiolates, and phosphonates, the side chains were bound by hydrophobic forces to the enzyme surface. The evidence was that each additional methylene (up to the optimum) increased the binding energy by between 600 and 870 cal, a value compatible with that calculated, for hydrophobic interaction of a methylene group, of 730 cal (Cohn and Edsall, 1943).

Several studies have examined the effects of progressive increases in chain length upon effectiveness of compounds as substrates or inhibitors of acetylcholinesterase. The results fall into two classes. Compounds of the first class show progressive increases in effectiveness with no indication of a cutoff; examples are inhibition by *N*-alkyltrimethylammoniums with R = 1–7 (Bergmann, 1955) and R = 8–12 (Paton, 1961) and by α,ω-bistrimethylammoniumalkanes with R = 4–10 (Bergmann, 1955); and hydrolysis of *n*-alkyl fluoroacetates with R = 2–6 (Bergmann and Shimoni, 1953).

Substrates and inhibitors of the second class show quite different patterns. An example is hydrolysis of *n*-alkyl acetates, with an optimum for R = 4 (Mounter and Whittaker, 1950). The four series of organophosphates of Figure 1 show a sharp leveling off when the straight part of the carbon chain is greater than C_6; this is true for our *n*-alkyl phosphates and phosphorothiolates and for the two ethyl methylphosphonate series of Brestkin *et al.* (1964). Identical behavior has been found in diethyl ω-(3-pentyl)alkyl phosphates and phosphorathiolates (Bracha and O'Brien, 1968). Thus, six different series of organophosphates show identical behavior.

The two patterns described above suggest that different compounds bind to quite different areas around the active sites. A remarkably clear proof of this view is the finding by Purdie and McIvor (1966) that 2.5×10^{-5} M DPA can inhibit by 50–70% the hydrolysis of

TABLE III: Toxicity of Phosphates and Phosphorothiolates to Mice and Houseflies.



	(a) Phosphates			(b) Phosphorothiolates			
	Mice		Houseflies	Mice		Houseflies	
n	LD_{50} (mg/kg)	Limits of Confidence (95%)	LD_{50} (mg/kg)	LD_{50} (mg/kg)	Limits of Confidence (95%)	LD_{50} (mg/kg)	Limits of Confidence (95)
1	>100		>250	58.3	51.2–66.4	77.1	62.5–95.1[a]
2	>100		>250	28.1	23.4–33.8[a]	102.8	89.4–118.0[a]
3	>100		>250	47.9	45.0–50.9	250	
4	>100		>250	31.7	30.1–33.3	250	
5				14.6	12.5–17.1	101.2	91.0–112.6
6			Not prepared	12.9	11.5–14.4	68.6	62.4–75.5
7	45.4	41.7–49.4	>250	16.7	14.0–20.0	74.5	70.4–78.9
8	26.7	24.8–28.8	>100	>100		250	

[a] A χ^2 test at the 5% level showed that the data on which this value was based deviate significantly from linearity.

phenyl acetate, indoxyl acetate, acetylcholine, and acetylthiocholine by acetylcholinesterase; under identical conditions it activates hydrolysis of indophenyl acetate by 100%. Furthermore, the DPA-treated enzyme had lost its ability to react with Amiton. One may conclude that indophenyl acetate binds to one zone, and that Amiton, DPA, and the four other substrates all bind elsewhere.

The data quoted above clearly suggest that alkyl phosphates, phosphorothiolates, and phosphonates can all bind to a hydrophobic patch which can accommodate only six methylenes. Such a patch could be a narrow strip, one carbon wide, extending 10 Å from the esteratic site; or roughly circular with a diameter of about 3.4 Å, centered about 4 Å from the esteratic site; or some intermediate form. If this patch is the one to which Amiton itself binds, it is therefore the same one to which DPA, indoxyl acetate, phenyl acetate, acetylcholine, and acetylthiocholine all bind. But it is equally possible that the nonionic phosphates bind to the above hydrophobic patch; whereas Amiton and acetylcholine bind to a quite different area, perhaps involving a true anionic site. Such a possibility makes it very difficult to evaluate the coulombic role in binding by comparing affinities of ionic and nonionic isosteres.

Becker *et al.* (1963) have described changes in anticholinesterase activity with changing alkyl groups in aromatic phosphonates. In one series, *O-p*-nitrophenyl *O*-ethyl phenylalkylphosphonates, the findings are in harmony with the above conclusions, if one takes the phenyl group as being equal in length to three methylene groups (Stuart–Briegleb models show both to be 4.7 Å, measuring carbon atoms only). It is very surprising that in the precisely analogous series of *O-p*-nitrophenyl *O*-ethyl alkylphosphonates, Becker *et al.* found that progressive increase of the alkyl group from C_3 to C_{10} gave a decrease in potency until C_5 and a leveling thereafter.

Let us return to consideration of diethyl *n*-alkyl phosphates and phosphorothiolates. Variations in k_i (or I_{50}) can only be due to variations in the phosphorylation constant k_2 or the affinity, $1/K_a$, since $k_i = k_2/K_a$. Probably, additional methylene groups do not substantially alter k_2, because k_2 is primarily sensitive to electronic effects. Consequently, the k_2 of the smallest compounds should give a guide to the k_2 of the whole series, *i.e.*, the members of the phosphate series should all be as poor as triethyl phosphate in their intrinsic phosphorylating ability. It follows that most of the 50,000-fold improvement observed in this series when n is increased from 1 to 5 (*i.e.*, m increased from 2 to 6) is due to increased affinity. These observations might suggest that these phosphates do not phosphorylate cholinesterase, but merely complex with it. However, the assay method employed uses a high substrate concentration (5×10^{-3} M) which would displace complexed inhibitor. Therefore, all the inhibition reported in this paper is due to phosphorylation of the enzyme.

The contribution of hydrophobic forces can be large. Clearly, weak inhibitors can be converted into potent inhibitors simply by adding appropriate hydrophobic groups. In simple *n*-alkyl phosphates (and, to a lesser extent, phosphorothiolates) the hydrophobic interaction is the principal contribution to potency. Very different approaches (O'Brien, 1963) have suggested that in

some commercial organophosphates, hydrophobic forces (originally thought to be Van der Waals' forces) play a substantial role, whereas in other compounds such as diisopropyl phosphorofluoridate or diethyl dichlorovinyl phosphate, they play a negligible role.

The Thiolo Effect. The extra potency of phosphorothiolates as compared with their phosphate analog has been called "the thiolo effect"; in trialkyl phosphates with branched chains, the effect is only large with a few specific pairs of compounds (Bracha and O'Brien, 1968). The same is true in the present series, in which the difference between the phosphate and phosphorothiolate is large for $n = 1$, *i.e.*, $m = 2$ (more than 710-fold) and $n = 3$, *i.e.*, $m = 4$ (33-fold) but in all other cases is sixfold or less, as shown in Table II. As in the branched compounds, the reason for the specificity of the effect is unknown.

Toxicity. Only the phosphorothiolates had measurable toxicity for mice, and these compounds were considerably more toxic to mice than to houseflies. The reasons for this selectivity are unknown.

Experimental Section

Anticholinesterase Inhibition and Toxicity. Anticholinesterase activity was determined by the ferric chloride–hydroxylamine colorimetric assay method (Hestrin, 1949). Procedures for estimation of anticholinesterase inhibition and toxicity to mice and houseflies were as previously described (Bracha and O'Brien, 1968). The values of k_i were calculated by the relationship $k_i = 0.695/I_{50}t$ (Aldridge, 1950).

Preparation of Trialkyl Phosphates. To a 0.1-mole dispersion of sodium in 100 ml of benzene was added dropwise 0.15 mole of the appropriate alcohol or thiol. After the initial reaction had slackened, the mixture was refluxed until all the sodium was consumed. Diethyl chlorophosphate (0.1 mole) was added dropwise at 0°. After the addition was complete, the mixture was refluxed for 4 hr and left overnight, and emptied into 200 ml of water. The benzene layer was separated, the aqueous phase was extracted with ether, and all organic phases were combined, washed, dried, and fractionated. The physical data, yields, and analyses of the esters prepared are given in Table I. The Eisnlohr molar refraction, $MR_e = M \cdot n_D^{20}$, was compared with the calculated value obtained upon addition of individual bond refraction constants (Sayre, 1958; Vogel *et al.*, 1952).

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