site; in such cases, differences of K_a for two inhibitors do not reflect, as is normally assumed, differences in affinity for a common binding site.

Acknowledgments

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References

Aharoni, A. H. (1967), Ph.D. Thesis, Cornell University, Ithaca, N. Y.

Aksnes, G., and Froyen, P. (1966), *Acta Chem. Scand. 20*, 1451.

Aldridge, W. N., and Davison, A. N. (1953), *Biochem. J. 55*, 763.

Berends, F., Posthumus, C. H., Sluys, I. V. D., and Dierkauf, F. A. (1959), *Biochim. Biophys. Acta 34*, 576.

Bracha, P., and O'Brien, R. D. (1968), *Biochemistry 7*, 1545 (this issue; following paper).

Bray, H. G., and White, K. (1957), Kinetics in Thermodynamics and Biochemistry, New York, N. Y., Academic, p 182.

Heilbronn-Wikström, E. (1965), *Svenska Kemisk Tidskrift 77* (11), 3.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Laidler, K. J. (1955), *Discussions Faraday Soc. 20*, 83.

Main, A. R. (1964), *Science 144*, 992.

Main, A. R., and Hastings, F. L. (1966), *Biochem. J. 101*, 584.

Main, A. R., and Iverson, F. (1966), *Biochem. J. 100*, 525.

Moorefield, H. H. (1957), *Contrib. Boyce Thompson Inst. 18*, 463.

O'Brien, R. D. (1960), Toxic Phosphorus Esters, New York, N. Y., Academic.

O'Brien, R. D. (1965), *Ann. N. Y. Acad. Sci. 123*, 156.

Scaife, J. F. (1959), *Can. J. Biochem. Physiol. 37*, 1301.

Snyder, J. A. (1960), U. S. Patent 2,922,739.

Uchida, T., Rahmati, H. S., and O'Brien, R. D. (1965), *J. Econ. Entomol. 58*, 831.

Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, New York, N. Y., Academic, pp 558–561.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Wilson, I. B. (1951), *J. Biol. Chem. 190*, 111.

Trialkyl Phosphate and Phosphorothiolate Anticholinesterases. I. Amiton Analogs*

P. Bracha and R. D. O'Brien

ABSTRACT: Carbon isosteres of *O,O*-diethyl *S*-(2-diethylaminoethyl) phosphorothiolate (Amiton) and its quaternary analog, as well as their homologs, show high inhibitory power against acetylcholinesterase, and high toxicity to mice. It was shown that the inhibitory potency results from excellent affinity for the enzyme surface, which compensates for poor phosphorylating ability. The affinity is due to hydrophobic interaction.

Affinity increases steadily with increasing side-chain length until a six-carbon length, after which it remains constant. This effect is interpreted in terms of a hydrophobic patch of limited size. Phosphorothiolates differ profoundly in potency from phosphates only in short-chain compounds. Charge is not an absolute necessity for binding to the esterase, and contributes only 18% to the binding of Amiton to acetylcholinesterase.

The organophosphates inhibit cholinesterase by phosphorylating that part of its active site called the esteratic site. Most potent organophosphates are relatively good phosphorylating agents, as judged, for instance, by the acid character of their leaving groups (O'Brien, 1960; Ooms, 1961). Exceptions to this generalization include those compounds whose leaving group resembles acetylcholine, which is the normal substrate. *O,O*-Diethyl *S*-(2-diethylaminoethyl)-phosphorothiolate (Amiton;[1] Ooms, 1961) and *O,O*-

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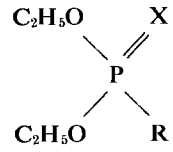
[1] Abbreviation used: Amiton, *O,O*-diethyl *S*-(2-diethylaminoethyl) phosphorothiolate.

TABLE I: Physical Constants, Yields, and Analyses of *O,O*-Diethyl (ω-3-Pentyl)alkyl Phosphates, Phosphorothionates, and Phosphorothiolates.

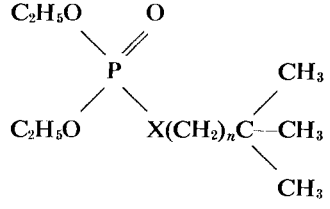


Compd	R	X	Yield (%)	Bp (mm)	n_D^{20}	MR_E[a]	BR_E[b]	Formula	Calcd (%)	Found (%)
IIIa	CH_2CH_3 | $OCHCH_2CH_3$	O	60	112–113 (10)	1.4172	317.80	318.06	$C_9H_{21}PO_4$	C, 48.21 H, 9.44 P, 13.81	C, 48.10 H, 9.15 P, 13.69
IIIb	CH_2CH_3 | $SCHCH_2CH_3$	O	52	130–132 (10)	1.4605	350.96	352.15	$C_9H_{21}PO_3S$	C, 44.98 H, 8.81 P, 12.89	C, 44.87 H, 8.70 P, 12.71
IVa	CH_2CH_3 | $OCH_2CHCH_2CH_3$	O	30	96–97 (0.3)	1.4194	338.20	338.66	$C_{10}H_{23}PO_4$	C, 50.41 H, 9.73 P, 13.00	C, 50.64 H, 9.70 P, 12.81
IVc	CH_2CH_3 | $OCH_2CHCH_2CH_3$	S	70	89–90 (0.05)	1.4554	370.16	370.75	$C_{10}H_{23}PO_3S$	C, 47.23 H, 9.12 P, 12.18	C, 47.64 H, 8.93 P, 12.20
IVb	CH_2CH_3 | $SCH_2CHCH_2CH_3$	O	70	93–94 (0.1)	1.4629	372.05	372.75	$C_{10}H_{23}PO_3S$	C, 47.23 H, 9.12 P, 12.18	C, 47.50 H, 9.12 P, 12.05
IVd	CH_2CH_3 | $SCH_2CHCH_2CH_3$	S	72	90–91 (0.02)	1.4892	402.68	404.84	$C_{10}H_{23}PO_2S_2$	C, 44.42 H, 8.52	C, 44.90 H, 8.88
IIa	CH_2CH_3 | $OCH_2CH_2CHCH_2CH_3$	O	67	93–94 (0.08)	1.4183	357.84	359.26	$C_{11}H_{25}PO_4$	C, 52.38 H, 9.92 P, 12.30	C, 52.47 H, 9.84 P, 12.49
IIc	CH_3CH_2 | $OCH_2CH_2CHCH_2CH_3$	S	75	98–99 (0.08)	1.4558	390.66	393.35	$C_{11}H_{25}PO_3S$	C, 49.23 H, 9.39 P, 11.54	C, 49.34 H, 9.37 P, 11.37
IIb	CH_2CH_3 | $SCH_2CH_2CHCH_2CH_3$	O	78	110–111 (0.3)	1.4573	391.07	393.35	$C_{11}H_{25}PO_3S$	C, 49.23 H, 9.39 P, 11.54	C, 49.02 H, 9.37 P, 11.80
IId	CH_2CH_3 | $SCH_2CH_2CHCH_2CH_3$	S	65	114–115 (0.25)	1.4727	418.87	421.44	$C_{11}H_{25}PO_2S_2$	C, 46.45 H, 8.86	C, 46.90 H, 9.24
Va	CH_2CH_3 | $O(CH_2)_3CHCH_2CH_3$	O	57	108–109 (0.1)	1.4223	378.76	379.86	$C_{12}H_{27}PO_4$	C, 54.12 H, 10.22 P, 11.63	C, 54.12 H, 10.43 P, 11.83

Vb	$S(CH_2)_3CHCH_2CH_3$ CH_2CH_3	O	55	115–116 (0.09)	1.4553	410.94	413.95	$C_{12}H_{27}PO_3S$	C, 51.04 H, 9.64 P, 10.97	C, 50.68 H, 9.64 P, 10.83
VIa	$O(CH_2)_4CHCH_2CH_3$ CH_2CH_3	O	55	111–112 (0.08)	1.4294	400.73	400.46	$C_{13}H_{29}PO_4$	C, 55.69 H, 10.43 P, 11.05	C, 55.63 H, 10.39 P, 10.97
VIb	$S(CH_2)_4CHCH_2CH_3$ CH_2CH_3	O	63	120–121 (0.05)	1.4550	431.24	434.55	$C_{13}H_{29}PO_3S$	C, 52.68 H, 9.86	C, 52.40 H, 9.72
VIIa	$O(CH_2)_5CHCH_2CH_3$ CH_2CH_3	O	45	118–119 (0.05)	1.4276	420.25	421.06	$C_{14}H_{31}PO_4$	C, 57.12 H, 10.62	C, 56.89 H, 10.31
VIIb	$S(CH_3)_5CHCH_2CH_3$ CH_2CH_3	O	60	126–127 (0.05)	1.4610	453.51	455.15	$C_{14}H_{31}PO_3S$	C, 54.16 H, 10.07	C, 53.78 H, 9.86

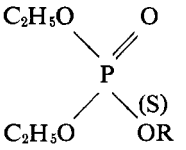
[a] Molar refraction (found). [b] Molar refraction (calcd) (see text).

TABLE II. Physical Constants, Yields, and Analyses of *O,O*-Diethyl (ω-*t*-Butylalkyl) Phosphates and Phosphorothiolates.



									Calcd (%)			Found (%)		
Compd	*n*	X	Yield (%)	Bp (mm)	n_D^{20}	MR_E	BR_E	Formula	C	H	P	C	H	P
VIIIa	0	O	35	87–87 (8)	1.4095	296.30	297.46	$C_8H_{19}PO_4$	45.71	9.11	14.73	45.68	9.07	14.59
IXa	1	O	55	72–73 (0.2)	1.4120	316.63	318.06	$C_9H_{21}PO_4$	48.21	9.44	13.81	48.06	9.30	13.58
Xa	2	O	52	83–84 (0.3)	1.4188	338.04	338.66	$C_{10}H_{23}PO_4$	50.41	9.73	13.00	50.63	9.72	12.95
Xb	2	S	57	92–93 (0.08)	1.4585	370.95	372.75	$C_{10}H_{23}PO_3S$	47.23	9.12	12.18	47.56	9.34	11.78
XIa	3	O	61	91–92 (0.1)	1.4219	358.73	359.26	$C_{11}H_{25}PO_4$	52.38	9.92	12.30	52.12	9.82	12.46
XIb	3	S	59	105–106 (0.1)	1.4618	392.30	393.35	$C_{11}H_{25}PO_3S$	49.23	9.39	11.54	49.61	9.72	11.22
XIIa	4	O	47	93–94 (0.08)	1.4240	378.86	379.86	$C_{12}H_{27}PO_4$	54.12	10.22	11.63	53.78	10.11	11.48
XIIb	4	S	70	112–113 (0.05)	1.4561	411.17	413.95	$C_{12}H_{27}PO_3S$	51.04	9.64	10.97	50.98	9.92	10.69

TABLE III: Inhibitory Power of Phosphates and Phosphorothiolates.



Compd	R	I_{50} (a) Phosphates	I_{50} (b) Phosphorothiolates	Ratio a:b
II	CH_2CH_3 $(CH_2)_2CHCH_2CH_3$	9.5×10^{-7}	3.0×10^{-7}	3.2
III	CH_2CH_3 $CHCH_2CH_3$	4.7×10^{-4}	6.0×10^{-6}	78
IV	CH_2CH_3 $CH_2CHCH_2CH_3$	$>10^{-3}$	5.6×10^{-7}	>1,780
V	CH_2CH_3 $(CH_2)_3CHCH_2CH_3$	8.5×10^{-7}	1.9×10^{-7}	4.5
VI	CH_2CH_3 $(CH_2)_4CHCH_2CH_3$	8.5×10^{-7}	1.6×10^{-7}	5.3
VII	CH_2CH_3 $(CH_2)_5CHCH_2CH_3$	4.8×10^{-7}	1.6×10^{-7}	3.0
VIII	$C(CH_3)_3$	5.2×10^{-5}		
IX	$CH_2C(CH_3)_3$	4.2×10^{-5}		
X	$(CH_2)_2C(CH_3)_3$	4.6×10^{-6}	1.6×10^{-6}	2.9
XI	$(CH_2)_3C(CH_3)_3$	2.4×10^{-6}	6.4×10^{-7}	3.8
XII	$(CH_2)_4(CH_3)_3$	8.4×10^{-7}	2.3×10^{-7}	3.6
XIII	C_2H_5 $CH_2CH_2NC_2H_5$	$>10^{-3}$	4.9×10^{-8}	>20,400

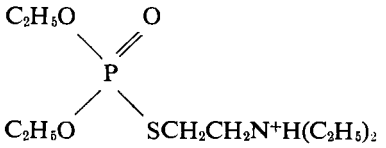
diethyl *O*-(3,3-dimethyl-1-butyl) phosphate (Fukuto, 1957) could serve as examples. It is assumed that the high affinity of such compounds for the enzyme surface compensates for the poor phosphorylating ability.

Recently, kinetic procedures were introduced (Main, 1964; Main and Iverson, 1966) to evaluate separately the affinity and the phosphorylating ability, based on reaction 1. Thus, K_a is a reciprocal measure of affinity

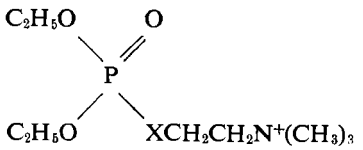
$$E + PX \underset{}{\overset{K_a}{\rightleftharpoons}} EPX \xrightarrow{k_2} EP + X \qquad (1)$$

and k_2 of phosphorylating activity. These procedures were extensions of those developed by Kitz and Wilson (1962) for methanesulfonate inhibitors.

It is widely held that the binding of the choline moiety of acetylcholine to the enzyme involves coulombic attraction to the so-called anionic site (Wilson, 1960), and it was natural to suppose that organophosphates, such as Amiton or its quaternary analogs (I), were similarly bound (Tammelin, 1958a,b). We were therefore surprised to find that *O,O*-diethyl *S*-(3-ethyl-1-pentyl) phosphorothiolate (IIb in Table I), the carbon

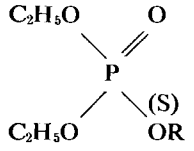


Amiton (at pH 7)



Ia, X = O
b, X = S

TABLE IV: Toxicity of Phosphates and Phosphorothiolates to Mice and House Flies.



Compd	R	(a) Phosphates				(b) Phosphorothiolates			
		Mice		House Flies		Mice		House Flies	
		LD_{50} (mg/kg)	Limits of Confidence (95%)	LD_{50} (mg/kg)	Limits of Confidence (95%)	LD_{50} (mg/kg)	Limits of Confidence (95%)	LD_{50} (mg/kg)	Limits of Confidence (95%)
III	$CH(CH_2CH_3)_2CH_2CH_3$	>100		>250		25.1	23.3–27.1	97.7	68.8–139.0[a]
IV	$CH_2CH(CH_2CH_3)CH_2CH_3$	>100		>250		16.1	13.9–18.7	>100	
II	$(CH_2)_2CH(CH_2CH_3)CH_2CH_3$	18.3	12.4–27.1	>250		9.8	6.9–14.0	102	90.2–115.6
V	$(CH_2)_3CH(CH_2CH_3)CH_2CH_3$	23.7	21.2–26.2[a]	>100		4.7	4.0–5.4	79.4	61.7–102.1
VI	$(CH_2)_4CH(CH_2CH_3)CH_2CH_3$	16.5	13.2–20.8[a]	>100		4.9	4.4–5.5	23.6	20.6–27.0
VII	$(CH_2)_5CH(CH_2CH_3)CH_2CH_3$	27.4	21.7–34.8[a]	81.9	67.3–99.7	4.5	3.4–5.8[a]	66.0	56.1–77.6
VIII	$C(CH_3)_3$	>100		>250				Not prepared	
IX	$CH_2C(CH_3)_3$	>100		>250				Not prepared	
X	$(CH_2)_2C(CH_3)_3$	82.7	75.2–91.1	>250		35.3	33.3–37.5	250	
XI	$(CH_2)_3C(CH_3)_3$	36.4	33.2–39.8	>250		18.5	14.8–23.0[a]	76.8	65.9–89.5
XII	$(CH_2)_4C(CH_3)_3$	22.5	20.4–24.9	>100		7.1	6.6–7.7	37	30.3–41.5
XIII	$CH_2CH_2N(C_2H_5)_2$	>100[b]		535[b]		0.3[b]		100[b]	

[a] A χ^2 test at the 5% level showed that the data on which this value was based deviate significantly from linearity. [b] From O'Brien and Hilton (1964).

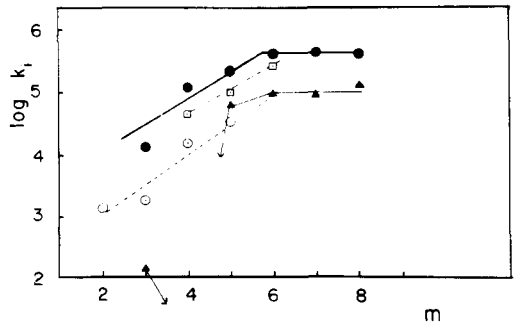


FIGURE 1: Relation between anticholinesterase potency and number of carbons in longest branch of main side chain (m). (●) *O,O*-Diethyl *S*-(ω-3-pentylalkyl)phosphorothiolates (series A), (▲) diethyl ω-(3-pentylalkyl) phosphates (series B), (⊡) *O,O*-diethyl *S*-(ω-*t*-butylalkyl) phosphorothiolates (series C), and (⊙) diethyl (ω-*t*-butylalkyl) phosphates (series D). Values of k_i below 10^2 cannot be determined accurately because of solubility limitations.

analog of Amiton, was only a little less potent than Amiton itself, in spite of the fact it had neither a formal charge on its polyalkylated carbon to bind it to the anionic site, nor any highly electrophilic groups to contribute toward the proper polarization of the P=O bond (Bracha, 1967). Presumably IIb, which would be an even worse phosphorylating agent than Amiton, must bind very well to the enzyme surface. In order to explore the nature of that surface and of the binding forces, we have prepared a variety of analogs of IIb and measured their anticholinesterase activity and toxicity.

Results

The compounds, whose syntheses are described under Experimental Section, are shown with their physical properties, yields, and analytical data in Tables I and II, and with their anticholinesterase potencies and toxicities to mice and houseflies in Tables III and IV. The compounds prepared included phosphorothionates, containing the P(S)O group; phosphorothiolates, containing the P(O)S group; and phosphates, containing the P(O)O group. The phosphorothionates had no significant anticholinesterase activity (*i.e.*, they were inactive at 10^{-3} M) nor did they have toxicity to mice or houseflies (*i.e.*, they were inactive at 100 mg/kg against mice or 250 mg/kg against flies). They will not be discussed further. The phosphates and phosphorothiolates were of two classes; class I (Table I) had a variety of side groups whose main chain was from three to eight carbons long of the general formula $(C_2H_5O)_2P(O)X(CH_2)_nCH(C_2H_5)_2$, (X = O, S). Class II (Table II) had a *t*-butyl head on its main side chain, separated from the P(O) or the P(O)S by from zero to four methylene groups.

Discussion

The Thiolo Effect. One unexpected finding (Table III) was that IIb, the carbon analog of Amiton, is only threefold more active than its phosphate analog IIa. This small difference is potency is generally found in the present series, with the dramatic exception of IV, in which the difference is more than 1780-fold, and III where it is 78-fold.

It has become widely held that in compounds of this class, phosphates are virtually inactive, being typically 10,000-fold less inhibitory than their phosphorothiolate analogs. Let us call this phenomenon the thiolo effect. The effect was first shown by Tammelin (1957) for 16 compounds with side chains of 2-trimethylammoniumethyl or 2-dimethylaminoethyl, and containing also $(CH_3)(C_2H_5O)P$ or $(CH_3)(C_3H_7O)P$ or $(C_2H_5O_2)P$. It was also observed for Amiton derivatives by O'Brien and Hilton (1964), with six compounds having side chains $CH_2CH_2NR_1R_2$, where R_1 and R_2 could be C_2H_5 or C_2H_4F. Phosphates and phosphorothiolates of this type differ also in susceptibility to alkaline hydrolysis, but the difference is relatively modest, being 16-fold (Tammelin, 1957) or 17-fold (Larsson, 1958) for $(CH_3O)_2P(O)XCH_2CH_2N^+(CH_3)_3$. A few phosphorothiolates hydrolyze two to eight times more slowly than their phosphates, *e.g.*, $(C_2H_5O)_2P(O)SC_2H_4SC_2H_5$ and its sulfone (Fukuto *et al.*, 1955), but, nevertheless, Heath (1961) provides data that in general the phosphorothiolates hydrolyze abnormally fast. Larsson (1958) points out that the inductive electrophilic effect for thioalkyl groups is actually smaller than that for alkoxy groups (Ingold, 1953). Nevertheless, the pK_a of SH compounds is much larger than of OH; thus ethanethiol and butanethiol are quite acidic, with pK_a's of about 10.7 (Ailman, 1965); and pK_a's of the leaving groups of phosphorus esters are excellently correlated with the alkaline hydrolysis rate constants of the phosphate esters (Heath, 1961).

The data of Table III show that in most cases (*i.e.*, seven pairs of compounds) the phosphorothiolates are three to five times more inhibitory than their phosphates. On the basis of the above discussion, it is plausible to assign this small thiolo effect to electronic effects which parallel differences in alkaline hydrolyzabilities. By contrast the 78-fold thiolo effect between IIIa and b and of more than 1800 between IVa and b would seem to require a special explanation. In this light the large thiolo effect described in the literature for 2-aminoethyl compounds seems to be a rather special case, and not a general phenomenon as has been previously thought.

An additional puzzle about the thiolo effect is that in the nitrogenous compounds, the effect is large when the nitrogen is separated from the PO group by a two-carbon chain; but in the precisely analogous all-carbon analog, the thiolo effect is small, and only when the above chain is shortened to one or to zero does one see a large thiolo effect. Unfortunately, for the nitrogenous compounds a series is not yet available which can enable one to study the thiolo effect as a function of leaving-group chain length. We will therefore restrict our discussion to the all-carbon compounds.

The phenomenon to be explained, then, is why the thiolo effect in compounds such as $(C_2H_5O)_2P(O)X$-

TABLE V: Kinetics of Cholinesterase Inhibition.[a]

Compound	$K_a \times 10^5$ (M)	k_2 (min^{-1})	k_i (min^{-1} mole^{-1})
Amiton (25°)	0.72 ± 0.18	6.66 ± 1.72	9.25×10^5
O,O-Diethyl *S*-(3-ethyl-1-pentyl) phosphorothiolate (IIb) (25°)	6.16 ± 0.53	2.58 ± 0.13	4.2×10^4
Diisopropyl phosphorofluoridate (25°)	117	40.7	3.48×10^4
O,O-Dimethyl *S*-1,2-bis(carbethoxy)ethyl phosphorothiolate (malaoxon) (37°)	77	11	1.42×10^4
3,5-Diisopropylphenyl *N*-methylcarbamate (37°)	0.34	1.38	4.07×10^5
o-Isopropoxyphenyl *N*-methylcarbamate (37°)	0.99	1.05	1.06×10^5
1-Naphthyl *N*-methylcarbamate (37°)	1.06	1.33	1.25×10^5

[a] Data for Amiton from Aharoni and O'Brien (1968), for malaoxon from Main (1964), for diisopropyl phosphorofluoridate from Main and Iverson (1966), and for carbamates from O'Brien *et al.* (1966). Data from Main's laboratory are for serum cholinesterase, remainder are for bovine erythrocyte acetylcholinesterase.

$(CH_2)_nCH(C_2H_5)_2$ is 78-fold when $n = 0$, 1800 when $n = 1$, and 3–5 for larger values of n. Inspection of Figure 1 shows that as n increases, the potency of the phosphorothiolates increases steadily, whereas that of the phosphates shows a profound drop when $n = 1$ (corresponding to $m = 4$). Presumably, therefore, the thiolo effect is due to some special aspect of the behavior of the phosphates which is not present in the phosphorothiolates. This behavior is not shown by all phosphate series; for example, the *t*-butylalkyl phosphates of Figure 1 show a fairly steady improvement up to the limiting size of a six-carbon main chain.

In considering series A alone, one possibility would be that there is a limited hydrophobic patch, A, within 5.4 Å of the esteratic site, and bounded by a barrier, such that alkyl groups extending more than 5.4 Å cannot bind to patch A. A second patch, B, is situated with its near edge about 7.5 Å from the esteratic site, so that only compounds with chains longer than 7.5 Å can bind to B. Thus the compound with $n = 1$ is too large to fit on to A, yet cannot extend to B.

A simple explanation of this type is insufficient to explain why the phosphorothiolates do not display a parallel phenomenon. The reason may lie in the substantially altered three-dimensional configuration of phosphorothiolates; given a fixed position of the phosphorus, the side chain of a phosphorothiolate is at an angle smaller by 9° than that of the corresponding phosphate, a difference caused by the valence angle of sulfur being 100° and of oxygen being 109°.

Structure–Activity Relations in the Nonbasic Compounds. The forces involved in binding the side chain can only be van der Waals or hydrophobic, and the latter are almost certainly more important in view of the relative nonspecificity of the structure–activity relation (Belleau and Lacasse, 1964). Figure 1 shows that for carbon analogs of Amiton (series A) and their phosphate analogs (series B) and that for ω-*t*-butylalkyl phosphorothiolates (series C) and phosphates (series D) the length of the principal carbon chain is of great importance. Extending it to six carbons gives a progressive increase. The change in free energy of binding per added methylene amounts to 660 cal for series A, 600 cal for series C, and 710 cal for series D. Brestkin *et al.* (1964) found for two series of *O*-ethyl methylphosphonthiolates an increase per added methylene of 840 cal for *S*-ω-*t*-butylalkyl compounds and 870 cal for *S*-*n*-alkyl derivatives. In a series of straight-chain *n*-alkyl phosphorothiolates we have found (Bracha and O'Brien, 1968) a value of 800 cal/methylene. Main and Hastings (1966) found for serum cholinesterase a value of 663 cal for malaoxon homologs and 685 cal for alkyl butyrates. Belleau and Lacasse (1964) calculate 650 cal for *n*-alkyltrimethylammoniums, and say that such effects are "entirely accountable on the basis of the contribution of hydrophobic interaction to binding, and not to van der Waals' forces." The free energy of transfer of a methylene group, under ideal conditions, from an aqueous to a nonpolar environment amounts to 730 cal (Belleau and Lacasse, 1964).

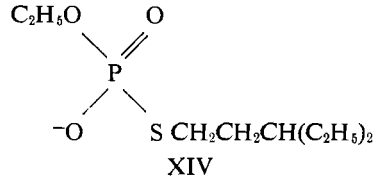
We conclude that in all the above cases, hydrophobic forces account entirely for the binding of the side chains to the enzyme. The cutoff at $m = 6$ in Figure 1 implies that the corresponding hydrophobic patch on the enzyme is of limited size.

Comparison of Amiton and Its Carbon Analog. Next let us consider the implications of the finding that at 38°, IIb is only six times less inhibitory than Amiton. As eq 1 indicates, both affinity factors (affecting K_a) and electronic factors (affecting k_2) must be considered. One would expect IIb to be a poor phosphorylator, for the pK_a of ethanethiol is 10.5–10.9 and of butanethiol is 10.7 (Ailman, 1965) and one would expect 3-ethylpentanethiol to have a similar value. By contrast, 2-diethylaminoethanethiol has a pK_a of 7.9 (Ooms, 1961). Main kinetic analysis (Table V), which had to be performed at 25° (see Experimental Section), shows that the k_2 for IIb is 2.6-fold less than that of Amiton. The K_a for IIb is 8.6-fold more than for Amiton, *i.e.*, the affinity is 8.6-fold less. The difference in the binding energies to cholinesterase of the two

compounds at 25° can be calculated (Webb, 1963), $\Delta\Delta F = 1.36 \log K_{a_1}/K_{a_2}$, and gives a value of 1.27 kcal/mole. Presumably this reflects primarily the contribution of the charge on Amiton (which is protonated at neutral pH). This is a very low contribution for an ionic bond, whose energy can be as high as 99 kcal/mole in sodium chloride (Weast *et al.*, 1964). One may calculate (Webb, 1963) that a charge separation of 8.7 Å is implied by a binding energy of 1.27 kcal/mole. An alternative possibility is that Amiton is bound to what we perhaps should call a second site (rather than a truly anionic site) by hydrogen bonding or by ion-induced dipole forces. The second possibility is attractive in that it might explain why, in the strictly analogous substrate situation, 3,3-dimethylbutyl acetate (the carbon isostere of acetylcholine) is almost as good a substrate for erythrocyte acetylcholinesterase as is acetylcholine itself (Adams, 1949), implying that the cationic nitrogen of choline does not make an extremely large contribution to binding.

From the K_a of Amiton one may calculate (from $\Delta F = RT \ln K_a$) a ΔF of 6.9 kcal/mole for the binding energy. The coulombic contribution, calculated above, is 1.27 kcal/mole, and therefore contributes only 18% to the total binding energy.

In spite of the fact that the variations discussed so far have all been due to steric variations, and have emphasized the binding aspect, these inhibitors act as phosphorylating agents, not as simple reversible competitive inhibitors. The evidence is, firstly, that inhibition is progressive; and secondly, that electronic features are of great importance, because desethylation of IIb to give XIV abolishes anticholinesterase activity; XIV has an I_{50} of 1.0×10^{-2} M (Aharoni and O'Brien, 1968). Such abolition is found in virtually all organophosphates, and is attributable to abolition of the electrophilic character of the phosphorus. Nevertheless,



XIV

under special conditions, the binding aspect can be emphasized. Thus, Heilbronn-Wikstrom (1965) found that at 0° with "phosphorylthiocholines and tertiary analogs" all of the inhibition could be reversed by dialysis or gel filtration, and was therefore probably due exclusively to reversible binding.

Table V also shows that Amiton and its carbon analog are unlike the familiar organophosphates, which have poor affinity and a high k_2, and more like the carbamates, which have high affinity and a low k_2. (Nevertheless, two carbamate analogs which were prepared, *i.e.*, $CH_3NHC(O)O(CH_2)_nCH(C_2H_5)_2$, with $n = 1$ and 2, were devoid of anticholinesterase activity at 10^{-2} M.) We conclude that the carbon analog of Amiton is a good inhibitor because its poor phosphorylating ability is compensated by its excellent affinity for acetylcholinesterase. This affinity is derived from hydrophobic interaction with a zone of limited size on the enzyme surface.

All of the discussion above has assumed that the changes in potency are associated with changes in binding energy caused by utilization of additional binding areas of the enzyme. In principle, it is hard to exclude an alternative view, that various changes in enzyme conformation are induced by various inhibitors. In addition, we have no proof that the K_a values are true thermodynamic dissociation constants, rather than complex ratios. Nevertheless, the finding that additional methylene groups add precisely the theoretical free energy for hydrophobic bonding, suggests that our assumptions are correct.

The toxicity of these compounds for mice was simply related to their anticholinesterase activity, the more inhibitory compounds being the more toxic. But only V and their higher homologs had any significant toxicity for houseflies. A similar selectivity has long been known for Amiton, and has been attributed to the ionizability of Amiton's side chain (O'Brien, 1963; O'Brien and Hilton, 1964). The finding that these carbon analogs behave similarly casts doubt upon that attribution.

Experimental Section

Anticholinesterase Inhibition. Anticholinesterase activity was determined by the ferric chloride–hydroxylamine colorimetric assay (Hestrin, 1949). Winthrop bovine erythrocyte cholinesterase was inhibited for 10 min at 38° at pH 7.4. Shorter inhibition periods and lower temperature were found necessary for performance of the main analysis (Main, 1964), because very high inhibitor concentrations were required. Enzyme solution was inhibited for 1 min at 25°, with the appropriate concentration of the organophosphorus compounds. In a typical experiment 0.4 ml of enzyme solution (about 16 units) in phosphate buffer (pH 7.4) was added at 37° to 0.1 ml of inhibitor solution of the appropriate concentration, mixed, and kept at 37° for exactly 10 min when 2 ml of 5.2×10^{-3} M solution of acetylcholine bromide in Tris buffer (pH 8.1) was added, mixed, and kept at 37° for 15 min. The resulting mixture (2 ml) was then pipetted to a Klett test tube, and 4 ml of a solution constituted of equal volumes of 14% hydroxylamine hydrochloride and 14% sodium hydroxide was added. Hydrochloric acid solution (2 ml) (1:2) was added followed by 2 ml of 10% ferric chloride in 0.1 N hydrochloric acid, and color intensity was measured at 540 mμ. K_a and k_2 and their standard errors were computed by the Wilkinson (1961) weighted regression procedure using a CDC 1604 computer. The bimolecular rate constants, k_i, were calculated from values of I_{50} (the concentration inhibiting by 50% in time t) by using Aldridge's expressions (1950).

$$k_i = \frac{0.695}{I_{50}t}$$

Toxicity. White mice were injected intraperitoneally

with the appropriate dose of inhibitor in 0.25 ml of propylene glycol. Toxicity to flies was determined by topical application of the appropriate dose of inhibitor in 1 μl of acetone, to duplicate groups of 20 insects. The insects were held at room temperature for 24 hr and mortalities were recorded. Failure to move spontaneously or upon stimulation was the criterion for death. The LD_{50} doses and 95% confidence limits (Table IV) were calculated by computer using an iterative process, involving a weighted linear regression based on probit analysis (O'Brien, 1967).

Synthesis. PREPARATION OF TRIALKYL PHOSPHATES, PHOSPHOROTHIOLATES, AND PHOSPHOROTHIONATES. To a 0.1-mole dispersion of sodium in 70 ml of benzene, was added 0.1 mole of the appropriate alcohol or thiol in 50 ml of benzene. Heat was provided when the initial reaction slackened and the mixture was refluxed until all the sodium was consumed. The resulting mixture was cooled to 0°, and 0.1 mole of diethyl chlorophosphate or diethyl chlorothiophosphate was added dropwise. After the addition was complete, the mixture was refluxed for 4 hr and then left overnight, and emptied into 200 ml of water. The benzene layer was separated and the water was extracted twice with ether. All organic phases were combined, washed with water, dried over magnesium sulfate, and fractionated. The physical data, yields, and analyses of the phosphorus compounds prepared, are summarized in Tables I and II. Infrared spectra were determined on a Perkin-Elmer Infracord spectrophotometer and the following peaks, at the wavelength (microcons) and intensity indicated, served to identify the individual compounds.

PHOSPHATES. Alkyl peaks were at 6.76 (medium), 7.12 (medium) (in ω-(3-pentylalkyl) phosphates), as well as 7.23 μ (medium) (in the ω-(*t*-butylalkyl) phosphates); 7.86 (strong, P=O), 8.50 (medium), 9.61, and 10.14 μ (strong, POC_2H_5).

PHOSPHOROTHIOLATES. Alkyl peaks unchanged were at 7.95 (strong, P=O), 8.50 (medium), 9.75, and 10.22 μ (strong, these peaks were usually better separated in phosphorothiolates than in the corresponding phosphates).

PHOSPHOROTHIONATES. Absence of P=O absorption was at 7.8–8.0. The physical data, yields, and analyses of the esters prepared are given in Tables I and II.

The Eisenlohr molar refraction, $MR_e = M \cdot n_D^{20}$, was compared with the calculated values obtained upon addition of individual bond refraction constants (Sayre, 1958; Vogel *et al.*, 1952).

INTERMEDIATES. The intermediate alcohols for the above reactions were obtained commercially in the case of 2-ethyl-1-butanol, which was purified according to Prout and Carson (1949); or made by treating the Grignard reagent of the appropriate bromoalkane with ethylene oxide in the cases of 4-ethyl-1-hexanol, 6-ethyl-1-octanol, and 5,5-dimethyl-1-hexanol; or made by reducing with lithium aluminum hydride (Sarel and Newman, 1956) the appropriate carboxylic acid (the latter being obtained by treatment of the Grignard reagent of the appropriate bromoalkane with CO_2, in the cases of 3-ethyl-1-pentanol and 5-ethyl-1-heptanol, and obtained commercially in the case of 3,3-dimethyl-1-butanol); or made from 4,4-dimethyl-1-bromopentane in the case of 4,4-dimethyl-1-pentanol, both of these compounds being described by Whitmore and Homeyer (1933).

The alkanethiols were prepared in every case by reacting the bromoalkane with thiourea in water, and then refluxing with sodium hydroxide. A typical procedure was as follows. A mixture of 25 g of 2-ethyl-1-bromobutane and 15 g of thiourea in 12 ml of water was stirred and refluxed. After 5 hr it became homogeneous; 3 hr later it was cooled, 12 g of sodium hydroxide in 120 ml of water was added, and reflux was resumed for 2 hr. After cooling, the upper layer was separated and dried; yield, 73%.

All the bromoalkanes were prepared by treatment of the corresponding alcohol with PBr_3, essentially as described by Noller and Dinsmore (1943). Analyses and boiling points of those alcohols and alkanethiols which were purified and have not previously been reported were as follows.

Anal. Calcd for 3-ethyl-1-pentanol: C, 72.39; H, 13.83. Found: C, 71.92; H, 13.47; bp 65–67° (15 mm); yield, 87%. Calcd for 4-ethyl-1-hexanol: C, 73.78; H, 13.93. Found: C, 73.64; H, 13.5; bp 179–183° (750 mm); yield, 57%. Calcd for 5-ethyl-1-heptanol: C, 74.93, H, 13.98. Found: C, 74.79; H, 13.88; bp 85–87° (9 mm); yield, 71%. Calcd for 6-ethyl-1-octanol: C, 75.88; H, 14.01. Found: C, 75.60; H, 13.96; bp 97–99° (9 mm); yield, 45%. Calcd for 5,5-dimethyl-1-hexanol: C, 73.78; H, 13.93. Found: C, 73.72; H, 13.89; bp 75–79° (18 mm); yield, 76%. Calcd for 2-ethyl-1-butanethiol: C, 60.95; H, 11.85. Found: C, 60.84; H, 11.79; bp 141–143° (750 mm); yield, 73%. Calcd for 3-ethyl-1-pentanethiol: C, 63.62; H, 12.17; S, 24.24. Found: C, 63.59; H, 12.02; S, 24.49; bp 92–93° (65 mm); yield, 78%. Calcd for 4-ethyl-1-hexanethiol: C, 65.68; H, 12.40. Found: C, 65.62; H, 12.28; bp 188–189° (750 mm); yield, 81%. Calcd for 3,3-dimethyl-1-butanethiol: C, 60.94; H, 11.94; S, 27.12. Found: C, 61.18; H, 11.84; S, 26.97; bp 62–63° (65 mm); yield, 74%. Calcd for 4,4-dimethyl-1-pentanethiol: C, 63.56; H, 12.19; S, 24.24. Found: C, 63.46; H, 12.10; S, 24.37; bp 154–156° (750 mm); yield, 45%.

O DESETHYLATION OF IIB. Desalkylation was performed by the method of Lecock and Todd (1954), yielding the desired product (XIV) in a 51% yield, along with 28% of unchanged starting material. The acidic ester was further purified by successive extractions with 0.05 N sodium hydroxide and showed a characteristic infrared pattern with peaks at 2.80, 4.23, 5.95, and 8.0 μ.

The slight shift of the P=O band absorption was characteristic and found in other similar desalkylations. By thin-layer chromatography on silica gel G plates, it was possible to further distinguish the desalkylated compound from its parent. Using the system *i*-propyl alcohol–water–ammonium hydroxide (75:24.5:05), R_F values were triester, 0.80–0.82; desalkylated compound, 0.58–0.60. Visualization was by bromine vapor.

Anal. Calcd for $C_9H_{21}O_3PS$: C, 44.98; H, 8.81. Found: C, 45.21; H, 8.91.

References

Adams, D. H. (1949), *Biochim. Biophys. Acta 3*, 1.

Aharoni, A. H., and O'Brien, R. D. (1968), *Biochemistry 7*, 1538 (this issue; preceding paper).

Ailman, D. E. (1965), *J. Org. Chem. 30*, 1074.

Aldridge, W. N. (1950), *Biochem. J. 46*, 451.

Belleau, B., and Lacasse, G. (1964), *J. Med. Chem. 7*, 768.

Bracha, P. (1967), *Israel J. Chem. 5*, 121.

Bracha, P., and O'Brien, R. D. (1968), *Biochemistry 7*, 1555 (this issue; following paper).

Brestkin, A. P., Godovikov, N. N., Godyna, E. I., Kabachnik, M. I., Mikhelson, M. Ya., Rozengart, E. V., and Yakovlev, V. A. (1964), *Dokl. Akad. Nauk SSSR 158*, 880.

Fukuto, T. R. (1957), *Advan. Pest Control Res. 1*, 147.

Fukuto, T. R., Metcalf, R. L., March, R. B., and Maxon, M. G. (1955), *J. Econ. Entomol. 48*, 347.

Heath, D. F. (1961), Organophosphorus Poisons, London, Pergamon.

Heilbronn-Wikstrom, E. (1965), *Svensk Kem. Tidskr. 77*, 3.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Ingold, C. K. (1953), Structure and Mechanism in Organic Chemistry, Ithaca, N. Y., Cornell University.

Kitz, R., and Wilson, I. B. (1962), *J. Biol. Chem. 237*, 3245.

Larsson, L. (1958), *Svensk Kem. Tidskr. 70*, 405.

Lecock, J., and Todd, A. R. (1954), *J. Chem. Soc.*, 2381.

Main, A. R. (1964), *Science 144*, 992.

Main, A. R., and Hastings, F. L. (1966), *Biochem. J. 101*, 584.

Main, A. R., and Iverson, F. (1966), *Biochem. J. 100*, 525.

Noller, C. R., and Dinsmore, R. (1943), Organic Synthesis, Coll. Vol. II, New York, N. Y., p 358.

O'Brien, R. D. (1960), Toxic Phosphorus Esters, New York, N. Y., Academic, p 84.

O'Brien, R. D. (1963), *J. Agr. Food Chem. 11*, 163.

O'Brien, R. D. (1967), *Bull. Environ. Contamination Toxicol. 2*, 163.

O'Brien, R. D., and Hilton, B. D. (1964), *J. Agr. Food Chem. 12*, 53.

O'Brien, R. D., Hilton, B. D., and Gilmour, L. P. (1966), *Mol. Pharmacol. 2*, 593.

Ooms, A. J. J. (1961), Ph.D. Thesis, University of Leiden, Leiden, Netherlands.

Prout, F. S., and Carson, J. (1949), *J. Org. Chem. 14*, 132.

Sarel, S., and Newman, M. S. (1956), *J. Am. Chem. Soc. 78*, 5416.

Sayre, R. (1958), *J. Am. Chem. Soc. 80*, 5438.

Tammelin, L. E. (1957), *Acta Chem. Scand. 11*, 1340.

Tammelin, L. E. (1958a), *Arkiv Kemi 12*, 287.

Tammelin, L. E. (1958b), *Svensk Kem. Tidskr. 70*, 157.

Vogel, A. I., Cresswell, W. T., Jeffrey, G. H., and Leicester, J. (1952), *J. Chem. Soc.*, 514.

Weast, R. C., Selby, S. M., and Hodgman, C. D., Ed. (1964), Handbook of Chemistry and Physics, Cleveland, Ohio, Chemical Rubber Co.

Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. I, New York, N. Y., Academic.

Whitmore, F. C., and Homeyer, A. H. (1933), *J. Am. Chem. Soc. 55*, 4555.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Wilson, I. B. (1960), *Enzymes 4*, 501.