Hydrophobic Bonding of Trialkyl Phosphates and Phosphorothiolates to Acetylcholinesterase*

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ABSTRACT: The effects of branching of an alkyl side chain at its head or on its intermediate carbons, on the anticholinesterase activity of trialkyl phosphates or phosphorothiolates is described. There is no evidence for any specially favorable kinds of locations of branching.

In general, added methylenes simply contribute to the total available for hydrophobic bonding; if they project away from the enzyme surface, they make little contribution, suggesting that folding of the enzyme does not occur during phosphorylation.

It has long been recognized that a full explanation of the variations in anticholinesterase activity among organophosphates must take into account at least two factors. One, upon which most work has been done, is the reactivity (usually as measured by its electronegativity) of the phosphorus atom, which determines the rate of the phosphorylation step. The second is the ease of binding between the inhibitor and enzyme, to form a complex prior to the phosphorylation step. The role of coulombic forces in binding to the anionic site of the enzyme in complex formation has usually been stressed, but recent work has emphasized the fact, clearly recognized in the early work of Wilson (1952), that noncoulombic forces may play a major role. It has been shown that there is, in the vicinity of the esteratic site of erythrocyte acetylcholinesterase, a hydrophobic area which is limited in size; for instance, in simple tri-*n*-alkyl phosphates or phosphorothiolates, progressive lengthening of one alkyl group led to a progressive increase in binding energy of about 700 cal/methylene until the group was six carbons long; further lengthening had no effect (Bracha and O'Brien, 1968b). As a result of these and related data it was proposed that acetylcholinesterase has a hydrophobic patch near its active site, of a limited size, large enough to accommodate six methylenes (Bracha and O'Brien, 1968b). Simultaneous and independent studies by Brestkin *et al.* (1964) and Abdubakhabov *et al.* (1968) have reached a similar conclusion for horse plasma cholinesterase, using data from five new series of phosphonothiolates. They note that at least a part of the hydrophobic area of the enzyme is separate from the "anionic site," for added methylenes have their predicted effects even in the series $(RO)(CH_3)P(O)SC_2H_4S^+(CH_3)C_2H_5$, in which the S^+ is probably bound to the anionic site.

Wilson (1952) showed that in inhibitory tetraalkylammoniums, one of the alkyl groups projects away from the enzyme, for it makes no bonding contribution. By contrast in analogous substrates, all the alkyl groups contributed; thus acetylcholine had about 560 cal more binding energy than dimethylaminoethyl acetate. The implication was that enzyme folding played a role in the case of substrates but not in the case of inhibitory alkylammoniums. Similarly, special efficacity has been attributed to strategically located *t*-butyl groups in organophosphates (Fukuto, 1957) and carbamates (Kolbezen *et al.*, 1954). Finally, the warfare agent soman is remarkable for its potency and for its pinacolyl group. All these considerations led us to explore the role of branching in the alkyl chain of diethyl alkyl phosphates and phosphorothiolates. In particular, we wanted to find if some particular kinds or locations of branching were unusually important in promoting potency; such a finding might throw light on the detailed topography of the active-site zone.

Results

We have prepared 13 new compounds. Eight of these (Table I) constitute two homologous series of four phosphates and four phosphorothiolates having an isopropyl head separated by one, two, three, or four carbons from the PO or PS groups. Two other compounds are "missing" members of an earlier series of phosphorothiolates, having a *t*-butyl head separated by zero or one carbon from the PS group. We therefore report herein on three newly completed series of compounds. The other three compounds (XI, XII, and XIII of Table II) were designed to have branching at the "base" rather than the "head" of the long alkyl group; one of them (XI) has a pinacolyl group, a constituent of one of the most toxic organophosphates, soman or pinacolyl methylphosphonofluoridate.

The structures, physical properties, and analyses of these compounds are presented in Tables I and II, their potency against acetylcholinesterase and their toxicity for mouse and houseflies in Table III; and their synthesis is described under Experimental Section. The corresponding data for other branched-chain compounds with which they are compared are from Bracha and O'Brien (1968a) and for unbranched compounds from Bracha and O'Brien (1968b). The variation of potency with chain length is shown in Figures 1 and 2.

In view of the very poor leaving-group character of the *O*-alkyl and *S*-alkyl groups, one has to consider whether these compounds are acting as phosphorylating agents like typical organophosphates, or as simple reversible inhibitors.

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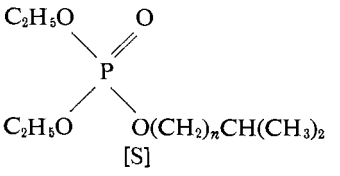
TABLE I: Physical Constants, Yields, and Analyses of *O,O*-Diethyl (ω-Isopropyl)alkyl Phosphates and Phosphorothiolates.



Compd	n	Yield (%)	Bp, °C (mm)	n_D^{20}	ΣMR_B[a]	ΣBR_E[b]	Formula	Calcd (%) C	Calcd (%) H	Found (%) C	Found (%) H
					A. Phosphates						
I	1	70	70–71 (0.09)	1.4125	296.93	297.46	$C_8H_{19}O_4$	45.71	9.11	45.99	9.08
II	2	65	75–76 (0.1)	1.4153	317.37	318.06	$C_9H_{21}O_4P$	48.20	9.44	48.34	9.45
III	3	55	84–85 (0.1)	1.4178	337.70	338.66	$C_{10}H_{23}O_4P$	50.41	9.73	50.25	9.78
IV	4	44	98–100 (0.1)	1.4202	358.31	359.26	$C_{11}H_{25}O_4P$	52.58	9.92	50.27	9.99
					B. Phosphorothiolates						
V	1	62	82–83 (0.07)	1.4550	329.24	331.55	$C_8H_{19}O_3PS$	42.46	8.46	42.41	8.24
VI	2	69	87–88 (0.1)	1.4583	350.44	352.15	$C_9H_{21}O_3PS$	44.98	8.81	45.37	8.76
VII	3	74	102–103 (0.1)	1.4585	370.95	372.75	$C_{10}H_{23}O_3PS$	47.23	9.12	46.98	9.10
VIII	4	64	109–111 (0.1)	1.4534	390.04	393.35	$C_{11}H_{25}O_3PS$	49.23	9.39	49.29	9.45

[a] Molar refraction found. [b] Molar refraction calculated from data of Sayre (1958) and Vogel *et al.* (1952).

Direct test (Figure 3) showed the inhibition reaction to be progressive; more specifically, it followed typical pseudo-first-order kinetics found with virtually all other organophosphates. Supporting evidence (Bracha and O'Brien, 1968a) that closely related compounds actually phosphorylate (presumably by an electrophilic attack) is (a) *O,O*-diethyl *S*-(3-ethyl-1-pentyl)phosphorothiolate inhibits in accordance with Main kinetics, *i.e.*, at high concentrations one reaches a saturation condition above which further concentration increases do not produce proportional increases in inhibition. Presumably, an equilibrium step is followed by a reactive step. (b) Replacement of P(O) by P(S) abolishes inhibitory activity in two alkoxy and two alkylthio compounds closely related to the present series. (c) Replacement of POC_2H_5 by PO^- abolishes the inhibitory activity of *O,O*-diethyl *S*-(3-ethyl-1-pentyl)phosphorothiolate (Aharoni and O'Brien, 1968).

Although phosphorylation probably occurs, experiments of the type described here cannot establish which is the leaving group. Probably it is the *S*-alkyl group in the case of phosphorothiolates.

As in the case of the previously reported compounds, all the present compounds show moderate toxicity to mice and very little toxicity to houseflies.

Discussion

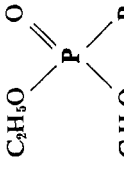
In the following discussion, it will be assumed that the variations in alkyl side chains have negligible effect upon the electronegativity of the phosphorus, but do modify the affinity for the enzyme surface. We shall use the designation m for the number of carbons in the longest continuous sequence in the alkyl side chain, counting from the PO; thus with a sequence $POCH(C_2H_5)_2$ we have $m = 3$. In Table I only, convenience requires that we use the term n for the number of methylenes separating the PO or PS from the isopropyl head.

In the two new series with an isopropyl head, progressive increase from $m = 3$ to $m = 6$ does indeed progressively increase potency, measured by k_i, the bimolecular reaction constant. If one assumes that all the improvement in k_i is due to improvement of binding, one may compute ΔF for each added methylene. In the phosphate series with an isopropyl head, the average ΔF was 780 cal, and for the phosphorothiolate series it was 580 cal. In the newly completed series of phosphorothiolates with a *t*-butyl head, the average ΔF was 760 cal. These are close to the value of 730 cal for transfer of a methylene from an aqueous to a nonpolar environment (Cohn and Edsall, 1943). Probably, therefore, the improvement caused by increasing m is due almost exclusively to hydrophobic bonding, as was concluded in the series previously examined.

In examining the four series of phosphorothiolates and four series of phosphates we have now synthesized, it is interesting that only in the phosphates with unbranched side chain or a 3-pentyl head does one see the dramatic trough in potency at $m = 4$. Perhaps "adverse binding" occurs in these two compounds, as shown for some chymotrypsin substrates (Huang and Niemann, 1952; Rapp *et al.*, 1966).

The phosphorothiolates are commonly two to five times more potent than their corresponding phosphates; for example, the potency ratios for the five pairs of *t*-butyl compounds are 1.6, 5.9, 2.9, 3.8, and 3.7. This small "thiolo effect" as we have called it, becomes large in a few cases only. The best example was the series with a 2-pentyl head when $m = 4$; as pointed out previously, the thiolo effect was over 1000-fold in this case. The new data confirm the prior conclusion that the thiolo effect, long known to be

TABLE II: Physical Constants, Yields, and Analyses of Branched *O,O*-Diethyl Alkyl Phosphates and Phosphorothiolates.



Compd	R	Yield (%)	Bp, °C (mm)	n_{20}^{D}	ΣMR_E	ΣBR_E	Formula	Calcd (%) C	Calcd (%) H	Found (%) C	Found (%) H
IX	$SC(CH_3)_3$	33	76–78 (0.1)	1.4600			$C_8H\ PO_3S$	42.46	8.46	42.26	8.36
X	$SCH_2C(CH_3)_3$	74	92–93 (0.5)	1.4574		352.15	$C_9H_{21}PO_3S$	44.98	8.81	45.15	8.78
XI	$OCH(CH_3)C(CH_3)_3$	39	75–76 (0.05)	1.4205	338.46	338.66	$C_{10}H_{23}O_4P$	50.41	9.73	49.96	9.97
XII	$SC(CH_3)_2CH_2CH_3$	48	79–80 (0.1)	1.4646		352.15	$C_9H_{21}PO_3S$	44.98	8.81	44.98	8.65
XIII	$OCH_2C(C_2H_5)_2CH_2CH_2CH_3$	47	117–119 (0.2)	1.4323	401.54	400.46	$C_{13}H_{29}O_4P$	55.69	10.43	55.28	10.49

TABLE III: Anticholinesterase Activity and Toxicity.

Compd	k_i (moles^{-1} min^{-1}) for Acetylcholinesterase	LD_{50} (mg/kg) Mice	LD_{50} (mg/kg) Houseflies
I	1.6×10^3	>100	>250
II	1.3×10^4	>100	>250
III	2.2×10^4	81	>250
IV	7.5×10^4	24	66
V	7.9×10^3	62	>250
VI	1.1×10^4	86	>250
VII	2.8×10^4	43	>100
VIII	1.4×10^5	7.3	17
IX	2.1×10^3	59	51
X	1.0×10^4	67	>250
XI	2.5×10^3	>100	>250
XII	3.9×10^4	21	89
XIII	1.7×10^5	8.2	>250

very large in closely related nitrogenous organophosphorus compounds with 2-dimethylaminoethyl (Tammelin, 1957) or 2-diethylaminoethyl (O'Brien and Hilton, 1964) leaving groups, is in fact a rather exceptional situation, rather than a general one as previously thought. In the new compounds having an isopropyl head, the thiolo effect was even smaller than we have previously encountered; the ratio of the potencies of PSR to POR was 4.9, 0.8, 1.3, and 1.9 for $m = 1, 2, 3$, and 4, respectively.

In spite of the very substantial variations in branching in the different series, all series tend to maximal potency of about log $k_i = 5.5$ for phosphorothiolates and log $k_i = 5.0$ for phosphates. This observation underlines the nonspecifity of the side-chain contribution, and speaks against any close matching of specially shaped side chain to specially shaped complementary surface in the enzyme's active site.

The compounds with isopropyl and with *t*-butyl heads show extremely similar activities for any value of *m*, both in the phosphate and the phosphorothiolate series. In the case of the phosphates, the potencies are in fact identical within the limits of experimental error. It follows that the additional methylene of the *t*-butyl compounds makes zero contribution to binding, nor does it block binding nor lead to adverse binding. It must project away from the enzyme surface as proposed by Wilson (1952) for the analogous alkylammoniums, but as was *not* found for the analogous substrates by Wilson. Wilson (1952) argued that "when we have binding at both the esteratic and anionic sites the 'extra' methyl group is not inert" (as judged by effects upon K_m). In the organophosphates described herein, it appears that the extra methyl group is "inert." We interpret this to mean that enzyme folding occurs in the binding or the acylation step with Wilson's substrates, but not in the binding or phosphorylation steps in our phosphates or phosphorothiolates or in Wilson's quaternary ammonium inhibitors.

Although one added methylene may be without effect (as in a comparison of isopropyl and *t*-butyl), two added methylenes may improve reactivity. For instance, the 3-pentyl series may be considered as an *n*-alkyl series with an ethyl

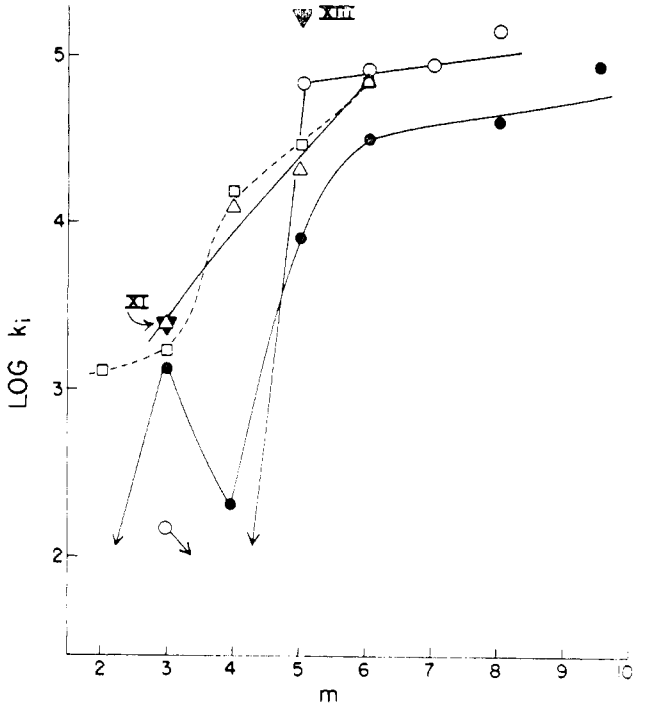


FIGURE 1: Relation between anticholinesterase potency and number of carbons in the longest chain (m) of diethyl alkyl phosphates. Alkyl group is ω-3-pentylalkyl (○), ω-t-butyl alkyl, ω-isopropylalkyl (△), n-alkyl (●), pinacolyl (▼, XI), or 2,2-ethylpentyl (▼, XIII). Values below log k_i = 2 could not be determined because of solubility limitation.

branch added, and Figure 2 shows for phosphorothiolates that (for any value of m) the 3-pentyl compound is superior to its n-alkyl analog. The same is true for the phosphorothiolates for values of m greater than 4; below that, the trough

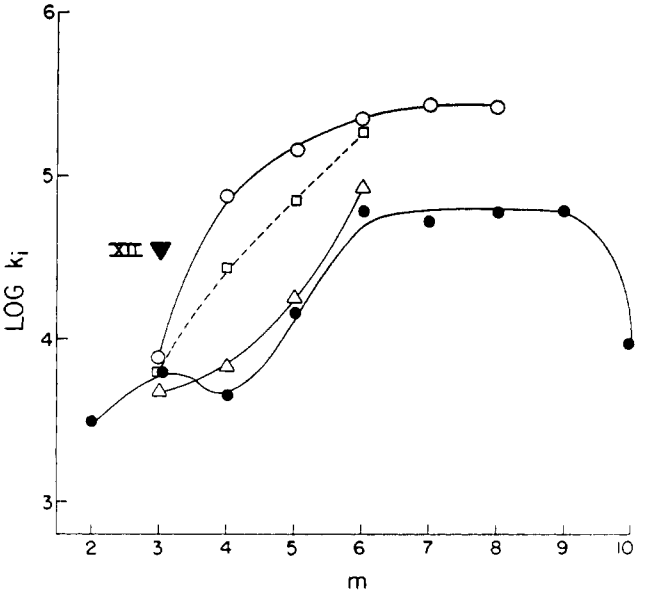


FIGURE 2: Values for diethyl S-alkyl phosphorothiolates. Compound XII has 1,1-dimethylpropyl as alkyl group. Remaining legend as for Figure 1.

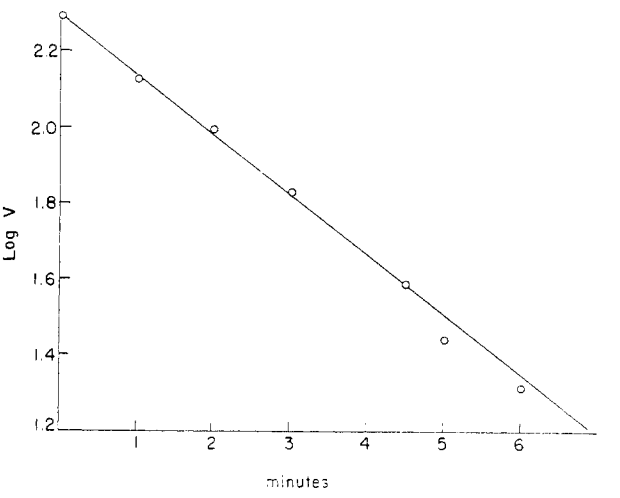


FIGURE 3: Progressive inhibition of acetylcholinesterase by VIII at 38° and pH 7.4 (pH-Stat method). V is velocity of reaction in arbitrary units.

effect is even more severe for the 3-pentyl than the n-alkyl series, perhaps because of very efficient adverse binding in the 3-pentyl compound. But the efficacy of two added methylenes does not imply folding of the enzyme during reaction; the ethyl group is long enough to permit its terminal methylene group to interact with the enzyme surface without such folding.

The phosphates (XI, XIII) with branches near the base of their alkyl side chain show no substantial improvement over analogs with terminal branching or with no branching; that is, they are not much better than compounds with equal m values. This constitutes evidence against the importance of any particular chain configuration. For the phosphorothiolate XII, its two basal methylenes improve it fourfold over analogs lacking them, giving a ΔF increment of 400 cal/methylene. They therefore make about half the contribution they would have if added terminally.

In general, the data presented herein argue against the existence of specially favorable kinds of locations of branching in conferring potency upon trialkyl phosphates. On the contrary, any differences in, for instance, an n-alkyl dialkyl phosphate and its t-butyl analog is attributable simply to the extra methylenes and hence extra hydrophobic forces in the t-butyl. And because of the limited extent of the postulated hydrophobic patch, the advantage of such extra methylenes is substantially lost if the n-alkyl portion is sufficiently large.

Experimental Section

Cholinesterase Inhibition and Toxicity Studies. Procedures for the estimation of anticholinesterase activity against bovine erythrocyte cholinesterase as well as for the evaluation of toxicity to mice and houseflies were previously described (Bracha and O'Brien, 1968a). It should be pointed out that the k_i values of Table III were derived from I_{50} values, as previously described.

Synthesis. Some of the compounds mentioned were described already in the previous parts of this work. Diethyl alkyl phosphates and the corresponding phosphorothiolates were prepared by reacting the appropriate alcohol or thiol

(as the sodium salt) with a molar quantity of diethyl chlorophosphate as described earlier by us. The physical data, yields and analyses of the phosphorus compounds prepared, are summarized in Table I and II.

The alcohols and the thiols for the above reactions were usually available commercially, except for 5-methyl-1-hexanol which was prepared by treating the Grignard reagent from 1-bromo-3-methylbutane with ethylene oxide. The unavailable thiols were usually prepared by reacting the corresponding bromoalkanes (Noller and Dinsmore, 1943) with thiourea. Neopentylmercaptan was prepared by the method of Bordwell *et al.* (1951).

Acknowledgments

Dr. Y. Chiu kindly performed the experiments summarized in Figure 3.

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