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Phosphorylation and Affinity Constants for the Inhibition of Acetylcholinesterase by Dimethoxon Analogs[1]

F. L. HASTINGS AND W. C. DAUTERMAN

USDA Forest Service, Southeastern Forest Experiment Station Forestry Sciences Laboratory, Research Triangle Park, North Carolina 27709 and Department of Entomology, North Carolina State University, Raleigh, North Carolina 27607

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Seventeen oxon-analogs of dimethoate were prepared and the *in vitro* cholinesterase inhibition constants (K_a, k_2, and k_i) and *in vivo* toxicities to mice and houseflies determined. The *in vitro* cholinesterase inhibition studies indictated that monosubstitution on the carbamoyl nitrogen primarily affected reactivity (k_2), whereas di-substitution affected affinity (K_a). Modification of the *O,O*-dialkyl moiety had little effect on affinity although reactivity increased proportionately with chain length. Conformational change in the enzyme surface was suggested as a possible explanation of these results. A distance of 2.7 to 4.7 Å between the carbonyl carbon and phosphorus atoms of these inhibitors appeared to best satisfy the spatial requirements of acetylcholinesterase. While most of the compounds investigated were highly toxic to mice, only compounds which were *N*-mono-substituted showed insecticidal activity. There was no definite correlation between *in vivo* and *in vitro* studies.

INTRODUCTION

A large number of carbamate and organophosphorus insecticides, acaricides, and nematocides contain the carbamoyl moiety either as an active inhibitory group or as part of the "leaving group." In most organophosphorus insecticides this moiety is associated with the leaving group, *i.e.*, dimethoate, Azodrin, Bidrin, phosphamidon, CIBA 2307, formothion, Fitios, Fac, and Tartan. These compounds have a broad spectrum of insecticidal properties; seven are also acaricidal, six possess systemic properties, and one, dimethoate, is both a systemic insecticide and systemic acaricide. The present investigation is restricted to organophosphorus compounds which are analogs of dimethoate, with the carbamoyl moiety in the leaving group. Our objective was to determine how variation in the *N*-alkyl and *N,N*-dialkyl portions of the carbamoyl moiety affected affinity (K_a) and rate of phosphorylation (k_2) of acetylcholinesterase. The measurement of these parameters was extended to include a series of substituted *O,O*-dialkyl analogs of dimethoxon and *O,O*-diethyl *S*-(*N*-methylcarbamoyl-1- or -2-ethyl) phosphorothiolate. An attempt was made to correlate the *in vitro* kinetic constants with the *in vivo* toxicities of these compounds to mice and houseflies.

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METHODS

Chemical Synthesis

The *O,O*-diethyl *S*-(*N*-alkylcarbamoylmethyl) phosphorothiolates and *O,O*-diethyl *S*-(*N,N*-dialkylcarbamoylmethyl) phosphorothiolates were prepared by refluxing in acetone equimolar amounts of sodium *O,O*-diethyl phosphorothioate with the various mono- and di-substituted *N*-alkyl chloroacetamides for 10 hr (1). The 2-chloro-*N*-alkyl and 2-chloro-*N,N*-dialkylacetamides were prepared by the method of D'Alelio and Reid (2). After cooling, the sodium chloride was filtered off and the acetone removed under vacuum. The residue was dissolved in chloroform and washed with water. The chloroform layer was dried over anhydrous sodium sulfate and removed under vacuum.

The *O,O*-dialkyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolates were prepared by refluxing the potassium salt of *O,O*-di-*n*-propyl phosphorothioate, *O,O*-di-*iso*-propyl phosphorothioate, and *O,O*-di-*n*-butyl phosphorothioate with 2-chloro-*N*-methylacetamide as described previously.

The *O,O*-diethyl *S*-(*N*-methylcarbamoyl-1-ethyl) phosphorothiolate and *O,O*-diethyl *S*-(*N*-methylcarbamoyl-2-ethyl) phosphorothiolate were prepared by reacting sodium *O,O*-diethylphosphorothioate with 2-bromo-*N*-methylpropionamide and 3-bromo-*N*-methylpropionamide, respectively, as described above.

The compounds were purified by molecular distillation and multimolecular column chromatography (3). The structure of the compounds was verified by infrared spectroscopy with a Perkin–Elmer 237B spectrophotometer. The purity of the compounds was determined by thin-layer chromatography on silica gel using benzene and methanol (9:1) as the solvent system. The compounds were detected using iodine vapors. The purity of the compounds was further checked by GLC using a column of 5% QF-1 on Diatoport S and a flame ionization detector. All compounds appeared to be >99% pure with the exception of compounds VII and XVI which were approximately 97%.

The percentage of phosphorus was determined as molybdovanadophosphoric acid (4) (Table 1). The generalized name and physical constants of the compounds are presented in Table 1.

Toxicity Evaluations

The toxicity of the dimethoxon analogs was determined by administering the compounds orally to 20- to 25-g female Dublin mice (ICR strain). The compounds were diluted in corn oil and force-fed at 0.2 ml per 25-g mouse. After treatment, the mice were checked hourly for about 6 hr and were observed for 2 days after the last deaths occurred. Contact insecticidal toxicity was based on 24-hr mortality counts after topical application of the insecticide solutions in 1.23 μl of acetone to individual 3- to 4-day-old female houseflies, *Musca domestica* (L.), NAIDM strain. The LD_{50} values for flies and mice were computed using the method of Finney (5) for probit analysis, which was adapted to a program for an IBM 360 computer.

Enzyme Assays

Acetylcholine hydrolase (EC 3.1.1.7) partially purified from bovine erythrocytes, was obtained from Sigma Chemical Company, St. Louis, Mo. Stock solutions were prepared in 10 m*M* sodium phosphate buffer, pH 7.6, to which 1 drop of toluene was added. Working solutions contained 4 units/ml (1 unit hydrolyzed 1 μmole acetylcholine per minute at pH 8.0, 37° C). No other salts were added. Acetylcholine chloride was obtained from Sigma Chemical Company. Assays were conducted in 3 m*M* substrate, pH 7.6, and 25° C.

TABLE 1

Physical constants of dimethoxon analogs

Compound	Percent yield	Phosphorus (%)		Boiling point	
		Theoretical	Found	C°	mm
N-alkyl analogs					
1 *O,O*-Diethyl *S*-(*N*-carbamoylmethyl) phosphorothiolate	30	13.63	12.84	120°	0.1
2 *O,O*-Diethyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate	52	12.84	12.53	130°	0.1
3 *O,O*-Diethyl *S*-(*N*-ethylcarbamoylmethyl) phosphorothiolate	41	12.13	12.71	135°	0.1
4 *O,O*-Diethyl *S*-(*N*-*n*-propylcarbamoylmethyl) phosphorothiolate	37	11.50	11.78	130°	0.05
5 *O,O*-Diethyl *S*-(*N*-*iso*-propylcarbamoylmethyl) phosphorothiolate	Supplied by Montecatini Edison, Milano, Italy				
6 *O,O*-Diethyl *S*-(*N*-*n*-butylcarbamoylmethyl) phosphorothiolate	38	10.93	10.19	140°	0.1
N,N-dialkyl analogs					
7 *O,O*-Diethyl *S*-(*N,N*-dimethylcarbamoylmethyl) phosphorothiolate	58	12.13	12.03	130°	0.05
8 *O,O*-Diethyl *S*-(*N,N*-diethylcarbamoylmethyl) phosphorothiolate	35	10.93	10.75	150°	0.2
9 *O,O*-Diethyl *S*-(*N,N*-*di*-*n*-propylcarbamoylmethyl) phosphorothiolate	32	9.48	9.44	120°	0.1
10 *O,O*-Diethyl *S*-(*N,N*-di-*iso*-propylcarbamoylmethyl) phosphorothiolate	41	9.48	9.28	150°	0.05
11 *O,O*-Diethyl *S*-(*N,N*-di-*n*-butylcarbamoylmethyl) phosphorothiolate	22	9.13	9.55	140°	0.15
O,O-dialkyl analogs					
12 *O,O*-Dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate	Supplied by American Cyanamid Co.				
13 *O,O*-Di-*n*-propyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate	43	11.47	10.60	120°	0.05
14 *O,O*-Di-*iso*-propyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate	21	11.47	10.85	130°	0.05
15 *O,O*-Di-*n*-butyl *S*-(*N*-methylcarbamoylmethyl) phosphorothiolate	28	10.42	9.75	130°	0.05
Branched analogs					
16 *O,O*-Diethyl *S*-(*N*-methylcarbamoyl-2-ethyl) phosphorothiolate	28	12.13	11.90	—	—
17 *O,O*-Diethyl *S*-(*N*-methylcarbamoyl-1-ethyl) phosphorothiolate	48	12.13	12.45	140°	0.05

Determination of K_a and k_2 values

The procedure of Main and Iverson (6) was followed. Inhibition rates were measured at pH 7.6 and 25° C in 5 m*M* sodium phosphate buffer. Enzyme (0.5 ml) was placed in one sidearm of the inhibition-reaction vessel and 0.5 ml of inhibitor in the other. Residual enzyme activity was determined using Brinkmann pH stats. Small amounts of ethanol were added to compounds IX, X, XI, XIII, and XV to enhance solubility. The alcohol never exceeded 1% in the enzyme–inhibitor mixture.

Statistics

The rates of the inhibition reactions with various concentrations of inhibitor were

followed by plotting the log of the velocity against time. The slopes were calculated by regression analysis yielding 2.3 $\Delta \log v/\Delta t$ or (ρ) and the standard errors. The (ρ) and (i) values were then substituted into the following equation and the K_a, k_2, and k_i values and their standard errors obtained by regression analysis according to Wilkinson (7):

$$\frac{i}{\rho} = \frac{i}{k_2} + \frac{1}{k_i}.$$

RESULTS AND DISCUSSION

Kinetic Constants

The K_a, k_2, and k_i values of the 17 analogs of dimethoxon are given in Table 2. Compounds I–XI were either mono- or di-substituted on the carbamoyl nitrogen. The substituents were hydrogen, methyl, ethyl, *n*-propyl, *iso*-propyl, and *n*-butyl or di-methyl, di-ethyl, di-*n*-propyl, di-*iso*-propyl, and di-*n*-butyl. Compounds XII–XV were substituted in the *O,O*-dialkyl moiety with similar substituents. Compounds XVI and XVII were analogs of compound II in which the bond distance between the sulfur and carbonyl carbon was increased by one methylene group (XVI), or an additional methyl group was added to the single methylene between the sulfur and carbonyl carbon (XVII).

TABLE 2

Affinity constants, phosphorylation rate constants, and biomolecular rate constants for dimethoxon analogs against acetylcholinesterase from bovine erythrocytes at 25° C, pH 7.6[a].

	Compound	K_a (mM)	SE (%)	k_2 (min^{-1})	SE (%)	k_i (M^{-1} min^{-1}) ($\times 10^2$)	SE (%)	Range (mM)	No. of concentrations
R = $(C_2H_5O)_2P(O)\cdot SCH_2C(O)$									
I	RNH_2	12.7	19.7	1.15	5.2	0.91	14.3	(5.6–54)	5
II	$RNHCH_3$	16.8	15.5	50.5	9.5	30.0	7.0	(2.5–20)	5
III	$RNHC_2H_5$	18.2	6.0	16.5	3.6	9.1	3.3	(1.0–30)	5
IV	$RNHn\text{—}C_3H_7$	16.1	10.6	14.7	6.1	9.1	4.4	(2.0–20)	5
V	$RNHiso\text{—}C_3H_7$	3.1	16.1	2.4	1.7	7.7	14.3	(5.0–49.5)	4
VI	$RNHn\text{—}C_4H_9$	18.2	10.4	20.5	6.3	11.3	4.4	(2.0–20)	5
VII	$RN(CH_3)_2$	2.90	12.1	15.5	2.6	53.8	10.0	(2.5–20)	5
VIII	$RN(C_2H_5)_2$	0.81	11.1	5.8	3.5	71.4	9.2	(0.1–3)	8
IX	$RN(n\text{—}C_3H_7)_2$	0.82	3.7	5.0	1.2	60.6	2.8	(0.5–3)	5
X	$RN(iso\text{—}C_3H_7)_2$	.28	53.6	.65	4.6	22.9	49.3	(0.5–5)	5
XI	$RN(n\text{—}C_4H_9)_2$	1.45	3.5	20.2	2.0	138.9	1.6	(0.25–1.5)	5
R = $P(O)SCH_2\text{—}C(O)\cdot NHCH_3$									
XII	$(CH_3O)_2R$	13.8	2.2	6.22	1.0	4.5	1.4	(5.0–30)	4
II	$(C_2H_5O)_2R$	16.8	15.5	50.5	9.5	30.0	7.0	(2.5–20)	5
XIII	$(n\text{—}C_3H_7O)_2R$	12.4	18.6	140.4	13.9	113.2	5.2	(.75–7.5)	6
XIV	$(iso\text{—}C_3H_7O)_2R$	20.9	24.9	1.46	17.8	0.69	7.2	(3.75–12.5)	5
XV	$(n\text{—}C_4H_9O)_2R$	6.9	13.0	188.4	7.5	273.3	6.7	(1.0–10)	5
XVI	$(C_2H_5O)_2P(O)S\text{—}CH_2\text{—}CH_2\text{—}C(O)NHCH_3$	17.2	33.1	0.79	11.4	0.47	21.3	(10.0–54)	6
XVII	$(C_2H_5O)_2P(O)S\text{—}CH(CH_3)\text{—}C(O)NHCH_3$	21.9	9.6	40.8	4.7	18.6	5.4	(5.0–40)	5

[a] Substrate velocity measurements were at pH 7.6 and 25° C using 3 mM acetylcholine chloride.

Aliphatic substituents in the leaving groups of both substrates and inhibitors have been demonstrated to contribute to binding by hydrophobic interaction (8–12). However, with the exception of the *N-iso*-propyl analog, the mono-substituted compounds (II–VI) showed uniformly low binding, as reflected by the high K_a values. This suggests that increasing the *N*-alkyl group has little influence on binding. The fact that the unsubstituted amine, compound I, bound better than the mono-substituted compounds lends support to this contention. By contrast, the lower K_a values of the di-substituted compounds (VII–XI) suggests that the second alkyl group contributed to better binding. On the average, these compounds bound 16-fold better than the mono-substituted compounds. Since the rate plots appeared to satisfy the criteria that inhibition was first order over the time course of the inhibition and further that the log v versus t plots passed through log v_0 at $t = 0$ (13), K_a was considered to be a measure of initial binding, *i.e.*, $K_a = k_{-1}/k_1$. Therefore, calculation of the free energy associated with each additional methylene for the di-substituted series seemed justified. The average ΔF/methylene was 700 cal, a value reasonably close to the theoretical binding energy expected from the hydrophobic binding of a methylene group (14). However, the effect of each additional methylene group on binding decreased as the chain length increased. Similar results have been reported for a series of alkoxycarbonyl malaoxon inhibitors and a comparably substituted alkyl butyrate substrate series (8).

In general, the phosphorylation rate constants (k_2) for the mono-substituted compounds (II–VI) were higher than the di-substituted compounds (VII–XI) and their overall inhibition, as reflected by k_i, was primarily dependent on k_2. The k_2 values of the di-substituted compounds (VII, VIII, IX, and X) were 3- to 4-fold lower than the comparable mono-substituted compounds while the K_a values were, respectively, 5.8, 22.5, 19.6, and 20 times smaller. These compounds are better inhibitors primarily due to good affinity. Compound XI, which was one of the best inhibitors studied, had a k_2 comparable to its mono-substituted analog while its K_a was approximately 13-fold smaller.

The similar K_a values of the *O,O*-dialkyl compounds (XII–XIV) indicated that lengthening of the phosphorylalkoxy portion of the molecule had only minor influence on binding. Compound XV, the di-*n*-butyl analog bound somewhat better than the other analogs, but its total binding energy was 3000 cal/mole or only 340 cal/mole higher than its closest analog, compound XIII. Thus, the two additional methylenes only accounted for a total of 240 cal/mole. Our results are similar to those of Chiu *et al.* (12) who measured the binding constants (K_a) for a series of similarly substituted *O,O*-dialkyl malaoxons and paraoxons. The uniform K_a values led them to suggest that the enzyme's acyl-binding site was fully occupied by one or both methyl groups in the phosphorylalkoxy portion of the molecule. This explanation seems equally plausible for our *O,O*-dialkyl analogs.

The phosphorylation rate constants (k_2), with the exception of the *iso*-propyl analog, increased with increasing chain length. This indicates that the larger phosphorylalkoxy members are able to influence reactivity although the acyl-binding site is completely occupied by the smaller substituent. There is apparently an attachment between the longer dialkyl members and the enzyme. It is doubtful that this attachment is hydrophobic since the energy associated with such bonding is much greater than that calculated from the equilibrium constants. At any rate, the attachment, whatever its nature, appears to modify the enzyme in a manner which affects the reactivity of the active site. The large variation in our phosphorylation rate constants (k_2) was somewhat surprising

since the *O,O*-dialkyl paraoxons and malaoxons (12) were remarkably similar. Another difference between the dialkyl malaoxons and paraoxons and our dimethoxon analogs involves the *iso*-propyl compound. Our *iso*-propyl analog bound as tightly as the other compounds yet its reactivity was almost nil (k_2 = 1.5 min^{-1}). The *iso*-propyl paraoxons and malaoxons bound less tightly than the other analogs, while their reactivity, in agreement with our compounds, was much lower. These results point out the dangers of generalizing between series of compounds, *i.e.*, what may be true for a series of malaoxons or paraoxons may not be true for a series of dimethoxons.

Compound XVI was prepared to determine whether the bond distance between the carbon of the carbonyl and phosphorus was critical to inhibition. In all compounds, except compound XVI, this distance was 2.7–4.7 Å. This distance closely approximates the 2.5–4.5 Å believed to be the distance between the anionic and esteratic sites of acetylcholinesterase (15). The distance between the carbonyl carbon and phosphorus atom for compound XVI was 3.3–6.0 Å. The 17.2 mM K_a of this compound indicated it bound as tightly as its closest analog, compound II, but once bound, it was quite unreactive (k_2 was almost nil). Apparently the carbonyl carbon became attached to or

TABLE 3

Toxicity of dimethoxon analogs to white mice and houseflies

Compound	k_i (M^{-1} min^{-1}) ($\times 10^2$)	Mice LD_{50} (mg/kg)	95% Confidence limit (mg/kg)	Housefly LD_{50} (mg/kg)	95% Confidence limit (mg/kg)	Mouse/housefly ratio
R = $(C_2H_5O)_2P(O)\cdot SCH_2C(O)$						
I RNH_2	0.91	90.9	(82.4–101.6)	38.35	(30.95–47.65)	2.4
II $RNHCH_3$	30.0	7.6	(5.0–11.8)	2.40	(1.80–3.15)	3.2
III $RNHC_2H_5$	9.1	16.1	(14.8–17.5)	8.05	(6.30–10.10)	2.0
IV RNH*n*-C_3H_7	9.1	24.4	(22.6–27.7)	64.65	(61.85–65.90)	.38
V RNH*iso*-C_3H_7	7.7	18.3	(17.5–19.1)	10.95	(8.45–13.45)	1.7
VI RNH*n*-C_4H_9	11.3	15.5	(9.8–18.9)	97.15	(74.75–118.20)	.16
VII $RN(CH_3)_2$	53.8	4.77	(4.30–5.13)	22.55	(18.45–27.80)	.21
VIII $RN(C_2H_5)_2$	71.4	17.8	(15.2–20.0)	21.85	(20.60–24.10)	.82
IX $RN(n\text{-}C_3H_7)_2$	60.6	29.2	(25.7–31.2)	29.65	(23.15–39.30)	.99
X $RN(iso\text{-}C_3H_7)_2$	22.9	52.3	(48.9–54.6)	64.00	(59.50–68.50)	.82
XI $RN(n\text{-}C_4H_9)_2$	138.9	20.5	(18.2–23.0)	34.90	(24.50–47.50)	.59
R = $P(O)SCH_2$—C(O)$NHCH_3$						
XII $(CH_3O)_2R$	4.5	24.2	(20.4–26.3)	0.345	(0.320–0.365)	70.1
II $(C_2H_5O)_2R$	30.0	7.6	(5.0–11.8)	2.40	(1.80–3.15)	3.2
XIII $(n\text{-}C_3H_7O)_2R$	113.2	84.2	(82.1–84.2)	>500	—	—
XIV $(iso\text{-}C_3H_7O)_2R$	0.69	414.7	(385.9–414.7)	>500	—	—
XV $(n\text{-}C_4H_9O)_2R$	273.3	61.2	(59.6–62.9)	>500	—	—
XVI $(C_2H_5O)_2\cdot P(O)S—CH_2—CH_2—C(O)NHCH_3$	0.47	126.2	(120.5–130.0)	>500	—	—
XVII $(C_2H_5O)_2\cdot P(O)S—CH(CH_3)—C(O)NHCH_3$	18.6	15.4	(10.2–16.7)	7.70	(3.10–20.35)	2.0

near the anionic site but the intervening distance to the phosphorus atom was too large for phosphorylation of the esteratic site. Chiu and Dauterman (16) reported similar results for the β-glutarate analog of malaoxon. Compound XVII, a branched-chain analog of compound II, showed only minor differences in binding and rate of phosphorylation. The results from compounds XVI and XVII demonstrate the importance of maintaining the P-S-C-carbonyl linkage for satisfying the spatial requirements of the enzyme.

Toxicity Data

In general, there is no definite relationship between the *in vitro* cholinesterase inhibition (k_i values) and the *in vivo* toxicity to mice and houseflies (Table 3). Such findings point out that simple relationships are rarely found between living and isolated systems.

A few points of interest regarding Table 3 are: (a) Except for compound XIV, all compounds are quite toxic to white mice. Compounds II and VII were the most toxic, with LD_{50} values of 7.6 and 4.8 mg/kg, respectively. (b) Only six compounds showed a mouse/fly ratio of greater than one (I, II, III, V, XII, and XVII). Compound XII (dimethoxon) was most selective with a ratio of 70 while the other compounds had ratios of only 2 to 3. With the exception of the amine (I), these compounds were all mono-substituted on the carbamoyl nitrogen. (c) The toxicity of compound I, the unsubstituted amine, was 12 and 16 times lower than compound II to mice and houseflies, respectively. In contrast, other workers (17, 18) reported that the unsubstituted amines of phosphamidon and Bidrin were more toxic than the parent compounds. In the case of dimethoate (19), the unsubstituted amine was more toxic than either the parent compound or its oxygen analog. The contrasting results might be explained by differences in the *O,O*-di-alkyl moieties (di-ethyl versus di-methyl). In any case, the *in vitro* cholinesterase inhibition of compound I corroborates the toxicity data.

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REFERENCES

1. O. Foss, Di-*O*-alkylmonothiophosphates and di-*O*-alkylmonoselenophosphates and the corresponding pseudohalogens, *Acta Chem. Scand.* **1,** 8 (1947).
2. G. F. D'Alelio and E. E. Reid, A series of *N*-methyl amides, *J. Amer. Chem. Soc.* **59,** 109 (1937).
3. G. G. Patchett and G. H. Batchelder, Multimolecular adsorption chromatography for purification of gram quantities of pesticides, *J. Agr. Food Chem.* **9,** 395 (1961).
4. C. J. Barton, Photometric analysis of phosphate rock, *Anal. Chem.* **20,** 1068 (1948).
5. D. J. Finney, "Probit Analysis, A Statistical Treatment of the Sigmoid Response Curve," Vol. 13, p. 256, Cambridge University Press (1952).
6. A. R. Main and F. Iverson, Measurement of the affinity and phosphorylation constants governing irreversible inhibition of cholinesterases by di-isopropyl phosphorofluoridate, *Biochem. J.* **100,** 525 (1966).
7. G. N. Wilkinson, Statistical estimations in enzyme kinetics, *Biochem. J.* **80,** 324 (1961).
8. A. R. Main and F. L. Hastings, A comparison of acylation, phosphorylation and binding in related substrates and inhibitors of serum cholinesterase, *Biochem. J.* **101,** 584 (1966).
9. P. Bracha and R. D. O'Brien, Trialkyl phosphate and phosphorothiolate anticholinesterases. I. Amiton analogs, *Biochemistry* **7,** 1545 (1968).
10. P. Bracha and R. D. O'Brien, Trialkyl phosphate and phosphorothiolate anticholinesterases. II. Effect of chain length on potency, *Biochemistry* **7,** 1555 (1968).
11. P. Bracha and R. D. O'Brien, Hydrophobic bonding of trialkyl phosphates and phos-

phorothiolates to acetylcholinesterase, *Biochemistry* **9,** 741 (1970).

12. Y. C. Chiu, A. R. Main, and W. C. Dauterman, Affinity and phosphorylation constants of a series of *O,O*-dialkyl malaoxons and paraoxons with acetylcholinesterase, *Biochem. Pharmacol.* **18,** 2171 (1969).
13. A. R. Main, Kinetics of cholinesterase inhibition by organophosphate and carbamate insecticides, *Can. Med. Ass. J.* **100,** 161 (1969).
14. C. Tanford, "Physical Chemistry of Macromolecules," p. 130. Wiley, New York (1961).
15. W. B. Neely, The use of molecular orbital calculations as an aid to correlate the structure and activity of cholinesterase inhibitors, *Mol. Pharmacol.* **1,** 137 (1965).
16. Y. C. Chiu and W. C. Dauterman, The affinity and phosphorylation constants of a series of branched-chain homologs of diethyl malaoxon and acetoxon with acetylcholinesterase, *Biochem. Pharmacol.* **18,** 1665 (1969).
17. G. P. Clemons and R. E. Menzer, Oxidative metabolism of phosphamidon in rats and a goat, *J. Agr. Food Chem.* **16,** 312 (1968).
18. R. E. Menzer and J. E. Casida, Nature of toxic metabolites formed in mammals, insects, and plants from 3-(dimethoxyphosphinyloxy)-*N,N*-dimethyl-*cis*-crotonamide (Bidrin) and its *N*-methyl analogue (SD-9129), *J. Agr. Food Chem.* **13,** 102 (1965).
19. G. W. Lucier and R. E. Menzer, Nature of oxidative metabolites of dimethoate formed in rats, liver microsomes, and bean plants, *J. Agr. Food Chem.* **18,** 698 (1970).