Helv. Chim. Acta 48, 1546.
Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed, New York, N. Y., Academic, p 327.
Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959), *J. Biol. Chem. 234*, 2627.
Flavin, M. (1965), *J. Biol. Chem. 240*, PC 2759.
Flavin, M., and Slaughter, C. (1964), *Biochemistry 3*, 885.
Glover, D. J., and Landsman, S. G. (1964), *Anal. Chem. 36*, 1690.
Gould, E. S. (1959), Mechanism and Structure in Organic Chemistry, New York, N. Y., Holt, Rinehart and Winston, p 372.
Horecker, B. L., Rowley, P. T., Grazi, E., Cheng, T., and Tchola, O. (1963), *Biochem. Z. 338*, 36.
Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.
Mehler, A. H. (1963), *J. Biol. Chem. 238*, 100.
Metzler, D. E., Ikawa, M., and Snell, E. E. (1954), *J. Am. Chem. Soc. 76*, 648.
Riordan, J. F., and Christen, P. (1968), *Biochemistry 7*, 1531 (this issue; preceding paper).
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966), *J. Am. Chem. Soc. 88*, 4104.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 358.
Rose, I. A., and O'Connell, E. L. (1967), *Arch. Biochem. Biophys. 118*, 758.
Rose, I. A., O'Connell, E. L., and Mehler, A. H. (1965), *J. Biol. Chem. 240*, 1758.
Rutter, W. J., Woodfin, B. M., and Blostein, R. E. (1963), *Acta Chem. Scand. 17, Suppl. 1*, 226.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

The Inhibition of Acetylcholinesterases by Anionic Organophosphorus Compounds*

A. H. Aharoni† and R. D. O'Brien

ABSTRACT: Data on seven pairs of organophosphates confirm the view that O desalkylation normally reduces anticholinesterase activity profoundly; the average reduction was 158,000-fold. By contrast, O desalkylation of certain tertiary amine-containing organophosphates only reduces activity by 60- to 292-fold. Thermodynamic and kinetic studies on the inhibition process, coupled with analog synthesis and studies on pK_a, on the nature of the inhibited enzyme, and on joint inhibition of cholinesterase by selected pairs of organophosphates, suggest that internal salt formation can reduce the anionicity of certain desalkylated organophosphates, and permit them to be effective anticholinesterases.

It is well established that the organophosphates inhibit AChE[1] by phosphorylating its esteratic site (Wilson, 1951; Aldridge and Davison, 1953; O'Brien, 1960). Recently the role of complex formation prior to phosphorylation has been indicated and evaluated (Main, 1964; Main and Hastings, 1966; Main and Iverson, 1966). The terminology of eq 1 will be employed, where P is the dialkyl phosphoryl group, X the leaving group, and E the enzyme. Main (1964) has shown that

$$E + PX \underset{}{\overset{K_a}{\rightleftharpoons}} EPX \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad (1)$$

the apparent bimolecular rate constant as normally measured, k_i, is equal to k_2/K_a, and would better be called the bimolecular reaction constant. The k_3 (reactivation) step is slow enough to be ignored in most cases, except where reactivators are employed.

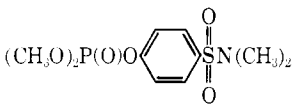
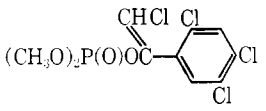
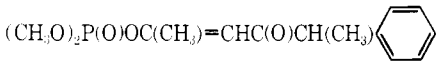
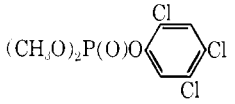
Because the phosphorylation step involves an electrophilic attack by the phosphorus, its rate is promoted by electrophilic substituents attached to the phosphorus. For the same reason, hydrolytic removal of one of the ester groups of these phosphotriesters profoundly reduces anticholinesterase activity, because it has the effect of attaching an anion to the phosphorus, for the POH group exists virtually entirely as PO^- at physiological pH. This fact, though very poorly documented, is the basis of the general view that such a hydrolysis (for instance, in metabolism of these compounds) constitutes virtually a total detoxication. We were therefore surprised to note that compounds such as $(C_2H_5O)(HO)P(O)SCH_2CH_2N^+(C_2H_5)_2(CH_3)$ had "unusual effectiveness" as insecticides (Snyder, 1960). Similarly, Heilbronn-Wikström (1965) reported good anticholin-

* From the Department of Entomology and Section of Neurobiology and Behavior, Cornell University, Ithaca, New York. *Received May 26, 1967.* This study was supported in part by Grant GM-07804 of the Public Health Service.

† This work was submitted for partial fulfillment of the requirements for the Ph.D. degree at Cornell University.

[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: AChE, acetylcholinesterase; 2-PAM, 2-pyridinealdoxime methiodide.

TABLE I: Loss of Anticholinesterase Activity[a] by Desalkylation.[b]

Compd	Formula	Enzyme	k_i (l. mole^{-1} min^{-1}) Parent	k_i (l. mole^{-1} min^{-1}) *O*-Desalkyl Derivative	Ratio k_iParent: k_iDesalkyl
	A. Compounds Not Containing Amino Group				
1		Fly heads	8.7×10^5	1.73	503,000
2		Fly heads		1.5×10^2	
		Red cells		4.0	
3	$(CH_3O)_2P(O)OC(CH_3)=CHC(O)N(CH_3)_2$	Fly heads	4.95×10^4	2.47	20,000
4		Fly heads	4.3×10^6	2.9×10^2	15,000
		Red cells	3.5×10^4	1.73	20,000
5	$(CH_3O)_2P(O)OCH=CCl_2$	Fly heads	6.9×10^6	6.3×10^2	10,950
		Red cells	2.77×10^4	0.99	28,000
6	$(CH_3O)_2P(O)SCH_2(O)NHCH_3$	Fly heads	9.24×10^5[c]	1.39	665,000
7		Red cells	2.3×10^4	3.85	6,000
8	$(C_2H_5O)_3PO$	Fly heads		0.7	
9	$(C_2H_5O)_2P(O)SCH_2CH_2CH(C_2H_5)_2$	Red cells	2.3×10^5	6.9	33,000
	B. Compounds Containing Amino Group(s)				
10	$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$	Fly heads	6.9×10^6	3.85×10^4	179
		Red cells	1.4×10^6	4.8×10^3	292
11	$(C_2H_5O)_2P(O)SCH_2\overset{+}{C}H_2N(C_2H_5)_3$	Red cells	6.9×10^6	86.6	80,000
12	$(C_2H_5O)_2P(O)SCH[CH_2N(CH_3)_2]_2$	Red cells	4.1×10^5	6.9×10^3	60
13	$(C_2H_5O)_2P(O)OCH_2CH_2N(C_2H_5)_2$	Red cells	1.98		

[a] American Cyanamid Co. kindly donated Famoxon (**1**), PO analog of dimethoate (**6**), and their *O*-desalkyl analogs. Shell Development Co. kindly donated SD8447 (**2**), dichlorvos (**5**), Bidrin (**3**), Ciodrin (**4**), and their *O*-desalkyl analogs. Dow Chemical Co. kindly donated ronnel (**7**) and its *O*-desmethyl analog. Chipman Chemical Co. kindly donated Amiton (**10**). C-Amiton (**9**), quaternary Amiton (**11**), amino-Amiton (**12**), *O*-Amiton (**13**), and their *O*-desethyl analogs were synthesized as described in the Experimental Section. [b] 38°, pH 7.4. [c] Calculated from Uchida *et al.* (1965).

esterase activity in the phosphonate $(C_2H_5)(HO)P(O)SCH_2CH_2N(C_2H_5)_2$.

The present study was initiated to explore these unexpected observations. We have used the compound Amiton, $(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$, which we also found to lose relatively little anticholinesterase activity upon O desethylation.

Experimental Section

Synthesis. Table I indicates that most of the compounds were donated by various companies. C-Amiton (Table I, **9**) and its *O*-desethyl analog were prepared by P. Bracha and are described by Bracha and O'Brien (1968). The detailed synthesis, purifications, and physical properties, including elemental analyses, infrared spectra, and chromatographic behavior of the following compounds are described by Aharoni (1967). Compound **11**, the quaternary salt of Amiton, was prepared by reacting Amiton with ethyl iodide; compound **12**, amino-Amiton, was prepared by chlorinating bis(dimethylaminomethyl)methanol with $SOCl_2$ and treating the product with $(C_2H_5O)_2POSK$. Desethylation of Amiton (**10**) and of compounds **11** and **12** are performed according to Snyder (1960).

Inhibition. The enzymes used were Winthrop bovine red blood cell AChE and homogenates of fly heads, harvested according to Moorefield (1957) from mixed sexes of the Wilson strain, 2- to 3-days old. Enzyme solutions were always iced prior to use. The buffer,

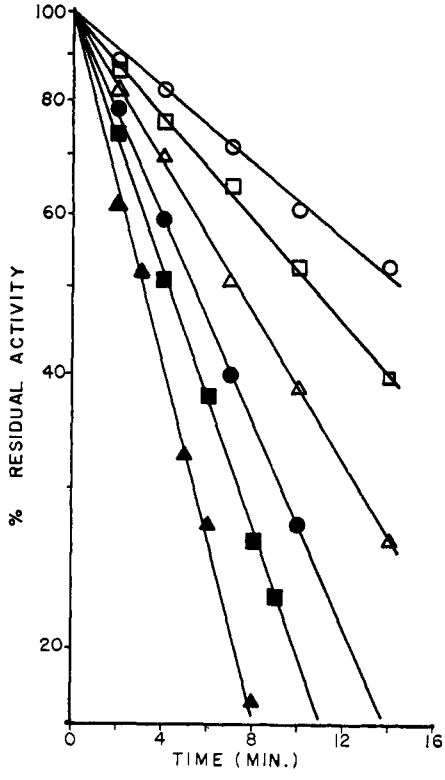


FIGURE 1: Inhibition of red blood cell AChE by Amiton (2.5×10^{-7} M) at various temperatures. (○) 5°, (□) 10°, (△) 15°, (●) 20°, (■) 25°, and (▲) 30°.

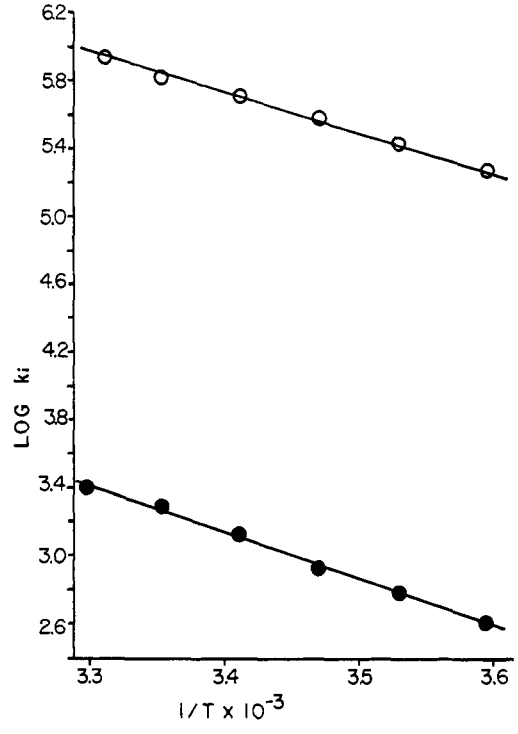


FIGURE 2: Temperature dependence of inhibition of red blood cell AChE by Amiton (2.5×10^{-7} M) (○) and *O*-desethyl-Amiton (6×10^{-5} M) (●).

except where otherwise stated, was phosphate (0.067 M, pH 7.4). For the data of Table I and Figures 6 and 7, 0.4 ml of enzyme in buffer (10% house fly heads or 0.8 mg/ml of red blood cell AchE) was added to 0.1 ml of prewarmed inhibitor in buffer, incubated for 10 min at 38°, and then the reaction was stopped by adding 2 ml of acetylcholine bromide to give a final concentration of 4.25×10^{-3} M. After 15 min at 38°, 2 ml of this mixture was assayed for acetylcholine by the Hestrin (1949) procedure.

All subsequent work used red blood cell AChE only. For thermodynamic studies, 0.3 ml of prewarmed inhibitor in buffer was added to 2.7 ml of prewarmed enzyme (1.12 mg/ml of buffer) giving final concentrations for Amiton of 2.5×10^{-7} M and for desethyl-Amiton of 6×10^{-5} M. After various times at the appropriate temperature, 0.4 ml of the mixture was added to 2 ml of prewarmed acetylcholine bromide (final concentration 4.25×10^{-3} M) and the same procedure as above was then employed.

For kinetic studies, 25° was employed for the inhibition phase rather than 38°, in order to permit the use of higher inhibitor concentrations. Prewarmed enzyme, 0.5 ml containing 0.8 mg/ml in buffer, was added to 0.5 ml of prewarmed inhibitor, and incubated for periods between 6 sec and 2 min, after which acetylcholine bromide was added for a final concentration of 4.25×10^{-3} M. When the period was less than 20 sec, the inhibition reaction vessel of Main and Iverson (1966) was used. The volume of substrate added varied between 4 and 18 ml, the larger volumes being needed in some cases to dilute the inhibitor in order to minimize further inhibition of the enzyme. Subsequent procedures were as described above, at 38°.

For the reactivation studies a modification of the method of Scaife (1959) was used. This employs Veronal buffer, which lessens the loss of activity which occurs in phosphate buffer with relatively long incubation times (thus at 25°, in the absence of substrate, the half-life of the enzyme was about 80 min, at which time only 10% of activity was lost in Veronal). Enzyme (10 mg of AChE in 0.8 ml of Veronal buffer) was mixed with 0.2 ml of inhibitor in Veronal buffer and incubated at 38°. Where possible, an inhibitor concentration 100 times greater than that which gave 50% inhibition in 10 min was used, and an incubation time of 5 min was employed. In other cases, an inhibitor concentration of 3 or 5×10^{-1} M was used with an incubation time of 10 min. Under either of these conditions, inhibition was virtually complete. The mixture (0.5 ml) was passed through a column of 0.5 g of Sephadex G-50 (fine) in a 10-ml graduated pipet and eluted with Veronal buffer under 1 psi, 0.5-ml fractions being collected. Amiton and the AChE were first examined separately, and it was shown that the third fraction contained most of the enzyme and no Amiton. The third fraction was therefore routinely selected, added to 4.5 ml of Veronal buffer, then mixed with 15 ml of 5×10^{-3} M acetylcholine bromide and 1 ml of 2.1×10^{-3} M 2-PAM. The reaction was followed at 25° in an automatic titrator (Radiometer TTTlc) using 0.1 M KOH. In each run, enzyme without inhibitor was first employed, then enzyme plus inhibitor was employed, using the same column; each point in Figures 4 and 5 represents the

activity of the inhibited enzyme sample at any time as a percentage of the activity of the uninhibited enzyme sample at that time.

In the work using mixed inhibitors the calculated line was obtained from the consideration (Bray and White, 1957) that when two simultaneous first-order reactions with constants k_1 and k_2 deplete a compound, its disappearance is governed by simple first-order kinetics with a composite first-order constant ($k_1 + k_2$).

pK_a Determination. For Amiton, 25 ml of 1 M compound was titrated with 10 M NaOH by buret, using a Radiometer PHM22V pH meter. For desethyl-Amiton, little material was available, and 5 ml of 0.1 M was titrated with 2.5 M NaOH using an Agla syringe.

Results

Inhibitory Potency. Table I shows the k_i values for a variety of organophosphates. Desalkylation reduces the inhibitory potency of compounds without an amino group, to the extent of 6000- to 665,000-fold. The k_i values for the desalkyl compounds should be regarded as upper limits (and therefore the ratios given are lower limits) because even minor contamination by parent compound would profoundly raise the k_i, *e.g.*, all the inhibitory potency reported for the desalkyl derivative of **7** could be caused by less than 0.1% of such contamination. In the case of diethyl phosphate also, the true k_i is probably lower than shown, because enzyme precipitation occurred with the high phosphate concentration required.

Table I shows that, by contrast, O desalkylation of Amiton (**10**) has a relatively small effect upon inhibitory activity, *e.g.*, 292-fold on red cell AChE. It was hypothesized that the reason for Amiton's behavior on desalkylation was that its amino group formed an internal salt with the phosphate anion, thus reducing the anionicity. One approach to confirmation of this hypothesis involved synthesis of various Amiton analogs, whose k_i's are given in Table I. Their significance will be discussed below. Because of the contamination problem described above, the absence of parent material in the *O*-desalkyl samples was important, and was achieved by separation of the compound on a thin-layer chromatograph.

Thermodynamics of Inhibition. Amiton and *O*-desethyl-Amiton both inhibit acetylcholinesterase in accordance with pseudo-first-order kinetics; an example is given in Figure 1. From studies at various temperatures, an Arrhenius plot was prepared (Figure 2). The graphs were interpreted according to Webb (1963) as

$$\ln k_1 = \ln (k/h) + \ln T + \Delta S^*/R - \Delta H^*/RT \quad (2)$$

where k is the Boltzmann constant, h the Planck constant, R the gas constant, T the absolute temperature, and ΔH^* and ΔS^* the enthalpy and entropy, respectively, of the activated complex. Regression analysis gave for Amiton: $\Delta H^* = 9.874 \pm 0.632$ kcal and $\Delta S^* = +1.16$ eu; for *O*-desethyl-Amiton: $\Delta H^* = 12.044 \pm 0.606$ kcal and $\Delta S^* = 3.23$ eu. The plus and minus values are the standard errors; as is customary the errors for ΔS^* are not given and the actual values are "not to be taken seriously" (Laidler, 1955); but the signs are of importance.

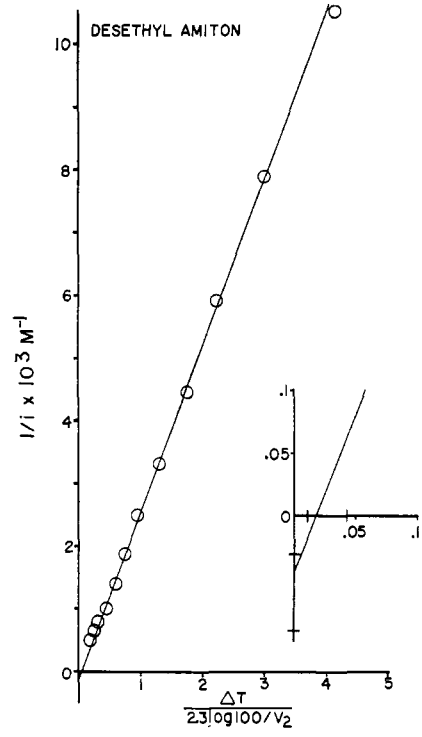


FIGURE 3: Main plot of inhibition of red cell AChE at 25° by *O*-desethyl-Amiton at various concentrations. V_2 is the percentage enzyme activity after incubation of enzyme and inhibitor for Δt (min). The right-hand-side plot is an enlargement of the x and y axes on which the standard errors are marked.

Kinetics of Inhibition. The procedure of Main (1964) was used to calculate K_a and k_2 of eq 1, by plotting $1/i$ against $t/2.3\Delta \log V$, since

$$\frac{1}{i} = \frac{t}{2.3\Delta \log V} \frac{k_2}{K_a} - \frac{1}{K_a}$$

where i is inhibitor concentration, t the incubation time, and $\Delta \log V$ the change in the logarithm of the

TABLE II: The Affinity Constant (K_a) and the Phosphorylation Constant (k_2).[a]

Compound	K_a (M)	k_2 (min^{-1})
Amiton	7.16×10^{-6} (26)	6.66 (26)
Desethyl-Amiton	2.18×10^{-2} (50)	54.0′ (56)
Quaternary Amiton	2.48×10^{-5} (46)	115.3′ (64)
C-Amiton	4.48×10^{-5} (24)	4.49 (31)
Amino-Amiton	1.2×10^{-4} (13)	55.33 (12)
Desethylamino-Amiton	1.14×10^{-3} (20)	4.1 (17)

[a] The standard errors are in parentheses, expressed as percentages.

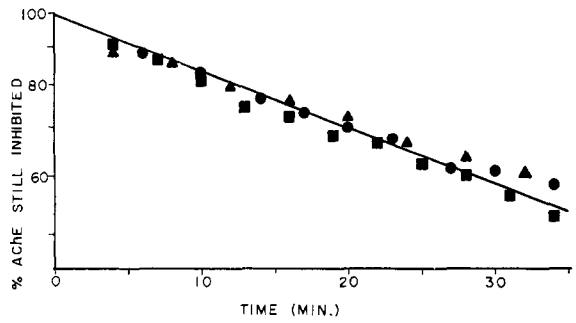


FIGURE 4: Reactivation of red cell AChE at 25° (pH 7.2) by 10^{-4} M 2-PAM in presence of 3.6×10^{-3} M acetylcholine initially, after inhibition by Amiton (●), *C*-Amiton (■), and diethyl *p*-nitrophenylphosphate (▲).

velocity caused by inhibition. The data were calculated by computer (control data 1604) using a program prepared by Mr. S. Rhine based on the weighted regression analysis of Wilkinson (1961), with results shown in Table II. Because a double-reciprocal plot is involved, and because the intercept is near the origin, as shown in the example in Figure 3, the standard errors of k_2 and K_a are high.

Reactivation and Aging. If the desalkylated organophosphates inhibit by phosphorylating the enzyme (as contrasted to only forming a reversible complex) they should yield a monoalkyl phosphorylated enzyme, having one POH group, which would be ionized at neutral pH. Such an enzyme is identical with what is believed (Berends *et al.*, 1959) to be "aged" enzyme, *i.e.*, enzyme which has become refractory to reactivation by oximes such as 2-PAM. Consequently one would expect that the desalkylated organophosphates should yield instantly aged inhibited enzyme. Figures 4 and 5 show indeed that three diethyl phosphates yield one form of enzyme (presumably diethylphosphoryl-cholinesterase) and that two dimethyl phosphates yield another form (presumably dimethylphosphoryl-cholinesterase) as judged by a different rate of induced

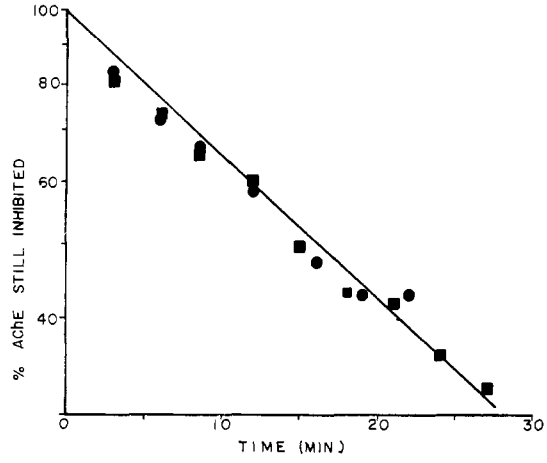


FIGURE 5: Reactivation of red cell AChE at 25° (pH 7.2) by 10^{-4} M 2-PAM in the presence of 3.6×10^{-3} M acetylcholine initially, after inhibition by two dimethyl phosphates compounds, **4** (■) and **5** (●).

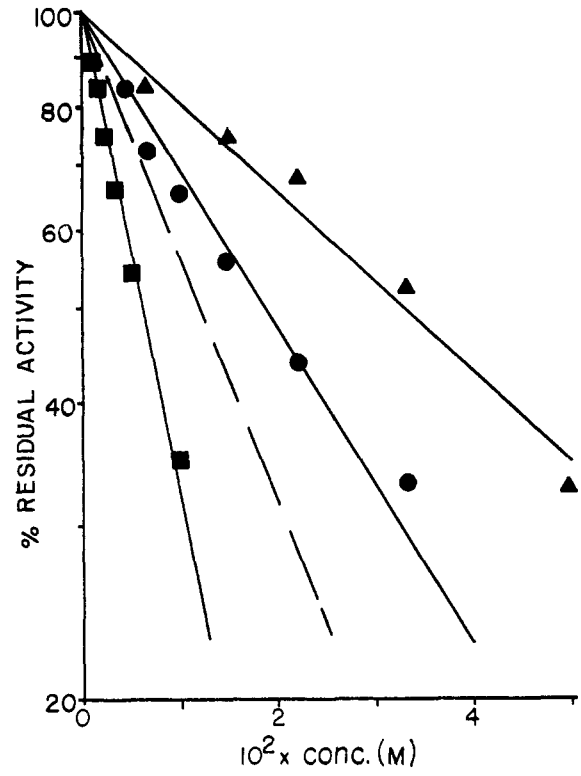


FIGURE 6: Observed inhibition of red blood cell AChE by *O*-Amiton (▲), *O*-desmethyl derivative of **7** (●), and a mixture of both, each at the indicated concentration (■). (———) The result calculated for the mixture in the absence of interaction.

reactivation. By contrast, under identical experimental conditions, enzyme inhibited by *O*-desalkyl derivatives of Amiton, of C-Amiton, of **4**, and of compound **5** could not be reactivated.[2]

Compound Interaction. The possibility was tested that salt formation between pairs of organophosphates could occur, using one *O*-desalkyl derivative of a potent inhibitor, mixed with an undesalkylated amine-containing compound that was a poor inhibitor. Figures 6 and 7 show two cases in which such interaction occurred, the mixture having a more potent effect than was predicted on the assumption of no interaction. The observed inhibition rate was 1.94-fold faster than that predicted for the compounds of Figure 6, and 4.54-fold for the compounds of Figure 7. These values are derived from regression analysis of the data.

pK_a Values. If it is the case that internal salt formation can occur in *O*-desethyl-Amiton, then the pK_a of the amine group should be increased by the influence of the anion brought into its vicinity by ring formation. Figure 8 shows that such is the case: the pK_a of the amino group of Amiton is 8.6, and that of desethyl-Amiton is 9.75.

[2] We previously reported (O'Brien, 1965) that $(C_2H_5O)(HO)P(O)SCH_2CH_2N^+(CH_3)(C_2H_5)_2$ yielded inhibited AChE that could be partially reactivated by 2-PAM. This observation was based on our earliest studies, using a commercial sample, later shown to contain extensive impurities, *i.e.*, eleven, three of which were anticholinesterases.

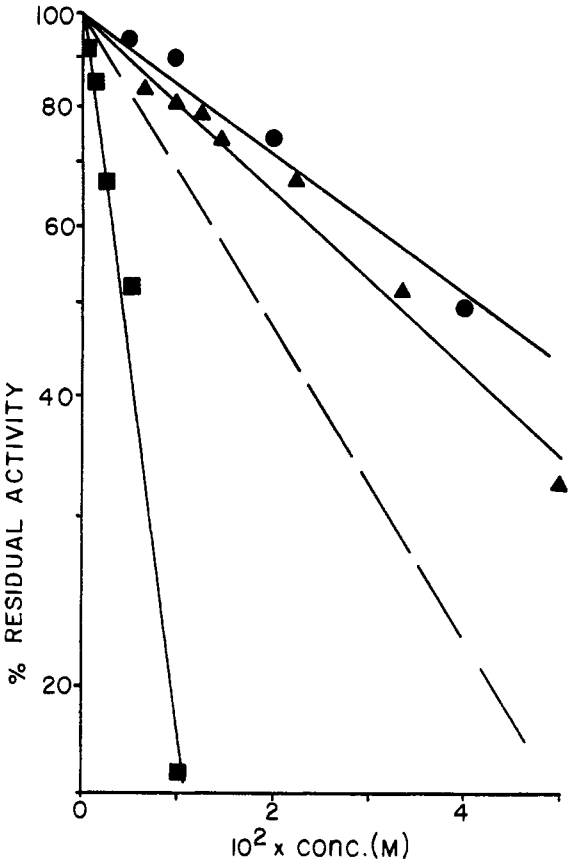


FIGURE 7: Observed inhibition of red blood cell AChE by *O*-Amiton (▲), *O*-desmethyl derivative of **4** (●), and a mixture of both, each at the indicated concentration (■). (— — —) The result calculated for the mixture, in the absence of interaction.

Discussion

Orthodox theory leads one to expect that O desalkylation should profoundly reduce the inhibitory effectiveness of organophosphates. Table I confirms that for "usual" compounds such is the case, the average reduction being 158,000-fold. Amiton, by contrast, suffers only a modest reduction by O desalkylation, to the extent of 179-fold for acetylcholinesterase from fly heads and 292-fold for red cell enzyme. There must be something unusual about Amiton; our first thought was that because Amiton is a fairly close analog of acetylcholine, the "natural" substrate, this similarity might bestow unusually good affinity on it. However, as Table II shows, although Amiton does indeed have excellent affinity, that affinity is reduced almost 10,000-fold by O desethylation. Furthermore, compounds like amino-Amiton, with a rather poor affinity, are even less affected by O desalkylation, as we shall describe below.

O-Desethyl-Amiton inhibits cholinesterase by phosphorylating it, rather than by forming only a reversible complex. The evidence is, firstly, that the reaction is a progressive one, *e.g.*, see Figure 6. Secondly, the reaction gives rise to an enzyme which cannot be reactivated by dilution or treatment by 2-PAM. Thirdly, the inhibition kinetics conform with eq 1, as Figure 3 shows, and indicate a large phosphorylation constant (k_2 in Table II). Fourthly, the ΔH of the reaction is 12 kcal/mole.

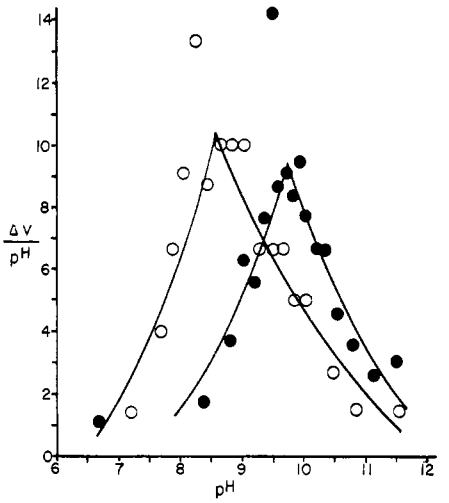


FIGURE 8: First derivative of titration curve of Amiton (○) and *O*-desethyl-Amiton (●) by NaOH.

Since *O*-desethyl-Amiton is a phosphorylating agent, then, one has to explain how an electrophilic attack can be accomplished by a phosphorus attached to an anion. A plausible hypothesis is that internal salt formation occurs, so that the anionicity of the PO^- group is greatly reduced.

Let us review the evidence supporting internal salt formation. Firstly, the increased pK_a of *O*-desethyl-Amiton over Amiton shows that the anion of *O*-desethyl-Amiton does indeed influence the nitrogen strongly, and because it is inconceivable that such an effect be inductive (for it would be transmitted through five intermediate, saturated atoms) it must be by a field effect, so that the anion does indeed approach the nitrogen closely.

Secondly, we will examine analogs. The term "ratio" will be used to apply to the ratio of the potencies of a parent to its desalkylated product; the size of the ratio quantifies the desalkylation effect. Typical compounds have a ratio averaging 158,000 (Table I) contrasted to 179 or 292 for Amiton. If the nitrogen of Amiton is replaced by carbon (**9**) the ratio jumps up to 33,000, making it clear that the nitrogen is all important. It is interesting that for quaternary Amiton (**11**) the ratio is also high, at 80,000. We interpret this as being due to hindrance of ring formation caused by the three *N*-ethyl groups. Supporting evidence (Table II) is that quaternary Amiton has 3.5 times less affinity for the enzyme than Amiton, and 17-fold better phosphorylating activity, presumably because of a field effect of the N^+ on the phosphorus. A similar failure of a quaternary to form a ring was reported by Aksnes and Froyen (1966): the protonated tertiary ester of acetylcholine was hydrolyzed by OH^-, in the pH range 7–9.5, ten times faster in alkali than was acetylcholine itself, presumably because only in the tertiary ester could ring formation occur, in this case between the proton on the nitrogen and the oxygen of the carbonyl. By contrast, when the carbon chain was shorter or longer, so that

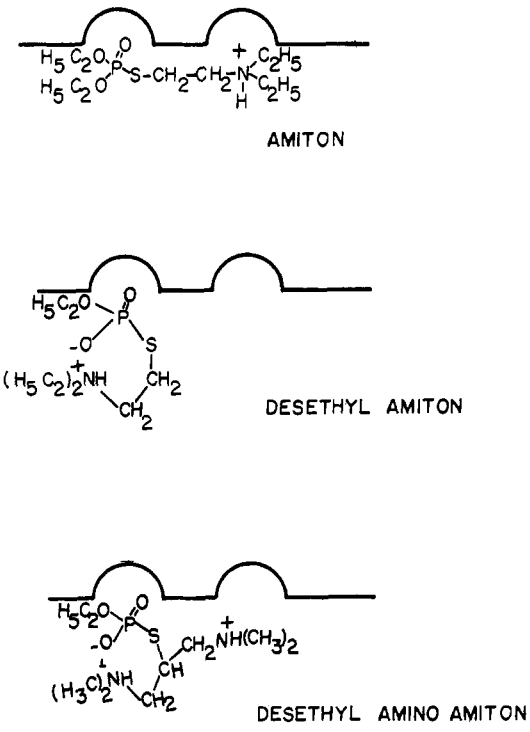


FIGURE 9: Proposed attachment to enzyme of Amiton, desethyl-Amiton, and desethylamino-Amiton.

ring formation could not occur, the tertiary form hydrolyzed at a rate less than or equal to that of the quaternary.

Assuming that some of the marked potency of Amiton itself is due to binding to some second site (possibly "the anionic site") by the $NH^+(C_2H_5)_2$, we wondered if the desethyl derivative lost potency because the NH^+ was occupied in ring formation and therefore not available for second-site binding. We therefore made a compound, amino-Amiton (Table I (**12**), which had an additional amino group located where it could bind to a second site, as shown in Figure 9. And indeed, this compound showed an extraordinarily small loss of potency (60-fold) on desalkylation. That this was due to improved binding is indicated by the fact (Table II) that desethylamino-Amiton has 20-fold more affinity for the enzyme than does desethyl-Amiton.

The same rationale which led to the above work with amino-Amiton suggested another test of our hypothesis. Laidler (1955) points out that extensive binding to an enzyme surface displaces water molecules formerly bound to that surface, and the consequent entropy increase can exceed the entropy loss associated with formation of the enzyme–substrate or enzyme–inhibitor complex. If Amiton is bound to a second site and its *O*-desethyl derivative is not, the entropy of activation of the inhibition should be large for the former, and small or negative for the latter. And indeed we found that the entropy of activation was +1.16 eu for Amiton and −3.23 eu for *O*-desethyl-Amiton.

Evidence was sought to support the possibility that appropriate cations can indeed enhance the phosphorylating ability of desalkylated compounds, by using one molecule for the desalkyl form and a second, different molecule (which would have to be a very poor anticholinesterase itself, yet preferably able to bind to the enzyme) to provide the cation. Two such cases were studied, both using *O*-Amiton (**13**) for the second molecule. This compound is virtually inactive as an anticholinesterase, for reasons which are unknown, but almost certainly not due to poor leaving-group character, since closely related compounds such as $(C_2H_5O)_2P(O)OCH_2CH_2CH(C_2H_5)_2$, with even worse leaving groups, are potent inhibitors (Bracha and O'Brien, 1968). It is probable that the N of *O*-Amiton fails to bind to the anionic site, and this failure would be advantageous as far as availability for interactions with other inhibitors is concerned. The desalkylated compounds used were the derivatives of potent inhibitors which were also commercial organophosphates (**4** and **7**). In both cases, a substantial interaction, in the predicted direction, was encountered (Figures 6 and 7) demonstrating that salt formation can occur, with consequent promoted phosphorylating ability, with two different molecules suitably juxtaposed on the enzyme.

All evidence, therefore, supports the hypothesis that internal salt formation accounts for the relatively small loss in anticholinesterase activity when Amiton is O desalkylated. Consequently, one can predict that such small losses will be found only in the rather rare case that an organophosphate has a tertiary basic group located in such a way as to approach closely to the PO^- group.

It would be misleading to complete this account without pointing to certain apparent anomalies, all appearing in Table II. (a) Why is *O*-desethyl-Amiton actually an eightfold better phosphorylating agent (larger k_2) than Amiton? Although salt formation should reduce the anionicity of *O*-desethyl-Amiton, one would not expect the joint effect of the anion and the cation to lead to enhanced electrophilic character of the phosphorus. One possibility is that hydrogen bonding occurs, within the esteratic site, to the proton on the amine; with Amiton, that amine is bound elsewhere. This possibility is lessened when one notes that desethylation of amino-Amiton leads to a 13-fold reduction of k_2. (b) Why does amino-Amiton have an affinity (measured by $1/K_s$) which is 167-fold less than Amiton, and is 3-fold less than carbon Amiton? Probably the answer is that amino-Amiton has dimethylamine instead of diethylamino groups, and therefore reduced hydrophobic interaction at the second site.

Finally, the fact that C-Amiton has only sixfold less affinity than Amiton for the enzyme, suggests that the binding of Amiton-like compounds to a "second site" involves a smaller coulombic contribution and a larger hydrophobic contribution than standard views on the anionic site would lead one to expect.

Some of these anomalies may have their basis in the relatively simple view that the binding (K_s) and phosphorylation (k_2) steps do not interact. It is quite conceivable that the binding modifies the susceptibility to phosphorylation, quite apart from its role in juxtaposing inhibitor and esteratic site. In addition, different binding sites may well be used by different inhibitors even though they all phosphorylate the same esteratic

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

We are most grateful to Mr. B. D. Hilton for synthesis of some compounds and for assistance in the synthesis of the others; to Dr. P. Bracha for providing the indicated compounds and for very helpful discussions; to Mr. S. Rhine for preparing the computer program; to E. I. du Pont de Nemours Co. for providing Du Pont 3323-57, whose existence provoked this study; and to Dr. R. J. Magee for drawing attention to its existence and importance.

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*-

* From the Section of Neurobiology and Behavior, Cornell University, Ithaca, New York. *Received July 5, 1967.* This work was supported in part by Grants No. EF 00171 and GM 07804 of the U. S. Public Health Service.

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