

is uncertain, since it may be initiated only by direct interaction between GSSG and GSH. Failure to keep glutathione sufficiently reduced does seem to be the basis for pathological effects in certain cells (Beutler, 1960; Mills, 1959). Another interesting possibility is that interaction between disulfides and thiol groups in enzyme systems may provide more reactive intermediates for rapid transfer of electrons. Possibly such interactions account for the especially labile form of sulfur reported to be present in xanthine oxidase, liver aldehyde oxidase, dihydroorotic dehydrogenase, and some other enzymes (Handler *et al.*, 1964).

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Structure and Activity of Some Aryl *n*-Methyl Methylphosphoramidates as Cholinesterase Inhibitors

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From the Dow Chemical Company, Midland, Mich.

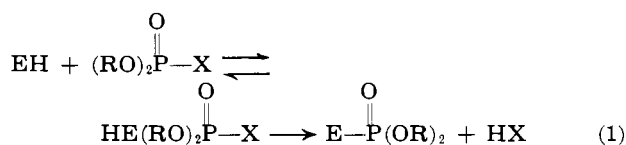
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A series of aryl *n*-methyl methylphosphoramidates were prepared and examined for inhibition of insect cholinesterase. This property was compared to the lability of the phosphorus-oxygen-aromatic bond. The correlation was excellent except for those compounds possessing a 4-*t*-butyl and the 3-*t*-butyl group on the phenyl ring. In these cases, enzyme inhibition was greater than could be accounted for by the lability of the P-O-phenyl linkage. A possible explanation is advanced for this discrepancy. An interpretation of the correlation between infrared stretching frequency and cholinesterase inhibition is also presented.

There has been a great deal of work connected with the mechanism of action of organophosphorus compounds as insecticides (see for example the work of Fukuto and Metcalf, 1956, and Metcalf and March, 1953, as well as the excellent books by O'Brien, 1960, and Heath, 1961). These workers have shown that toxicity of certain compounds to insects is sometimes associated with the cholinesterase enzyme system.

Following up these and earlier correlations, many investigators have studied the reaction between organophosphorus compounds and various esterases. Thus, Aldridge and Davison (1952) have shown that the inhibition of erythrocyte cholinesterase by paraxon and some of its analogs followed pseudo-first-order kinetics and was bimolecular. Furthermore, the bimolecular rate constants for this inhibition paralleled the rate of hydrolysis of these phosphates in water. These same authors proposed a mechanism for this reaction which

may be depicted as follows:



where EH is the enzyme, R is any alkyl group, and X is any displaceable group such as halogen, alkoxy, or aryloxy. Such a mechanism implied that the inhibitory powers of the organophosphates were directly related to the lability of P-X bond. Indeed many such correlations have been made (Aldridge and Davison, 1952; Fukuto and Metcalf, 1956; Wilson, 1951, 1952).

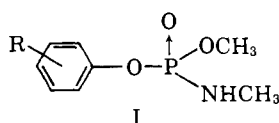
In the present series of organophosphoramidates, 4-*t*-butyl-2-chlorophenyl *n*-methyl methylphosphoramidate (Ruelene, insecticide of the Dow Chemical Co.), appeared to have greater cholinesterase-inhibitory powers than could be explained on the basis of the lability of the P-O-phenyl linkage. It is the purpose of this investigation to explore the relationships be-

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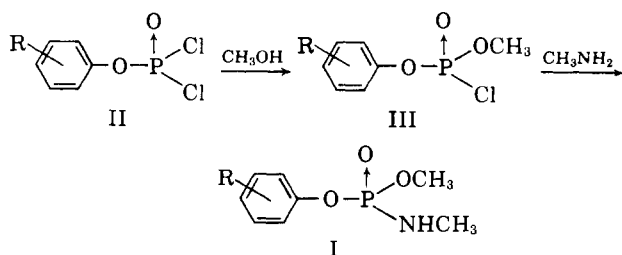
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tween structure and enzyme activity of a series of organophosphates possessing the following structures:

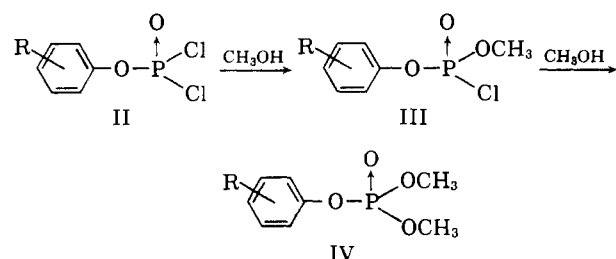


The question of the reversibility of the phosphorylation reaction will also be examined.

These materials were prepared by the reaction of methanol with aryl phosphorodichloridates (II) to form the aryl methylphosphorochloridates (III) followed by treatment with methylamine.



While the solvolytic reaction of aryl phosphorodichloridates with methanol is known to proceed in two stages to the formation of aryl dimethylphosphates (IV) (Orloff *et al.*, 1958) no attempt to limit the reaction to the first stage has been reported.



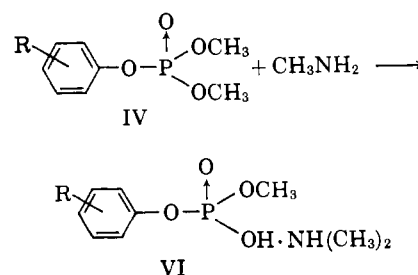
The reaction of equimolar amounts of a dichloridate II with methanol gave intermediate III containing variable amounts of unreacted starting material II and dimethyl ester IV. Treatment of this mixture with methylamine gave compound I contaminated with IV and aryl *N,N'*-dimethylphosphorodiamidate (V), the latter resulting from amidation of dichloridate II.

Since the aryl methyl methylphosphoramidates were to be examined as cholinesterase inhibitors it was necessary to remove contaminants IV and V, for these impurities would be expected to be cholinergic.

Some of the crystalline products were readily purified by repeated recrystallization from suitable solvents. The liquid products, however, could not be separated from impurities IV and V by vacuum distillation. It was necessary, therefore, to develop an improved method for their preparation.

The synthesis procedure was modified by using a sufficient excess of methanol to convert all dichloridate II into III and IV. The methanolysis products were then treated with a large excess of methylamine in order to amidate compound II to I and to bring about the dealkylation of the dimethyl ester IV.

While the amidation reaction was very rapid, the dealkylation required 1–24 hours depending on the concentration of methylamine and the particular dimethyl ester undergoing dealkylation. Compound I is much more resistant to dealkylation than IV. Since the dealkylated material VI existed in this particular system as a water-soluble salt it was readily removed. After



distillation of the solvent under reduced pressure, compound I was purified by recrystallization or by vacuum distillation in a molecular still. The aryl *n*-methyl methylphosphoramidates were found to be pure by vapor-phase chromatography, and by infrared and nuclear-magnetic-resonance spectroscopy. The physical properties and analytical data are listed in Table I.

EXPERIMENTAL

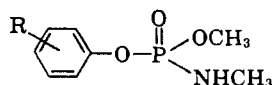
Chemicals.—Melting points were taken on a Thomas Hoover Capillary melting-point apparatus and are corrected. Vapor-phase-chromatographic analyses of compounds I, II, IV, and V were performed with a 61 × 0.6-cm column of 20% silicone gum rubber on Chromosorb P (60–80 mesh) in an F & M Model 500 programed-temperature gas-chromatographic apparatus. Compound III decomposed on the heated column and was not analyzed.

The arylphosphorodichloridates were prepared by a variation of the method of Orloff *et al.* (1958) using anhydrous magnesium chloride as the catalyst.

Aryl *n*-Methyl Methylphosphoramidates.—The following procedures are typical of the methods employed for the preparation of aryl *n*-methyl methylphosphoramidates. No efforts have been made to find the optimum conditions for each compound. The yields, physical properties, and analytical data are listed in Table I.

***n*-Methyl 2,4,5-Trichlorophenylmethylphosphoramidate.**—To a solution of 157 g (0.5 mole) of 2,4,5-trichlorophenylphosphorodichloridate in 200 ml of distilled chloroform was added with stirring 16 g (0.5 mole) of methanol. The addition was carried out over a period of 1 hour with stirring at a temperature of 60–64°. The evolution of hydrogen chloride ceased 15 minutes after the addition of the alcohol. Evaporation of the solvent under reduced pressure left 154 g of a non-crystallizable methanolysis product. The acid chloride III could not be distilled without extensive decomposition. Seventy-eight g of the methanolysis product (equivalent to 0.25 mole of compound III) was taken up in 300 ml of methylene chloride and treated with 16 g (0.5 mole) of methylamine at 10–20°. The reaction mixture was washed three times with 100 ml of water and dried, and the solvent was evaporated under reduced pressure. The residue (71 g) slowly solidified on standing, mp 92–96°. Recrystallization from 1 liter of cyclohexane gave 50 g of colorless crystalline ester amide, mp 99–100° (yield 68%). Repeated recrystallization raised the melting point to 99.5–100.5°.

***n*-Methyl Phenylmethylphosphoramidate.**—Eight g (0.25 mole) of methanol was added in 15 minutes to a stirred solution of 52.7 g (0.25 mole) of phenylphosphorodichloridate in 300 ml of carbon tetrachloride at 20–25°. After 1 hour of stirring at room temperature, the methanolysis product was purged with nitrogen to remove hydrogen chloride. An analysis of this product by gas chromatography indicated large quantities of unreacted phenylphosphorodichloridate. An additional 3.5 g (0.11 mole) of methanol was added. After

TABLE I
 ARYL *n*-METHYL METHYLPHOSPHORAMIDATES


Group R	Yield ^a (%)	mp or bp (°C)	Analysis						Frequency (cm ⁻¹) of Oxygen- Phenyl Stretching Mode
			Calcd			Found			
			P	N	Cl	P	N	Cl	
<i>p</i> -NO ₂	53	63.5–64.5 ^b	12.58	11.38		12.50	11.50		1237
2,4,5-Cl ₃	68	99.5–100.5 ^c	10.17	4.60	35.00	10.15	4.52	35.00	1259
2,4-Cl ₂	56	53.5–54.5 ^d	11.47	5.19	26.26	11.47	5.27	26.28	1245
<i>o</i> -Cl	65	50.5–51.5 ^d	13.15	5.95	15.05	13.05	6.03	15.28	1235
<i>p</i> -Cl	55	85–86 ^e (0.3)	13.15	5.95	15.05	12.95	5.88	15.03	1228
H	37	84–85 ^f (0.3)	15.41	6.97		15.21	7.23		1213
<i>p</i> -OCH ₃	37	104 ^g (0.1)	13.40	6.06		13.24	5.89		1207
<i>m</i> -C(CH ₃) ₃	55	100–101 ^h (0.2)	12.04	5.44		11.81	5.71		1209
<i>p</i> -C(CH ₃) ₃	72	62.5–63.5 ^d	12.04	5.44		11.81	5.72		1225
2-Cl,4-C(CH ₃) ₃	69	61.5–62 ^d	10.62	4.88		10.62	4.80		1243

^a Yield of purified material. ^b Recrystallized from carbon tetrachloride. ^c Recrystallized from methanol. ^d Recrystallized from petroleum ether (30–60°). ^e d_4^{25} 1.3059; n_D^{25} 1.5182. ^f d_4^{25} 1.2111; n_D^{25} 1.5083. ^g d_4^{25} 1.2402; n_D^{25} 1.5133. ^h d_4^{25} 1.1074; n_D^{25} 1.5017.

1 hour of stirring the methanolysis product was again purged with nitrogen, examined by vapor-phase chromatography, and found to be free of starting dichloridate. The methanolysis product was cooled to 10° and a 3-fold excess (32 g) of methylamine was added in 15 minutes. External cooling was necessary to keep the temperature below 29°. An analysis of the products 2.5 hours after the amine addition indicated considerable quantities of dimethyl ester. However, it was decomposed in 24 hours by the methylamine. The amidation product was washed three times with water to remove methylamine hydrochloride and the amine salt of the dealkylated dimethyl ester. Solvent was evaporated under reduced pressure leaving 18.6 g. (37% yield) of methyl phenylmethylphosphoramidate, which was further purified by molecular distillation (bp. 84–85° at 0.3 μ).

n-Methyl *p*-Nitrophenylmethylphosphoramidate.—Eleven g (0.34 mole) of methanol was added dropwise over a period of 20 minutes to a stirred solution of 70 g (0.27 mole) of *p*-nitrophenylphosphorodichloridate in 300 ml of carbon tetrachloride which was maintained at 20–25°. On completion of the alcohol addition, the solution was stirred at room temperature for an additional hour, then purged with nitrogen to aid removal of hydrogen chloride. The methanolysis product was examined by gas chromatography and was found to be free of *p*-nitrophenylphosphorodichloridate. The methanolysis product was then cooled to 5° and treated with an excess (32 g) of methylamine. An analysis of the reaction mixture by vapor-phase chromatography 15 minutes after the addition of the amine showed the presence of large quantities of dimethyl-*p*-nitrophenylphosphate. In 1.25 hours the dimethyl ester had decreased by more than 90%. After standing for an additional hour (2.5 hours after addition of methylamine) the dimethyl ester was reduced to less than 0.5%. The amidation product was then washed three times with 75 ml of 3% sodium bicarbonate solution, twice with 100 ml of 3% sodium chloride solution, and finally with 100 ml of water. The solvent was removed under reduced pressure leaving 35 g (52%

yield) of light-yellow liquid which solidified on standing overnight, mp 62.5–65.5°. After two recrystallizations from carbon tetrachloride, the product melted at 63.5–64.5°.

Cholinesterase Assay.—The anticholinesterase activities of the *n*-aryl methyl methylphosphoramidates were measured by the standard Warburg manometric method (Metcalf, 1949). The fly-brain cholinesterase was obtained by homogenizing 2.7 g of frozen fly-heads (*Musca domestica* L.) in buffer (300 ml) consisting of 8.76 g NaCl, 8.13 g MgCl₂·6 H₂O, and 2.10 g NaHCO₃/liter. The resulting solution was filtered through glass wool and used at the rate of 2.2 ml per Warburg flask. The substrate, 0.3 ml of acetylcholine solution (1.82 g of the chloride salt/50 ml of the above buffer), was added to the side arm of the Warburg flask. The inhibitors were prepared by diluting the acetone solution with water to the appropriate concentration and adding at the rate of 1 ml/Warburg flask. After incubation of the inhibitor with the enzyme for 60 minutes at 37° the substrate was added and the volume of CO₂ produced in 30 minutes was measured and compared with the standard without inhibitor. The bimolecular rate constant for the reaction between cholinesterase enzyme and the phosphate was determined from equation (2) (Aldridge and Davison, 1952).

$$k = \frac{2.3}{tI} \log \frac{100}{b} \quad (2)$$

where t = time in minutes, I = molar concentration of inhibitor, and b = percentage residual activity at time t .

Irreversibility of Inhibition by n-Methyl 4-t-Butyl-2-chlorophenyl Methylphosphoramidate.—The question of the reversible and irreversible nature of the inhibition was studied using a dialysis technique. The enzyme was inhibited with 4-*t*-butyl-2-chlorophenyl methyl methylphosphoramidate, dialyzed for 24 hours against water, and reassayed. In addition, the dialyzed inhibited enzyme was reacted with an equimolar amount of pyridine-2-aldoximemethiodide (2-PAM)¹

¹ Abbreviation used in this work: 2-PAM, pyridine-2-aldoximemethiodide.

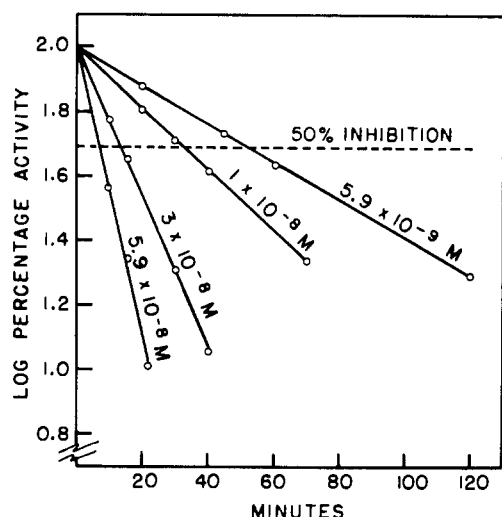


FIG. 1.—Rate of inhibition of fly-head cholinesterase by *n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate. Concentration of inhibitor is shown against each curve.

for 15 minutes prior to assaying for enzyme activity. It was discovered that fly-head cholinesterase lost most of its activity when dialyzed against buffer. The reversibility studies, therefore, were conducted on a commercial preparation of bovine erythrocyte cholinesterase (Winthrop Laboratories, New York City). This material was shown to be stable to dialysis.

Infrared Measurements.—The infrared spectra were recorded with a Herscher KBr foreprism-grating spectrometer with 1 cm^{-1} resolution (Herscher, 1959) of $10 + 2\%$ solution in CCl_4 ($3800\text{--}1333\text{ cm}^{-1}$) and CS_2 ($1333\text{--}450\text{ cm}^{-1}$).

RESULTS

The rate of reaction of *n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate was determined with fly-head cholinesterase using double-armed Warburg flasks so that inhibitor could be added at 37° and the reaction stopped at the desired time by the addition of substrate from the other side arm. The results of this experiment are shown in Figure 1 where the logarithm of the percentage of activity is plotted against time in minutes. On plotting the times for 50% in-

TABLE II
VELOCITY CONSTANTS FOR THE BIMOLECULAR REACTION
BETWEEN FLY-HEAD CHOLINESTERASE AND THE ARYL
n-METHYL METHYLPHOSPHORAMIDATES^a

Aryl Group	k (liter mole ⁻¹ min ⁻¹) 37°	pK_a^b
4- <i>t</i> -Butyl-2-chloro-phenyl	1.10×10^6	8.7
2,4,5-Trichlorophenyl ^c	1.07×10^6	7.2
2,4-Dichlorophenyl	1.07×10^5	7.7
<i>p</i> -Nitrophenyl	7.45×10^4	7.1
<i>p</i> - <i>t</i> -Butylphenyl	7.83×10^3	10.6
<i>o</i> -Chlorophenyl	7.40×10^3	8.4
<i>m</i> - <i>t</i> -Butylphenyl	7.11×10^3	10.2
<i>p</i> -Chlorophenyl	6.02×10^3	9.2
Phenyl	6.94×10^2	9.9
<i>p</i> -Methoxyphenyl	4.28×10^2	10.2

^a The pK_a of the corresponding phenol is also included.

^b The pK_a values were obtained from the Special Services Laboratory, the Dow Chemical Co., Midland, Mich. ^c Our value agrees quite well with the value of 1.1×10^6 liter moles⁻¹ min⁻¹ reported by Fukoto *et al.* (1963) for this particular aryl *n*-methyl methylphosphoramidate.

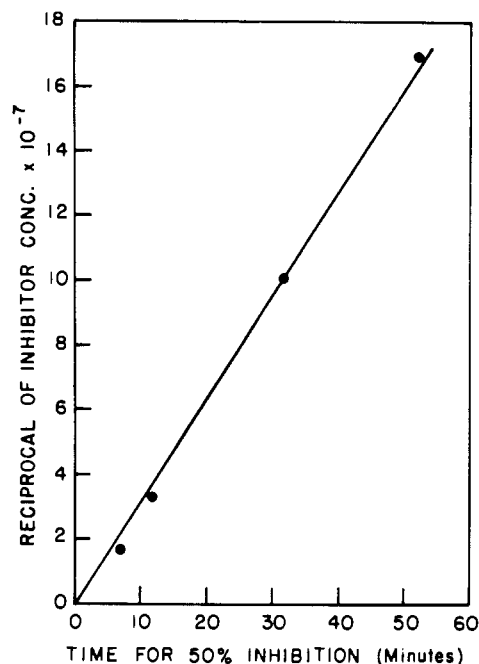


FIG. 2.—Demonstration that the time for 50% inhibition is inversely proportional to concentration of *n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate. Data taken from Fig. 1.

hibition against the reciprocal of inhibitor concentration a straight line (Fig. 2) is obtained, indicating that the slowest reaction is bimolecular.

The bimolecular rate constants for the remaining inhibitors were determined by means of equation (2). These data are given in Table II. Table I and II list the infrared stretching frequency of the oxygen-carbon (aromatic) bond and the pK_a of the corresponding phenol for the aryl phosphoramidates used in this investigation.

Figure 3 shows the correlation between the log of the bimolecular rate constant and stretching frequency of O-phenol bond (Fig. 3).

The results in Figure 4 were obtained by adding inhibitor (*n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate) and substrate at zero time. After the substrate was utilized, as evidenced by the plateau in the CO_2 curve, the flasks were allowed to incubate for 60 minutes, at which time a second portion of substrate was added. The results of the studies on the irreversible nature of the phosphorylation are shown in Table III.

DISCUSSION

The rate data (Fig. 1) for the inhibition of fly-head cholinesterase indicate that for each particular concentration of inhibitor (*n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate) the reaction shows unimolecular properties. However, the fact that the time for 50% inhibition is inversely proportional to the concentration of inhibitor demonstrates that the reaction follows second-order kinetics. A similar kinetic pattern was reported by Aldridge (1950). This author went on to say that for inhibitors possessing this type of kinetics the rate-determining step must be the formation of the complex in equation (1). The subsequent breakdown of the complex with the formation of the phosphorylated enzyme is a rapid nonreversible reaction.

Figure 4, again, demonstrates that the rate of inhibi-

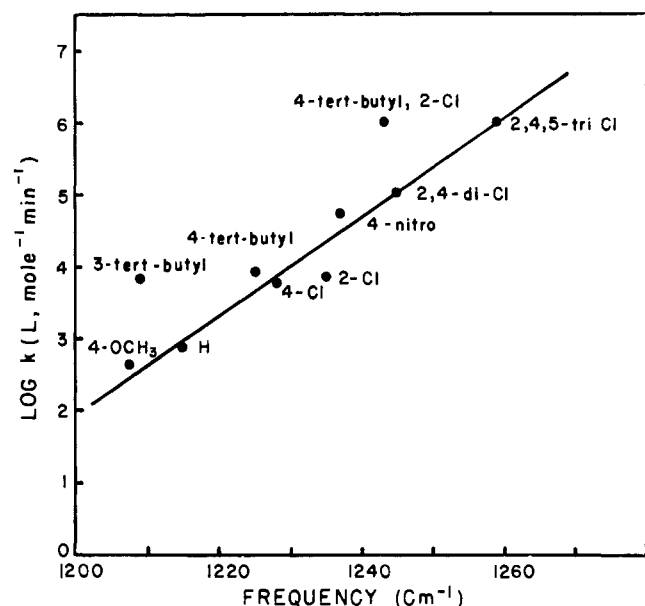


FIG. 3.—Relation of $\log k$ for cholinesterase inhibition to frequency of phosphorus-oxygen-aromatic stretching vibration for the series of substituted phenylphosphoramidates.

TABLE III
RESULTS OF DIALYSIS EXPERIMENTS USING ERYTHROCYTE CHOLINESTERASE AND *n*-METHYL 4-*t*-BUTYL-2-CHLOROPHENYL METHYLPHOSPHORAMIDATE (RUELENE)

Treatment Prior to Assaying	Activity (%) ^a
(1) Erythrocyte cholinesterase (EC)	100
(2) EC after dialysis	100
(3) EC incubated with Ruelene 1.48×10^{-5} M for 60 minutes	76
(4) Sample (3) dialyzed for 24 hours	39
(5) Sample (4) incubated with 2-PAM for 15 minutes	73
(6) Sample (2) incubated with 2-PAM for 15 minutes	100

^a Activity determined by adding the substrate, acetylcholine, after the indicated treatment and measuring the μ l of CO_2 evolved over a 60-minute time interval. The results are based on per cent activity, assigning 100% to the control (treatment 1).

tion is a time-dependent nonreversible reaction. When the inhibitor and substrate were added simultaneously, the inhibitor had no effect on the rate of the reaction compared to the control. This implies that the substrate has a greater affinity for the enzyme than the phosphate, i.e., the slow reaction in the inhibition is the bimolecular addition of the phosphate to the enzyme. However, once the substrate is used up and the enzyme is allowed to react with the phosphoramidate for a period of time, inhibition is demonstrated when a second portion of substrate is added to the reaction. In other words, the rate-determining step is the electrophilic attack of the phosphate on the esteratic site of the enzyme. On this basis any substituent which tends to make the phosphorus atom more electrophilic will increase the enzyme-inhibiting ability. Thus, the activity will increase with the acid character of the corresponding phenol. This relation is shown in Table II where the log of the bimolecular rate constant is tabulated with the pK_a of the phenol. Except for the compounds possessing a 4-*t*-butyl and a 3-*t*-butyl group on the phenol, a good correlation exists.

The dialysis experiments are in accord with the

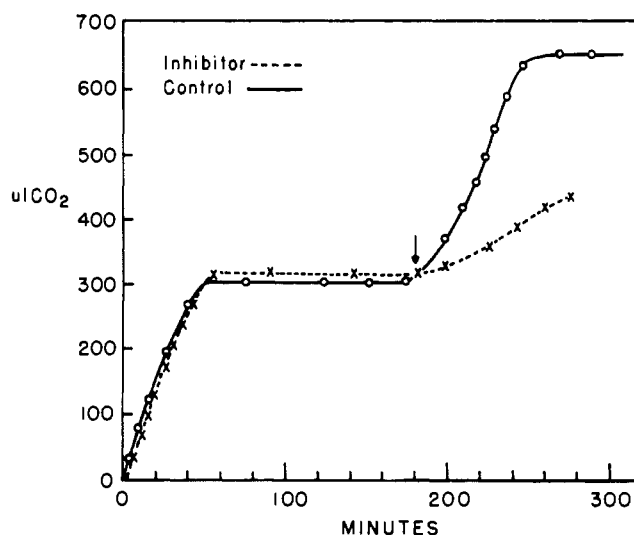
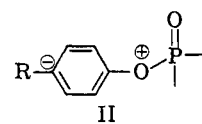
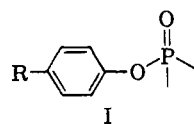


FIG. 4.—Demonstration of the time-dependent noncompetitive nature of the inhibition of cholinesterase by *n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate. Inhibitor and substrate were added simultaneously. At the time indicated by the arrow a second portion of substrate was added.

theory that the phosphorylation of the cholinesterase is a nonreversible reaction. In the foregoing experiment dialysis of the inhibited enzyme did not restore activity; in fact greater inhibition was observed due to the longer time of exposure of phosphate with enzyme. This is contrasted with the carbamates where dialysis has been shown to restore the activity of the inhibited enzyme (Aldridge, 1950). Furthermore, we have shown that the inhibition of erythrocyte cholinesterase by *n*-methyl 4-*t*-butyl-2-chlorophenyl methylphosphoramidate can be overcome by reaction with 2-PAM. This observation tends to confirm the theory that the inhibition of the enzyme does indeed involve an actual phosphorylation.

Fukuto and Metcalf (1956) found that the shift in frequency of the infrared-absorption band attributed to the phosphorus-oxygen-phenyl stretching vibration was a linear function of the ability of the substituted phenyldiethylphosphates to inhibit fly-brain cholinesterase. Plotting the frequencies in Table I against $\log k$ for the phosphoramidates used in this study reveals a similar relationship (Fig. 3). As O'Brien (1960) has pointed out, there is a puzzling anomaly in these results. One would expect that as the phenyl became more electrophilic it would draw electrons away from the P-O-phenyl bonds. This would tend to weaken the bonds and thus reduce the frequency. Ingraham *et al.* (1952) found this to be the case for the O-H stretching frequency of substituted phenols. The opposite situation was found by Fukuto and Metcalf (1956), namely, the more electrophilic the substituted phenol the higher the stretching frequency of the P-O-phenyl bond. This so-called anomaly in the above correlation may be explained in the following manner. The frequency of the absorption band observed is owing primarily to phenyl-oxygen stretching (Nyquist, 1957) and not to a phosphorus-oxygen-aryl stretching vibration. With this reassignment it may be argued that increasing the electrophilic nature of the substituent group R makes structure II more important:



The contribution from structure II would make the phenyl-oxygen bond stronger owing to increased resonance and thus increase its stretching frequency. At the same time, we would expect the oxygen-phosphorus bond to become weaker and consequently the organophosphate would be more susceptible to base hydrolysis. Therefore we believe that the anomaly pointed out by O'Brien (1960) about previous work (Fukuto and Metcalf, 1956) is clarified by the more recent assignment of this infrared-absorption band.

As noted earlier, there were three main exceptions to the correlation between enzyme-inhibitory powers and the electrophilic nature of the P atom. These were the materials possessing a *t*-butyl group in the 3 or 4 position of the benzene ring, which were more potent inhibitors than the parameter measuring the positive nature of the phosphorus would lead us to believe. This lends support to the hypothesis put forward by Wilson (1958) and O'Brien (1963) that the increased activity might be owing to binding by additional areas of the enzyme. The *t*-butyl group occupies about the same space as the methylated quaternary nitrogen of acetylcholine and it is conceivable that Van der Waals forces might hold the *t*-butyl group of the phosphate at the anionic site of the enzyme, thus enhancing the rate of hydrolysis over and above that which would occur if such close fit did not exist. One other interesting observation is that the compounds possessing the *t*-butyl group appear to be forming a new series, where the inhibitory activity is a function of both the geometry and the electronic nature of the molecule. An investigation of further phosphoramidates will be required to establish this trend unequivocally.

No explanation for the lack of correlation between the *p*-nitrophenylphosphoramidate and the *pK* of the corresponding phenol can be given at this time. It should be mentioned, however that the nitrophenyl derivative was quite susceptible to hydrolysis. When the incubation time for the phosphate with the enzyme

was shortened, a higher value for the bimolecular inhibition constant was found. In other words, the rate of reaction between the phosphate and enzyme was linear for the first 15 minutes. After this time, the extent of hydrolysis became significant in lowering the actual concentration of phosphate. The value for *k* reported in Table II takes this fact into account; however it is still not sufficient to correct the deviation observed. Other factors must be operating in the action of this particular phosphoramidate.

One conclusion that emerges from this study is that, with certain organophosphates, geometry plays a role which may be of equal importance to the electronic character of the molecule.

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