Model Studies for the Reactivation of Aged Phosphonylated Acetylcholinesterase. Use of Alkylating Agents Containing Nucleophilic Groups

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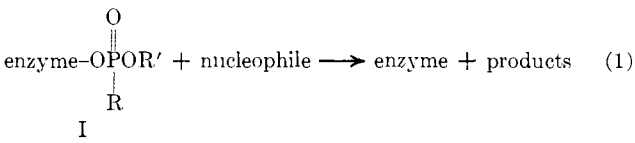
Using sodium *p*-nitrophenyl methylphosphonate as a model for aged (dealkylated) phosphonylated cholinesterase, a study was made of its rate of reaction with various alkylating reagents containing nucleophilic groups. In DMF the alkylation rate ranged widely between a second-order rate constant, $k_2 = 190\ M^{-1}\ hr^{-1}$, at 25°, to no observable reaction in 96 hr at 60° corresponding to $k_2 < 0.02\ M^{-1}\ hr^{-1}$. Aqueous hydrolysis rates at pH 7.15, 25°, were determined for 20 of the mixed esters. The hydrolysis rates also varied extensively, with $t_{1/2}$ ranging from 10^5 sec to less than 5 sec. The exceptionally rapid aqueous hydrolysis of several activated α-bromo ketones strongly supports a mechanism involving attack by OH^- on the CO group. The first example of the intramolecular participation of an amide group in phosphonate hydrolysis by attack on P was observed in carbamoylmethyl *p*-nitrophenyl methylphosphonate. An exceptionally small rate ratio of 0.025 was found for the reaction of phenacyl methylsulfonate *vs.* phenacyl bromide with sodium *p*-nitrophenyl methylphosphonate in DMF. It is suggested that this ratio reflects steric interference in the reaction of the sulfonate and indicates that caution must be exercised in using the Hoffman method for estimating the position of the transition state on the reaction coordinate in SN_2 reactions. Finally, it was found that the spontaneous reactivation of phenacyl methylphosphonylated eel acetylcholinesterase occurs about 100 times more rapidly than that of the corresponding ethyl methylphosphonylated enzyme.

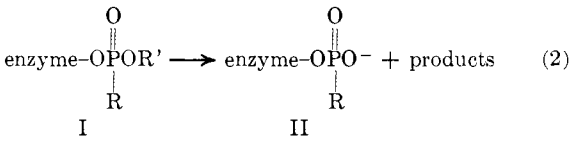
Poisoning by the organophosphorus anticholinesterases has been shown to involve the "irreversible" inhibition of the enzyme acetylcholinesterase (acetylcholine hydrolyase, EC 3.1.1.7), AChE. Inhibition is accompanied by the formation of a covalent P–enzyme linkage.[1,2] The phosphonylated enzyme spontaneously regains its activity slowly, with displacement of the phosphonyl group.[1] However, for purposes of therapy a rapid return of activity is important, and nucleophilic reagents such as oximes or hydroxamic acids are employed (eq 1). Among these, 2-formyl-1-methyl-



(1)

I

pyridinium oxime chloride (2-PAM) is one of the compounds of choice. Upon storage, the phosphonylated enzyme (I) becomes progressively resistant to reactivation. This phenomenon, termed aging, has been shown to involve loss of an alkyl group from the phosphonate moiety[1,3] (eq 2). The negatively charged

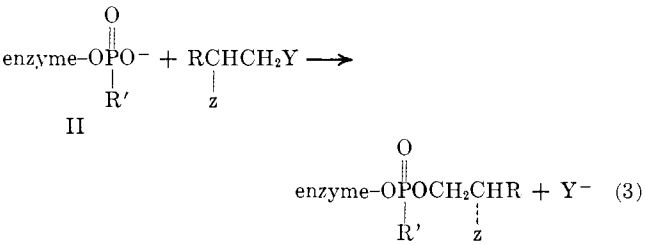


(2)

I II

phosphonate anion in II is no longer subject to displacement by nucleophilic reaction. Accordingly, it would be highly desirable to be able to reactivate this aged inhibited enzyme.

One approach to reactivating the aged enzyme (II) would be through realkylation of the phosphonate anion.[4] This would return it to I, which then would be subject to nucleophilic displacement by 2-PAM or some other oxime. Alternatively, one could incorporate *into the alkylating reagent* a group z which would cause displacement of the phosphonate through propinquity catalysis[5,6] (IV) and obviate the need for the second reagent[7] (eq 3 and 4). Actually, the steps represented



(3)

II

by eq 3 and 4 may be consecutive or concerted. In the latter case, since only a single transition state is

(1) For a review of the phosphorylation and phosphonylation of AChE, of its spontaneous reactivation and reactivation that is speeded by nucleophilic reagents, and the chemistry of aging of the phosphonylated enzyme see G. B. Koelle, "Handbuch der Experimentelles Pharmakologie," Vol. 15, Springer–Verlag, Berlin, 1963, pp 229 and 921.

(2) "Phosphorylation" of the enzyme gives $(RO)_2PO$–enzyme or RO(R)PO–enzyme depending on the structure of the inhibiting reagent, *i.e.*, $(RO)_2POX$ or RO(R)POX. In the first case we obtain a phosphorylated and in the second a phosphonylated enzyme [see *Chem. Eng. News*, **30**, 4515 (1952)]. The chemistry is substantially the same in the two cases. It is common practice to refer to the general case with the term phosphorylation, which is obviously ambiguous. An inclusive term would be desirable. One suggestion might involve the use of phospho-V-ylation to include esterification with the acids $(RO)_2P(O)OH$ and RO(R)P(O)OH. Such a term would be unambiguous, consistent with the general scheme of phosphorus nomenclature, applicable to the trivalent phosphorus acids, and clear in print. However, it would be difficult to use orally. In this paper, such explicit definitions are not required and we continue to use the conventional terms. Thus, phosphorylation is used for both the general and specific cases and phosphonylation where it is specifically applicable.

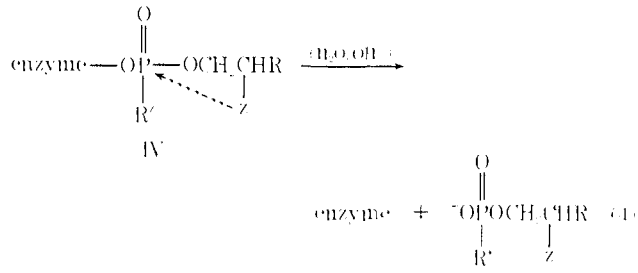
(3) H. O. Michel, B. E. Hackley, Jr., L. Berkowitz, G. List, E. B. Hackley, W. Gillilan, and M. Pankau, *Arch. Biochem. Biophys.*, **121**, 29 (1967).

(4) A. B. Ash, *et al.*, *J. Org. Chem.*, **34**, 4065, 4070 (1969).

(5) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, Inc., New York, N. Y., 1966, p 119.

(6) B. Capon, *Quart. Rev.*, **18**, 45 (1964).

(7) J. I. G. Cadogan and J. A. Maynard, *Chem. Commun.*, 854 (1966).



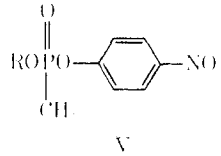
IV

involved, each of the "two" component parts of the overall reaction must accelerate the other. In either case, the electrophilic (CH_2Y) and z groups must be compatible to permit the desired reaction. In addition, reaction 4 is obviously subject to steric constraints as imposed by the required geometry of the transition state. Further, we would predict that the alkylating reagent must bind strongly and with proper orientation at the phosphonylated active site if it is to react preferentially with a poor electrophile such as the phosphonate anion[4] rather than with some other more electrophilic site in the enzyme.

There is, of course, a considerable latitude in the choice of the participating groups (z) for testing the above approach. Study of these reactions with the aged inhibited enzyme (II), even if it were available pure and in large quantity, would be complicated by problems of enzyme stability and possible competitive or parallel reactions with other sites on the enzyme or with aqueous solvent. Negative results could be misleading. Likewise positive results might give no clues for the design of better compounds.

This paper reports an in depth exploratory study into the several facets of the problem using model compounds. It includes studies on (a) alkylation of phosphonate in aqueous and nonaqueous media, (b) hydrolysis rates of alkylating compounds in aqueous solution, and (c) the relative effectiveness of a variety of groups z in speeding displacement of a good leaving group (*p*-nitrophenyl) from phosphonate mixed esters. It also reports (d) the acceleration of reaction 4, spontaneous reactivation of phosphonate inhibited enzyme, through incorporation of the carbonyl group as the propinquity catalytic group z.

Criteria for Choice of Model Compounds.—Our choice of model compounds was based upon the following considerations. They should have chemical properties that are related as closely as possible to those of the enzyme's active site. They should be relatively easy to prepare so that an appreciable range in compound types could be examined. Hydrolysis rates of the models should be subject to convenient assay. The models chosen for study were mixed phosphonate esters having the structure V, which reasonably satisfy these criteria.

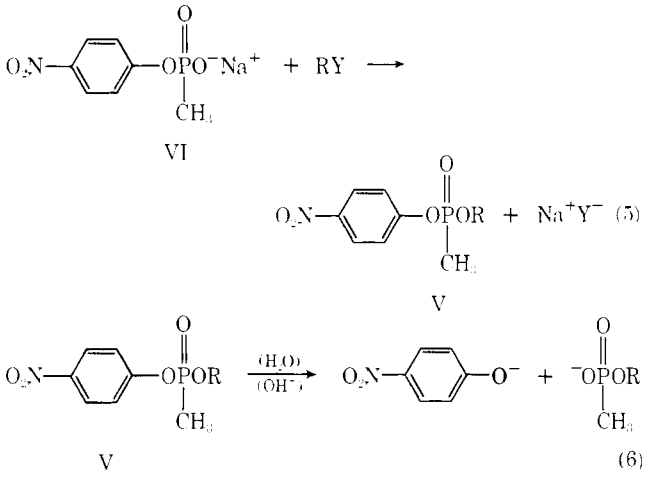


V

A. Chemical Properties Related to the Enzyme Active Site.—In the case of AChE, the active site is believed to include a serine OH and a histidine-derived imidazole.[1,5] There is evidence that phosphonylation occurs at the serine OH to give phosphonyl serine.[7] Detailed information on the composition and structure of the active site are lacking, so that design of an identical model is not possible. The detailed mechanism of displacement of phosphonate is also not known. However, by analogy with the postulated deacylation steps in AChE[7] and chymotrypsin,[10] it is believed to involve a general base assisted attack of a water molecule on P. The ability of the phosphonylated enzyme, I, to reactivate spontaneously and to be rapidly reactivated by nucleophilic reagents[1] suggests that the enzyme active site can act as a good leaving group (eq 1) and that the active site is restored intact.[11] Presumably this occurs through liberation of the serine OH. Serine esters are subject to several modes of cleavage in neutral or alkaline aqueous solution,[12,13] some of which result in loss of the OH group. The general base-catalyzed water-mediated hydrolysis to yield serine and carboxylic acid (or analogously phosphonic acid) requires attack either on the CO group or on the terminal C atom of serine. We feel that the latter pathway is highly unlikely. Hence reactivation (dephosphonylation) of the inhibited enzyme most probably involves displacement on P with resultant P–O cleavage. This mechanistic criterion mode of cleavage was used as a basis for choice of models. All of the compounds could not be tested to establish their conformity to this criterion. However, several of the compounds having a very wide range in hydrolysis rates have been shown to hydrolyze exclusively with loss of the *p*-nitrophenyl group. These include V in which R = Et (*vide infra*), phenacyl,[14] and *syn*-phenacyl oxime[15] which have relative hydrolytic reaction rates with aqueous OH^- of 1, 10^4, and 10^6, respectively. The inclusion of a good leaving group such as *p*-nitrophenyl in V is also important in minimizing complications in the kinetic analysis. A parallel reaction involving displacement of the alcohol moiety containing the group z would produce an error in stoichiometry. In our calculations we have assumed in each case that hydrolysis of the mixed esters, V, occurred exclusively through loss of *p*-nitrophenyl to give $(RO)(CH_3)(OH)PO^-$ (as Na salt).

B. Ease of Preparation.—The preparation, isolation, and purification of readily hydrolyzed mixed esters is often difficult because of the very property that one wishes to measure, their instability. Hence, for the preliminary comparative measurement of hydrolysis rates, we elected to avoid, if possible, the problems involved in isolation and purification of the models, V. Using our spectrophotometric assay methods as analytical tools, we were able to obtain yield and hydrolysis rate data on mixed esters, V, that were prepared *in situ*. For synthesis, we used sodium *p*-nitrophenyl

(8) R. M. Krupka, *Biochemistry*, **6**, 1183 (1967).
(9) N. K. Schaffer, C. S. May, and W. H. Summerson, *J. Biol. Chem.*, **206**, 201 (1954).
(10) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).
(11) It has been possible to obtain yields of as high as 93% of restored enzyme (AChE) from its isopropyl methylphosphonyl derivative by reaction with 1,1'-trimethylenebis(4-formylpyridinium bromide) dioxime, TMB-4; pH 7.4, 25°, 10^{-3} *M* TMB-4. R. Rice and G. M. Steinberg, unpublished.
(12) J. Photaki, *J. Amer. Chem. Soc.*, **85**, 1123 (1963).
(13) S. Ginsburg and I. B. Wilson, *ibid.*, **86**, 4716 (1964).
(14) C. N. Lieske, E. G. Miller, Jr., J. J. Zeger, and G. M. Steinberg, *ibid.*, **88**, 188 (1966).
(15) C. N. Lieske, J. W. Hovance, and P. Blomberg, *Chem. Commun.*, 976 (1969).



methylphosphonate, VI, and RY. The reactions involved are depicted in eq 5 and 6. Further, since the desired esters were prepared by an alkylation reaction similar to that envisioned for the aged inhibited enzyme, II, this approach gave information on comparative alkylation rates.

C. Convenience of Assay.—Hydrolysis of the model compounds V could be easily and conveniently measured spectrophotometrically at near neutral or at elevated pH by measurement of absorbance of the anion of *p*-nitrophenol at 402 mμ[16] (V and VI do not absorb at this wavelength).

Two assay procedures were used to determine the concentrations of V and *p*-nitrophenol that were produced in the reaction vessel. In method A an aliquot of the *in situ* preparation was added to 0.1 *M* carbonate buffer, pH 10, and heated on the steam bath for 10 min. This treatment gives complete hydrolysis of mixed esters V to *p*-nitrophenol and presumably $ROPCH_3(O)(O^-Na^+)$, yet gives no appreciable hydrolysis of unreacted VI (see Experimental Section). Therefore, the absorbance at 402 mμ is a measure of V plus free *p*-nitrophenyl present in the sample. In method B, the aliquot of synthesis mixture was mixed rapidly with 0.1 *M* Tris buffer of pH 7.15, 25°, transferred quickly to the spectrophotometer cuvette (total time 20 sec) and a continuous record made of the increase in absorbance at 402 mμ to completion of the hydrolysis reaction. With this method, the absorbance at end point gives a measure of mixed ester, V (assuming 100% hydrolysis to *p*-nitrophenyl), plus free *p*-nitrophenol present in the aliquot. From the slope of the linear plot of log $A_\infty/(A_\infty - A_t)$ *vs.* time (first-order reaction) one computes the hydrolysis rate constant. The *y*-intercept gives a measure of free *p*-nitrophenol present in the original aliquot, so that the concentration of V can be computed from the combined end point and *y*-intercept information (*vide infra*).

Results and Discussion

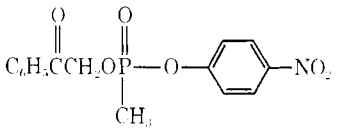
Alkylation. (1) Validation.—Initial tests were made to determine whether the alkylation of VI to yield mixed esters of type V would take place in aqueous media. In order to minimize the hydrolysis of V and also the alkylating compounds, yet to maintain VI in its anionic form,[17] a solution of pH ~3.4 was used. An aqueous solution, pH 3.4, of VI was mixed with alkylating compound dissolved in dioxane to give a final solution containing 71.5% (v/v) H_2O with the concentration of VI and alkylating compound each at 5×10^{-3} *M*. The mixture was refluxed for 4 hr. A control mixture, without alkylating agent, was treated in an identical fashion. Analyses were performed by assay method A. The results are given in Table I.

TABLE I
TRANSIENT ALKYLATION OF SODIUM *p*-NITROPHENYL METHYLPHOSPHONATE IN AQUEOUS SOLUTION

Alkylating agent	Per cent alkylation[a]
Phenacyl bromide (VII)	28.7, 22.5, 26.7, 24.6, 25
Benzyl bromide (XII)	34.4, 31
Phenacyl bromide oxime (XXIII)	17.7, 21
α-Bromophenylacetic acid (XXX)	33, 35
Styrene oxide (XXXII)	0, 0
2-Pyridohydroxamyl chloride hydrochloride (XXXIII)	61, 62
Phenyldimethylsulfonium methanesulfonate (XXXIV)	0, 0

[a] Represents extent of alkylation achieved under the experimental conditions. The mixed esters formed had decomposed completely under the conditions of reaction. See text for conditions and discussion.

These have been corrected for 13% hydrolysis of VI which was observed in the control experiment. Utilizing assay method B, it was found that the reaction mixtures contained no hydrolyzable ester.[18] Accordingly, the mixed ester formed had decomposed completely under the reaction conditions. This was confirmed in the case of *p*-nitrophenyl phenacyl methylphosphonate III, using a sample of the pure compound. Under the



III

test conditions, pure III decomposed completely (to give 100% free *p*-nitrophenol). Further support for the ester formation–decomposition route is given by the marked differences in yield of *p*-nitrophenol (indicated as per cent alkylation in Table I) for the various alkylating compounds together with the close reproducibility of the replicate runs. It was shown, also, that mixed phosphate esters could be prepared by reaction of phenacyl bromide with diethyl and dibutyl phosphates in aqueous EtOH, albeit in low yields (see Experimental Section). The use of completely nonaqueous solvents gave superior results. In particular, it was found that various mixed esters V could be readily formed at room temperature by reaction in MeCN or DMF. The alkylation reactions were 50–100 times more rapid in DMF than in MeCN. Accordingly, DMF was used as solvent for the alkylations performed in the rest of these studies.

(16) A. I. Biggs, *Trans. Faraday Soc.*, **50**, 800 (1954).

(17) The pK_a of phenyl methylphosphonic acid is 1.39 (J. O. Edwards, unpublished data). Hence the pK_a of *p*-nitrophenyl methylphosphonic acid is probably somewhat lower, but certainly not higher than 1.39.

(18) The same result was obtained when alkylation was performed at pH 5.

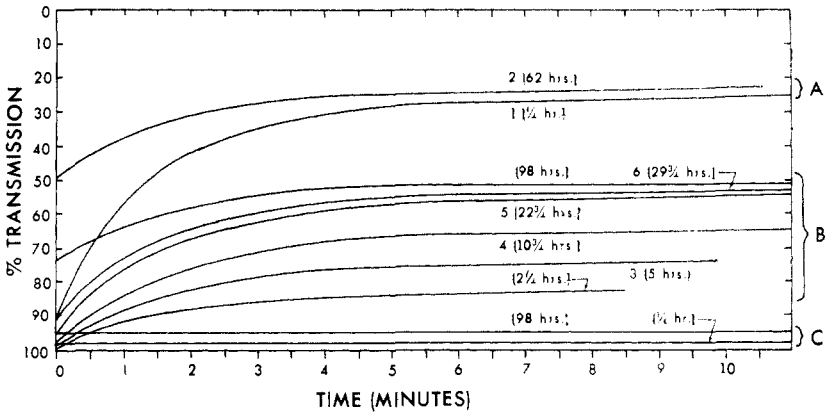


Figure 1.—Hydrolysis of *p*-nitrophenyl phenacyl methylphosphonate, III, pH 7.15, 25°, 0.1 *M* Tris: (A) pure ester, 5×10^{-5} *M*; (B) aliquots of DMF reaction mixture; original concentration, 5×10^{-3} *M* in phenacyl bromide (VII) and in VI. Equivalent concentration in cuvette, 4.45×10^{-5} *M*. (C) Identical with B, except that VII was omitted. Times indicated to the right of each curve represent the period of incubation in DMF, 25°, prior to assay. Curve identification numbers are placed in front of incubation periods. See Table I.

Table II
Hydrolysis Rates of Product of Reaction of Phenacyl Bromide and VI in DMF, 25°. Comparison with Authentic III

Curve in Figure 1	Incubation (hr)	Hydrolysis rate[a] k(sec^{-1}) $\times 10^5$	Hydrolysis rate[a] $t_{1/2}$	Absorbance[b] A_0	Absorbance[b] A_∞
		Pure III			
1	0.25	7.56	91.6	0.06	0.602
2	62	7.63	90.8	0.310	0.636
		Reaction product			
	0.5			0	0.032
	2.25			0	0.076
3	5	7.99	86.7	0	0.131
4	10.75	7.39	93.8	0.013	0.194
5	22.75	7.53	92.2	0.022	0.268
6	29.75	7.98	86.8	0.041	0.276
7	98			0.134	0.292

[a] Hydrolysis in 0.1 *M* Tris, pH 7.15, 25°. Concn, pure III = 5×10^{-5} *M*; reaction pdt. = nominal concn, 4.45×10^{-5} *M*. [b] Absorbance values computed from percent transmission in Figure 1. A_0 and A_∞ represent the initial and final values for each curve.

The reaction between phenacyl bromide, VII, and VI to give the known III[14] was examined in some detail to ensure that method B could be used for the required analyses. Compounds VII and VI were treated at 25° in DMF as described in the Experimental Section. Periodically, aliquots were taken and assayed for the *p*-nitrophenol produced in aqueous solution at pH 7.15 (assay B). A record of the progressive hydrolysis reaction with each of the aliquots is shown in Figure 1. This figure also includes hydrolysis results from a control study in which pure III was incubated in DMF at 25° for periods of 0.25 and 62 hr, and also VI alone for periods of 0.25 and 98 hr. The hydrolysis record is presented as a tracing of per cent transmission (402 mμ) *vs.* time (min). Data taken from this record were used to compute the hydrolysis rate constants listed in Table II. This table also includes the absorbance values (computed from % transmission) for each mixed ester hydrolysis run in Figure 1. A_0 and A_∞ represent the initial and final absorbance values, respectively, for each curve. Note that III is quite stable during short term (0.5 hr) incubation in DMF, but that upon long term incubation (62 hr) there is considerable decomposition (49%) to *p*-nitrophenol. Compound VI, alone, is quite stable in DMF (Figure 1). After 98 hr of incubation the *p*-nitrophenol absorbance rose from zero to 0.02, which represents only (approximately) 3% decomposition. Further, VI does not undergo hydrolysis under the conditions of method B. Note that in Figure 1, the two "hydrolysis" records (C) for VI are both perfectly horizontal straight lines.

First-order kinetic plots are given in Figure 2 for the hydrolysis of the pure III that had been incubated in DMF for the two time periods: 0.25 and 62 hr, and also for 4 of the aliquots taken from the synthesis mixture over the incubation time range of 5 to 29.75 hr. The latter have been grouped together and bracketed in Figure 2. It can be seen from Figure 2 and also from Table II that III hydrolyzes to give *p*-nitrophenol in a good linear first-order manner for more than 3 half-times. Also, it shows that extended incubation of III in DMF decomposes it, in part, to *p*-nitrophenol but that the remainder hydrolyzes to give *p*-nitrophenol in a manner identical with that of the undecomposed compound. Further, incubation of VI and VII in DMF gives progressive formation of III and with it concomitant (but minor) production of *p*-nitrophenol.

Thus, from the linearity of the hydrolysis rate plots over a period of several half-lives, the constancy of the computed rate constants, and their essential identity with that for authentic III, we conclude (1) that III was formed upon incubation in DMF, (2) its formation occurred progressively, and (3) it was the only substance formed which hydrolyzed to yield *p*-nitrophenol under the experimental assay conditions. Finally, its presence was also confirmed by tlc (see Experimental Section). It also can be seen from the progressive increase in A_0 that both the authentic III, and that prepared in DMF, solvolyzed to give *p*-nitrophenol to some extent during prolonged incubation. It is not known whether this represents "pure" solvolysis or hydrolysis by traces of H_2O present in the DMF. But, at least with this compound, decomposition in DMF was sufficiently slow to permit determination of its hydrolysis rate in aqueous solution. Similar results were obtained when the phenacyl bromide (VII) concentration was increased. Since VI is stable under assay conditions, one can calculate the second-order rate constant for

alkylation of VI from the slope of the plot of $1/[R]$ *vs.* time (where [R] = concentration of phenacyl bromide and also of VI; see Figure 3).

It can be seen from Figure 1 that the alkylation reaction does not go to completion since A_∞ for the 98-hr curve should approach that of the pure ester, III. One can speculate on a variety of side reactions that might be involved, including alkylation of liberated *p*-nitrophenol and reaction with DMF.[19] However, the side reactions did not interfere with the determination of the hydrolysis rates and therefore were not investigated. The concentration of phenacyl bromide for each incubation time was calculated from the A_∞ values (which measures the degree of conversion of VII into III), *i.e.*, $[RY] = A_{\infty(th)} - A_{\infty(obsd)}$, where $A_{\infty(th)}$ is the *p*-nitrophenol absorbance that one would obtain if all of the phenacyl bromide had been converted into III and $A_{\infty(obsd)}$, the *p*-nitrophenol absorbance actually observed. The plot of $1/[RY]$ *vs.* time was found to be linear for approximately one half-life. The computed second-order rate constant was 12 M^{-1} hr^{-1}. Upon repetition, using a larger number of short time points, shown in Figure 3, the rate constant was 18.8 M^{-1} hr^{-1}. It appears therefore that the alkylation reaction rate can be estimated to well within one order of magnitude from initial rate data (where side reactions of the alkylating agent are minimized). Since in these experiments the hydrolysis of the product ester III was carried out as a second step (essentially independent of the alkylation step in DMF), the extent of conversion of VI into ester should be primarily a function of the concentration of the reactants. This was found to be the case. At 5×10^{-3} M with respect to each VI and VII, a maximum conversion of 35% was obtained. When the concentration of VII was increased by a factor of 3, the conversion into III was increased to 56%.[20] Since III is a comparatively unstable phosphonate ester, achievement of such high conversion values suggests that the preparation of mixed phosphonate (phosphate) esters by alkylation of salts in DMF should have synthetic utility. The starting materials are comparatively simple and readily available and the reaction conditions are mild. In particular, for complex compounds of biological interest, where large quantities are not required and purification, if required, can be performed by chromatographic techniques, this procedure should be advantageous.

When the reaction of VI with 1-bromo-3-phenyl-2,3-propanedione (VIII) was examined in DMF, a fairly dark yellow–brown color appeared in the DMF solution immediately after mixing. As before, the DMF mixture was placed in the constant temperature bath at 25° and aliquots were taken periodically for assay at pH 7.15 (method B). Here, the curves indicating *p*-nitrophenol formation were straight lines parallel to the *x*-axis, *i.e.*, $A_0 = A_\infty$ for each line. However, the value of A_0 increased progressively with time of incubation in DMF. Thus, all of the *p*-nitrophenol was present at the time that the spectrophotometric recording was begun (approximately 20 sec after mixing of the DMF solution with the aqueous buffer). Control experiments showed that the increasing values of A_0 were not due to impurities in VIII or to HBr that might have been produced by hydrolysis of VIII. Thus, incubation of 5×10^{-3} M VI with an equal concentration of HBr in DMF for 6 hr gave no *p*-nitrophenol in the assay; nor did a reaction mixture of 2.5×10^{-3} M each, VI,

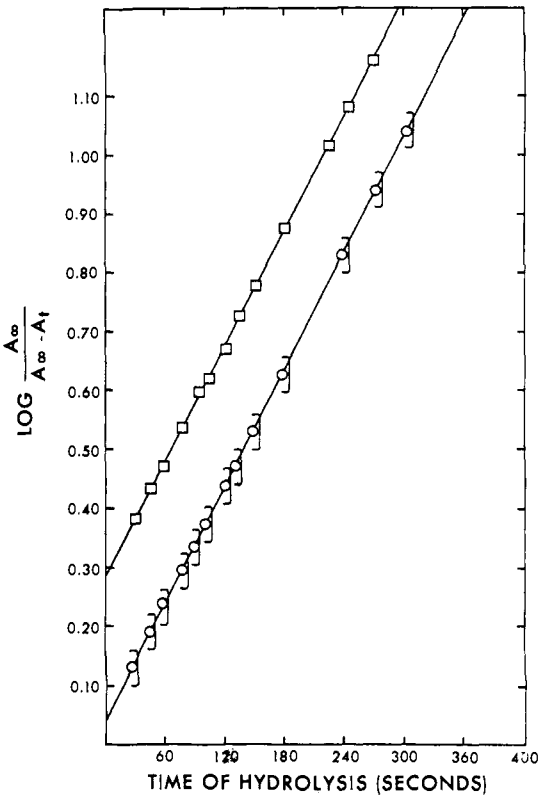


Figure 2.—First-order plot for hydrolysis of III, pH 7.15, 0.1 M Tris, 25°: (○) pure III, after 15-min incubation in DMF (Figure 1, curve 1); (|) range in values computed from raw data taken from curves 3 to 6 in Figure 1 (periods of incubation of VII and VI in DMF ranged from 5 to 29.75 hr); (□) pure III, after 62-hr incubation in DMF (Figure 1, curve 2).

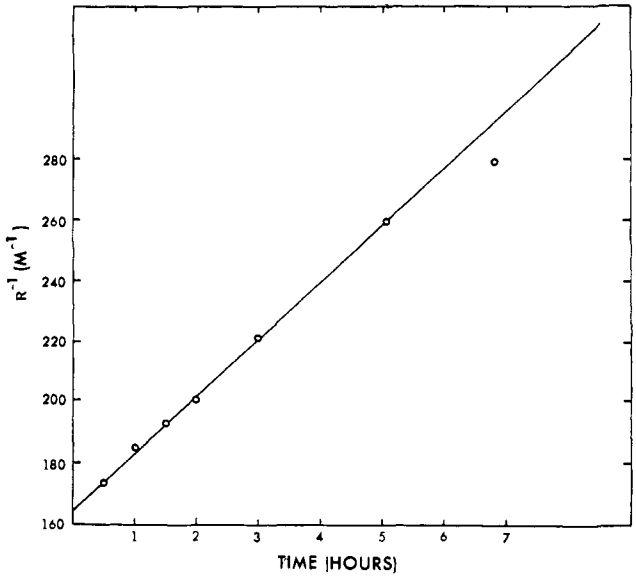


Figure 3.—Reaction between phenacyl bromide and VI in DMF, 25°. Initial concentration of each reactant, 5.0×10^{-3} M. R represents the concentration of each of the reactants determined "by difference" using assay A.

(19) G. M. Coppinger, *J. Amer. Chem. Soc.*, **76**, 1327 (1954).

(20) Percent conversion was determined from the value of $A_\infty - A_0$ in assay B. In each case this reached a maximum value and then declined. With the 1:1 ratio of reactants, the maximum value was reached in 11.25 hr, and with the 3:1 ratio, in 7 hr. In both cases, the same product was obtained, as determined from hydrolysis rate measurement.

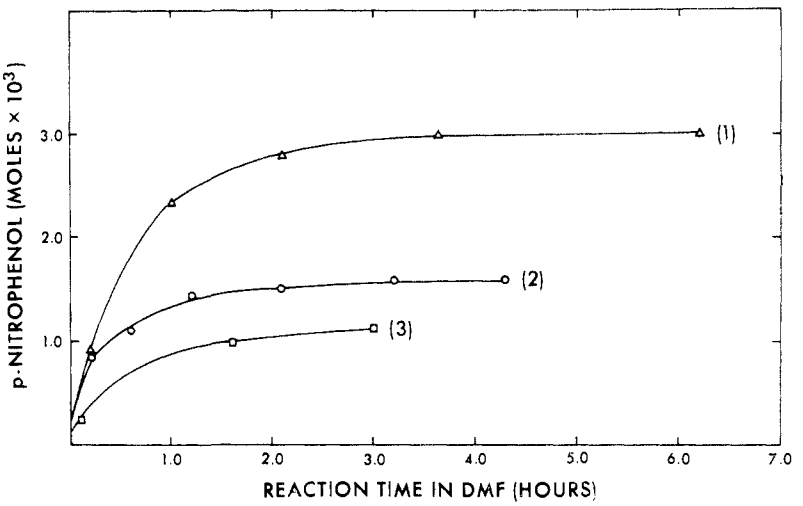


Figure 4.—Reaction between VI and VIII in DMF. Ordinate, quantity of *p*-nitrophenol produced during reaction, determined from absorbance at 402 mμ by assay B. In each case, the concentration of VI = 10^{-3} *M*. Concentration of VIII, in (1), 1.5×10^{-2} *M*; (2) 5×10^{-3} *M*; (3) 2.5×10^{-3} *M*.

VIII, and HBr in DMF give any *p*-nitrophenol. Accordingly, VI must be present in anionic form for alkylation to occur. Further, the rate and extent of *p*-nitrophenol formation, A_0, were both related to the initial concentration of VIII (Figure 4). It can therefore be concluded that this alkylating agent reacts with the phosphonate anion to produce mixed ester, V. However, the mixed ester must either hydrolyze so rapidly at pH 7.15 that hydrolysis is complete in less than 20 sec or solvolysis (or other mode of decomposition) of the ester must occur completely in the DMF prior to the addition to aqueous buffer.

(2) **Kinetics.**—In Table III the results of the reaction of VI with a selected group of alkylating agents are presented. Except where otherwise noted the alkylation reaction was run in DMF at 25° with VI and alkylating agent each at 5×10^{-3} *M*. Rate constants were computed from initial slopes. Derivation of the kinetic equations are given in the Experimental Section. Hydrolysis rates of the resultant mixed esters were measured by assay B. "Percentage conversion to mixed ester" was computed from the quantity of *p*-nitrophenol produced upon hydrolysis, using method A or method B, compared with the theoretical quantity of *p*-nitrophenol that would have been found if alkylation had gone to completion. No efforts were made to achieve maximum conversion to the mixed esters, V.

The accuracy of the alkylation rate constants given in Table I are directly related to the stability of the resultant mixed esters (*vide infra*). For the slow hydrolysis group, good linear plots were obtained for the entire period of reaction. With the *intermediate* group, rate constants were computed from the early linear part of the kinetic plot. In each case, the rate fell off after an initial satisfactory linear period due to side or sequential reactions in DMF. For the *fast* group, only very approximately estimates of the alkylation rate could be made. In general, the order of alkylation rates was as expected. It is noteworthy that "remote" electron-withdrawing groups such as the second carbonyl group in the ester corresponding to VIII and the pyridinium ring in that corresponding to XXV, which contribute markedly to the carbonyl assisted phosphonate hydrolysis reaction, also speed the phosphonate alkylation reaction (Table III).

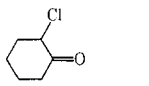
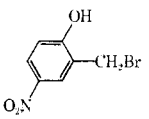
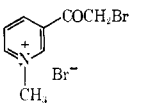
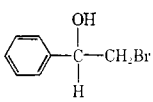
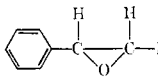
The relative reaction rates of the methylsulfonates XI and XV and the corresponding halides with phosphonate in DMF are worthy of note. Hoffman[21] has made the valuable suggestion that the S_N2 reaction rate ratio of an ion with a neutral tosylate (which we extend to the methylsulfonate since this change has little effect on its reaction as an electrophile) and its corresponding bromide gives an estimate of the position of the transition state on the reaction coordinate. His results have indicated a very wide range in k_{TOs}/k_{Br} from less than 1 to 7000. The ratio is small (large), if the nucleophile is powerful (weak) and the solvent has poor (strong) ionizing tendency (in terms of Hoffman's comparisons; see also Parker).[22] Hence, we would expect the rate ratio with several of our compounds to be large. Instead, the rate ratio is about 1 for $k_{XI}:k_{(IX \text{ or } X)}$ and 0.025 for $k_{XV}:k_{VII}$. It is possible that the transition states in these reactions in very close to the structures of reactants as per Hoffman's argument. However, with such a very poor nucleophile this appears improbable. Accordingly, we suggest that the retardation of the alkylation reaction of the methylsulfonates is due to steric interference to attack by the bulky phosphonate nucleophile. The rate ratio of 0.025 for $k_{XV}:k_{VII}$ in aqueous solution, *vide infra* (Table IV), *i.e.*, $k_{XV}:k_{VII}$ = *ca.* 4, is, on the other hand, quite normal as would be expected in the absence of such steric effects.[21]

(3) **Hydrolysis of Mixed Esters (V).**—Hydrolysis rates of the mixed esters V are given in Table III. Except where noted otherwise, method B was used with measurements made at pH 7.15 and 25°. In certain selected cases, hydrolyses were run at pH 10.0 and at pH 4.6. Based upon the rates of hydrolysis, the mixed esters fall into three groups: (1) slow reactants; those containing no intramolecular participating groups, z; (2) intermediate; those containing moderately effective participating groups, z, which increased hy-

(21) H. M. R. Hoffman, *J. Chem. Soc.*, 6753 (1965).
(22) A. J. Parker, *Advan. Phys. Org. Chem.*, **5**, 173 (1967).

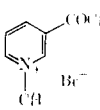
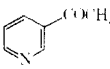
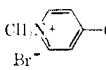
TABLE III

ALKYLATION OF SODIUM *p*-NITROPHENYL METHYLPHOSPHONATE, VI, IN DIMETHYLFORMAMIDE. HYDROLYSIS RATE OF THE RESULTANT MIXED ESTERS, V, IN AQUEOUS SOLUTION, pH 7.15, 25°

Alkylating agent		Rate of alkylation of VI;[a] k_2 (M^{-1} hr^{-1})		Hydrolysis rate of the resultant mixed ester, V		Conversion to V (%)[b]
No.	Compd	25°	60°	k_{obsd} (sec^{-1})	$t_{1/2}$ (sec)	
VII	$C_6H_5COCH_2Br$	18.8 (10.6)	166 (0.6)	7.6×10^{-3} 4.63×10^{-4}[c]	91.2 1.49×10^{3}[c]	35
VIII[d]	$C_6H_5COCOCH_2Br$	Approx 190		Instantaneous[e]		50
IX	C_2H_5Br	0.12		2.12×10^{-4}[f,g]	3.27×10^{3}[f,g]	7
X	CH_3I	1.0		3.86×10^{-4}[f]	1.79×10^{3}[f]	47
XI	$CH_3OSO_2CH_3$	0.3		3.78×10^{-4}[f]	1.83×10^{3}[f]	38
XII	$C_6H_5CH_2Br$	1.76 (113)	43	1.4×10^{-6} 4.90×10^{-4}[f] 3.70×10^{-4}[f,g]	5.0×10^{5} 1.44×10^{3}[f] 1.87×10^{3}[f,g]	20
XIII	*o*-$O_2NC_6H_4CH_2Br$		86	1.4×10^{-6} 8.3×10^{-4}[f]	5.0×10^{5} 8.4×10^{2}[f]	
XIV	$C_6H_5COCBr(CH_3)_2$	N.r. (150 hr)		5.22×10^{-6}[h] (3.77×10^{-6})	1.33×10^{5}[h]	7.5[h]
XV	$C_6H_5COCH_2OSO_2CH_3$	0.48 (417)		7.6×10^{-3}	91.2	27
XVI	*p*-$CH_3OC_6H_4COCH_2Br$			2.76×10^{-3}	251	50
XVII	*o*-$MeOC_6H_4COCH_2Br$			1.6×10^{-3}	440	11
XVIII				7.3×10^{-4}	8.6×10^{2}	
XIX	CH_3COCH_2Br		Approx 128	8.57×10^{-3} 1.97×10^{-3}[c]	80.9 355[c]	57
XX			27	1.31×10^{-3}	530	
XXI[i]	H_2NCOCH_2Br	0.3		4.2×10^{-4}	1.69×10^{3}	
XXII[j]	$C_6H_5NHCOCH_2Br$		13.8			
XXIII[k]	$C_6H_5C(=NOH)CH_2Br$			Instantaneous[e]		30
XXIV	$EtOCOCOCH_2Br$			Instantaneous[e]		
XXV[l]		Approx 160		Instantaneous[e]		28
XXVI	$Et_2NCH_2CH_2Br$		40% reaction in 24 hr	Instantaneous[e]		40
XXVII	$(HO)_2POCH_2Cl$		40% reaction in 24 hr	Instantaneous[e]		40
XXVIII	$BrCH_2COOH$		7% in 5 hr			7
XXIX	α-Bromosuccinic acid		N.r. (48 hr)			
XXX	$C_6H_5CHBrCO_2H$		N.r. (48 hr)			
XXXI			N.r. (92 hr)			
XXXII			N.r. (48 hr)			

[a] Alkylation at 25°: VI and alkylating agent 5×10^{-3} *M* each. At 60°: 10^{-2} *M* each. Reaction half-times are given in parenthesis. N.r. = no reaction for time given. [b] For conditions cited under a: $A_\infty \times 100$/theoretical absorbance for complete reaction. [c] Hydrolysis at pH 4.6; 0.1 *M* acetate. [d] J. Wegmann and H. Dahn, *Helv. Chim. Acta*, **29**, 1247 (1946); Calcd: C, 47.6; H, 3.1; Found: C, 48.6; H, 3.6. [e] $k_{obsd} > 0.15$ sec^{-1}. [f] Hydrolysis at pH 10.0, 25°; carbonate buffer (0.1 *M*). [g] Measurement made on isolated, purified, and characterized sample of the mixed ester. [h] 1.1×10^{-2} *M* XIV plus 10^{-3} *M* VI in MeCN, refluxed 3 hr. Hydrolysis rate measured at pH 7.29 in 0.1 *M* Tris. k_{obsd} computed for pH 7.15 in parenthesis. [i] Mp 87–88°; Buchner, *Ber.*, **25**, 1160 (1892), mp 86–88°. [j] Mp 133°, Abenius, *J. Prakt. Chem.*, **40**, 428 (1900); mp 131°, *Beilstein*, **12**, 245 (1900). [k] H. Korten and R. Scholl, *Ber.*, **34**, 1901 (1901). "Compound" was mixture of bromide and chloride. See M. Masaki, K. Fukui, and M. Ohta, *J. Org. Chem.*, **32**, 3564 (1967). [l] Prepared by Ash-Stevens, Inc., mp 166–167.5°; Calcd: C, 32.57; H, 3.08; Br, 54.18. Found: C, 32.51; H, 3.18; Br, 54.39.

TABLE IV
HYDROLYSIS RATES IN AQUEOUS SOLUTION

No.	Compound	pH	$t_{1/2}$ (min)	Rate[a] k_{OH} (M^{-1} min^{-1})	Ethanol content (% by volume)
VII	$C_6H_5COCH_2Br$	10.0	18.4	3.8×10^2	2
		9.0	122	6.2×10^2	2
VIII	$C_6H_5COCOCH_2Br$	8.0	3.38	2.05×10^5	5
		7.5	10.6	2.04×10^5	5
		7.0	22.5	3.04×10^5	5
XIV	$C_6H_5COC(CH_3)_2Br$	10.0	0.72	0.96×10^3	10
		9.5	1.90	1.14×10^3	10
		9.0	5.87	1.18×10^3	10
XV	$C_6H_5COCH_2OSO_2CH_3$	9.5	12.05	1.8×10^3	3
		9.0	35.0	2.0×10^3	3
XXV		8.0	1.9;	3.4×10^5	0
		7.53	5.8	3.4×10^5	0
		7.15	18.5	3.4×10^5	0
XXXV		8.0	36	1.9×10^4	0
		7.4	60	4.5×10^5	0
XXXVI		6.5	38	5.7×10^5	0
		6.0	99	7.0×10^5	0

[a] $k_{OH} = k_{obsd}/[OH^-]$.

drolysis rate by 3 or 4 orders of magnitude; (3) fast; compounds which had liberated all of the *p*-nitrophenol in less than 20 sec under assay conditions.

Slow.—These included the Me, Et, benzyl, and *o*-nitrobenzyl esters. It is of interest to note that in going from Me to *o*-nitrobenzyl the reaction rate is increased by a factor of only 2. This indicates the comparative lack of sensitivity of the rate of the hydrolytic reaction (nucleophilic attack on phosphorus) to changes in the nonleaving ester group. Hydrolysis of the two benzyl esters corresponding to XII and XIII are each substantially first-order in phosphonate and OH^-.[23] It is striking that the CO-containing compound corresponding to XIV also falls into the slow group. The *gem*-dimethyl groups prevents enolization of the carbonyl group and can also sterically hinder access by OH^- or H_2O to either (or both) the phosphonate and CO groups. It has been shown from deuterium incorporation data that the acceleration of hydrolysis of III is not due to enolization, but most probably involves the formation of carbonyl hydrate.[24] The Stewart and Breigleb molecular model of the mixed ester corresponding to XIV shows little or no crowding around the P atom. There appears to be ample space for entry of OH^- for attack on P. On the other hand, the two Me groups cause considerable crowding about the adjacent CO. Thus, it would appear that they eliminate CO acceleration of the hydrolysis reaction by interferring with OH^- (or H_2O) addition to the CO, which is required for its participation in the reaction.[14]

Intermediate.—These include the unsubstituted and *p*- and *o*-methoxyphenacyl, 2-ketocyclohexyl, acetonyl, 5-nitro-2-hydroxybenzyl, and the carbamoylmethyl esters. Reduction in the rate of hydrolysis by OMe substitution on the benzene ring in the phenacyl esters is in keeping with the participation of the CO group in the hydrolysis reaction. The slight additional retardation caused by introduction of a MeO group into the *ortho* position of the phenacyl moiety (esters corresponding to compound XVII *vs.* XVI) and a 10- to 15-fold retardation on passing from III to *o*-methylphenacyl *p*-nitrophenyl methylphosphonate[24] further support this suggestion. In view of the sensitivity of the hydrolysis of the CO-containing mixed esters V to bulking near the CO group, the near identity in hydrolysis rates of the phenacyl and acetonyl compounds (esters corresponding to VII and XI) is somewhat surprising.

At pH 4.6, the predominant reaction in CO-assisted hydrolysis no longer involves the hydroxide ion. Instead of the calculated (based upon $[OH^-]$ reduction in rate of 350 times in passing from pH 7.15 to pH 4.6, one observes only 16.4 and 4.35 times, respectively, for the phenacyl and acetonyl compounds.

The CO-participation reaction is sensitive to other structural changes about the CO group. Thus, in comparing the ester corresponding to XVIII with that of XIX, there is a 12-fold reduction in rate. The phenolic OH in the ester corresponding to XX is also able to participate in the hydrolysis reaction. However, it participates much less effectively than does CO. Although it is approximately 50% ionized[25] at pH 7.15, so that a high concentration of anion is available for reaction, the degree of acceleration is only of the same order of magnitude. The carbamoyl group also contributes to the hydrolysis reaction. Reaction of the ester corresponding to XXI gives displacement on P to yield *p*-nitrophenol at a rate which is about 20 times less rapid than that of the acetonyl compound (ester corresponding to XIX). The participation of the amide group in *displacement on P* at near neutral pH is particularly noteworthy because of possible implication of the peptide bond in the catalytic function of enzymes involving phosphonyl group transfer. Previously, Schmir and Zioudrou[26] observed that with amide con-

(23) The concentration of OH^- is 715 times greater at pH 10 than at pH 7.15. Hence, the rate ratios should give this value, for a reaction that is first-order in $[OH^-]$. If, however, as is often the case in hydrolysis reactions, the buffer components contribute to reaction velocity, modest deviations from the calculated ratio may be expected. See, for example ref 14.

(24) C. N. Lieske, to be published.

(25) D. E. Koshland, Jr., Y. D. Karkhams, and H. G. Latham, *J. Amer. Chem. Soc.*, **86**, 1448 (1964).

(26) G. L. Schmir and C. Zioudrou, *Biochemistry*, **2**, 1305 (1963).

taining mixed esters of diphenyl and dibenzyl phosphoric acid the amide group participates in cleavage of phosphate, but only through attack on the ester C atom with the formation of heterocycles. No P–O cleavage was detected by them. The present result suggests that with a sufficiently good leaving group attached to P, such as *p*-nitrophenyl *or possibly an enzyme active site*, the peptide group may also participate by this simpler, less disruptive pathway for phosphate displacement.

Fast.—These include the esters corresponding to VIII, XXIII, XXIV, XXV, XXVI, and XXVII. In each case the hydrolytic reaction to give *p*-nitrophenol was complete at the beginning of the assay. With each, there was a progressively increasing amount of *p*-nitrophenol found upon periodic assay of the DMF synthesis mixture. It is probable that alkylation took place in DMF and that the mixed esters, being unstable, decomposed in the DMF. Alternatively, they might have hydrolyzed in the assay solution too rapidly to have been noted. A further possibility is that the *p*-nitrophenol may have been formed in the DMF through a concerted displacement reaction.

Support for the alkylation, rapid hydrolysis sequence comes from hydrolysis rate studies with the purified mixed ester corresponding to XXIII. The hydrolysis rate is exceptionally rapid. At pH 4.90, $t_{1/2}$ = 1.34 min; calculated $t_{1/2}$ at pH 7.41 = 0.5 sec.[27] Several attempts at the synthesis of pure samples of each of the mixed esters corresponding to VIII, XXIV, and XXV were unsuccessful, suggesting their instability. The three compounds share as a common feature a strong electron sink, which greatly activates the CO group.[28,29] Such activation would be expected to enhance its overall contribution to the phosphonate hydrolysis reaction.

(4) **Hydrolysis of Alkylating Agents.**—Hydrolysis rates of several of the alkylating agents in aqueous solution are given in Table IV. At constant pH, the hydrolysis reactions were first-order in each case. The k_{obsd} was calculated by the method of Guggenheim[30] from the record of alkali titrant required to maintain constant pH. At the pH values recorded in Table IV, reaction end-points approach 1 mol of base consumed per mol of compound, indicating simple hydrolysis (eq 7). At higher pH values, the quantity of alkali

$$RCOCH_2Y + OH^- \longrightarrow RCOCH_2OH + Y^- \qquad (7)$$

consumed increases. The increase is at least in part due to further decomposition of $RCOCH_2OH$.[31] Favorski rearrangement[32] may also be a contributing factor to the additional alkali consumption at higher pH values. However, this matter was not investigated.

(27) C. N. Lieske, J. W. Hovanec, G. M. Steinberg, and P. Blumbergs, *Chem. Commun.*, 13 (1968). J. W. Hovanec, C. N. Lieske, G. M. Steinberg, J. N. Pikulin, A. B. Ash, and P. Blumbergs, First Northeast Regional Meeting of the American Chemical Society, Boston, Mass., Oct. 1968, Abstracts, p 97.

(28) D. L. Hooper, *J. Chem. Soc. B*, 169 (1967).

(29) Y. Pocker, J. E. Meaney, and B. J. Nist, *J. Phys. Chem.*, **71**, 4509 (1967).

(30) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).

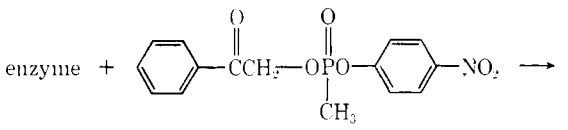
(31) Support for the assumption of simple hydrolysis at "lower" pH, where 1 mol of acid is produced, was obtained with phenacyl bromide. Upon refluxing for 5–6 hr at pH 8 in 1 *M* borate there was 70–75% conversion into phenacyl alcohol (determined by vpc). At higher pH, phenacyl alcohol decomposes. Thus, at pH 13, the K band, λ_{max} 248 mμ (in MeCN, λ_{max} 244.5 mμ; E, 1.13×10^4) disappears instantaneously. At pH 11, a similar loss in K band occurs, but, it takes place over a period of several hours.

(32) R. B. Loftfield, *J. Amer. Chem. Soc.*, **76**, 35 (1954).

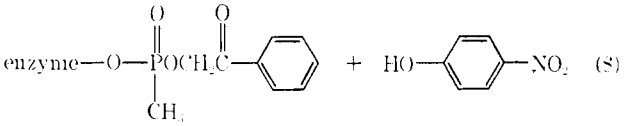
The much higher hydrolysis rate of XIV compared with that of VII is certainly indicative of an elimination reaction rather than OH replacement (eq 7).

It is particularly striking that the compounds containing "remote" electron-withdrawing groups (*vide supra*) such as VIII, XXV, and XXXVI hydrolyze so much more rapidly than does phenacyl bromide, VII. An increase of this magnitude is difficult to explain in terms of simple added inductive effect on the CH_2 carbon.[33] The mechanism of hydrolysis of α-halo ketones, which are among the most rapid of the S_N2 hydrolysis reactions known, is still not settled.[34] We would like to suggest that their hydrolysis may be similar in mechanism to that of the CO-acclerated phosphonate hydrolysis. This would involve OH^- attack on CO to give either a preequilibrium hydrate or a concerted displacement reaction by the carbonyl O. Such a mechanism would be compatible with the very large increase in hydrolysis rate in VIII and XXV, since the CO group is greatly activated (marked increase in degree of hydration) by the adjacent CO group[28] and the pyridinium ring.[29]

(5) **Enzyme Reactivation.**—*p*-Nitrophenyl phenacyl methylphosphonate (III) provided a means for examining the premise that incorporation of a propinquity catalytic group, z, into the phosphonate moiety would increase the rate of spontaneous reactivation (dephosphonylation) of the phosphonylated enzyme (reaction 4). Like other *p*-nitrophenyl phosphates and phosphonates, III is a potent inhibitor of AChE. Inhibition is progressive (see Figure 5), it is not diminished by dilution of the inhibited enzyme and reactivation occurs upon treatment with the oxime, TMB-4 (note ref 11). The inhibition reaction is depicted in eq 8.[1] In this case the propinquity catalytic group, z, is the carbonyl. It was reported earlier[14] that the hydrolytic



III



(8)

reaction of III to give *p*-nitrophenyl and phenacyl methylphosphonic acid (Na salt) is approximately 10^4 times faster than the corresponding reaction for ethyl *p*-nitrophenyl methylphosphonate (ester corresponding to IX) which lacks the CO group. A comparison of the rates of spontaneous reactivation, pH 7.4, 25°, of enzyme inhibited by the two phsophonates is shown in Figure 6. Both reactivations follow first-order kinetics. The ethyl methylphosphonylated enzyme reactivates with $t_{1/2}$ = 12,000 min, while the phenacyl methylphosphonylated enzyme (Bio-Gel 10 separated from impurities after inhibition) reactivates with $t_{1/2}$ = 180 ± 57 min. Generally, spontaneous return of activity occurs more rapidly when the groups attached to P

(33) F. G. Bordwell and W. T. Brannen, Jr., *ibid.*, **86**, 4645 (1964).

(34) See, for example: D. J. Pasto, K. Garves, and M. P. Serve, *J. Org. Chem.*, **32**, 774 (1967); K. Okamoto, H. Kushiro, I. Nitta, and H. Shingu, *Bull. Chem. Soc., Jap.*, **40**, 1900 (1967).

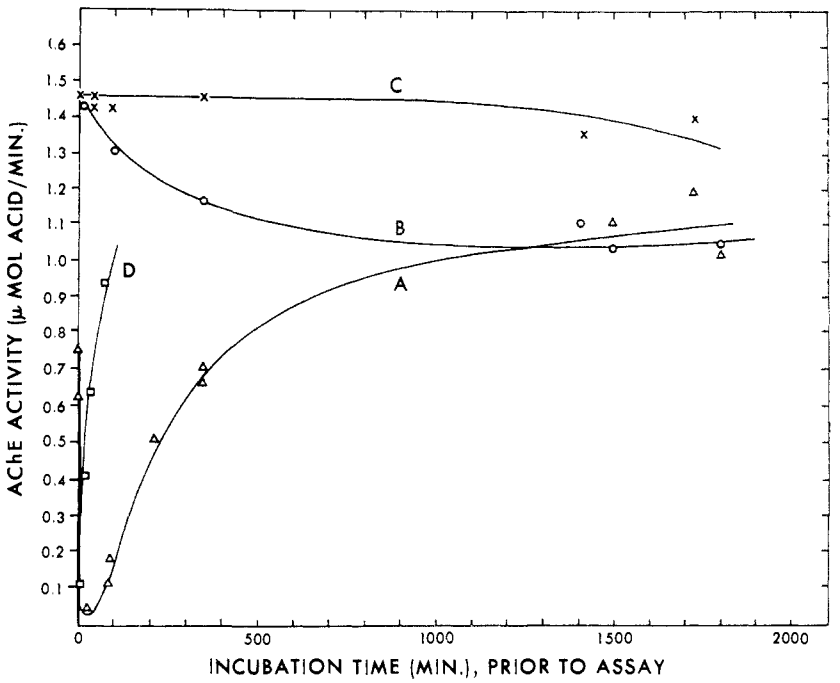


Figure 5.—Reaction of III with AChE; inhibition and reactivation, pH 7.4, 25°: (A) compound III (in dioxane) added directly to enzyme buffer mix; (B) compound III incubated for 1 hr at pH 7.4 prior to addition of enzyme; (C) untreated AChE control; (D) return of activity in the presence of 10^{-3} *M* TMB-4.

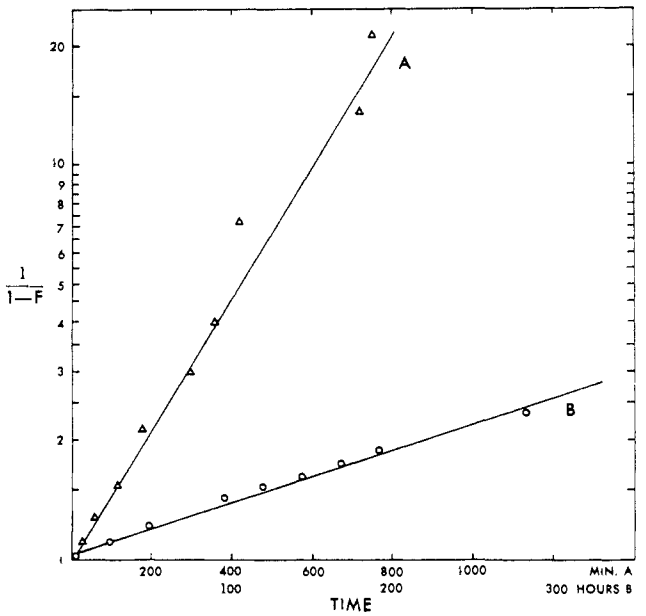


Figure 6.—Spontaneous reactivation of inhibited eel AChE, pH 7.4, 25°. Inhibitors: (A) compound III; (B) ethyl *p*-nitrophenyl methylphosphonate. (F) represents the fraction of restored enzymatic activity. In each case, the inhibited enzyme was Sephadexed to remove residual inhibitor prior to reactivation study.

are small.[35] Thus, the properly positioned CO group would appear to contribute considerably to speeding the reactivation process.[36] That propinquity catalysis is not the only contributor to rapid spontaneous reactivation is evidenced by a recent report that bis(β-chloroethyl)phosphorylated bovine erythrocyte cholinesterase reactivates spontaneously with a half-time of 41 min.[37a] These results indicate the need for a study of the mechanism of spontaneous reactivation.

(6) Alkylation of Aged Inhibited Enzyme.—Seven of the compounds (VII, VIII, XXV, XXXIII, XXXIV, XXV, and XXXVI) were tested for reactivation of aged inhibited enzyme. These have a wide range in phosphonate alkylation rates and also in the hydrolysis rates of the corresponding mixed esters, V. Further, some members of the group contain the "needed" positive charge for enzyme binding while others do not. None was effective. Each compound was found to be a progressive inhibitor of the active enzyme (suggesting covalent reaction with the enzyme).[37b] Conditions for test of reactivation were limited to those which produced only partial inactivation of the intact enzyme. Typical conditions, time of incubation and extent of inhibition of the intact enzyme (in parenthesis) were as follows: VII, pH 7.4, 10^{-2} *M*, 30 min (83%); VIII, pH 6.0, 10^{-3} *M*, 108 min (24%); XXV, pH 6.0, 10^{-2} *M*, 90 min (23%). All measurements were made at 25°. Finally, ^{32}P-labeled aged inhibited enzyme was treated for 4 days with VII. There was no removal of the ^{32}P label.

Evidently, even highly reactive alkylating agents fail to remove the methylphosphonate group from the aged inhibited enzyme, II. Why? We consider two general possibilities. (1) The phosphonate group, which is formed initially upon aging[3] (aging occurs *via* a carbonium ion pathway with C–O cleavage) has become unavailable for alkylation. This could occur for a variety of reasons, including a conformational change which might cause imbedding of the phosphonate group into a "protected" area of the enzyme, deactivation of the phosphonate group through charge neutralization by a nearly positively charged group on the enzyme, or even reaction with another of the enzymes amino acids to give a dicovalently bound struc-

(35) Reference 1, p 340.

(36) Even more rapid reactivation is observed with ring substituted phenacyl methylphosphonylated eel AChE. C. Gunter, unpublished data.

(37) (a) E. Reiner and W. N. Aldridge, *Biochem. J.*, **105**, 171 (1967); (b) G. M. Steinberg and J. A. Cramer, *Biochem. Pharmacol.*, **19**, in press.

ture. (2) Alkylation does take place, but the enzyme active site is no longer a "good" leaving group. This might result from a conformational change concurrant with the occurrence of aging or perhaps as a result of a separate nonspecific reaction of the alkylating agent with another site in the enzyme. If this were so, the group z activated hydrolysis, if it occurred at all, would lead to self cleavage of the inserted R group rather than separation of the phosphonate moiety from the enzyme. Clearly, only further studies can establish the validity of any of these speculations.

Experimental Section

All reagents were analytical grade or equivalent. Most of the test compounds were obtained from commercial sources or were prepared by literature methods and gave the reported physical properties. Purification by distillation or recrystallization was performed as required.

Sodium *p*-Nitrophenyl Methylphosphonate (VI). A. Bis(*p*-nitrophenyl) Methylphosphonate.—To a rapidly stirred suspension of 143 g (0.8 mol) of rigorously dried finely powdered sodium *p*-nitrophenolate in 1.4 l. of dry Et_2O containing 45 g (0.45 mol) of Et_3N, 49 g (0.38 mol) of freshly distilled methyl phosphonodichloridate was added slowly. The mixture was stirred at room temperature for 30 min, refluxed for 2 hr and allowed to stand overnight. The solid was filtered and washed (H_2O) until the wash was colorless. It was then suspended, with stirring, three times in 1 l. of ice–water to remove residual sodium *p*-nitrophenolate. After drying *in vacuo* the material was crystallized twice (Me_2CO) to give 61 g (67%) of product mp 122–123° (lit.[38] 155°).

B. Sodium *p*-Nitrophenyl Methylphosphonate.—To 36 g (0.106 mol) of bis(*p*-nitrophenyl)methylphosphonate in 1 l. of MeCN, 1.85 l. of 1 *N* KOH was *slowly* added with stirring. The reaction mixture was acidified with AcOH to pH 5.8, the MeCN removed *in vacuo*, and the residue extracted (Et_2O, 4 X) to remove the *p*-nitrophenol formed in the reaction. The remaining colorless liquid was evaporated to a small volume and passed over a Dowex-50 (acid) ion-exchange resin. The free acid was recrystallized from $CHCl_3$–pentane to mp 107–108° and converted into the Na salt by slow addition of an equivalent quantity of NaOMe in MeOH. MeOH was removed and the product recrystallized from EtOH, mp 265–270°, yield, 23 g (90%). *Anal.* ($C_7H_7O_5NNaP$) C, H, P.

Alkylation Reaction.—Phenacyl bromide is used to exemplify the standard procedure. DMF (Baker Anal.) was purified by treatment with CaH_2 and distilled at 50 mm directly into a storage vessel containing type 4A molecular sieve (Linde Div., Union Carbide Corp.).

A. Standard Alkylation Procedure.—Phenacyl bromide (Eastman White Label, α-bromoacetophenone) and VI were mixed in DMF in equal concentrations of 5×10^{-3} *M* and the solution placed in a constant temperature bath at 25°. Periodically, 25-μl aliquots were taken for assay of the resultant mixed ester. With the less reactive alkylating compounds reaction was conducted at 60°.

B. Assay of Mixed Esters. Assay A.—The sample was added to pH 10, 0.1 *M* carbonate buffer, refluxed 10 min, cooled to room temperature and the *p*-nitrophenolate content determined from the absorbance at 402 mμ (Beckman DB-2 spectrophotometer). The absorbance gave a linear Beer–Lambert relationship over the useful concentration range. Under these conditions, VI gave a negligible blank (2%), while the most stable ester, ethyl *p*-nitrophenyl methylphosphonate was completely hydrolyzed in less than 3 min. Hence, this assay gave a quantitative measure of the conversion of VI into mixed ester.

Assay B.—The sample was added to temperature-equilibrated aqueous 0.1 *M* Tris, pH 7.15, 25°, rapidly mixed and transferred to a cuvette mounted in the thermostated compartment of the spectrophotometer, and a recording made of the progressive hydrolysis of the mixed ester by measurement of the absorbance at 402 mμ (*p*-nitrophenoxide). The time elapsed from initial mixing to start of the spectrophotometer recording could be reduced to a minimum of 20 sec. From this assay, one could determine (a) the initial *p*-nitrophenol content (a measure of decomposition of mixed ester during the alkylation period), (b) the total *p*-nitrophenol available, *i.e.*, the total amount of mixed ester that had been formed *via* alkylation, and (c) the hydrolysis rate of the prepared mixed ester. In every case, the hydrolysis reactions followed good first-order kinetics and thus strongly indicated the presence of a single hydrolyzable species.

C. Tlc of Mixed Ester.—To further substantiate the formation of III from the reaction of phenacyl bromide with VI, in DMF, an aliquot of the reaction mixture was spotted on a silica gel tlc plate next to authentic III. The spots were moved with 95% $CHCl_3$–5% Me_2CO and developed with I_2 vapor. Authentic III gave a single spot at R_f 0.6. The reaction mixture gave a large spot at R_f 0.6, some unreacted phenacyl bromide (R_f, 1.0) and VI at R_f 0. There also were observed a few minor spots of unknown composition.

D. Alkylation in Aq EtOH.—To $(EtO)_2P(O)OH$ (3.061 g, 0.020 mol), dissolved in 20 ml of H_2O, there was added 50 ml of 0.2 *M* phosphate buffer, pH 7.0 and 50 ml of EtOH containing 4.075 g (0.020 mol) of phenacyl bromide. The clear mixture was refluxed for 3.25 hr, cooled (pH now 1.9), and vacuum stripped to remove most of the EtOH. The remaining solution was extracted with three 30-ml aliquots of $CHCl_3$, the aliquots combined and vacuum stripped. There remained 2.86 g of oil. Analysis by vapor phase chromatography (2% SE-30 on 100–120 mesh Gas Chrom S, 6 ft × 1/4 in., column temperature 168°, injector temperature 250°, argon flow rate 75 ml/min., detector–argon diode) gave 0.29 g (5.1%) of a peak at t = 13.75 min which corresponds to that of pure diethyl phenacyl phosphate. With $(BuO)_2P(O)OH$, under the same conditions the yield of dibutyl phenacyl phosphate was 6%. No effort was made to maximize yields.

Enzyme Studies. A. Inhibition and Spontaneous Reactivation.—To a solution of eel AChE (Sigma, type III), approx 5×10^{-9} *M* in 0.5 *M* KCl, 0.15% gelatin, 1.5×10^{-3} *M* Tris, pH 7.4, 25°, there was added III[39] dissolved in dioxane to give 10^{-5} *M* III and 1% dioxane. III was added last to minimize its hydrolysis prior to reaction with the enzyme. Aliquots (0.2 ml) were periodically taken and assayed for enzymatic activity by adding to 2.8 ml of a mixture contained in the thermostated reaction vessel (25°) of a Radiometer TTT1 Titrator, fitted with Autoburette ABU-1 and Titrigraph SBR-2. The resulting 3.0 ml of assay solution had the composition: 5×10^{-4} *M* Tris (pH 7.4), 0.3 *M* KCl, 0.1% gelatin, 0.037 *M* acetylcholine chloride. Enzyme activity was determined from initial slope of the record of rate of addition of 0.015 *N* NaOH to maintain constant pH. N_2 was passed over the assay solution in the covered titration vessel to prevent CO_2 absorption.

For measurement of reaction kinetics, the enzyme was incubated with inhibitor for 15 min and the inhibited enzyme separated from residual inhibitor by passage through Sephadex G-50 or Bio-Gel 10. Reactivation of the enzyme contained in the protein fractions followed first-order kinetics. Controls of uninhibited enzyme were run concurrently and their values (which did not change by more than 20% from their initial activity values) were used in computing reactivation results.

For the reaction A → B, where A represents inhibited enzyme and B, reactivated enzyme, the first-order kinetic relationship is $\ln (B_\infty/B_\infty - B_t) = kt$, where B_t and B_∞ represent enzyme activities at times t and ∞, respectively. Actually, B_∞ was determined from the activity of a control enzyme solution identical with the incubation mixture except that it contained no III. To correct for changes in B_∞, let the fraction of activity restored, $F = B_t/B_\infty$. Then, $\ln [1/(1 - F)] = kt$.

Reactivation study of ethyl methylphosphonyl AChE was performed as above. Instead of III, ethyl *p*-nitrophenyl methylphosphonate[40] (ester corresponding to IX) was used to inhibit the enzyme.

B. Reactivation with TMB-4.—The incubation mixture containing III, composition as cited above, was maintained for 15 min. Then, 0.53 ml was mixed with 0.06 ml of 10^{-2} *M* TMB-4. This solution was kept at 25° and aliquots were taken for assay. Correction was made for inhibition (reversible) of the enzyme by TMB-4.

C. Alkylation of Aged Inhibited Enzyme. Test for Return of Activity.—AChE (Sigma, type III) diluted 1 to 1000 (to ap-

(38) H. Tolkmith, U.S. Patent 2,668,845, Feb. 9, 1954; *Chem. Abstr.*, **49**, 5519 (1955).

(39) C. N. Lieske, J. W. Hovanec, G. M. Steinberg, J. N. Pikulin, W. J. Lennox, A. B. Ash, and P. Blumbergs, *J. Agr. Food Chem.*, **17**, 255 (1969).

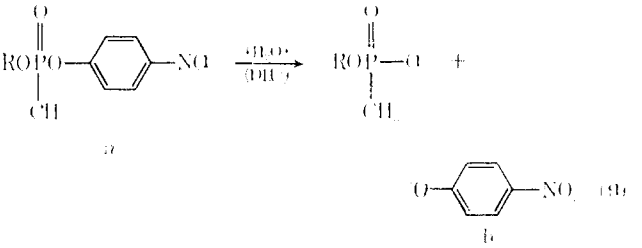
(40) A. M. DeRoos, *Rec. Trav. Chim. Pays-Bas*, **78**, 145 (1959).

proximately 2×10^{-8} *M*) in 1.08 ml of standard Tris–gel buffer (10^{-3} *M* Tris, 0.3% gelatin, 1 *M* KCl, pH 7.4) was mixed with 0.12 ml of 10^{-5} *M* pinacolyl methylphosphonofluoridate (soman) and the mixture incubated at room temperature overnight. This treatment yields aged inhibited enzyme, II.[3] The solution (1 ml) was placed on a Sephadex G-50 (11 × 1 cm) or Bio-Gel P-10 (14 × 1 cm; 50–150 mesh) column and eluted with standard Tris–gel buffer. Effluent (3 ml) containing the protein fraction was collected. The effluent (2.5 ml) was mixed with 2.0 ml of H_2O and 0.5 ml of test compound (dissolved in H_2O or other appropriate solvent). The reaction solution was maintained at 25°, at constant pH, usually 6.0, with the Radiometer Autotitrator. Aliquots were taken, passed through the gel column to separate the enzyme from residual test compound (and its hydrolysis products). Assays were then made for enzymatic activity both at once and after incubation for 24 hr at the pH employed during the first incubation to permit additional time for possible self-reaction. "Control" measurements of inhibition of intact enzyme by test compounds were performed in the identical manner, except that uninhibited enzyme was used as the enzyme source.

Test for Release of ^{32}P.—A 0.1-ml sample of 10^{-5} *M* AChE was incubated with 1 μl of ^{32}P labeled 0.01 *M* soman (Defense Research Board of Canada) in 10^{-3} *M* Tris (pH 7.4), 0.3% gelatin, and 1 *M* KCl. The inhibited enzyme was allowed to age overnight and the mixture was then passed through a Bio-Gel P-10 column to remove nonprotein bound ^{32}P. A 0.5-ml aliquot of the inhibited enzyme was mixed with 0.5 ml of 2×10^{-2} *M* phenacyl bromide (VII) in 50% aq acetone and the mixture incubated for 4 days. The treated AChE was divided into two parts, one of which was treated for 1 hr with 2×10^{-2} *M* TMB-4 (an oxime reactivator of unaged phosphonylated AChE).[11] A control sample of inhibited untreated enzyme and each of the two treated fractions were solvent extracted. Each was made acidic with two drops of 5 *N* H_2SO_4 and extracted three times with 1.0-ml portions of water saturated 1:1 BuOH–C_6H_6. The entire organic phase was transferred to a 2.5-cm planchet and made alkaline to phenolphthalein with 10 *N* NaOH then dried with moderate heat from a heat lamp. The planchet was counted with Tracerlab low background counter (Omni/guard).

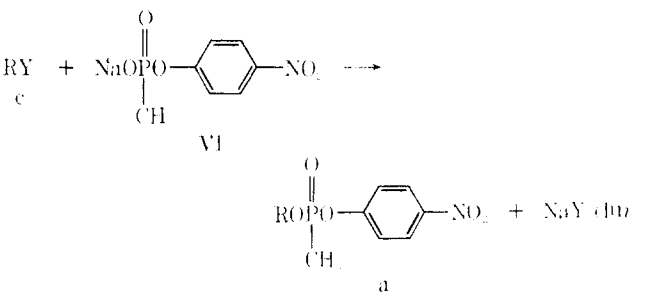
Hydrolysis of Alkylating Compound.—Each of the compounds listed in Table IV was made up to 10^{-3} *M* in a mixture of 0.1 *M* aq KCl and EtOH. The quantity of EtOH was limited to the minimum required to insure complete solution of the test compound. The rate of hydrolysis was determined from the rate of delivery of 0.0146 *N* NaOH required to maintain constant pH with the Radiometer Autotitrator TTT1. Titration was performed on 3 ml of test solution in a covered vessel using the 0.25-ml capacity burette. N_2 was passed over the solution to minimize CO_2 absorption. The reaction in each case was first-order. The rate constant, k_{obsd}, was calculated using the Guggenheim procedure.[30]

Kinetics.[41,42] **A. For Assay B. First-Order Kinetics.**—The hydrolysis reactions of the mixed phosphonate esters are typified by III.[14] The reaction is first-order each in III, [OH^-], and buffer base. At constant pH, therefore, the reaction is first-order (eq 9) since [OH^-] and [buffer base] remain constant.



The hydrolysis was followed by spectrophotometric measurement of *p*-nitrophenoxide ion (b). Hence, $\ln [b_\infty/(b_\infty - b_t)] = k_{obsd}\, t$; and $\log (A_\infty/A_\infty - A_t) = k_{obsd}\, t/2.303$, where b_t and b_∞ represent the concentration of *b* at times *t* and ∞, respectively, and A_t and A_∞ the corresponding absorbance values measured on the spectrophotometer. A plot of $\log [A_\infty/(A_\infty - A_t)]$ *vs.* *t* should give a straight line of slope = $k_{obsb}/2.303$ and *y* intercept = 0. If the hydrolysis solution contains a mixture of a and b, *i.e.*, free *p*-nitrophenol produced during the incubation period in DMF, the $\log [A_\infty/(A_\infty - A_t)]$ *vs.* *t* plot will still be linear. However, its intercept will not be zero but equal to $\log [A_\infty/(A_\infty - A_0)]$. With all of the compounds examined under assay B conditions, the plotted data gave good straight lines for several reaction half-lives.

B. For Alkylation Reaction. Second-Order Kinetics.—The alkylation reactions in DMF were found to follow second-order kinetics during the early stages of the reaction. For convenience, the reactants, c and VI were present in equal concentration, eq 10. Under these conditions the integrated form of the rate



equation is: $1/C_t - 1/C_0 = k_2 t$, where C_t and C_0 are the concentrations of *c* (and also of VI) at times *t* and *0*, respectively. Since $C_t + a_t = C_0$, the value of C_t can be determined by assay of a_t (concentration of a at time *t*) using either assay procedures A or B, above. A plot of $1/C_t$ *vs.* *t* yields a straight line with slope equal to k_2 and *y* intercept equal to $1/C_0$. Reaction half-time, $t_{1/2} = 1/k_2 C_0$.

Acknowledgment.—We are indebted to Drs. Ash and Blumbergs, Ash-Stevens, Inc. for providing several test samples, to Dr. George Davis, Research Laboratories, Edgewood Arsenal, for valuable discussions concerning the mechanism of alkylation reactions and to PFC Warren Gillilan for measurement of the rates of solvolysis of several of the alkylating compounds.

(41) A. A. Frost, and R. G. Pearson, "Kinetics and Mechanism," John Wiley & Sons, Inc., New York, N. Y., 1953.

(42) W. J. Moore, "Physical Chemistry," Prentice–Hall, Inc., Englewood Cliffs, N. J., 1962.