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Reaction of 4-Formyl-1-methylpyridinium Iodide Oxime with Isopropyl Methylphosphonofluoridate*

J. C. Lamb,† G. M. Steinberg,‡ S. Solomon, and B. E. Hackley, Jr.

ABSTRACT: The reaction of isopropyl methylphosphonofluoridate (GB) with 4-formyl-1-methylpyridinium iodide oxime (4-PAM), a model treatment compound for anticholinesterase poisoning, in near-neutral aqueous solution yields *O*-(isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime (4-PPAM). Its rate of formation is defined by the relationship $dx/dt = k[GB][4\text{-PAM anion}]$, where $k = 885 \pm 70$ M^{-1} min^{-1}, 30°, pH 7.6, 0.1 M KNO_3. *O*-(Isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime is quite stable in acidic and neutral aqueous solution. Its decomposition in alkaline solution is defined by the relationship $dx/dt = k[OH^-][4\text{-PPAM}]$, where $k = 86 \times 10^3$ M^{-1} min^{-1}, 30°, 0.1 M KCl.

O-(Isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime is a potent anticholinesterase. Its decomposition in near-neutral aqueous solution is speeded by imidazole, 4-formyl-1-methylpyridinium iodide oxime, bicarbonate, and two hydroxamic acids.

In the past two decades the class of organophosphorus compounds demonstrating anticholinesterase properties has become increasingly important. Among these we find a wide range of insecticides, certain medicinals, and the chemical compounds known as nerve agents. These compounds exhibit a spectrum of pharmacological effects; however, their principal actions closely resemble those of the neurohumor acetylcholine. This, together with their ability to rapidly and irreversibly inhibit the enzyme acetylcholinesterase, has suggested that their toxic action is a direct result of the inhibition of this enzyme. Inhibition of the enzyme has been shown to involve the actual phosphorylation of the enzyme, presumably at its active site. In general, the anticholinesterases are relatively resistant to aqueous hy-

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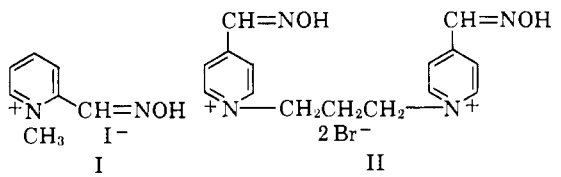
drolysis. However, several groups of compounds have been found which react rapidly with many of the anticholinesterases in near neutral aqueous solution. Among these rapid reactants the oximes are also outstanding for their ability to remove the phosphate (or phosphonate) moiety from the inhibited enzyme and so restore enzymatic activity. Within the family of oximes two are of major interest because of their effectiveness (when administered together with the alkaloid atropine) in saving the lives of experimentally poisoned animals. These are 2-formyl-1-methylpyridinium iodide oxime (2-PAM; I)[1,2] and 1,1′-trimethylenebis(4-formylpyridinium bromide oxime) (TMB-4; II).[2]

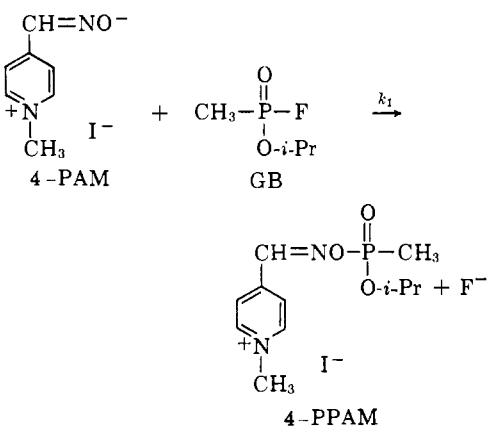


I II

These oximes, as well as many others, react rapidly under physiological conditions of pH and temperature with such organophosphonates as the nerve agent, isopropyl methylphosphonofluoridate (GB). In the course of a study of the kinetics of the reaction of oximes with GB (Hackley, 1958; Green *et al.*, 1958) in near-neutral aqueous solution, it was observed that the oximes fall roughly into two groups: one group which yields 2 or more moles of acid/mole of GB destroyed in the rate-controlling step, and a second group which gives approximately 1 mole of acid in a first rapid step followed by a slow acid-producing second step. Compounds I and II fall into the latter group. It seemed likely that there was intermediate formation of a phosphonylated oxime. In the case of the phosphonylated pyridinium oximes, one might expect, by analogy with the reported properties of other quaternary phosphates, that these compounds might themselves be potent anticholinesterases, and this was indeed found to be the case. A preliminary report of this observation has been published (Hackley *et al.*, 1959). For additional background information pertinent to the present report, reference may be made to several comprehensive recent reviews on the organophosphate anticholinesterases (O'Brien, 1960; Heath, 1961; Koelle, 1963).

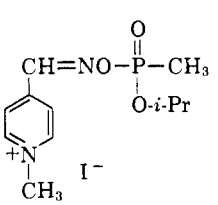
It was the purpose of this study to examine in detail the sequence of reactions which occur under physiological conditions of pH and temperature, *i.e.*, near-neutral aqueous solution, between GB and a quaternary pyridinium aldoxime. For the latter, 4-formyl-1-methylpyridinium iodide oxime (4-PAM) was chosen instead of I or II. Its phosphonylation product is more stable than that of I and with it one avoids the complexity of two reactive oximino groups, as with II.

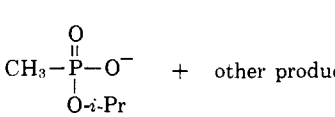
By analogy with its reaction with isonitrosoacetophenone (Hackley, 1958), GB should give with 4-PAM the sequential reactions 1 and 2. If this were indeed the reaction sequence, reaction 1 should follow a second-order kinetic pattern with first-order dependence upon GB and oximate, respectively. The formation of fluoride ion, at a constant pH, near neutrality, represents an increase in the quantity of acid in the system. Hence, reaction rate could be measured not only by direct determination of the concentration of reactants or products, but also by following the rate of acid production at constant pH. 4-PAM was maintained in large excess and therefore its concentration varied by a relatively small factor during the course of reaction. Determination of fluoride is a difficult and time-consuming procedure. Hence, with the availability of a Beckman Autotitrator which can be set to deliver alkali to maintain constant pH, the rate of acid production was measured as a means of studying the kinetics of reaction 1. To confirm the validity of this assumption, independent determinations were made of GB loss by the Schoeneman reaction, of *O*-(isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime (4-PPAM) appearance by the formation of a nonchloroform-extractable anticholinesterase, and by independent measurement of the stoichiometry of acid production.



4-PAM GB

4-PPAM (1)





(2)

The rate of reaction 2 was measured by loss of anticholinesterase activity. Support for the hypothesis that the nonchloroform-extractable anticholinesterase was in fact 4-PPAM came from parallel studies of the rate of decomposition of an independently synthesized sample

[1] Abbreviations used: 2- or 4-PAM, 2- or 4-formyl-1-methylpyridinium iodide oxime; TMB-4, 1,1′-trimethylenebis(4-formylpyridinium bromide oxime); GB, isopropyl methylphosphonofluoridate; 4-PPAM, *O*-(isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime.

[2] 2-PAM and TMB-4 are symbols which are in common use in the biological and biochemical literature. The former is generally used as its chloride or methylsulfonate salts. These are referred to in the biological literature as PAM chloride and P2S, respectively.

of 4-PPAM, from comparison of the reaction rates of both samples with eel cholinesterase, and from ultraviolet absorption spectra. For convenience, the nonchloroform-extractable inhibitor is referred to in the remainder of the paper as 4-PPAM.

The discovery that, under "physiological" conditions of pH and temperature, the product of reaction between the highly toxic nerve agent and its treatment compound resulted in the formation of a new equally toxic compound prompted a further study of the effect of added nucleophiles upon the decomposition rate of the latter.

Experimental Section

Materials. All stock reagents were of C.P. grade. Hexanohydroxamic acid and benzohydroxamic acid were obtained from Mr. J. Epstein, Protection Research Branch, Edgewood Arsenal. The 4-PPAM was prepared by methylation, with methyl iodide, of the product of reaction of 4-PAM and isopropyl methylphosphonochloridate (Hackley and Owens, 1959). The sample was stored in a freezer under vacuum over P_2O_5.

Anal. Calcd for $C_{11}H_{18}PIN_2O_3$: C, 34.6; H, 4.69; I, 33.1; N, 7.29; P, 8.07. Found: C, 33.8; H, 4.8; I, 34.3; N, 7.5; P, 7.47; moisture (Karl Fischer), 0.04.

The agreement between calculated and found values would appear to be reasonably satisfactory when one considers that 4-PPAM is strongly hydroscopic and unstable to handling, and that it resisted all attempts at purification by recrystallization. A single 4-PPAM sample was used for the entire study. This sample, which will be referred to as *crys* 4-PPAM to distinguish it from 4-PPAM prepared *in situ*, reacted with electric eel cholinesterase with a second-order rate constant of 4.7×10^7 M^{-1} min^{-1} (for conditions see under enzymatic procedure).[3] The sample showed no decline in cholinesterase reaction rate during the course of the study.

Kinetics

Acid Production. Each of the eight kinetic runs was carried out under identical conditions. In a typical run, 60 ml of 0.5 M KNO_3 and 100 ml of CO_2-free water were placed in a 400-ml jacketed beaker maintained at 30°. 4-PAM, 78.3 mg (final concentration 9.89×10^{-4} M), was dissolved in this solution and the pH was adjusted to 7.6 by means of a Beckman Autotitrator. The volume was adjusted to 294 ml by addition of water and the pH again was adjusted to 7.6. Four milliliters of a stock GB solution (5 μg/ml) was added, and the volume then was brought to 300 ml; final concentration of GB, 6.70×10^{-5} M. Reaction rate was calculated by the Guggenheim procedure (Frost and Pearson, 1953) from a record of the volume of 0.01 N sodium hydroxide delivered by the Autotitrator as a function of time at a constant pH of 7.6 (Swidler and Steinberg, 1956).[4] Due to fluctuations in the control by the Autotitrator, the pH oscillated between the extremes of 7.56 and 7.62 during several runs.

GB and Nonchloroform-Extractable Inhibitor Determination. Direct determination of GB was made by the Schoeneman reaction as adapted for analytical purposes by Rosenthal *et al.* (1956). This reaction involves conversion of GB to a perphosphonate which in turn oxidizes an amine, such as *o*-toluidine, to a colored compound. In each of the two kinetic runs, there was added to 300 ml of 0.01 M phosphate, pH 7.6, 30.0°, 4-PAM and GB to give initial concentrations of 3.0×10^{-4} M and 6.7×10^{-5} M, respectively. At suitable intervals 10-ml aliquots were withdrawn and extracted with 2 ml of chloroform, 1 ml of which was analyzed by the Schoeneman reaction. The aqueous layer was removed, adjusted to pH 4–5 with dilute HCl to stop 4-PPAM hydrolysis (see Results), then extracted with three 30-ml portions of chloroform to completely remove residual GB.[5] The layer was then diluted appropriately for determination of anticholinesterase activity as described below.

Decomposition of 4-PPAM. PREPARED *in situ.* 4-PPAM was prepared in water solution at pH 7.5 by treating GB, 6.7×10^{-5} M, with a very large excess of oxime, 3.0×10^{-3} M. Reaction was carried out in 300 ml of 0.01 M KCl in a jacketed beaker with magnetic stirring. pH was continuously monitored with a Beckman Model G pH meter. After 5 min, when reaction was approximately 98% complete, 2-ml aliquots were withdrawn periodically and extracted with 60 ml of chloroform to be absolutely certain of GB absence, and the declining inhibitor concentration was determined enzymatically.

CRYSTALLINE SAMPLE. All of the reagents except 4-PPAM were added to 300 ml of CO_2-free distilled water contained in a jacketed reaction vessel fitted with motor stirrer. The pH was adjusted with the Autotitrator and checked on a previously standardized Beckman Model G pH meter. Crystalline 4-PPAM was added directly to start the reaction. Since the 4-PPAM is very soluble and the reaction period quite long (2–7 hr), no appreciable zero time error resulted. The temperature was maintained at 30.0° by circulating thermostatically controlled water through the jacketed beaker.

In those instances where oxime was present it served as a buffer and no further pH adjustment was required. In the absence of oxime, pH was maintained by the Autotitrator. At selected time intervals 1-ml aliquots were withdrawn and serially diluted to a proper concentration range for measurement of rate of enzyme inhibi-

[3] The sample was estimated to contain approximately 18% unreacted oxime; hence, the corrected value of the reaction rate constant is 5.7×10^7 M^{-1} min^{-1}. For details concerning purity and methods of handling, the paper by G. M. Steinberg and S. Solomon (in preparation) may be consulted.

[4] A detailed study is reported of the pitfalls of following reaction rates with the Beckman Autotitrator, a machine which titrates incrementally.

[5] Rosenthal *et al.* (1956) give the distribution coefficient of GB in $CHCl_3$–H_2O as 31.

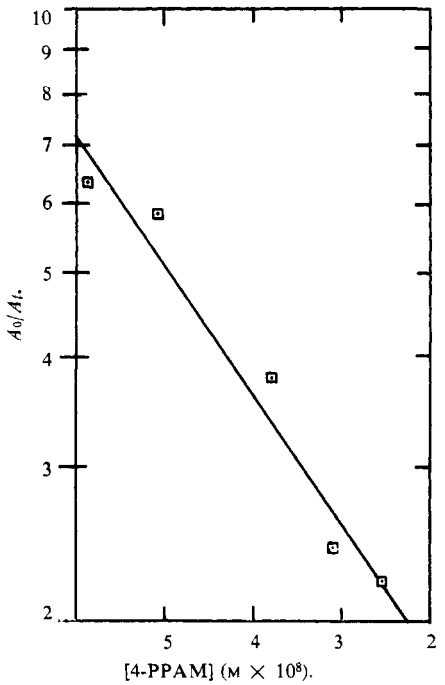


FIGURE 1: Acetylcholinesterase inhibition: first-order rate dependence upon 4-PPAM concentration. Enzyme concentration, 3.94×10^{-10} M; incubation time (with inhibitor), 1 min; 25°; pH 7.40.

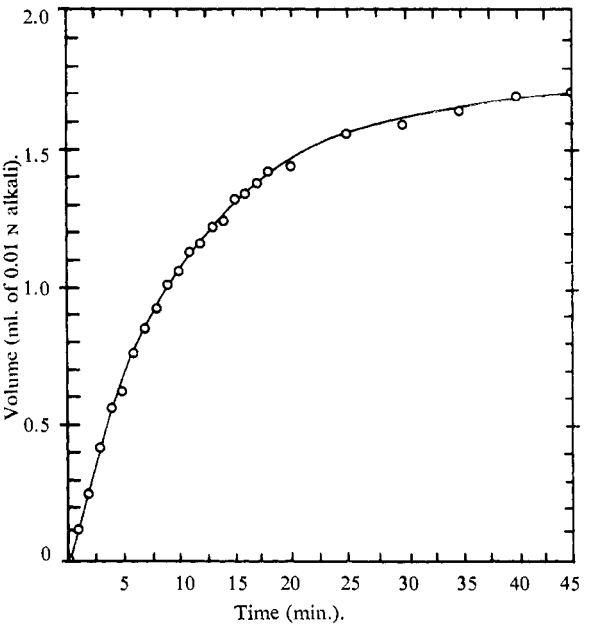


FIGURE 2: Reaction of GB with 4-PAM: typical kinetic run in Autotitrator. pH 7.60, 30°, 0.1 M KNO_3, 9.89×10^{-4} M 4-PAM, 6.70×10^{-5} M GB; rate of delivery of 0.01 N NaOH to maintain constant pH.

tion (value of A_0/A_t between 2 and 10; see below). Since the concentration range of inhibitor added to the enzyme in the microanalytical procedure is limited and precision falls off sharply outside the range, "improper" dilutions were discarded and new aliquots were taken.

Enzymatic Determination of Inhibitor Concentration. The concentration of 4-PPAM either appearing during the course of the direct reaction of 4-PAM and GB or that remaining during studies of the rate of breakdown was estimated by its rate of inhibition of the enzyme eel acetylcholinesterase. Relative enzymatic activities were determined from constant pH titrations similar to the procedure of Glick (1937) as modified by Dr. H. Michel of these laboratories. The apparatus has been described by Schwartz and Meyers (1958). The method consists of a rapid microtitration at constant pH of the acetic acid produced upon enzymatic hydrolysis of acetylcholine chloride. This substrate is maintained in such excess that for short time intervals, 1 min in this case, its rate of hydrolysis is directly proportional to enzyme concentration, *i.e.*, reaction is of zero order.

It had been shown previously (Michel and Krop, 1951) that the rate of inhibition of the enzyme upon reaction with an organophosphorus compound is first order with respect to each. Its first-order dependence on 4-PPAM concentration was confirmed by us (Figure 1). Under the condition of excess inhibitor, I, its concentration is given by eq 3, where E_0 and E are the concentrations of active enzyme at times t_0 and t, respectively, k_1 is the second-order reaction rate constant,

$$I = \frac{2.303}{k_1 t} \log \frac{E_0}{E} \qquad (3)$$

and t, the time of reaction. Since the concentration, E, was determined in each case by measuring the quantity of acid, A, produced in 1 min, we may set the relation: $E_0/E = A_0/A_t$, where A_0 and A_t are the quantities of acid produced in 1 min after incubation with inhibitor for 0 and t min, respectively. For convenience the time, t, in eq 3 was uniformly set at 1 min. Thus, I is directly proportional to $\log A_0/A_t$ and in those cases where the rate of change of inhibitor concentration was the function of interest, the value of $\log A_0/A_t$ was used directly in the computations.

In practice, A_0 was determined as the volume of standard alkali required to maintain constant pH for 1 min immediately upon addition of inhibitor and substrate. A_t was measured in the same manner except that in this case all substances were mixed and incubated for 1 min *in the absence of substrate*. At that time, substrate was added and now the quantity of acid produced during the next 1-min interval was measured.

Standard conditions employed in each assay were 25.0°, pH 7.40, in aqueous solution containing 0.1 M KCl, 0.025 M Tris buffer, and 0.3% gelatin. Acetylcholine chloride 0.025 M (Merck) served as substrate for the electric eel acetylcholinesterase[6] which was set at 3.94×10^{-10} M.[7] pH was determined by use of the

[6] Obtained from Dr. D. Nachmanson, Columbia University Medical School.

[7] As determined by Dr. H. Michel.

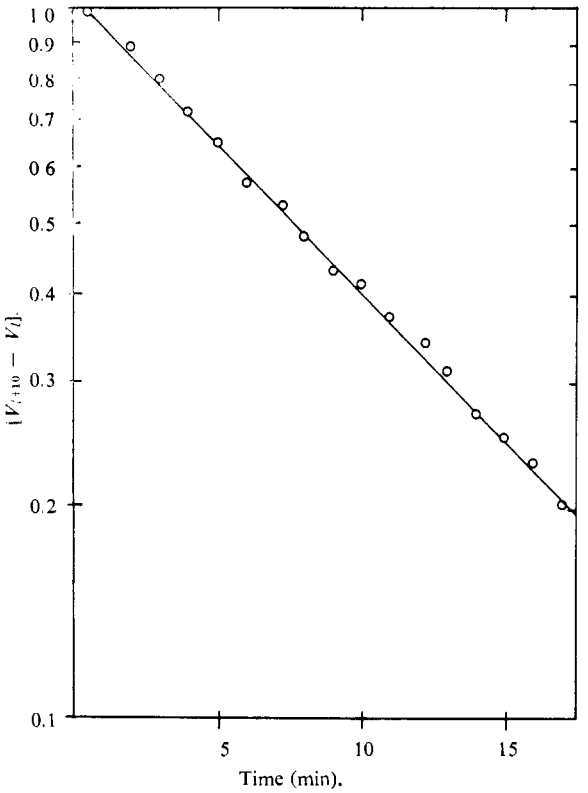


FIGURE 3: Guggenheim plot of typical acid production run. Intervals of 10 min, $k_{obsd} = 10.49 \times 10^{-2}$ min^{-1}.

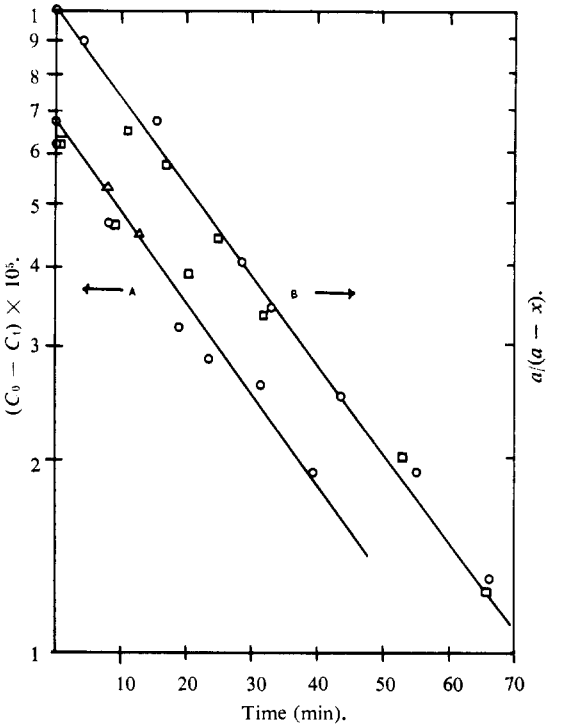


FIGURE 4: Comparison of rate of disappearance of GB with rate of formation of nonchloroform-extractable cholinesterase inhibitor. Curve A, appearance of inhibitor; curve B, disappearance of GB; run 59, ○; 60, ⊡; 66, △.

Beckman Model G pH meter and was maintained constant by manual titration during the 1-min interval. Because of the logarithmic nature of the response, A_0/A_t values had to lie within the range of 2–10 in order to obtain reasonably precise estimates of I.

To distinguish between GB and the organic solvent insoluble product of its reaction with 4-PAM (4-PPAM), GB was removed by extraction with chloroform. The $CHCl_3$–H_2O distribution coefficient previously reported for GB was confirmed in this work.[5] It was also established that water saturated with chloroform had no effect upon enzyme activity and that 4-PPAM was not removed from water by repeated chloroform extraction.

Results

Kinetics of the Initial Reaction

Acid Production. In each run the concentration of 4-PAM was maintained in excess over that of the GB. Figure 2 is a typical graph of acid production *vs.* time. Since there was a slow, but definite, production of acid for a considerable time period, an empirical end point could not be certainly obtained. Pseudo-first-order rate constants were calculated by the Guggenheim procedure (Frost and Pearson, 1953) for which the end point value need not be known. A graph of a typical run using this procedure is shown in Figure 3. The first-order rate constant for each run was determined from the slope of the straight line fitted by inspection to eq 4, where V_t and V_{t+10} are volumes of standard

$$\log (V_{t+10} - V_t) = \frac{k_{obsd}t}{2.303} \qquad (4)$$

alkali delivered to maintain constant pH at times t and $t + 10$ min, respectively. The average value for the first-order rate constant, k_{obsd}, obtained under identical conditions in 8 runs (6.70×10^{-5} M GB, 9.89×10^{-4} M 4-PAM, 0.1 M KNO_3, pH 7.60, 30.0°), was $11.53 \pm 0.91 \times 10^{-2}$ min^{-1} (average $t_{1/2} = 6.01 \pm 0.45$ min). Since it had been previously established (Hackley, 1958; Green *et al.*, 1958; Green and Saville, 1956) that oxime reactions with phosphonofluoridates are first order each with respect to the oximate anion and to phosphonofluoridate, a second-order rate constant was calculated for the reaction and found to be 885 ± 70 M^{-1} min^{-1}.

For purposes of calculation the pK_a value of 4-PAM was taken as 8.39, based upon spectrophotometric data.[8,9] Using the rate constant for the early part of the reaction estimated by the Guggenheim method, it is calculated that 1.68 ± 0.13 ml of 0.01 N alkali is re-

[8] In another paper, G. M. Steinberg and S. Solomon (in preparation) have found a value of 8.50 determined by potentiometric titration in 0.02 M sodium iodide.

[9] Ginsburg and Wilson (1957) reported the pK_a as 8.6.

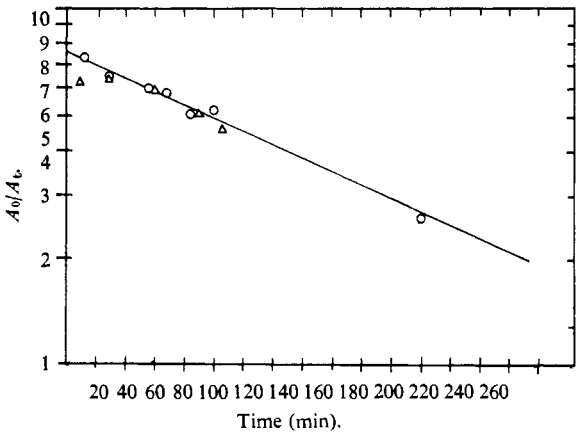


FIGURE 5: Hydrolytic decomposition of *in situ* 4-PPAM, pH 7.5, 30°, 0.1 M KCl, 3×10^{-3} M 4-PAM.

quired for the completion of reaction 1, *i.e.*, at $t = \alpha$. This corresponds to 0.81 mole of acid liberated/mole of GB. The theoretical value is 0.86 mole, when correction is made for consumption of partially neutralized oxime (Swidler and Steinberg, 1956).

GB Disappearance. Direct determination of the rate of disappearance of GB was followed in two identical runs using the Schoeneman reaction adapted for analytical purposes (Rosenthal *et al.*, 1956) (6.70×10^{-5} M GB, 3.00×10^{-4} M 4-PAM, 0.01 M phosphate, pH 7.60, 30.0°). Figure 4 shows the data plotted according to the conventional first-order rate relationship, eq 5, where a represents the initial concentration of GB and x that remaining at time t. The value of

$$k_{obsd} = \frac{2.303}{t} \log \frac{a}{a - x} \qquad (5)$$

k_{obsd} was found to be 3.12×10^{-2} min^{-1} ($t_{1/2}$ = 22.2 min). This gives a second-order rate constant, k_2, of 938 M^{-1} min^{-1} based on the oximate anion concentration, which agrees very well with that found acidometrically.

Appearance of a Nonchloroform-Extractable Inhibitor of Cholinesterase. After chloroform extraction of the GB for its determination in the kinetic runs above, there remained in the aqueous phase a powerful inhibitor of eel cholinesterase. Figure 4 shows that the rate of appearance of the nonextracted enzyme inhibitor closely parallels the fall in GB concentration. Data from a third independent run, made under identical conditions, in which only nonextractable inhibitor was determined, have been added to curve A, Figure 4. The k_{obsd} for the reaction followed in this manner was found to be 3.20×10^{-2} min^{-1} ($t_{1/2}$ = 21.5 min) with a k_2 of 959 M^{-1} min^{-1}, in very good agreement with the rates of GB loss and acid production.

4-PPAM. A. *In situ.* The latter part of the direct reaction between GB and 4-PAM gives a slow but discernible production of acid above the theoretically expected value of 0.86 mole. This acid production might reasonably be ascribed to the decomposition of the phosphonylated intermediate. Since such a process might be expected to give a complicated pattern of acid production, the rate of disappearance of the intermediate was measured by the enzymatic procedure. To assure rapid and complete conversion of the GB to 4-PPAM, reaction was carried out at a high concentration of 4-PAM (6.7×10^{-5} M GB, 3.0×10^{-3} M 4-PAM, pH 7.5, 0.1 M KCl, 30.0°). The inhibitory product formed in the reaction disappeared slowly under these conditions. In Figure 5, where composite data from two runs are combined, the log plot of inhibitor concentration *vs.* time gives a straight line consistent with first-order kinetics, with reaction half-time $t_{1/2}$ = 162 min. *Based upon the assumption of complete conversion of GB to 4-PPAM*, the concentration of the latter at $t = 5$ min is therefore $0.98 \times 6.7 \times 10^{-5}$ M = 6.6×10^{-5} M. From the rate of inhibition of the enzyme by an aliquot of the $t = 5$ min solution, the second-order rate constant for the reaction of *in situ* 4-PPAM with the enzyme under the standard assay conditions (see Experimental Section: Enzymatic Determination of Inhibitor Concentration) was found to be 4×10^7 M^{-1} min^{-1} (eq 3). A solution of 4-PPAM prepared *in situ* by reaction of 4-PAM with an excess of GB, with subsequent removal of the latter by chloroform extraction, absorbed in the ultraviolet region with a maximum at 256 mμ; calculated E_{max} 8.4×10^3. A comparison of the properties of the 4-PPAM prepared in aqueous solution at near neutrality, *in situ*, with that of the separately prepared crystalline 4-PPAM is given in Table I.

TABLE I: Properties of *in Situ* and Crystalline 4-PPAM.

4-PPAM	λ_{max}, mμ (E_{max})	Reaction Rate[a] (M^{-1} min^{-1})	Decompn. Rate,[b] $t_{1/2}$ (min)
In situ	256 (8400)	4×10^7	162
Crystalline	255 (12,000)[c]	5.7×10^7	157[d]

[a] Rate with eel acetylcholinesterase; for conditions, see Experimental Section. [b] Decomposition at constant pH in near-neutral aqueous solution; both were first-order reactions; 30°, pH 7.5, 0.1 M KCl, 3.0×10^{-3} M 4-PAM. [c] See footnote 3. [d] Calculated from eq 7.

B. CRYSTALLINE 4-PPAM. Physical properties are given in Table I. The reaction rate constant of 5.7×10^7 M^{-1} min^{-1} is corrected for 4-PAM content.[3] The decomposition of 4-PPAM in aqueous solution is strongly pH dependent. As shown in Table II, its rate is very slow below pH 6, whereas at pH above 8 it is too fast for

TABLE II: Decomposition of 4-PPAM (Crystalline); 30°, 0.100 M KCl, pH Maintained Constant by Autotitrator.

Run No.	[4-PPAM] (M × 10^5)	pH	[OH^-] (M × 10^7)	$t_{1/2}$ (min)	k_{obsd} (min^{-1} × 10^3)	k_2[a] (M^{-1} min^{-1} × 10^{-3})
XIV	3.95	7.23	1.70	427	1.62	9.52
III	2.56	7.60	3.98	225	3.08	7.73
IIIa	2.56	7.60	3.98	225	3.08	7.73
XV	3.96	7.80	6.31	113	6.14	9.73
XII	3.77	8.00	10.00	75	9.25	9.25
IX	1.61	8.00	10.00	91	7.62	7.62
						Av 8.60
XIII	3.44	6.00	0.1	>16,000		
XVII	3.36	6.00	0.1	>16,000		
XVIII	3.36	2.92	...	...[b]		

[a] k_2 is second-order reaction rate constant as defined in eq 6. [b] No measurable decomposition over a 7-day period

TABLE III: Decomposition of 4-PPAM (Crystalline); 2.56 × 10^{-5} M, 30°, pH 7.6. Effect of Added Salts.

Run No.	[KCl] (M)	[4-PAM] (M)	$t_{1/2}$ (min)	k_{obsd} (min^{-1} × 10^3)	k_2[a] (M^{-1} min^{-1})
I	0	3.13 × 10^{-3}	22	31.5	63.5
II	0	3.13 × 10^{-4}	52	13.3	23.3
IV	0.1	3.13 × 10^{-3}	130	5.33	4.52
VII	0.02	7.50 × 10^{-4}	136	5.10	16.5

[a] k_2 is second-order rate constant defined in eq 7.

convenient determination by the enzymatic method.[10] At constant pH, reaction follows first-order kinetics (Figure 6). Since the first-order reaction rate constants are proportional to hydroxide ion concentration, the reaction is second order as defined by eq 6, there being no significant H_2O or [H^+] components in the rate equation over the measured range.

$$\frac{dx}{dt} = 8.6 \times 10^3\ M^{-1}\ min^{-1}\ [OH^-][4\text{-PPAM}] \quad (6)$$

Effect of Added Compounds. A. 4-PAM. In the absence of other salts 4-PAM markedly accelerated the decomposition of 4-PPAM, the reaction being kinetically first order, and its rate varying very roughly with the inverse of the square root of the oxime concentration (Table III), suggesting a salt effect. Addition of salts such as potassium chloride or phosphate reduced the effect of 4-PAM, and for all further studies of the effect of 4-PAM and other compounds on the decomposition rate, swamping concentrations of neutral salt, *e.g.*, 0.1 M KCl, were maintained. In the presence of added salt, reaction kinetics, at constant pH, remained first order (Figure 7) with rate proportional to oxime concentration (Table IV). Through variation of pH (Table V) it was ascertained that acceleration of 4-PPAM decomposition by 4-PAM was dependent on the concentration of 4-PAM anion. In the presence of 4-PAM, the rate of decomposition of 4-PPAM in aqueous solution, 30°, 0.1 M KCl, fits the more general eq 7, where [A] represents the concentration of unprotonated species of 4-PAM, *i.e.*, the anion, and k_2 the rate constant for reaction between the 4-PAM anion and 4-PPAM. For 4-PAM, k_2 = 4.63 M^{-1} min^{-1}.

$$\frac{dx}{dt} = [4\text{-PPAM}](8.6 \times 10^3\ M^{-1}\ min^{-1}\ [OH^-] + k_2\ M^{-1}\ min^{-1}\ [A]) \quad (7)$$

B. OTHER BASES. The decomposition of 4-PPAM in near neutral aqueous solution is accelerated by several other bases including the hydroxamic acid anion, imidazole, and bicarbonate. The data are furnished in Tables VI and VII. Studies with bicarbonate were performed at 30° in a Warburg apparatus, the rate being measured by manometric determination of evolved

[10] A minimum of 20 min was required for each determination of concentration.

TABLE IV: 4-PPAM Decomposition. Acceleration by 4-PAM at pH 7.6.[a]

Run No.	4-PAM[b] Concn (M × 10^3)	[A^-] (M × 10^3)	$t_{1/2}$ (min)	k_{obsd} (min^{-1} × 10^3)	k_2[c] (M^{-1} min^{-1})
III	0	0	225	3.08	...
XXI	2.16	0.294	157	4.41	3.40
IV	3.13	0.425	130	5.33	4.52
IVb	3.13	0.425	125	5.55	5.03
XXII	5.00	0.68	110	6.28	4.22

[a] 30.0°, 0.100 M KCl, 2.62 × 10^{-5} M 4-PPAM, pH maintained by Autotitrator. [b] [A^-] is the concentration of 4-PAM anion. [c] k_2 is second-order rate constant defined in eq 7.

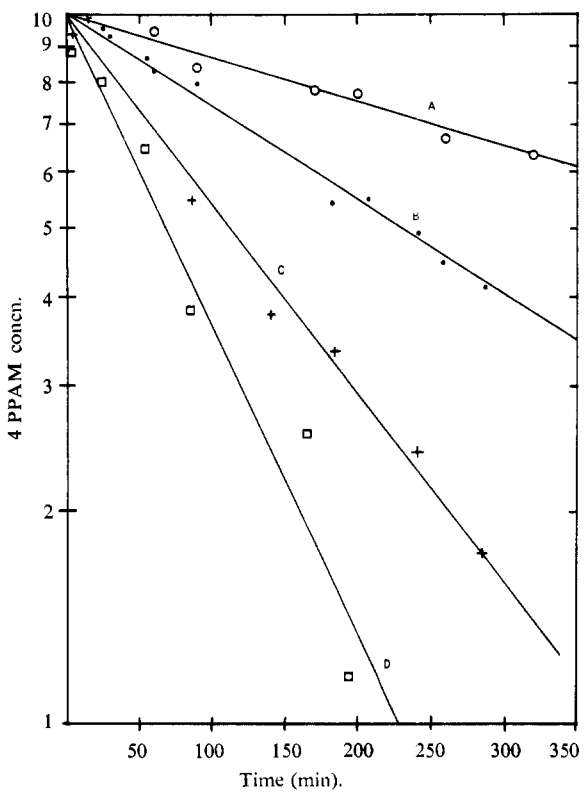


FIGURE 6: 4-PPAM decomposition in aqueous solution, 30°, 0.100 M KCl. Values of 4-PPAM concentration are comparative, the "curves" having been adjusted to meet a single origin. Curve A, pH 7.23 (run XIV); B, pH 7.60 (runs III and IIIa); C, pH 7.80 (run XV); D, pH 8.00 (run IX).

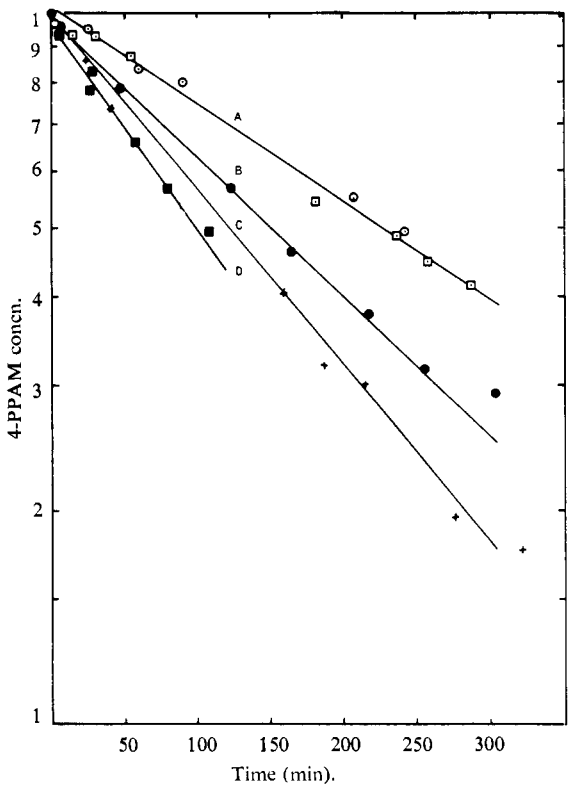


FIGURE 7: 4-PPAM decomposition: acceleration by 4-PAM, 30°, pH 7.6, 0.100 M KCl. Values of 4-PPAM concentration are comparative, the "curves" having been adjusted to meet a single origin. Curve A, no added 4-PAM; B, 2.16 × 10^{-3} M 4-PAM; C, 3.13 × 10^{-3} M 4-PAM; D, 5.00 × 10^{-3} M 4-PAM.

carbon dioxide (Wagner-Jauregg and Hackley, 1953).[11] In 0.1 M sodium bicarbonate, k_2 for 4-PAM is 6.07 M^{-1} min. The corresponding k_2 value for "bicarbonate," based upon its total molar concentration rather than on the concentration of an individual species, is 1.2 × 10^{-2} M^{-1} min^{-1}.

Discussion

It is seen that 4-PAM indeed falls into that second class of oximes which have an initial rapid acid-produc-

[11] These determinations were made by O. O. Owens.

TABLE V: 4-PPAM Decomposition. Acceleration by 4-PAM at Several pH Levels.[a]

Run No.	pH	[OH⁻] (M × 10⁷)	4-PAM[b] Concn (M × 10³)	4-PAM[b] [A⁻] (M × 10⁴)	$t_{1/2}$ (min)	k_{obsd} (min⁻¹ × 10³)	k_2[c] (M⁻¹ min⁻¹)
XXV	7.20	1.58	50	2.94	257	2.70	4.59
XXVI	7.40	2.51	25	2.26	204	3.40	5.97
XXVII	7.53	3.38	34.9	4.11	145	4.79	4.65

[a] 30.0°, 0.100 M KCl, 2.62 × 10⁻⁵ M 4-PPAM, pH maintained by Autotitrator. [b] [A⁻] is the concentration of 4-PAM anion. [c] k_2 is second-order rate constant defined in eq 7.

TABLE VI: 4-PPAM Decomposition. Acceleration by Imidazole and Hydroxamic Acids.[a]

4-PPAM (M × 10⁵)	pH	Reagent	Reagent pK_a	Reagent Total Concn (M)	Reagent Concn of Unprotonated Species (M)	$t_{1/2}$ (min)	k_{obsd} (min⁻¹)	k_2[b] (M⁻¹ min⁻¹)
4.25	7.6	Imidazole	7.0	4.99 × 10⁻²	3.98 × 10⁻²	163	4.25 × 10⁻³	2.11 × 10⁻²
3.24	7.6	BHA[c]	8.8	2.50 × 10⁻²	0.148 × 10⁻²	<5	...	...
3.37	7.6	BHA[c]	8.8	5.00 × 10⁻⁴	2.96 × 10⁻⁵	107	6.47 × 10⁻³	103
3.20	7.6	HHA[d]	9.4	8.20 × 10⁻⁴	1.28 × 10⁻⁵	100	6.93 × 10⁻³	274
3.43	7.4	HHA[d]	9.4	1.00 × 10⁻³	1.00 × 10⁻⁵	130	5.33 × 10⁻³	319

[a] For conditions see Table IV, footnote *a*. [b] Second-order rate constant calculated for unprotonated form of the reagent (eq 7). [c] Benzohydroxamic acid. [d] Hexanohydroxamic acid.

TABLE VII: 4-PPAM Decomposition. Acceleration by Bicarbonate and 4-PAM at pH 7.6.[a]

[4-PAM] (M)	$t_{1/2}$ (min)	k_{obsd} (min⁻¹ × 10³)
0	160	4.35
2.3 × 10⁻³	102	6.30
1.15 × 10⁻²	50	13.9

[a] 9.4 × 10⁻⁴ M 4-PPAM, 0.1 M $NaHCO_3$. Rate determination by CO_2 evolution in Warburg manometric apparatus (Wagner-Jauregg and Hackley, 1953).

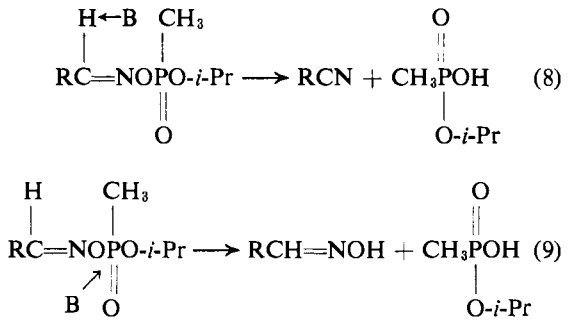
ing reaction with GB and which yield relatively stable phosphonylated products (eq 1). This is supported by the close similarity in rates of acid production, GB disappearance, and formation of the water-soluble nonchloroform-extractable anticholinesterase. It also conforms to the stoichiometry of acid production.

The identity of the water-soluble nonchloroform-extractable anticholinesterase as 4-PPAM, although highly probable, cannot be stated with certainty without direct isolation of the product. This has not been attempted in this work. Table I shows that the reaction product is very similar to crystalline 4-PPAM in its decomposition rate in aqueous solution and that its absorption maximum is closely similar. The low values for E_{max} and reaction rate with eel acetylcholinesterase must be ascribed to incomplete reaction.[12]

The decomposition rate of 4-PPAM in aqueous solution is proportional to [OH⁻] but is not susceptible to a discernible [H_3O^+]-catalyzed or water reaction. In addition, the decomposition rate is speeded by a variety of other bases, including the anions of 4-PAM, benzo- and hexanohydroxamic acids, imidazole, and bicarbonate.

Aldoxime carboxylic esters of this type decompose in alkaline solution by Beckmann elimination (reaction 8) or by hydrolysis (reaction 9) depending, in part, on whether the oxime configuration is *trans* or *cis* to the "aldehydic" proton (Hauser and Jordan, 1935). Reaction 8 fits into the class of general base catalyzed reac-

[12] In this connection it is important to note both the extremely high velocity constant and the very great specificity of the enzyme so that if an entirely different product were formed its rate of reaction with the enzyme would probably be different by *orders of magnitude*.



(8)

(9)

tions, whereas reaction 9 probably involves nucleophilic attack (Green *et al.*, 1958).

Catalysis of 4-PAM anion can take place only *via* reaction 8 since nucleophilic attack by reaction 9 would simply give exchange. For the other catalysts either route would be available, the two pathways leading to different products. A study of this matter is the subject of a separate paper (G. M. Steinberg and S. Solomon, in preparation). Since increasing acid strength favors reaction 8 for both *syn* and *anti* oxime esters, the occurrence of reaction 8, *per se*, does not establish the stereochemical configuration of 4-PPAM (although 4-PAM is known to exist in the *syn* configuration; Poziomek *et al.*, 1961).

Implications in Therapy. In this study it has been shown that under physiological conditions of pH and temperature the highly toxic anticholinesterase GB is converted by a model treatment compound, 4-PAM, into an equally potent and toxic anticholinesterase, 4-PPAM. As suggested in an earlier report (Hackley *et al.*, 1959), improved therapy might result if means were found for rapidly destroying the toxic products. The hydroxamic acids (anions) are the most rapid nontoxic reactants known for organophosphate esters and anhydrides. Although far more effective in speeding the decomposition of 4-PPAM than the other compounds tested, they are still several orders of magnitude less than required to be practically useful.

Alternate approaches to overcoming this difficulty need be sought. One such area to be considered for investigation is an examination of the effect of neighboring group participation on speeding the destruction of the phosphonylated oxime.

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