Phenyl *N*-Methylacetimidate in H_2O and D_2O and Its Reactions with Nucleophiles[1]

Y. Pocker,*[2a] M. W. Beug,[2b] and K. L. Stephens[2c]

Contribution from the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received June 21, 1973*

Abstract: The chemical decomposition of phenyl *N*-methylacetimidate (PNMA) is examined over the pH (pD) range 2–13 in both H_2O and D_2O; the major hydrolysis products above pH 5 are phenol (or phenolate) and *N*-methylacetamide. Throughout the range examined, the protonated imido ester appears to be the primary reactive species. Its water reaction is faster in H_2O than in D_2O, $k_{H_2O}/k_{D_2O} = 2.17$, whereas catalysis by nucleophilic components is found to be the same in both solvents. Azide and cyanide ions are powerful nucleophiles toward the protonated imido ester, the product from reaction with azide being 1,5-dimethyltetrazole which does not hydrolyze further. On the other hand, nucleophilic catalysis by imidazole leads to the formation of the unique compound imidazyl *N*-methylacetimidate which subsequently hydrolyzes to *N*-methylacetamide and imidazole.

Considerable research effort has been devoted to the kinetics of hydrolysis and aminolysis of imido esters because they provide information about the modes of formation and decomposition of tetrahedral intermediates.[3–5] Phenyl *N*-methylacetimidate (PNMA) is a particularly interesting imido ester because phenolate is a much better leaving group than the alcoholates of most other acetimidates that have been investigated, and, except under quite acidic conditions, the tetrahedral addition intermediate breaks down preferentially with its expulsion.[6–8] The carbon of the C=N group of PNMA is known to be quite susceptible to nucleophilic attack,[6] and the similarity of this compound to phenyl esters provides a fruitful area for mechanistic investigation of tetrahedral intermediates. The reaction of phenyl *N*-methylacetimidate with azide ion was particularly intriguing, and the product provides direct evidence for a nucleophilic attack by azide ion.

In this study, we describe the pH–rate profile for PNMA hydrolysis in H_2O and D_2O in an attempt to obtain important information about the role of water and the timing of the proton transfer(s) in the rate-determining step of this reaction. Kandel and Cordes[6] have reported that phosphate buffers strongly catalyzed the rate of hydrolysis of PNMA. We therefore determined catalytic coefficients for a number of buffer components in both H_2O and D_2O in order to determine whether the base-promoted hydrolysis was due to nucleophilic attack or to general base catalysis.

(1) Support of this work by U. S. Public Health Service Grant AM 09221 from the National Institute of Arthritis and Metabolic Diseases is gratefully acknowledged.

(2) (a) Author to whom correspondence should be addressed. (b) Predoctoral Trainee (1968–1971) of the National Institute of General Medical Sciences (GM 39680), U. S. Public Health Service. (c) National Science Foundation Trainee.

(3) (a) E. S. Hand and W. P. Jencks, *J. Amer. Chem. Soc.*, 84, 3505 (1962); (b) B. H. DeWolfe and F. B. Augustine, *J. Org. Chem.*, 30, 699 (1965); (c) G. L. Schmir and B. A. Cunningham, *J. Amer. Chem. Soc.*, 87, 5692 (1965); (d) B. K. Chaturvedi and G. L. Schmir, *ibid.*, 90, 4413 (1968).

(4) M. L. Bender, *Chem. Rev.*, 60, 53 (1960); "Mechanisms of Homogeneous Catalysis from Protons to Proteins," Wiley-Interscience, New York, N. Y., 1971, Chapter 6.

(5) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, Chapter 10.

(6) M. Kandel and E. H. Cordes, *J. Org. Chem.*, 32, 3061 (1967).

(7) T. C. Pletcher, S. Koehler, and E. H. Cordes, *J. Amer. Chem. Soc.*, 90, 7072 (1968).

(8) W. P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, 90, 2622 (1968).

Imido esters are known to react readily with amino residues of proteins.[9–11] The reaction has been shown to occur almost exclusively with ε-aminolysyl residues leading to amidine formation.[10] We were therefore interested in the potential utility of PNMA as an enzyme modification agent. For this purpose, PNMA has two clear advantages: (1) phenolate is a much better leaving group than ethoxide, hence the rate of reaction of PNMA is much faster than that of ethyl acetimidate, the amidination agent of general use;[9–11] and (2) the rate of reaction can be readily monitored by following phenol (or phenolate) appearance in the uv. Significant rates of PNMA decomposition were noted both with albumin and with carbonic anhydrase.[12] Although the imido ester behaved both as a substrate and as an enzyme modifier, the data indicate that it should not be grouped with the ester substrates of carbonic anhydrase[13,14] since a different site is apparently involved.

Because specific histidine residues often play an important role in the active side of enzymes, we took this opportunity to delineate the reactivity of PNMA toward imidazole. Also, in order to better elucidate the probable pH dependence of the amidination of ε-aminolysyl residues, a study was made of the aminolysis of PNMA by *n*-butylamine, L-lysine, and *N*-α-CBZ-L-lysine. The present paper shows that all these reactions exhibit a bell-shaped pH dependence.

Experimental Section

Materials. Phenyl *N*-methylacetimidate was prepared by the method of Oxley and Short.[15] Hydroxylamine hydrochloride (0.4 mol) was added with constant stirring to a mixture of reagent acetone (1.2 mol) and 5 *N* sodium hydroxide (200 ml). When oxime formation was complete, the reaction mixture was cooled to 11° and freshly distilled benzenesulfonyl chloride (0.4 mol), bp 80–82° (0.1 mm), was added dropwise with stirring over a period of 1 hr with the temperature being maintained near 11°. The solution was then allowed to come slowly to room temperature with continual stirring. After an additional 1–10-hr period, the reaction was complete as evidenced by the disappearance of ben-

(9) M. J. Hunter and M. L. Ludwig, *J. Amer. Chem. Soc.*, 84, 3491 (1962).

(10) L. Wofsy and S. J. Singer, *Biochemistry*, 2, 104 (1963).

(11) A. Nilsson and S. Lindskog, *Eur. J. Biochem.*, 2, 309 (1967).

(12) Y. Pocker, M. W. Beug, and F. Ann Beug, *Biochemistry*, 12, 2483 (1973).

(13) Y. Pocker and J. T. Stone, *Biochemistry*, 7, 3021 (1968).

(14) Y. Pocker and M. W. Beug, *Biochemistry*, 11, 698 (1972).

(15) P. Oxley and W. F. Short, *J. Chem. Soc.*, 1514 (1948).

zenesulfonyl chloride and formation of a white crystalline product. The whole mass was poured into ice-water. The solid was immediately isolated by suction filtration, washed several times with distilled ice-water, and then dried *in vacuo* for 1 week over P_2O_5. The yield of white crystalline acetoxime benzenesulfonate was 80–95%.

Acetoxime benzenesulfonate (0.3 mol), mp 52° (lit.[6] mp 52°), and phenol (0.3 mol) were refluxed in dry toluene (200 ml dried over sodium wire) for 1 hr and allowed to stand overnight. A heavy oil, which crystallized on cooling, separated from the toluene. An additional 200 ml of toluene was added and the whole mass was shaken with 250 ml of 3 *N* NaOH. Within 10 min, all of the solid was dissolved and the toluene layer turned deep brown. The toluene layer was isolated and dried (Na_2SO_4) and the toluene removed by rotary evaporation at reduced pressure. The remaining dark oil was fractionally distilled. The major fraction, bp 45–50° (0.5 mm), was redistilled to give pure phenyl *N*-methylacetimidate, $n^{20}D$ 1.5185, bp 74–76° (3.5 mm) [lit.[15] bp 65° (1.5 mm)]; nmr and ir spectra were in accord with the assigned structure.

Synthesis of 1,5-Dimethyltetrazole. The procedure of Markgraf, Bachmann, and Hollis[16] was used with slight modification. To a cold solution of acetoxime (0.18 mol) in 170 ml of 1 *N* NaOH was added freshly distilled benzenesulfonyl chloride (0.18 mol) dropwise, and the heterogeneous mixture was stirred at room temperature for 6 hr. Sodium azide (0.18 mol) was added; the mixture was refluxed 6 hr and evaporated to dryness at reduced pressure. The solid residue was extracted with $CHCl_3$, the extract was dried (Na_2SO_4), and the $CHCl_3$ was removed at reduced pressure. The crude product was recrystallized from benzene–ether: mp 70–71° (lit.[17] mp 73–74°); nmr ($CDCl_3$)[16] δ 4.02 (s, 1) and 2.58 ppm (s, 1); mass spectrum (70 eV) *m/e* for $C_3H_6N_4$ 98.0588 (calcd *m/e* 98.0592).

1,5-Dimethyltetrazole from Phenyl *N*-Methylacetimidate and Sodium Azide. A solution of the imido ester (13 mmol) and sodium azide (26 mmol) in water (25 ml) at pH 8 was stirred at room temperature for about 12 hr. The solution was brought to pH 4 with HCl and extracted with $CHCl_3$. The aqueous layer was made basic to pH 11 with NaOH and again extracted with $CHCl_3$. The basic $CHCl_3$ extract was dried (Na_2SO_4), filtered, and evaporated under reduced pressure leaving a white residue: mp 69–71°; nmr ($CDCl_3$) δ 4.02 (s, 1) and 2.58 ppm (s, 1); mass spectrum (70 eV) *m/e* for $C_3H_6N_4$ 98.0590 (calcd *m/e* 98.0592).

Imidazyl *N*-Methylacetimidate from Phenyl *N*-Methylacetimidate and Imidazole. A solution of the imido ester (6.7 mmol) and imidazole (14 mmol) in water–acetonitrile (8:1) was stirred at room temperature for 45 min. The reaction mixture was extracted with $CHCl_3$; the extract was washed with dilute NaOH, dried (Na_2SO_4), filtered, and evaporated at reduced pressure leaving a liquid residue: nmr (CCl_4) δ 7.93 (unresolved m, 1, >NCH=N–), 7.45 (t, 1, J = 1.3 Hz, >NCH=C), 6.89 (m, 1, >C=CHN=), 3.15 (s, 3), and 2.24 ppm (s, 3); mass spectrum (70 eV) *m/e* for $C_6H_9N_2$ 123.0792 (calcd *m/e* 123.0796). If the reaction is allowed to run for 12 hr, no imidazyl *N*-methylacetimidate can be detected, only *N*-methylacetamide and imidazole.

The buffers employed in these studies were carefully selected for their good buffering capacity coupled with low nucleophilicity. They produced no side reactions with the reactants or products of the system and did not excessively catalyze the hydrolysis of substituted phenyl esters or PNMA. Phosphate buffers were specifically avoided in the study of the PNMA hydrolysis because of their powerful catalytic action.[6]

Tris was obtained from Aldrich and purified by sublimation. Triethylamine was obtained from Eastman Kodak Co. and purified by fractional distillation, bp 89–90° (760 mm). Malonic acid (Aldrich) was recrystallized from ethanol–ether, mp 134–135°. Diethylmalonic acid was prepared by alkaline hydrolysis of the diethyl ester (Aldrich) in absolute ethanol and recrystallized from ether–benzene, mp 128–129°. Reagent grade hydrochloric acid, acetic acid, and sodium acetate were products of Baker and Adamson. Reagent grade monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, and sodium hydroxide were obtained from Allied Chemical Co. and used without further purification.

Reagent grade acetonitrile (containing less than 0.1% water as analyzed by vapor phase chromatography), sodium chloride, sodium bromide, sodium iodide, and sodium cyanide were from J. T. Baker Co. Sodium azide and *n*-butylamine were from Eastman Organic Chemicals. Sigma supplied L-lysine and *N*-α-CBZ-L-lysine. Imidazole (Aldrich) was recrystallized from diethyl ether, mp 89–90°.

Instrumentation. Spectrophotometric determinations were performed using a Beckman DU-2 spectrophotometer equipped with an insulated cell compartment consisting of a specially constructed bath thermostated to 25.0 ± 0.02° by means of a Sargent Model SV (S-82060) thermonitor. Recently the instrument was replaced with an automated spectrophotometric system with the temperature controlled at 25.0 ± 0.05° by a Forma-Temp Jr. (Model 2095) circulating bath attachment. A deuterium lamp was used for all measurements at less than 350 nm and a tungsten lamp at greater wavelengths.

Reaction rates were measured in 1-cm silica cells from Precision Cells, Inc. (Herasil, Type 21). Rates employing D_2O were run in 0.5-cm silica cells (Herasil, Type 21) in order to reduce solvent consumption. A Cary Model 14 was used for obtaining uv spectral data.

Determinations of pH and pD were made on a Beckman 101900 research pH meter equipped with a Beckman 39071 calomel internal frit junction electrode and a Beckman 41263 glass electrode. All reported pH values are obtained directly from the pH meter reading. The reported pD values are obtained by adding 0.40 to the pH meter reading.[18]

Nmr spectra were determined with the Varian Associates A-60 and T-60 NMR instruments. Weights were measured on a Sartorius Model 2700 balance which has an absolute accuracy of 0.1 mg. Weighings of less than 10 mg of material were performed on a Cahn Model 10 electro-balance. Thin-layer chromatography was done using plates made with acid-washed alumina, neutral alumina, or silica gel G. Elution was performed with hexane, ether, and ethyl acetate used either singly or in varying combinations. Analytical gas chromatography was performed either on a Hewlett-Packard 5750 Research Chromatograph using helium for the carrier gas (50 psi, 15 ml/min flow rate at exit) and employing a flame ionization detector; or on a Hewlett-Packard 700 Laboratory Chromatograph equipped with a thermal conductivity detector and using helium carrier gas (120 psi, 25 ml/min flow rate at exit). The following columns were used: $^1/_8$ in. × 6 ft, 10% U.C. W98 on 80–100 Chromosorb C; $^1/_8$ in. × 24 ft Apiezon L on silylated Chromosorb G; and $^1/_8$ in. × 24 ft Carbowax on silylated Chromosorb G. Mass spectra were determined on an Associated Electrical Industries Ltd. Model MS9 mass spectrometer and the data were recorded and analyzed with a PDP-12 computer.

Kinetics. Phenyl *N*-methylacetimidate (PNMA) reaction mixtures for rate determinations were prepared by using a calibrated Hamilton syringe to inject 30 μl of acetonitrile containing PNMA into 2.97 ml of the appropriate buffer solution giving a reaction mixture with 1% (v/v) acetonitrile and an ionic strength of 0.55. The high ionic strength was necessary to allow use of a wide range of buffer concentrations (for Bell–Darwent plots)[19] and high anionic inhibitor concentrations, while maintaining ionic strength constant. For kinetic runs with some nucleophiles and with D_2O, 5-mm cells were employed. In these cells, we used 1.3 ml of buffer, and initiated the runs by injecting 13 μl of stock solutions of PNMA in acetonitrile.

The chemical hydrolysis of PNMA was followed spectrophotometrically at 275 nm, the isosbestic point for phenol–phenolate ion. PNMA does not absorb appreciably at 275 nm (Figures 1 and 2), but under acidic pH the disappearance of its protonated form can be followed at 247.5 nm.[6] In the present work it proved advantageous to follow the rate of nucleophilic attack by imidazole and azide on PNMA both at 240 nm and at 275 nm (see Figures 1 and 2). The two wavelengths gave similar results (see Table III) and plots of $-\log (A_\infty - A_t)$ *vs.* time were found to be linear for at least 1 half-life. At 275 nm, values of A_∞ were experimentally determined and were found to reach a stable value. However, when working at 240 nm, it was necessary to use extrapolated infinities since the *N*-methylacetimidoimidazole and azido *N*-methylacetimidate (formed by attack of the respective nucleophile on PNMA) underwent subsequent reaction leading to a decrease with time in the observed A_∞ (Figures 1 and 2). At pH's above neutrality, the reaction went to greater than 99% completion, producing phenol and *N*-methylacetamide. In the acidic region, increasing amounts of phenyl acetate and methylamine are also

(16) J. H. Markgraf, W. T. Bachmann, and D. P. Hollis, *J. Org. Chem.*, 30, 3472 (1965).
(17) E. K. Harvill, R. M. Herbst, E. C. Schreiner, and C. W. Roberts, *J. Org. Chem.*, **15**, 662 (1950).
(18) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(19) R. P. Bell and B. Darwent, *Trans. Faraday Soc.*, **46**, 34 (1950).

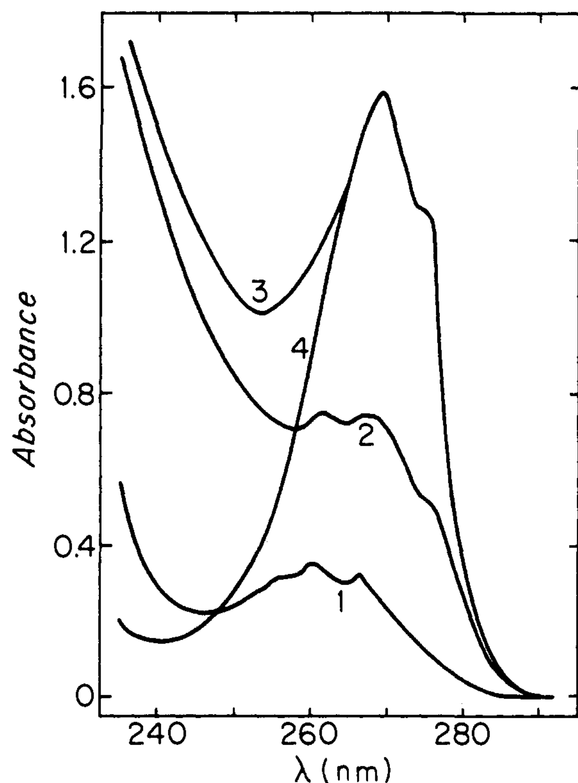


Figure 1. Time dependent uv spectra for nucleophilic attack on PNMA by imidazole at 25.0°. Reaction studied in 0.05 *M* Tris buffer (pH 7.5), 10% (v/v) acetonitrile, ionic strength 0.55, [PNMA] = [imidazole] = 1×10^{-3} *M*. Uv spectra at: (1) 30 sec, (2) 5 min, (3) 2 hr, (4) 24 hr.

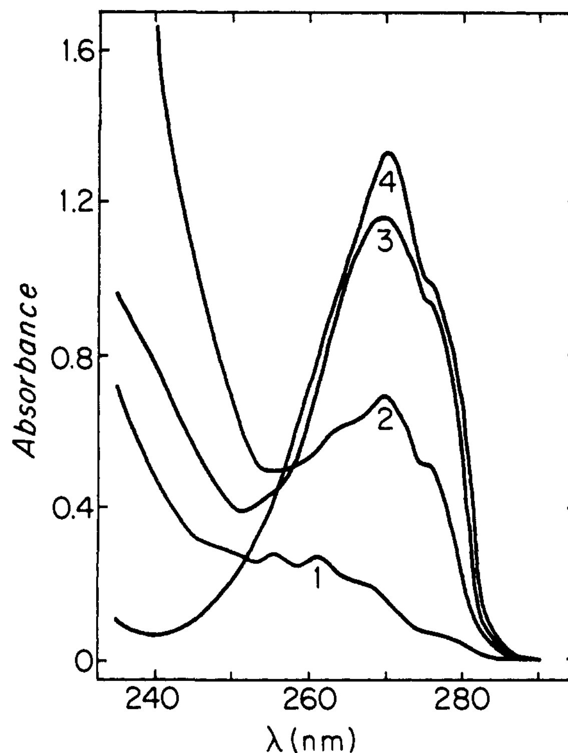


Figure 2. Time dependent uv spectra for nucleophilic attack of azide ion on PNMA at 25.0°. Reaction studied 0.05 *M* malonate buffer (pH 5.5), 10% (v/v) acetonitrile, ionic strength 0.55, [PNMA] = [azide] = 7.5×10^{-4} *M*. Uv spectra at: (1) 0 (no azide present), (2) 30 sec, (3) 15 min, (4) 6 hr.

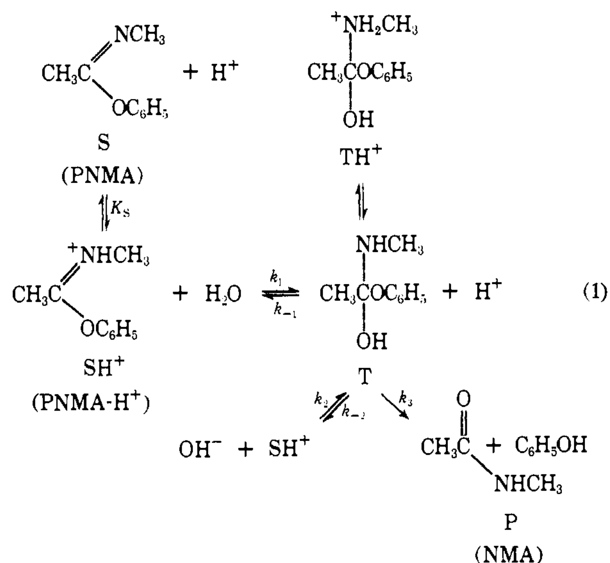
produced (comprising about 12% of the total reaction at pH 2.8—the most acidic pH examined).[7,8]

Pseudo-first-order rate constants for PNMA hydrolysis were evaluated using a Fortran IV computer program executed on a CDC 6400 digital computer. The program was written by Dr. N. Watamori to calculate the best slope for first-order plots by means of the least-squares method. The program gave the rate constants for plots of $-\log (A_\infty - A_t)$ *vs.* t and calculated reaction half-life, standard deviation of the slope, and the correlation coefficient. In addition, when the correlation coefficient was less than 0.999, the data were graphically plotted so that the operator could quickly pinpoint the cause of the discrepancy.

Rate constants for second-order nucleophilic attack of azide and imidazole on PNMA were also obtained. In this case, reactions were run using equal quantities of nucleophile and PNMA and plots of $1/(c_\infty - c_t)$ *vs.* t were obtained. Dr. N. Watamori's least-squares program was modified to plot $1/(A_\infty - A_t)$ *vs.* t and rate constants were obtained by multiplying the slope of the line by ϵb where ϵ was the appropriate extinction coefficient and b was the cell path length in cm.

Results

Buffer Catalysis. The pH–rate profile for the hydrolysis of PNMA was determined in buffered H_2O and D_2O solutions. The results are given in Tables I and II and are graphically displayed in Figure 3. The pH–rate profile in H_2O obtained in this study was in close agreement with that published by earlier workers.[6] They propose the following hydrolytic pathway (eq 1).[6,7]



From this scheme, the values for the various rate constants involved in rate-limiting steps can be obtained by proper application of the steady-state approximation to obtain the general equation for k_{buff}. By extrapolating to zero buffer concentration, the following expression for the first-order rate constants was derived (eq 2).[6] Under basic conditions, assuming $k_3 \gg k_{-2}$, the expression reduces to $k_0 = k_2K_w/K_S$. Under sufficiently acidic conditions, the substrate is completely protonated, and eq 2 reduces to $k_0 = k_1$.

$$k_0 = \left(\frac{k_3[\mathrm{H}^+]}{[\mathrm{H}^+] + K_S}\right)\left(\frac{k_2[\mathrm{OH}^-] + k_1}{k_3 + k_{-2} + k_{-1}[\mathrm{H}^+]}\right) \quad (2)$$

From the pH–rate profile shown in Figure 3, values of K_S, k_1, and k_2 can readily be obtained. The results are summarized in Table II and show excellent agreement with previously reported values.[6]

Since PNMA exists both in protonated and unprotonated forms in the pH region under investigation, the observed chemical hydrolysis rate in the presence of

Table I. Values of k_{buff} for the Hydrolysis of Phenyl N-Methylacetimidate as a Function of Buffer Concentration in H_2O and D_2O at 25.0°

pH	[Buffer],[a] M: 0.0[b]	0.05	0.10	0.15	0.20
	$k_{buff} \times 10^2$, M^{-1} min^{-1}				
	Malonate Buffer (H_2O)				
2.3	5.9	5.9	5.9	5.9	
2.8	6.1	6.2	6.3	6.4	
3.2	6.2	6.3	6.5	6.65	
3.6	6.3	6.5	6.7	6.8	7.0
4.6	6.4	6.6	6.9	7.1	7.3
5.1	6.0	6.6	7.2	7.8	8.4
5.6	5.5	6.3	7.1	7.9	8.7
6.0	4.5	5.5	6.5	7.5	8.5
6.4	3.4	4.3	5.2	6.1	7.0
	Diethyl Malonate Buffer (H_2O)				
6.5	3.5	3.8 (3.2)[c]	4.1	4.4	4.7
6.8	2.6	2.9	3.2	3.5	3.8
7.2	1.75	2.1	2.5	2.8	3.2
7.6	1.55	1.8 (1.6)[c]	2.1	2.35	2.65
8.2	1.45	1.65 (1.4)[c]	1.8	2.0	2.25
	Tris Buffer (H_2O)				
7.4	1.65	1.65	1.60	1.70	1.70
8.6	1.4	1.4	1.4	1.5	1.45
9.1	1.36	1.36	1.40		1.45
	Triethylamine Acetate Buffer (H_2O)				
10.0	1.35	1.35	1.30		
10.3	1.35 (1.40)[d]	1.35 (1.35)[e]	1.40		
10.6	1.35 (1.38)[d]	1.30 (1.35)[e]	1.40		
11.2	1.35	1.35	1.38		

pD	[Buffer],[a] M: 0.0[b]	0.05	0.10	0.15	0.20
	$k_{buff} \times 10^2$, M^{-1} min^{-1}				
	Malonate Buffer (D_2O)				
2.6	2.8	2.8	2.8	2.8	2.8
3.2	3.0	3.0	3.0	3.1	3.0
4.0	2.9	3.0	3.0	3.1	3.0
5.0	2.8	2.9	3.2	3.1	3.3
5.4	2.65	2.8	2.9	3.1	3.3
6.0	2.5	2.7	2.9	3.2	3.3
6.5	2.2	2.4	2.6	2.9	3.1
	Diethyl Malonate Buffer (D_2O)				
6.7	1.85	1.9	1.9		2.1
7.0	1.5	1.9	1.9		2.1
7.6	1.2	1.3	1.4		1.55
8.2	1.10	1.15	1.20		1.30
	Tris Buffer (D_2O)				
7.4	1.3	1.3	1.25	1.30	1.35
9.1	1.0	1.0	1.05	0.95	1.05
	Triethylamine Acetate Buffer (D_2O)				
10.3	0.96 (0.96)[d]	0.90 (1.00)[e]	0.96		
10.6	0.94 (0.94)[d]	0.92 (0.96)[e]	0.96		

[a] Ionic strength maintained at 0.55 with added sodium chloride. [b] All values in this column refer to k_0 and are obtained by extrapolating k_{buff} to zero buffer concentration. [c] Obtained using PNMA-2-^{14}C. [d] Obtained at 0.025 M buffer concentration. [e] Obtained at 0.075 M buffer concentration.

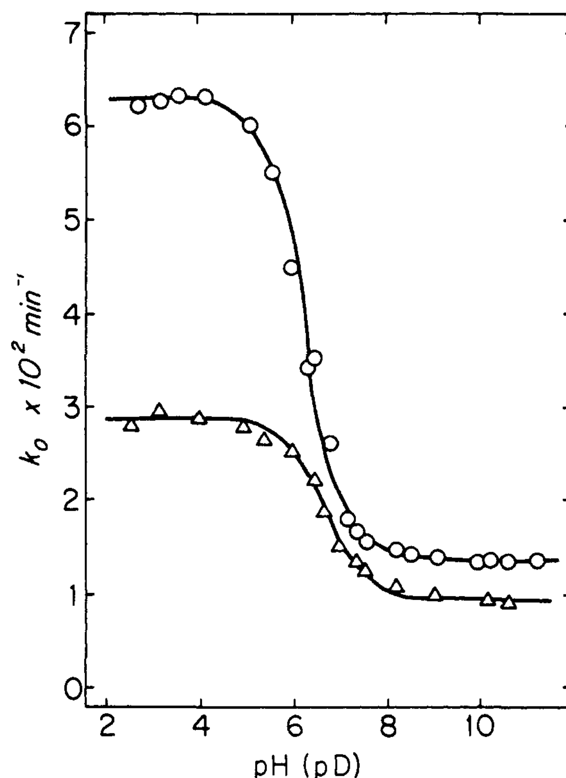


Figure 3. First-order rate constants for phenyl N-methylacetimidate hydrolysis extrapolated to zero buffer concentration at 25.0°; ionic strength 0.55, 1% (v/v) acetonitrile, and [PNMA] = 1.5 × 10^{-3} M. Reaction in (○) H_2O and (△) D_2O.

Table II. First-Order Rate Constants for Hydrolysis of PNMA at 25.0°

Physical constant	This work[a]: In H_2O	This work[a]: In D_2O	Kandel and Cordes[6] in H_2O[b]
pK_a	6.2	6.7	6.2
K_S	6.3 × 10^{-7}	2 × 10^{-7}	6.3 × 10^{-7}
k_2K_w/K_S	1.3 × 10^{-2} min^{-1}	9.4 × 10^{-3} min^{-1}	1.1 × 10^{-2} min^{-1}
k_2	8.2 × 10^5 M^{-1} min^{-1}	1.4 × 10^6 M^{-1} min^{-1}[d]	6.8 × 10^5 M^{-1} min^{-1}
k_1	6.3 × 10^{-2} min^{-1}	2.9 × 10^{-2} min^{-1}	6.5 × 10^{-2} min^{-1}
k_1/[H_2O][c]	1.15 × 10^{-3} M^{-1} min^{-1}	5.3 × 10^{-4} M^{-1} min^{-1}	1.18 × 10^{-3} M^{-1} min^{-1}
k_{MAL^-}[e]	0.04 M^{-1} min^{-1}		
$k_{MAL^{2-}}$[e]	0.2 M^{-1} min^{-1}		
k_{DEM^-}[e]	0.1 M^{-1} min^{-1}		
$k_{DEM^{2-}}$[e]	0.64 M^{-1} min^{-1}		

[a] With 1% (v/v) acetonitrile, ionic strength 0.55. [b] With 3% (v/v) acetonitrile, ionic strength 0.50. [c] The molar concentration of H_2O in 1% (v/v) acetonitrile is 54.9 and that of D_2O is 54.5 assuming $\rho_{D_2O}^{25°}$ = 1.104 g/ml [R. C. Weast, Ed., "Handbook of Chemistry and Physics," 49th ed, Chemical Rubber Co., Cleveland, Ohio, 1968, p F-4]. [d] The value of K_w in D_2O is 1.35 × 10^{-15}: A. K. Covington, R. A. Robinson, and R. G. Bates, *J. Phys. Chem.*, **70**, 3820 (1966). [e] MAL^- and MAL^{2-} refer to malonic acid mono- and dianions, respectively. DEM^- and DEM^{2-} refer to diethylmalonic acid mono- and dianions, respectively.

buffers must in theory include terms for buffer catalysis of both the protonated and the unprotonated forms of PNMA. The situation is simplified at the more extreme values of pH where one component is quite small and its contribution can be neglected. In addition, the protonated form of PNMA is subject to catalysis by the buffer species present in the medium while the unprotonated ester is quite unreactive. In the acidic region, where there is an appreciable concentration of protonated substrate, both malonate and diethyl malonate proved to be significant catalysts (see Figure 4). However, at high pH, where the concentration of protonated substrate is negligible, plots of k_{obsd} *vs.* buffer concentration for Tris and triethylamine acetate buffers give straight lines of near-zero slope.

Similar procedures were carried out to determine the magnitude of k_1 and k_2 in D_2O using Tris,

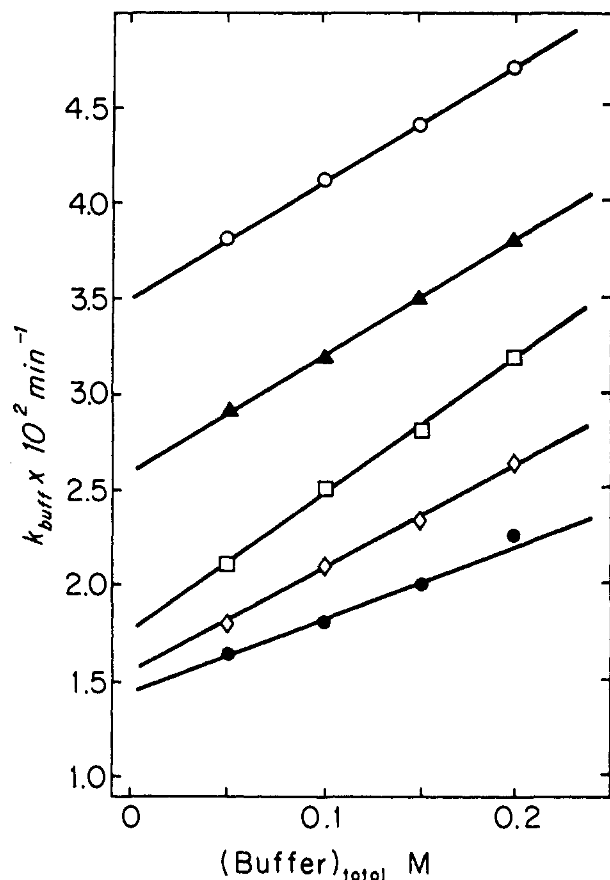


Figure 4. Buffer-catalyzed hydrolysis of PNMA in the region of the second ionization of diethyl malonate at 25.0°. Buffer concentration is varied between 0.05 and 0.20 *M*, [PNMA] = 1.5×10^{-3} *M*, 1% (v/v) acetonitrile and ionic strength 0.55; (○) pH 6.5; (▲) 6.8; (□) pH 7.2; (◇) pH 7.6; (●) pH 8.2.

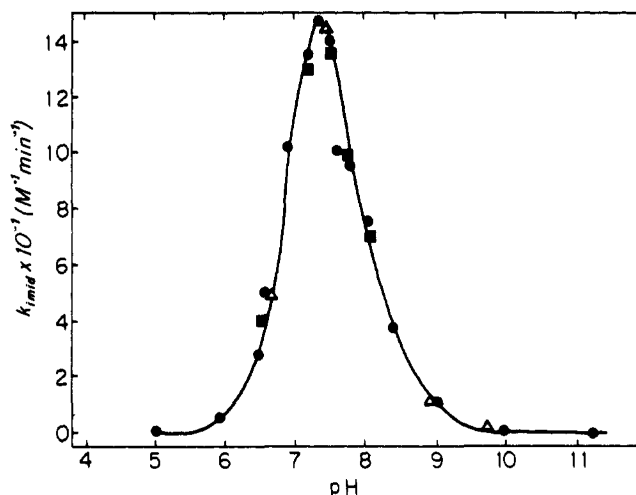


Figure 5. pH-rate profile for imidazole catalysis of PNMA hydrolysis at 25.0°; PNMA (5×10^{-4} *M*) in 0.05 *M* buffer with 1% (v/v) acetonitrile, ionic strength 0.55 and 1×10^{-2} *M* added imidazole. In general, for organic acids of pK_a less than 7, $pK_a^{D_2O} = pK_a^{H_2O} + 0.55$ (see references in footnote *c*, Table III). For $PNMAH^+$, we find ΔpK_a to be 0.5. Therefore, the value of $k_{Im}^{H_2O}$ at a given pH is comparable to the value of $k_{Im}^{D_2O}$ at a pD that is 0.5 unit greater than the pH in question. For easy visualization when D_2O is used as a solvent, the points are not plotted against pD, but are placed so that $[SH^+]/[S] = [SD^+]/[S]$. Reaction followed in (●) H_2O at 275 nm; (△) D_2O at 275 nm; (■) H_2O at 240 nm.

triethylamine acetate, malonate, and diethyl malonate buffers. The results are summarized in Table II.

Nucleophilic Catalysis of PNMA Decomposition. During a study of the bovine carbonic anhydrase (BCA) catalyzed hydrolysis of PNMA,[12] two potent anionic inhibitors of carbonic anhydrase, azide and cyanide, were added to the solution and a remarkably faster disappearance of PNMA was observed. This apparent susceptibility of PNMA to nucleophilic attack prompted an examination of PNMA hydrolysis in the presence of imidazole, *n*-butylamine, L-lysine, and *N*-α-CBZ-L-lysine, since similar nucleophilic residues are found in proteins. All of these compounds were capable of enhancing the decomposition of PNMA. In each case, the pH profile appeared as a bell-shaped curve (see, for example, Figure 5). The results are summarized in Table III. In the presence of bovine serum albumin (BSA) the pH-rate profile was also bell shaped.[12] However, with carbonic anhydrase, the pH-rate profile showed a more complicated pH dependence indicating that the assumption of simple nucleophilic catalysis was not sufficient to account for the decomposition of PNMA in the presence of enzyme.[12] Further, it was observed that the value of k_{BCA} was lower in D_2O than in H_2O. For all of the other nucleophiles, including albumin, the catalytic rate constant was the same in H_2O and in D_2O.

A detailed examination of the azide attack on PNMA proved interesting. The reaction could be monitored by following the release of phenol at 275 nm or the production of azido *N*-methylacetimidate (ANMA) at 240 nm. Initially, the rate of phenol release was equal to the rate of ANMA production, but after release of phenol was complete, ANMA reacted further as evidenced by a decrease in the absorption at 240 nm (Figure 2). However, when a second injection of 5×10^{-4} *M* PNMA was made into solutions containing 5×10^{-4} *M* azide and PNMA that had reacted completely, the reaction proceeded at the same rate as in the buffer indicating that no free azide ions remained.

That azide was acting as a nucleophile was indicated by the absence of a solvent effect for the azide catalysis. Had azide been acting as a general base catalyst for water attack on PNMA, instead of as a nucleophile, a solvent isotope effect would have been observed in D_2O. But, in fact, $k_{N_3^-}{}^{H_2O} = k_{N_3^-}{}^{D_2O}$, indicating that azide was indeed acting as a nucleophile. The unprotonated intermediate, ANMA, then rearranges to a tetrazole which does not react further. This tetrazole has been isolated from the reaction mixture and has been identified as 1,5-dimethyltetrazole; the melting point and nmr and mass spectra are identical with authentic 1,5-dimethyltetrazole and in accord with literature values.[16,17] ANMA would be expected to absorb in the uv (Figure 2) giving the initial absorption observed at 240 nm. However, once rearrangement to the tetrazole occurred, the absorbance at 240 nm should drop to near zero (as observed);[20] tetrazoles are reported to exhibit only small end absorption in the 200–220-nm region,[20] and our data confirm this. The total disappearance of the uv absorption at 240 nm indicated that the reaction of ANMA went to completion. The proposed reaction mechanism is as shown in Scheme I. The mechanism is consistent with the following observations: (1)

(20) C. Fallon and H. Herbst, *J. Org. Chem.*, **22**, 933 (1957).

Table III. Effect of Nucleophiles on Phenyl *N*-Methylacetimidate at 25.0° [a]

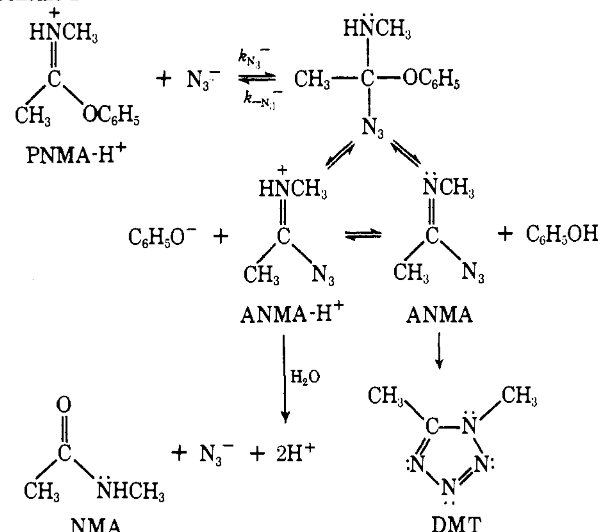
Wavelength, nm (solvent used)	Azide pH (pD)	Azide $k_{N_3^-}$,[b] M^{-1} min^{-1}	Imidazole pH (pD)	Imidazole k_{IM},[b] M^{-1} min^{-1}	Cyanide pH	Cyanide k_{CN^-},[b] M^{-1} min^{-1}
275 (H_2O)	2.20	13	5.00	0.5	5.05	0.25
	3.55	310	5.91	5.3	5.91	3.65
	4.31	1100	6.48	30	6.64	15.4
	4.65	1480	6.57	50	7.63	20.6
	5.19	1840	6.88	105	7.96	18.0
	5.60	1820	7.19	135	8.63	13.3
	5.90	1600	7.36	147	9.33	5.9
	6.14	1300	7.47	140	10.05	3.3
	6.35	1060	7.59	100	11.23	0.22
	7.36	230	7.76	95	12.10	0.01
	8.99	80	8.03	75		
	9.90	12	8.37	37		
			8.95	11.9		
			9.00	11.6		
			9.94	0.9		
			11.20	0.15		
240 (H_2O)	3.40	210	6.48	40		
	4.45	1360	6.88	100		
	4.65	1500	7.19	130		
	6.40	980	7.47	135		
			7.76	100		
			8.03	70		
275 (D_2O)	5.25[c]	1600	7.20[c]	50		
	6.73[c]	1080	7.95[c]	145		
	7.93[c]	200	9.48[c]	11.9		
	9.40[c]	90	10.24[c]	1.1		
	10.28[c]	10				

Wavelength, nm (solvent used)	*n*-Butylamine pH	*n*-Butylamine k_{NBA},[b] M^{-1} min^{-1}	L-Lysine pH	L-Lysine k_{Lys},[b] M^{-1} min^{-1}	*N*-α-CBZ-L-lysine pH	*N*-α-CBZ-L-lysine k_{NCL},[b] M^{-1} min^{-1}
275 (H_2O)	5.88	0.01	5.45	0.02	6.23	0.04
	6.65	0.05	6.20	0.2	7.18	0.76
	7.60	0.9	7.17	2.6	7.41	1.25
	7.95	1.3	7.70	4.3	7.80	2.0
	8.72	2.4	8.34	5.2	8.34	2.6
	9.51	2.9	8.90	5.2	9.03	3.15
	10.04	2.8	9.51	5.3	9.45	3.15
	10.50	2.5	10.10	4.9	10.03	3.0
	11.28	1.1	10.80	3.0	11.0	1.7
	12.03	0.4	12.01	0.8	12.00	0.4

[a] 1% (v/v) acetonitrile in 0.050 *M* buffers at an ionic strength of 0.55. The buffers used were malonate (pH 2–6.2), diethyl malonate (pH 6.2–7.5), Tris (pH 7.5–9.5), and triethylamine acetate (pH 9.5–12). [b] Second-order rate constants for nucleophilic attack. The subscripts are: N_3^- (azide); Im (imidazole); CN^- (cyanide); NBA (*n*-butylamine); Lys (L-lysine); NCL (*N*-α-CBZ-L-lysine); and k_N (general nucleophile). [c] The pD values listed for the runs in D_2O are equal to meter reading pH + 0.40.[3] It should be noted that in general for organic acids of p*K* less than 7, $pK_a^{D_2O} \sim pK_a^{H_2O} + 0.55$. [P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960); A. O. McDougall and F. A. Long, *ibid.*, **66**, 429 (1962); and R. A. Robinson, M. Paabb, and R. G. Bates, *J. Res. Nat. Bur. Stand., Sect. A*, **73**, 299 (1969)]. In this study, the ΔpK_a was found to be 0.5 (Table II). Therefore, the value of $k_N^{H_2O}$ at a given pH is comparable to the value of $k_N^{D_2O}$ at a value of pD that is 0.5 unit greater than the pH in question. For example, if one wants to determine the value of $k_{N_3^-}$ at the point where PNMA is 50% in the acidic form, one would compare $k_{N_3^-}{}^{H_2O}$ at pH 6.2 to $k_{N_3^-}{}^{D_2O}$ at pD 6.7.

the low rates of azide attack on PNMA in the basic region indicate that unprotonated PNMA (pK_a = 6.2) was not measurably subject to nucleophilic catalysis; (2) the low rates of reaction in the strongly acidic solutions when the pH was below the pK_a for azide (pK_a = 4.72) indicate that hydrazoic acid is not a nucleophile in this reaction; and (3) both NMA (30%) and DMT (70%) are formed around pH 5 indicating that H_2O attack of ANMA is competitive with rearrangement to form the tetrazole.

Scheme I



With imidazole the results are more straightforward. The attack by imidazole on PNMA could be monitored following phenol release at 275 nm or formation of imidazyl *N*-methylacetimidate (INMA) at 240 nm. The rate of phenol release was equal to the rate of formation of imidazyl *N*-methylacetimidate (Table III); however, most rates were monitored at 275 nm since a stable A_∞ was obtained. The A_∞ value at 240 nm reached a maximum and then decreased with time (Figure 1) so that A_∞ values used for rate calculations at 240 nm had to be estimated. The decrease in A_∞ at 240 nm is observed to arise from subsequent hydrolysis of INMA to regenerate imidazole and produce NMA. If the reaction is quenched when the absorption at 240 nm is near its maximum, imidazyl *N*-methylacetimidate can be isolated and has been identified by nmr and mass spectrum. No INMA could be detected when the absorption at 240 nm had disappeared. The initial formation of INMA is in accord with the fact that no solvent isotope effect was observed when D_2O was used as the solvent instead of H_2O ($k_{Im}^{H_2O} = k_{Im}^{D_2O}$, Table III).

Discussion

The mechanism proposed for the reaction of water with PNMA (eq 1) requires, depending upon the pH, that the rate-determining step of the reaction be either the attack of water or of hydroxide ion on the protonated imido ester. The unprotonated ester is presumed to be unreactive.[6] Additional mechanistic information comes from the observation of a solvent isotope effect in the hydrolysis of PNMA. From Table II it can be seen that the first-order rate constant, k_1, for attack by water on protonated PNMA drops from a value of 0.063 min^{-1} in H_2O to 0.029 min^{-1} in D_2O, ($k_1^{H_2O}/k_1^{D_2O}$) = 2.17. Based on this solvent isotope effect, we favor for k_1 a concerted mechanism involving a water-catalyzed attack by water.[21–23] Such a mechanism may involve

(21) H. H. Huang, R. R. Robinson, and F. A. Long, *J. Amer. Chem. Soc.*, **88**, 1866 (1966).
(22) Y. Pocker and J. E. Meany, *J. Phys. Chem.*, **71**, 3113 (1967); **73**, 1857 (1969).
(23) Y. Pocker and E. Green, *J. Amer. Chem. Soc.*, **95**, 113 (1973).

the direct formation of a neutral tetrahedral intermediate[23] *via* a transition state in which a proton transfer from one water molecule to another (possibly involving a one proton bridge[24]) is an integral part of the rate-determining attack on the protonated imido ester.

The solvent isotope effect on k_2 (Table II, Figure 3) also has interesting mechanistic implications. The rate constant can be considered either to reflect a rate-determining attack of water on the unprotonated PNMA or a rate-determining attack of hydroxide on protonated PNMA. Earlier authors have argued[3a,6,7] that the hydroxide attack on protonated PNMA provides a more reasonable mechanism for the "neutral" hydrolysis. The observed ratio, $(k_2^{H_2O}/k_2^{D_2O}) = 0.58$, tends to confirm this. Deuteroxide is both a stronger base and a stronger nucleophile than hydroxide so that one might anticipate the rate constant for the reaction of the deuterated imido ester with OD^- to be somewhat larger than that for the protonated imido ester with OH^-. Indeed, it has been noted earlier that the ratio k_{OH^-}/k_{OD^-} is *ca.* 0.7 in the enolization of acetone,[25a] in the racemization of mandelate ion,[25b] and in the decomposition of diacetone alcohol.[25c] A value of 0.72 for k_{OH^-}/k_{OD^-} was also noted more recently by Walters and Long[26] in the detritiation of 1,4-dicyano-2-butene-*1-t*. Had water attack on unprotonated PNMA been rate determining, effects on k_2 would have been either near unity for the water acting as a nucleophile, or they would have paralleled k_1, giving $k_2^{H_2O} > k_2^{D_2O}$, with water acting as a general base.

In all cases, an examination of the pH–rate profiles for the PNMA decomposition catalyzed by cyanide, azide, imidazole, *n*-butylamine, L-lysine, *N*-ε-CBZ-L-lysine, or bovine serum albumin[12] reveals a bell-shaped curve with a fairly sharp pH of maximum rate. The maximum occurs at a pH characteristic of the nucleophile (Table III). However, except in the case of azide, the maximum rate does not occur at a pH half-way between the pK_a of the nucleophile and that of PNMA. This unusual phenomenon was also observed by Hand and Jencks[3a] with other imido esters. Their explanation is that the reaction between amine and protonated imido ester is rate determining on the alkaline side of the pH–rate profile and that the decomposition of the tetrahedral intermediate is rate determining on the acid side of the pH–rate profile. It was of some interest to note that Hand and Jencks found that the only detectable path for formation of a tetrahedral addition intermediate involved reaction of the free amine with protonated imido ester. Further evidence that the rate-determining steps of the reaction are different in the alkaline and acidic pH regions came from their observation that general base catalysis was significant only on the acid side of the pH–rate profile. This observation fits well with the buffer catalysis found in this study. The malonate and diethyl malonate buffers used in the acid region both exhibited significant catalysis, whereas Tris and triethylamine acetate, used in the basic region, were found not to contribute measurably to the observed buffer rate.

The observation that the attack by azide ions exhibits a maximum at a pH equal to the average of the pK_a's for HN_3 and protonated imido ester is consistent with the fact that this nucleophile has a maximum catalytic rate in the acidic region so that the rate-determining step is the same on both sides of the maximum. The concentration of the tetrahedral intermediate should be at a maximum when the product of the concentrations of azide anion and protonated PNMA is maximized. This occurs at the average of the respective pK_a values, as observed.

Deuterium oxide solvent isotope effects have been shown to be significantly smaller for nucleophilic reactions than for general base catalyzed reactions.[27] Thus, the fact that these compounds are acting as nucleophiles rather than as general base catalysts in their reaction with PNMA was confirmed by running the parallel reactions in D_2O. For azide and imidazole it was found that $k_{N_3}^{H_2O} = k_{N_3}^{D_2O}$ and $k_{Im}^{H_2O} = k_{Im}^{D_2O}$, Table III. Similar results were obtained with bovine serum albumin.[12]

In conclusion, the protonated imido ester appears to be the reactive species in the hydrolysis of PNMA. It is also subject to nucleophilic attack by a large number of nucleophiles, the reaction with azide ion providing direct evidence for a tetrahedral intermediate.[28] The increased reactivity of PNMA compared to other imido esters previously studied makes it a valuable protein and enzyme modification agent.[12]

Acknowledgment. The authors wish to thank Dr. Anna Pocker for many helpful discussions.

(24) S. S. Minor and R. L. Schowen, *J. Amer. Chem. Soc.*, **95**, 2279 (1973).

(25) (a) Y. Pocker, *Chem. Ind. (London)*, 1383 (1959); (b) *ibid.*, 1117 (1958); (c) *ibid.*, 89 (1959).

(26) E. A. Walters and F. A. Long, *J. Phys. Chem.*, **76**, 362 (1972).

(27) M. L. Bender and G. H. Hamilton, *J. Amer. Chem. Soc.*, **84**, 2570 (1962).

(28) It is interesting that the water reaction exhibits general catalysis while imidazole acts as a nucleophile. A similar observation was made by T. C. Bruice and B. Holmquist [*J. Amer. Chem. Soc.*, **89**, 4028 (1967)] in their studies of activated oxalate esters.