The Reaction of Phenyl *N*-Methylacetimidate with Bovine Serum Albumin and Bovine Carbonic Anhydrase†

Y. Pocker,* M. W. Beug,‡ and F. Ann Beug

ABSTRACT: The kinetics of the reaction of phenyl *N*-methylacetimidate with bovine carbonic anhydrase and with bovine serum albumin were examined over the pH range 5–12 in both H_2O and D_2O. Bovine serum albumin (BSA) showed no turnover but was found to react irreversibly with phenyl *N*-methylacetimidate. The rates in H_2O and D_2O were the same, exhibiting a bell-shaped pH–rate profile with a maximum (k_{BSA} = 720 M^{-1} min^{-1}) near pH 8.7 (pD 9.2). In this reaction, the ϵ-aminolysyl residues are amidinated. With carbonic anhydrase, phenyl *N*-methylacetimidate was found to react as a modification agent leading to amidination of ϵ-aminolysyl residues of the enzyme. From competitive inhibition studies, it is shown that modification of all of the enzyme's lysine residues does not lead to any decrease in enzymatic activity. Therefore a lysine residue cannot be a part of the active site of bovine carbonic anhydrase. Isotopic labeling studies showed that while bovine serum albumin only underwent modification by phenyl *N*-methylacetimidate, bovine carbonic anhydrase exhibited both modification and turnover. Also, contrary to the observations with bovine serum albumin, the rate of reaction of phenyl *N*-methylacetimidate with carbonic anhydrase was slower in D_2O than in H_2O. In both H_2O and D_2O, the pH (pD) rate profile exhibited a maximum at 7 (7.5) and a lower plateau of activity above 9 (9.5). At pH 7, k_{modif} = 155 M^{-1} min^{-1} and k_{enz} = 135 M^{-1} min^{-1}, while at pH 8.9, k_{enz} = 55 M^{-1} min^{-1} and k_{modif} = 145 M^{-1} min^{-1}. The enzyme-catalyzed hydrolysis of phenyl *N*-methylacetimidate is not inhibited by acetazolamide, ethoxzolamide, or anions, nor does this imidate inhibit the enzymatic hydrolysis of *p*-nitrophenyl acetate. Evidently, the binding site and the turnover mechanism are distinctly different from that of CO_2, aldehydes, and phenyl esters.

A general characteristic of carbonyl compounds is their ability to react with primary amines with the loss of water to form Schiff bases (azomethines or imines). During the last ten years the importance of such Schiff bases as intermediates in certain enzymic transformations of carbonyl compounds has become apparent as in the case of fructose diphosphate aldolase (Grazi *et al.*, 1962; Lai *et al.*, 1965) and acetoacetate decarboxylase (Westheimer, 1963; Warren *et al.*, 1966). Consequently, in investigating carbonic anhydrase activity, it proved desirable to ascertain whether or not Schiff base intermediates are likely to play a role in the enzymic hydration of aldehydes (Pocker and Meany, 1964, 1965a,b, 1967a,b; Pocker and Dickerson, 1968) and of pyruvate, or other keto acids (Pocker and Meany, 1970). Since lysine residues are located near the active site of carbonic anhydrase, we have initiated an investigation of potential carbonic anhydrase substrates that also possess the capacity to interact irreversibly with exposed lysine residues.

In the present paper we examine the interaction of a novel modifier, phenyl *N*-methylacetimidate (PNMA)[1] with the enzyme, bovine carbonic anhydrase (BCA) and with bovine serum albumin (BSA), a protein that does not possess enzymic activity. With both proteins, PNMA is shown to react with lysyl residues to form amidines in a manner similar to the reaction of ethyl acetimidate with proteins (Hunter and Ludwig, 1962; Wofsy and Singer, 1963; Nilsson and Lindskog, 1967). PNMA has the advantage that its reaction can be followed spectrophotometrically by observing the rate of phenoxide ion production. It is a particularly interesting imide ester since the phenoxide group is a much better leaving group than the alcoholates of most other substrates that had been investigated, and except under acidic conditions, the tetrahedral intermediate decomposes preferentially with its expulsion.

We were led to investigate the possibility that PNMA was also a substrate of carbonic anhydrase because of the close resemblance between PNMA and phenyl acetate, a known substrate of the enzyme (Pocker and Beug, 1972). Indeed, we can now report that the enzyme catalyzes the hydrolysis of PNMA; this being the first reported case of apparent carbonic anhydrase versatility with respect to the addition of water across a C=N bond.[2] However, all inhibition data indicate that this activity is abnormal, possibly because the substrate is not bound at the same site as phenyl acetate.

† From the Department of Chemistry, University of Washington, Seattle, Washington. *Received December 20, 1972.* Support of this work by Grant AM 09221 from the National Institutes of Health of the U. S. Public Health Service is gratefully acknowledged. Taken in part from the dissertation presented by M. W. B. to the University of Washington in partial fulfillment of the requirements for the Ph.D. degree.

‡ Predoctoral trainee (GM 39680) of the National Institute of General Medical Sciences, U. S. Public Health Service.

[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: BCA, bovine carbonic anhydrase; BCA A and BCA B denote the isozymes A and B of BCA; BSA, bovine serum albumin; PNMA, phenyl *N*-methylacetimidate.

[2] The carbonic anhydrase catalyzed addition of water across C=N has not been reported previously. The reversible hydration of Schiff bases has been noted, but attempts to follow the reaction kinetically are often frustrated by the ready breakdown of the adduct (Dimroth base) to aldehyde and amine. The phenomenon of reversible hydration appears to be quite common in the pteridine and quinazoline series and has the advantage that it is hardly ever followed by the fission of the C–N bond. The addition of water is time dependent, but usually very rapid at room temperature. Unfortunately, a combination of poor enzyme–substrate binding and very low turnover prevents us from reporting on these hydrations (Y. Pocker, M. W. Beug, D. Bjorkquist, and C. Henderson, unpublished data).

Experimental Section

Materials

Phenyl N-methylacetimidate was prepared by the method of Oxley and Short (1948). The fractionally distilled product [n_D^{20}] 1.5185; bp 74–76° at 3.5 Torr; lit., 65° at 1.5 Torr (Oxley and Short, 1948)] had infrared (ir) and nuclear magnetic resonance (nmr) spectra (Kandel and Cordes, 1967) in accord with the assigned structure.

Phenyl N-methylacetimidate-2-^{14}C was prepared with minor modifications by the method of Oxley and Short (1948). Reagent acetone (2.0 ml) was added to 0.5 mCi of acetone-2-^{14}C (Mallinckrodt Nuclear, specific activity 4.2 Ci/mol). One-half milliliter (0.0068 mol) of the diluted acetone-2-^{14}C was added to 4 ml of 5 N NaOH and 0.5 g (0.0069 mol) of hydroxylamine hydrochloride. After 2 hr, the reaction was chilled to 0° and 1 ml (0.007 mol) of freshly distilled benzenesulfonyl chloride was added dropwise. The reaction was allowed to come to room temperature and the white crystalline acetoxime benzenesulfonate was washed with cold distilled water and dried *in vacuo* for 1 week over P_2O_5. It was then dissolved in 15 ml of dry toluene, refluxed for 2 hr with 0.67 g (0.0068 mol) of reagent phenol. After standing over night, 10 ml of 3 N NaOH was added with shaking. The toluene layer was isolated, dried over anhydrous sodium sulfate, and then the toluene was removed by vacuum evaporation. Kinetic runs with the resulting material (PNMA-2-^{14}C) and with unlabeled PNMA gave the same values (see Results).

Bovine carbonic anhydrase was prepared from commercial samples obtained from Mann Research Laboratories as described earlier (Pocker *et al.*, 1971). Concentrations based on 1.00 ± 0.05 atom of zinc/molecule (determined by atomic absorption spectrophotometry), assuming a molecular weight of 30,000, were in accord with a molar extinction coefficient, ϵ_M, at 280 nm of 54,000 M^{-1} cm^{-1}. The electrophoretic pattern obtained in 7% polyacrylamide gels on a Canalco Model 12 EPH apparatus and chromatography on DEAE-cellulose revealed that the bovine enzyme was a mixture of A and B isomers in the ratio *ca.* 1:2. While these two isozymes differ in electrophoretic mobility they have essentially identical amino acid composition and catalytic properties (Nyman and Lindskog, 1964; Pocker and Stone, 1967; Pocker and Dickerson, 1968). The bovine enzyme showed no loss of activity when stored at −20° under anhydrous conditions. Although aqueous stock solutions of the enzyme appeared to be quite stable at 25°, freshly prepared solutions were always used.

Bovine serum albumin was 100% electrophoretically pure material from Behringwerke AG.

Buffer Components and Solutions. The buffers employed in this study have been previously described (Pocker and Beug, 1972). 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 135°). Diethylmalonic acid was prepared by hydrolysis of the diethyl ester (Aldrich) with alcoholic KOH, followed by treatment with HCl in ether. The acid was recrystallized from ether–benzene (mp 128–129°). Reagent grade monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, sodium hydroxide, and acetic acid were obtained from Allied Chemical and used without further purification. The total buffer concentration used in this work was varied from 0.05 to 0.2 M over the pH range studied. The ionic strength was maintained constant at 0.55 by adding an appropriate amount of NaCl.

Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, was obtained from American Cyanamide Co. (Lederle Laboratory Division) and ethoxzolamide, 6-ethoxy-2-benzothiazolesulfonamide, from the Upjohn Co. Reagent grade acetonitrile (Baker analyzed) was used as a solvent for the preparation of stock solutions of phenyl *N*-methylacetimidate, phenyl acetate, and *p*-nitrophenyl acetate.

Instrumentation. The initial kinetic studies 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. The instrument has recently been replaced with an automated spectrophotometric system. The temperature is controlled at 25.0 ± 0.05° by a Forma-Temp Jr. (Model 2095) circulating bath attachment. All pH and pD determinations were made with a Beckman 101900 research pH meter equipped with a Beckman calomel internal 39071 frit junction reference electrode and a Beckman glass electrode 41263. The reported pD values are obtained by adding 0.40 to the pH meter reading (Glasoe and Long, 1960). Nuclear magnetic resonance spectra for structure verification were obtained with a Varian Associates A-60 instrument. Ultraviolet spectra were obtained on a Cary 14 recording spectrophotometer. Infrared spectra were obtained on a Perkin-Elmer 137 sodium chloride spectrophotometer. The determination of zinc content in the enzyme was done on a Beckman atomic absorption accessory (130000) attached to a Beckman Du-2 spectrophotometer. Radioisotope counting was done with a Beckman liquid sinctillation system equipped with a modified Model 33 teletypewriter printout device. The 1-ml aqueous samples were counted in polyethylene vials (Packard) with 15 ml of scintillation fluid containing naphthalene (60 g/l.), Omnifluor (8 g/l.), methanol (1%), and ethylene glycol (0.2%) in a dioxane base.

Kinetics. Kinetic runs for determination of buffer rates were performed in a 10-mm rectangular silica cell. A solution (0.03 ml) of acetonitrile containing substrate was injected by means of a calibrated Hamilton syringe into 2.97 ml of the appropriate buffer giving a final solution of acetonitrile 1% (v/v) and ionic strength, μ = 0.55. The acetonitrile stock solutions of PNMA could be prepared and stored for long periods of time with no decomposition if kept in the dark. While the acetonitrile served to increase the solubility of the PNMA and was transparent at wavelengths above 270 nm, it had the disadvantage of slightly inhibiting carbonic anhydrase activity (Pocker and Stone, 1965, 1967).

For the determination of enzymatically catalyzed rates of hydrolysis, high enzyme concentrations (10^{-4} M) were required. Because carbonic anhydrase absorbs (ϵ_M 54,000 M^{-1} cm^{-1} at 280 nm) in the region of the isosbestic point of phenol (275 nm), a solution of the enzyme equal in concentration to that employed in the reaction cell was used in the reference cell. In addition, 2- and 5-mm cells were used in order to reduce total absorption. With these narrower cells reproducibility was greatly improved because it became possible to balance the instrument using slit widths as narrow as 0.6 mm.

The hydrolysis and amidination rates were followed spectrophotometrically by monitoring the appearance of phenol or phenolate anion at 275 nm, the isosbestic point (where $\epsilon_{phenol} = \epsilon_{phenolate\ anion}$, log ϵ = 3.12). PNMA does not absorb appeciably at 275 nm, but under acidic conditions the disappearance of its protonated form can be followed at 247.5 nm (Kandel and Cordes, 1967). The two wavelengths gave similar results. At pH values above neutrality, the re-

action went to greater than 99% completion, producing phenol and *N*-methylacetamide. In the acidic region, small but increasing amounts of phenyl acetate and methylamine are also produced (Jencks and Gilchrist, 1968; Pletcher *et al.*, 1968).

PNMA concentrations were generally held between 1×10^{-3} and 1.5×10^{-3} M. In all cases buffer rates were linear for at least two half-lives. However in a few cases the enzymatic rates were not completely linear over one half-life. When this occurred, initial rates of reaction were calculated. Pseudo-first-order rate constants were determined by plotting $-\log (A_\infty - A_t)$ *vs.* time and were evaluated using a Fortran IV computer executed on a CDC 6400 digital computer. The program was written by Dr. Nori Watamori to calculate the best slope for first-order plots by means of the least-squares method. It gives the rate constant, the reaction half-life, the standard deviation of the slope, and the correlation coefficient. In addition, when the correlation coefficient is less than 0.999, the data is graphically plotted so that the operator can quickly pinpoint the cause of the discrepancy.

Inhibition Studies. Per cent inhibition of carbonic anhydrase activity by acetazolamide, ethoxzolamide, and selected anions was determined as a function of added inhibitor. The concentration of enzyme was varied from 5×10^{-5} to 1×10^{-4} M, PNMA concentration was 1×10^{-3} M; acetazolamide and ethoxzolamide concentrations were varied from 5×10^{-6} to 5×10^{-4} M; and concentrations of bromide, iodide, chloride, cyanide, and azide up to 1.2 M were employed. Using *p*-nitrophenyl acetate as a substrate, PNMA was checked for inhibitory action.

Determination of ^{14}C Incorporation into Protein. In an experiment designed to test for enzyme modification, phenyl *N*-methylacetimidate-2-^{14}C was added to a solution containing 3 mg/ml of bovine enzyme (10^{-4} M) or bovine serum albumin (4.4×10^{-5} M) and allowed to react to completion. The proteins were dissolved in 0.05 M diethyl malonate (pH 7.08, $\mu = 0.55$) in one set of experiments and in 0.05 M Tris (pH 8.92, $\mu = 0.55$) in the other set. The amount of PNMA-2-^{14}C added varied between 1×10^{-4} M (1800 cpm/ml) and 5×10^{-3} M (88,000 cpm/ml). For control purposes, the PNMA-2-^{14}C was also added to solutions containing no protein. In addition, in order to distinguish equilibrium binding of the reaction products with the protein from covalent bonding (amidination of the protein occurring during PNMA hydrolysis), one set of reactions was performed where enzyme or albumin was added after the PNMA-2-^{14}C had been allowed to hydrolyze completely.

Four 1-ml aliquots were removed from each solution and placed in 8-mm Union Carbide dialysis bags (prepared by boiling for 0.5 hr in deionized water containing 10^{-3} M EDTA and rinsing three times in deionized water). These quadruplicate samples were placed in a beaker and dialyzed with constant stirring against 150 ml/(set of four) of the same buffer that was contained in the bag.

After 4-hr dialysis, one bag from each set was removed and emptied into a polyethylene scintillation vial. One milliliter of each dialysate solution was simultaneously measured into a second set of vials. Then 15 ml of scintillation fluid was added to each vial and the vials counted in the Beckman liquid scintillation instrument. This procedure was repeated after 12-, 24-, and 72-hr dialysis.

Results

Buffer Catalysis. In order to determine the kinetics of the PNMA reaction with bovine carbonic anhydrase or bovine serum albumin it was first necessary to accurately know the buffer-catalyzed pH–rate profile for phenyl *N*-methylacetimidate. The pH-dependent rate of hydrolysis of PNMA was determined in both H_2O and D_2O. The results appear in Table I. The extrapolation to zero buffer concentration (Figure 1) is in good agreement with the results of earlier workers who studied the hydrolysis in H_2O (Kandel and Cordes, 1967). Indeed, mechanistic pathways for such hydrolyses have attracted a fair amount of attention (Kandel and Cordes, 1967; Jencks and Gilchrist, 1968; Pletcher *et al.*, 1968). A description of some novel aspects in the break-

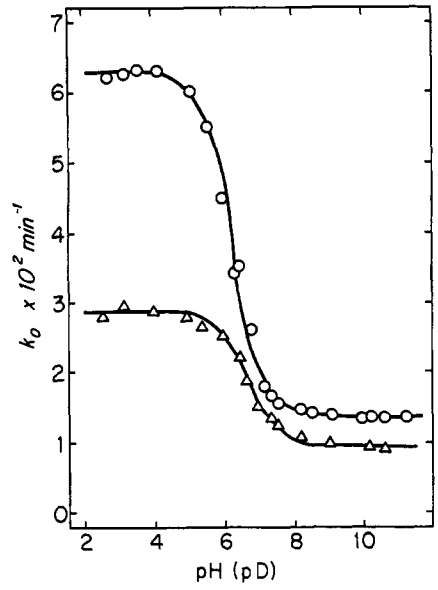


FIGURE 1: First-order rate constant for phenyl *N*-methylacetimidate hydrolysis extrapolated to zero buffer concentration at 25.0°; with $\mu = 0.55$, 1% (v/v) acetonitrile, and [PNMA] = 1.5×10^{-3} M: (○) in H_2O; (△) in D_2O.

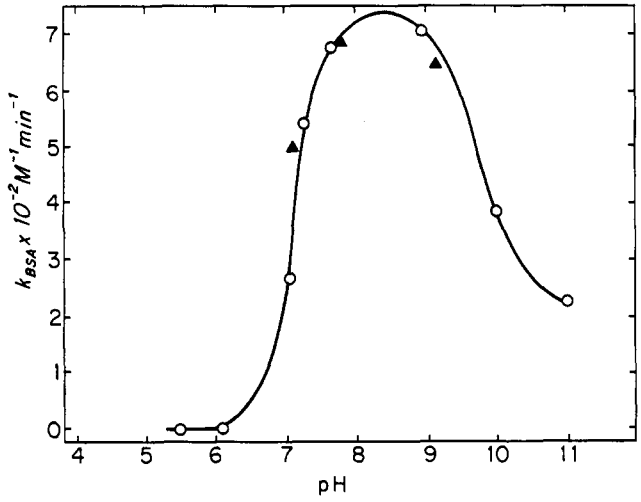


FIGURE 2: pH dependency of the bovine serum albumin reaction with PNMA at 25.0°; in 0.05 M buffers with 1% (v/v) acetonitrile, $\mu = 0.55$, [PNMA] = 1.0×10^{-3} M and [BSA] = 4.4×10^{-5} M 3.0 mg/ml). It should be noted that in general for organic acids of pK_a less than 7, $pK_a^{D_2O} = pK_a^{H_2O} + 0.55$ (Glasoe and Long, 1960; McDougall and Long, 1962; Robinson *et al.*, 1969). For PNMA, we found ΔpK_a to be 0.5. Therefore, the value of $k_{BSA}^{H_2O}$ at a given pH is comparable to the value of $k_{BSA}^{D_2O}$ at a pD that is 0.5 unit greater than the pH in question. For easy visualization when D_2O was used as a solvent, the points were not plotted against pD, but were placed so that $[SH^+]/[S] = [SD^-]/[S]$: (○) in H_2O; (▲) in D_2O.

TABLE I: Values of k_{buff} for the Hydrolysis of PNMA in H_2O and D_2O at 25.0°.

Buffer[a]	pH	$k_{buff} \times 10^{2}$ [b] min^{-1}	Buffer[a]	pH	$k_{buff} \times 10^{2}$ [b]
MAL	2.3	6.1	DEM	7.2	2.1
MAL	2.8	6.2	DEM	7.6	1.8
MAL	3.2	6.3	DEM	8.2	1.65
MAL	3.6	6.3	Tris	7.4	1.65
MAL	4.5	6.6	Tris	8.9	1.4
MAL	5.1	6.6	Tris	9.1	1.36
MAL	5.6	6.3	TEAA	10.0	1.35
MAL	6.0	5.5	TEAA	10.3	1.35
MAL	6.4	4.3	TEAA	10.6	1.35
DEM	6.5	3.8	TEAA	11.2	1.35
	pD[c]			pD[c]	
MAL	2.6	2.8	DEM	6.7	1.9
MAL	3.2	3.0	DEM	7.0	1.9
MAL	4.0	3.0	DEM	7.6	1.3
MAL	5.0	2.9	DEM	8.2	1.15
MAL	5.4	2.8	Tris	7.4	1.3
MAL	6.0	2.7	Tris	9.1	1.0
MAL	6.5	2.4	TEAA	10.3	0.92
			TEAA	10.6	0.90

[a] MAL (malonate), DEM (diethyl malonate), TEAA (triethylamine-acetate). The buffer strength was 0.05 M maintained at μ = 0.55 with added sodium chloride. [b] Results with PNMA-2-^{14}C were similar. [c] pD = pH_{mr} + 0.40 where pH_{mr} = pH meter reading (Glasoe and Long, 1960).

TABLE II: Modification of Bovine Serum Albumin by Reaction with PNMA-2-^{14}C.[a]

PNMA Concn (M)	pH	Dialysis[b] Time (hr)	icpm[c]	fcpm[d]	No.[e] of Groups Modified
5×10^{-3}	8.92	4	87,060	27,420	35
		12		25,770	33
		24		24,840	32
5×10^{-3}	7.00	4	88,030	17,900	23
		12		17,700	22.7
		24		16,500	21.6
1×10^{-3}	8.92	4	18,010	9,120	11.5
		12		9,310	11.7
		24		9,090	11.4
1×10^{-3}	7.00	4	18,230	5,290	8.6
		12		4,870	6.1
		24		5,000	6.2
1×10^{-3} [f]	8.92	4	18,370	127	
		12		0	
		24		156	
1×10^{-3} [f]	7.00	4	1,820	83	
		12		0	
		24		14	
1×10^{-4}	8.92	4	1,790	900	1.1
		12		890	1.1
		24		900	1.1
1×10^{-4}	7.00	4	1,820	560	0.70
		12		490	0.61
		24		510	0.64

[a] At 25.0° in 0.05 M diethyl malonate (pH 7.0) or Tris (pH 8.92) buffers containing 1% (v/v) acetonitrile with μ = 0.55. The concentration of BSA was 4.4×10^{-5} M (based on 3.0 mg/ml of BSA weighed into solution). [b] Each dialysis was performed at the same pH as the reaction. [c] Counts per minute (icpm) per milliliter of solution prior to dialysis = $(PNMA)_{initial}$ × activity of PNMA in cpm per mole. [d] fcpm = cpm (per ml in bag) − cpm (per ml of dialysate) after indicated elapsed time. [e] Number of groups modified = (fcpm/icpm) × ([PNMA]/[BSA]). A slight decrease was anticipated in the number of groups modified as a function of dialysis time since it has been shown (Nilsson and Lindskog, 1967; Wofsy and Singer, 1963) that protein acetimidated residues undergo a slow hydrolysis in aqueous solution. [f] With these runs, instead of adding protein and then PNMA, the BSA was added after the PNMA had been completely hydrolyzed.

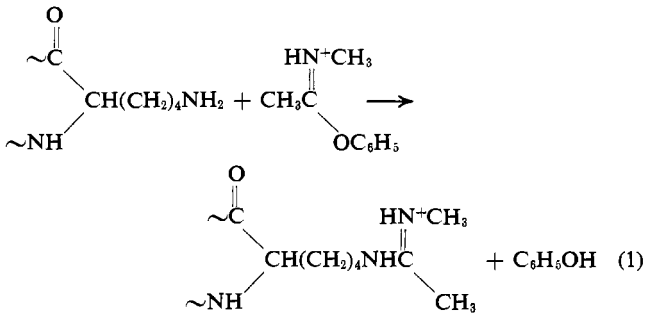
down of various tetrahedral intermediates formed from PNMA is being submitted for publication (Y. Pocker, M. W. Beug, and K. L. Stephens, unpublished results).

Reaction with Bovine Serum Albumin (BSA). If PNMA acts as an enzyme modifier by reacting with the ε-aminolysine groups [as would be predicted from the results obtained with other acetimidates (Hunter and Ludwig, 1962; Wofsy and Singer, 1963; Nilsson and Lindskog, 1967)], then it should readily interact with any protein containing lysine. Bovine serum albumin, a protein which does not act as an enzyme, was observed to "catalyze" the decomposition of PNMA (see Figure 2). The fact that the magnitude of this "catalysis" was the same in H_2O as in D_2O (Figure 2) was taken to indicate that general acid–base-catalyzed hydrolysis is not taking place and that the reaction under study actually involves a nucleophilic attack.[3] In support of this, decomposition of PNMA-2-^{14}C in the presence of bovine serum albumin was found to lead to ^{14}C incorporation into the protein. Injection of successive portions of PNMA led to parallel decreases in k_{obsd} consistent with a modification of the ε-aminolysyl residues to form amidines. The addition of a total of 5×10^{-3} M PNMA-2-^{14}C to 5×10^{-5} M albumin led to the amidination of approximately 33 ε-aminolysyl residues.

Isotopic Labeling of Bovine Serum Albumin and Bovine Carbonic Anhydrase with PNMA-2-^{14}C. The albumin was treated with uniformly labeled PNMA-2-^{14}C and ^{14}C incorporation was determined after dialysis. Control experiments showed that dialysis was virtually complete after four hours and that only covalently bonded ^{14}C was bound by either serum albumin or carbonic anhydrase. These results are summarized in Tables II and III and show that under mild

[3] Similar results have been obtained for the reaction of the albumin with *p*-nitrophenyl acetate. At pH 8.82 with 4.4×10^{-5} M albumin the rate of *p*-nitrophenol release is sixfold larger than that in buffer alone; $k_{BSA} = (k_{obsd} - k_{buff})/[BSA] = 1.7 \times 10^{3}$ M^{-1} min^{-1}. In D_2O solvent pD 9.3 (the same ratio of buffer components as at pH 8.82) the rate of *p*-nitrophenol release was also $k_{BSA} = 1.7 \times 10^{3}$ M^{-1} min^{-1}. The absence of a solvent isotope effect indicated that the rate enhancement in the presence of bovine serum albumin is due to a nucleophilic attack on the *p*-nitrophenyl acetate ester.

kinetic conditions it is possible to modify at least 5 residues/enzyme molecule and at least 33 residues/molecule of albumin. The modification is assumed to proceed almost exclusively *via* reaction with lysyl residues to form the amidine.



(1)

It was noted that over a 72-hr period, some hydrolysis of the *N*-methylacetimidated lysine residues occurred (~10%). As a result, ε-aminolysyl residues were regenerated and *N*-methylacetamide was produced. This observation was in general agreement with the findings of Nilsson and Lindskog (1967) using ethylacetimidate. Under conditions leading to an exhaustive acetimidation (Wofsy and Singer, 1963), Nilsson and Lindskog (1967) were able to modify 19 ε-aminolysyl residues in the enzyme. Similar modifications of lysyl residues have been carried out in these laboratories (Y. Pocker and D. G. Dickerson, unpublished observations).

Modification and Catalysis. Carbonic anhydrase enhances the rate of phenol release from PNMA. The value of $k_{BCA} = (k_{obsd} - k_{buff})/[E_0]$ was determined over the pH (pD) range 5.2–11.3 in both H_2O and in D_2O. The pH (pD) dependency of k_{BCA} is graphically depicted in Figure 3. In these runs, the concentration of PNMA was 150-fold in excess of the enzyme. Since it was clear from the isotopic substitution studies that enzyme modification was taking place, only initial rates were calculated (so the number of sites on the enzyme available for modification could be considered constant).

In contrast to albumin, the reaction between enzyme and PNMA was found to be slower in D_2O than in H_2O. This lead us to suspect that PNMA was acting both as an enzyme modifier and as a substrate. Indeed, the isotopic substitution studies with PNMA-*2*-^{14}C showed that in the decomposition of 1.5×10^{-3} M PNMA in the presence of 10^{-4} M enzyme at pH 7, *ca.* 55% of the total reaction resulted in ^{14}C incorporation into the enzyme. From this, k_{BCA} is best expressed as a sum, eq 2

$$k_{BCA} = k_{modif} + k_{enz} \quad (2)$$

At pH 7, $k_{modif} = 165$ and $k_{enz} = 135$ M^{-1} min^{-1}. At pH 8.92, 73% of the rate of phenol release arose from enzyme modification and 27% from hydrolysis. From this, one calculates that at pH 8.9, $k_{enz} = 55$ M^{-1} min^{-1} and $k_{modif} = 145$ M^{-1} min^{-1}.

Inhibition Studies. If the enzyme-catalyzed hydrolysis of PNMA is analogous to ester hydrolysis, then PNMA should be a competitive inhibitor of *p*-nitrophenyl acetate (Pocker and Stone, 1968b) provided its binding is strong enough to produce sufficient saturation of the enzyme to induce inhibition (K_m at least 3×10^{-2} M). To test this, varying concentrations (up to 3.3×10^{-2} M) of PNMA were added to a 0.05 M Tris buffer (at pH 7.71 or at pH 9.5) containing 1% (v/v) acetonitrile and 1×10^{-5} M enzyme. The run was initiated by injecting *p*-nitrophenyl acetate. No inhibition by PNMA could be observed. Since under virtually identical conditions, phenyl acetate (Pocker and Stone, 1968b) was found to be a competitive inhibitor of *p*-nitrophenyl acetate hydrolysis ($K_i = 2 \times 10^{-2}$ M), it is obvious that K_m for PNMA is considerably larger than 3×10^{-2} M.

TABLE III: Modification of BCA by Reaction with PNMA-*2*-^{14}C.[a]

PNMA Concn (M)	pH	Dialysis[b] Time (hr)	icpm[c]	fcpm[d]	No. of[e] Groups Modified
5×10^{-3}	8.92	4	85,600	10,740	6.2
		12		8,320	4.8
		24		9,267	5.5
		72		7,600	4.4
5×10^{-3}	7.00	4	88,300	9,808	5.5
		12		6,650	3.8
		24		6,500	3.7
		72		5,010	
1×10^{-3}	8.92	4	18,000	5,580	3.1
		12		5,380	3.0
		24		5,104	2.8
1×10^{-3}	7.00	4	18,300	4,021	2.2
		12		3,220	1.8
		24		3,020	1.7
1×10^{-3} [f]	8.92	4	18,000	121	
		12		0	
		24		0	
1×10^{-3} [f]	7.00	4	18,300	61	
		12		0	
		24		50	
1×10^{-4}	8.92	4	1,920	627	0.33
		12		551	0.29
		24		530	0.28
1×10^{-4}	7.00	4	1,820	526	0.27
		12		480	0.24
		24		345	0.18

[a] At 25.0° in 0.05 M diethyl malonate (pH 7.0) or Tris (pH 8.92) buffers containing 1% (v/v) acetonitrile with $\mu = 0.55$. The concentration of BCA was 1×10^{-4} M (based on 3.0 mg/ml of enzyme weighed into solution). [b] Each dialysis was performed at the same pH as the reaction. [c] Counts per minute (icpm) per ml of solution prior to dialysis = $[PNMA]_{initial} \times$ activity of PNMA in cpm per mole. [d] fcpm = cpm (per ml in bag) − cpm (per ml of dialysate) after indicated elapsed time. The dialysate procedure was described in the Experimental Section. [e] Number of groups modified = (fcpm/icpm) × ([PNMA]/[BCA]). The value calculated is only approximate, but should be a good indication of the minimum number of groups modified. On dialysis, there appeared to be a significant initial decrease in degree of acetimidation followed by the expected slower decrease (see Table II, footnote *e*). Considering the fact that the amidines of histidyl residues are more labile than those of lysyl residues further study of this phenomenon is indicated. [f] With these runs instead of adding enzyme and then PNMA, the BCA was added after the PNMA had been completely hydrolyzed. This showed that the PNMA hydrolysis products were not bound very strongly to the enzyme.

Anionic and sulfonamide inhibition of the enzyme-catalyzed hydrolysis of PNMA was carefully examined. Cyanide and azide, two of the most potent known anionic inhibitors of carbonic anhydrase activity (Mann and Keilin, 1940; Roughton and Booth, 1946; DeVoe and Kistiakowsky, 1961; Kernohan, 1965; Pocker and Stone, 1968a) proved to be such powerful nucleophiles for the reaction with PNMA that they were not suitable for study as anionic inhibitors (Y. Pocker, M. W. Beug, and K. L. Stephens, unpublished results). Iodide and bromide ions did not appreciably catalyze the decomposition of PNMA. Therefore, inhibition was studied in 0.05 M diethyl malonate buffers (pH 6.74, μ = 0.55) and 0.05 M Tris buffers (pH 8.55, μ = 0.55) using bromide and iodide ion concentrations up to 0.4 M. No bromide or iodide inhibition of enzyme-catalyzed PNMA hydrolysis was observed over the pH range investigated. In addition, inhibition by acetazolamide and ethoxyzolamide was examined. Using 5×10^{-5} M enzyme and up to 1×10^{-4} M acetazolamide or ethoxzolamide, we did not observe any inhibition of carbonic anhydrase catalyzed hydrolysis of PNMA over the pH range 6.7–10.41.

Discussion

PNMA as a Substrate. It is clear that PNMA plays a dual role, that of a substrate and enzyme modifier. However, it is difficult to quantitatively assess the meaning of the profile since it represents the combined effects of modification and the turnover of the protonated substrate. Use of phenyl *N*-methylacetimidate-2-^{14}C allowed us to show that in the basic region (pH 9) the pH–rate profile (Figure 3) results largely from enzyme modification. Around neutrality, the observed initial rates of phenol release must be attributed to both modification (55%) and turnover (45%).

Structural similarities between PNMA and phenyl acetate led us to believe that the two compounds may be hydrolyzed at the same site within the enzyme. However, phenyl acetate is an inhibitor of the enzyme-catalyzed *p*-nitrophenyl acetate hydrolysis while PNMA is not. In addition, carbonic anhydrase catalyzed hydrolysis of PNMA is not inhibited by either monovalent anions or sulfonamides. Inhibition by aromatic sulfonamides of the type $ArSO_2NH_2$ is considered characteristic of carbonic anhydrase. Indeed, all other reported substrates of carbonic anhydrase are inhibited both by sulfonamides and by monovalent anions. Thus, we must conclude that the active site for the enzyme-catalyzed hydrolysis of PNMA is different from that of CO_2, aldehydes, or esters. Furthermore, the attempted inhibition studies show that the binding site for PNMA is different probably from that of the "normal" substrates. It seems likely that PNMA is bound at a secondary hydrophobic site farther away from zinc and yet close enough to one of the reactive histidyl residues so that imidazole catalysis can be invoked.[4] Pocker and Meany (1967a,b) postulate that structural differences between 2- and 4-pyridinecarboxaldehydes may lead to differences in binding site for the two substrates and other evidence has been found recently for changes in binding site resulting from structural differences in the substrate (Pocker and Dickerson, 1968; Pocker and Guilbert, 1972; Pocker and Watamori, 1971). Factors that govern where and how the substrate is bound within the hydrophobic cleft of the enzyme are now under investigation in these laboratories, but are as yet unclear. With PNMA, binding may occur towards the outer edge of the cleft containing the zinc. The PNMA is then far enough from the zinc not to be affected by anionic inhibitors bound to zinc or zinc-bound acetazolamide or ethoxzolamide. Similarly, it would not be affected by any high pH titration of a group near zinc, nor would it compete with PNPA for a binding site. Yet, it would still be near an imidazole and in an area of ordered water structure, though not in a region possessing as high a degree of hydrolytic efficiency as the region in the immediate vicinity of the zinc. This picture has some appealing aspects. Considering the reactivity of PNMA, if it were bound in the ester binding site, one would expect higher values of k_{enz} than those observed. With the substituted phenyl esters, k_{enz}/k_{OH^-} was between 1 and 40. With PNMA the ratio of k_{enz}/k_{OH^-} was estimated to be less than 6×10^{-3} for protonated PNMA, and $< 1 \times 10^{-4}$ for unprotonated PNMA. Also, since imidazole has been shown to be a potent catalyst for PNMA decomposition (see footnote 4), the rate enhancement observed with enzyme could readily be accounted for by participation of an oriented imidazole group with an associated ordered water structure. The solvent isotope effects shown in Figure 3 cannot be interpreted rigorously,[5] although inferences can be drawn concerning the catalytic component responsible for the lower rate in deuterium oxide. Thus, it appears that the relative effectiveness of the enzyme in H_2O as compared to D_2O is associated with PNMA hydrolysis, k_{enz}, rather than with amidination, k_{modif}.

Specifically, $k_{BCA}^{H_2O}/k_{BCA}^{D_2O}$ for the enzyme-catalyzed hydrolysis of PNMA goes from a maximum of 1.8 at pH 7 to 1.6 above pH 9.5. For reactions such as nucleophilic attack of azide or imidazole on PNMA (Y. Pocker, M. W. Beug, and K. L. Stephens, unpublished results), the value of $(k_{N_3^-}^{H_2O}/k_{N_3^-}^{D_2O}) \simeq (k_{Im}^{H_2O}/k_{Im}^{D_2O}) \simeq 1$. Similarly, when PNMA acts as a protein modification agent on albumin, the value of $(k_{BSA}^{H_2O}/k_{BSA}^{D_2O} \simeq 1$. It should be noted that as discussed (wee Figure 2), the catalytic coefficients, k, are compared at pH and pD

[4] In a study of potential nucleophiles for the decomposition of PNMA (Y. Pocker, M. W. Beug, and K. L. Stephens, unpublished results), imidazole was found to be a potent catalyst in the decomposition of PNMA (at pH 7.4, k_{Im} = 150 M^{-1} min^{-1} and by comparison k_{BCA} = 155 M^{-1} min^{-1} at 7.49). The similarity in rate of nucleophilic attack by imidazole and rate of enzyme catalyzed decomposition of PNMA was striking, but may be fortuitous. Also, imidazyl *N*-methylacetimidate formed in the reaction of PNMA with imidazole was found to hydrolyze rapidly in aqueous media consistent with our observation that only lysyl residues are permanently modified by PNMA. We found no evidence for the involvement of other residues in the enzyme.

[5] Isotope effects in D_2O as solvent can, with caution, be used to distinguish between general base and nucleophilic catalysis. On the other hand, the observation of a difference in rate of an enzyme catalyzed reaction in water and deuterium oxide has, in the absence of a sufficiently detailed study, only a qualitative significance with regard to mechanism. Thus, while the difference in the ionization constant of the conjugate acid of PNMA in water and in deuterium oxide has been measured directly, the corresponding differences in the active form of carbonic anhydrase must be inferred either from a dependence of the activity on pH (pD) or from isotope effects on the acidity of the ionizing groups which are known to affect enzymatic activity. Another serious uncertainty in the interpretation of such isotope effects arises from the possibility that the solvent change from water to deuterium oxide changes not only the binding chracteristics of the enzyme but also its conformation through a change in the properties of hydrogen bonds, hydrophobic interaction and other less specific factors. Although such effects may be individually quite small, they may be amplified within an enzyme which exists in a delicate balance between conformations of differing activity. Consequently, the kinetic isotope effects of carbonic anhydrase in deuterium oxide must still be interpreted with the reserve with which respect for the long arm of coincidence should always engender.

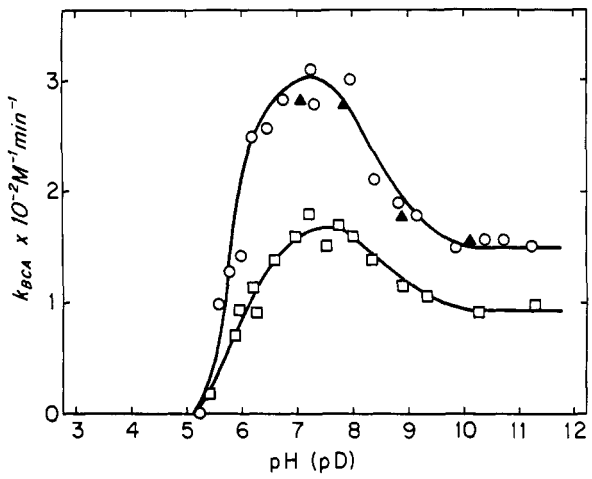


FIGURE 3: pH dependency of k_{BCA} for the BCA-catalyzed hydrolysis of PNMA in H_2O and in D_2O at 25.0°; 0.05 M buffers with 1.5×10^{-3} M PNMA, 1% (v/v) acetonitrile, $\mu = 0.55$, and 1×10^{-4} M BCA. pD = $pH_{mr} + 0.40$; $k_{BCA} = (k_{absd} - k_{buff})/[BCA]$: (○) in H_2O; (▲) in H_2O with PNMA-2-^{14}C; (□) in D_2O.

values such as $[SH^+]/[S] = [SD^+]/[S]$. Thus, the absence of a solvent isotope effect in purely nucleophilic reactions, especially in serum albumin modification, and the presence of the solvent isotope effect with carbonic anhydrase, was additional evidence for enzymatic catalysis occurring in the hydrolysis of PNMA.

PNMA as a Modification Agent. The observation that PNMA was a very potent modification agent of both serum albumin and carbonic anhydrase was in itself quite interesting. The high reactivity of the compound enables one to use small amounts of material under mild kinetic conditions to modify ε-aminolysine residues. The rate of modification can be followed spectrophotometrically by observing the rate of phenol appearance. Maximum rates of modification are obtained in neutral to slightly basic solutions.

The reaction with the enzyme also had important mechanistic implications. After treatment with PNMA under conditions sufficient to produce complete conversion of the lysine residues to amidines, we did not observe any alteration of enzymatic activity with respect to catalysis of *p*-nitrophenyl acetate hydrolysis. Thus it could be concluded that a lysine residue is not involved in the active site of carbonic anhydrase.

References

DeVoe, H., and Kistiakowsky, G. B. (1961), *J. Amer. Chem. Soc. 83*, 274.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Grazi, E., Cheng, T., Horecker, B. L. (1962), *Biochem. Biophys. Res. Commun. 7*, 250.

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

Jencks, W. P., and Gilchrist, M. (1968), *J. Amer. Chem. Soc. 90*, 2662.

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

Kernohan, J. D. (1965), *Biochim. Biophys. Acta 96*, 304.

Lai, C. Y., Tchola, O., Cheng, T., Horecker, B. L. (1965), *J. Biol. Chem. 240*, 1347.

Mann, J., and Keilin, D. (1940), *Nature (London) 146*, 164.

McDougall, A. O., and Long, F. A. (1962), *J. Phys. Chem. 66*, 429.

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

Nyman, P. O., and Lindskog, S. (1964), *Biochim. Biophys. Acta 85*, 141.

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

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

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

Pocker, Y., Beug, M. W., and Ainardi, V. R. (1971), *Biochemistry 10*, 1390.

Pocker, Y., and Dickerson, D. G. (1968), *Biochemistry 7*, 1995.

Pocker, Y., and Guilbert, L. J. (1972), *Biochemistry 11*, 180.

Pocker, Y., and Meany, J. E. (1964), *Abstr. 132, 6th Intern. Congr. Biochem., IV, New York, N. Y.*, 327.

Pocker, Y., and Meany, J. E. (1965a), *J. Amer. Chem. Soc. 87*, 1809.

Pocker, Y., and Meany, J. E. (1965b), *Biochemistry 4*, 2535.

Pocker, Y., and Meany, J. E. (1967a), *Biochemistry 6*, 239.

Pocker, Y., and Meany, J. E. (1967b), *J. Amer. Chem. Soc. 89*, 631.

Pocker, Y., and Meany, J. E. (1970), *J. Phys. Chem. 74*, 1486.

Pocker, Y., and Stone, J. T. (1965), *J. Amer. Chem. Soc. 87*, 5497.

Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6*, 668.

Pocker, Y., and Stone, J. T. (1968a), *Biochemistry 7*, 2936.

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

Pocker, Y., and Watamori, N. (1971), *Biochemistry 10*, 4843.

Robinson, R. A., Paabo, M., and Bates, R. G. (1969), *J. Res. Nat. Bur. Stand., Sect. A, 73*, 299.

Roughton, F. J. W., and Booth, V. H. (1946), *Biochem. J. 40*, 319.

Thorslund, A., and Lindskog, S. (1967), *Eur. J. Biochem. 3*, 117.

Warren, S., Zerner, B., and Westheimer, F. H. (1966), *Biochemistry 5*, 817.

Westheimer, F. H. (1963), *Proc. Chem. Soc. London*, 253.

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