Atypical Deacylation of the Acyl Enzymes Formed in the Reaction of α-Chymotrypsin with Bis(4-nitrophenyl) Carbonate and o-(4-Nitrophenylene) Carbonate

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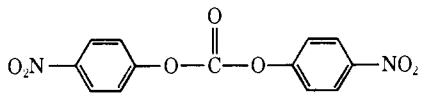
Abstract: The deacylation reactions of acyl enzyme derivatives prepared by reaction of α-chymotrypsin with bis(4-nitrophenyl) carbonate and o-(4-nitrophenylene) carbonate have been studied at 25°. With bis(4-nitrophenyl) carbonate, reaction of an equivalent amount of substrate with the enzyme results in release of 2 equiv of p-nitrophenol. The enzyme is then partially inhibited toward p-nitrophenyl acetate and the specific ester substrate N-acetylphenylalanine ethyl ester, suggesting that an acyl derivative is still present, presumably formed by reaction of an intermediate p-nitrophenyl carbonate acyl enzyme with a nucleophile in the active site. The return of activity was followed by (a) reaction with proflavin; (b) following the decrease in absorbance at 245 nm; and (c) a specific ester rate assay with N-acetylphenylalanine ethyl ester. With all of these methods the return of activity followed first-order kinetics and gave nearly identical rate constants. The absorbance at 245 nm is suggestive of an acyl histidine derivative, and the pH–rate constant profile for reactivation from pH 5 to 8 corresponds in shape to that for ring opening of the model acyl imidazole derivative formed from intramolecular imidazole attack in β-(4,5-imidazole)ethyl 4-nitrophenyl carbonate. The cyclic carbonate ester o-(4-nitrophenylene) carbonate reacts rapidly and quantitatively with α-chymotrypsin to give a large increase in absorbance with a maximum at 379 nm. The acylated enzyme is not active toward p-nitrophenyl acetate or N-acetylphenylalanine ethyl ester. The absorbance at 379 nm decreases slowly in a first-order process which releases 4-nitrocatechol and gives a reactivated enzyme. A specific substrate rate assay with N-acetylphenylalanine ethyl ester gave an identical rate constant. Hydrolysis of the acyl enzyme is pH independent from pH 6.12 to pH 8.79. High concentrations of urea produce a small rate enhancement. Thus the configuration of the active site is not a factor, and the histidine ($pK_a \sim 7$) in the active site of α-chymotrypsin is not involved in hydrolysis of this intermediate acyl enzyme. It is probable therefore that deacylation is a spontaneous reaction unassisted by the enzyme. This is possibly due to a conformation change resulting from production of the negatively charged phenoxide ion during the acylation reaction.

It is generally accepted that the α-chymotrypsin catalyzed hydrolysis of esters follows the kinetic scheme in eq 1 where ES is an enzyme–substrate complex, and

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

ES′ is an acylated enzyme, although little evidence exists for Michaelis–Menten complex formation in the case of nonspecific p-nitrophenyl esters.[2,3] There is very good evidence indicating that the amino acids histidine and serine form part of the active site of α-chymotrypsin,[4,5] and it has been well established that an acyl enzyme intermediate is formed during the course of the hydrolysis of both specific and nonspecific ester and amide substrates. This acyl enzyme intermediate is undoubtedly a serine ester.[4,5]

Deacylation reactions are of special interest since substrate binding to the enzyme is not a complicating factor as in acylation. Structural variations in the acyl group have been extensively studied in deacylation reactions. The usual types of substrates all give rise to acyl enzymes in which the serine hydroxyl is the leaving group in the subsequent deacylation. Appropriate carbonate diester substrates could, however, give acyl enzyme diester derivatives with leaving groups of much lower pK_a. The study of such compounds could therefore be of considerable mechanistic importance. Hydrolysis of the acyl enzymes produced by reaction of α-chymotrypsin with bis(4-nitrophenyl) carbonate (I) and o-(4-nitrophenylene) carbonate (II) has now been studied since these intermediates are labile nitrophenyl esters.



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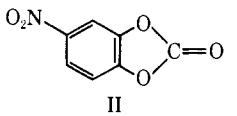

II

Experimental Section

Materials. α-Chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp. Identical kinetic results were obtained with this preparation and enzyme chromatographed on Sephadex-G25 by the procedure of Yapel, *et al.*[6] Acetonitrile was Eastman-Kodak Spectrograde which was twice distilled over P_2O_5 and once over K_2CO_3. Bis(4-nitrophenyl) carbonate and o-(4-nitrophenylene) carbonate were prepared as previously reported.[7,8] Ethyl (2-hydroxy-5-nitrophenyl) carbonate (mp 88°) was recrystallized from an ether–hexane mixture.[9]

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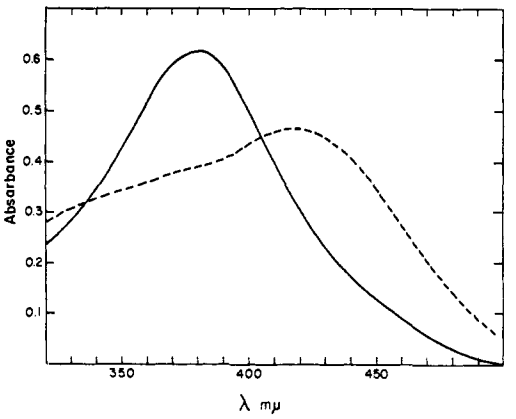

Figure 1. Spectrum at 25° (solid line) of the acyl enzyme prepared by adding 20 μl of a 0.01 *M* acetonitrile solution of *o*-(4-nitrophenylene) carbonate to a solution of 400 μl of 6.68×10^{-4} *N* α-chymotrypsin in 3.0 ml of phosphate buffer (pH 6.95 and $\mu = 0.09$). The dotted line is the spectrum of the solution upon completion of the deacylation reaction. This spectrum did not change with time (4 hr).

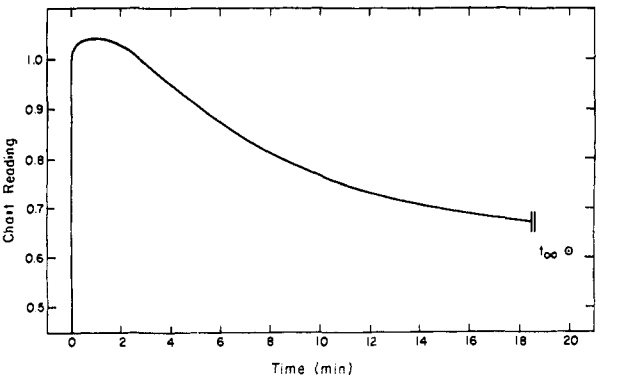

Figure 2. Plot of absorbance at 379 nm *vs.* time at 25° and pH 6.51, $\mu = 0.09$ after addition of 100 μl of a 1.17×10^{-3} *M* solution of *o*-(4-nitrophenylene) carbonate in acetonitrile to a solution of 100 μl of 1.13×10^{-3} *N* α-chymotrypsin in 3.0 ml of buffer. The Gilford chart scale is 0 to 0.3 OD full scale.

β-(4,5-Imidazole)ethyl 4-nitrophenyl carbonate hydrochloride was prepared by reaction of 4,5-(2-hydroxyethyl)imidazole, obtained by the procedure of Pandit and Bruice,[10] with *p*-nitrophenyl chloroformate (K and K Laboratories). After recrystallization from an ethanol–chloroform–ether mixture the product melted at 101–102°. *Anal.* Calcd for $C_{12}H_{12}ClN_3O_5$: C, 45.94; H, 3.86; N, 13.39. Found: C, 45.70; H, 4.00; N, 13.09.

Proflavin dihydrochloride (Mann assayed 70.9% free base) was used without purification. Buffers were prepared with appropriate A. R. grade materials to give ionic strengths of 0.1.

Kinetic Measurements. Rate constant determinations were made with a Gilford Model 2000 recording spectrophotometer. Constant temperature (±0.1°) was maintained by circulating water from a Precision Scientific Lo Temptrol 154 circulating water bath around the cell compartment. pH measurements were made with a Radiometer 22 pH Meter. Operational normality of enzyme stock solutions was determined by the titrimetric procedure of Schonbaum, *et al.*[11] The enzyme stock solution was made up at pH 5.05 (acetate buffer, $\mu = 0.1$). To initiate the reactions, substrate solutions in acetonitrile were added by means of a Hamilton syringe to the cuvette containing 3 ml of buffer and 100 μl of enzyme solution. The concentration of acetonitrile in experiments with bis(4-nitrophenyl) carbonate was 2.26%; in the experiments with *o*-(4-nitrophenylene) carbonate it was 3.13%.

When enzyme and equivalent amounts of bis(4-nitrophenyl) carbonate are mixed, a rapid 2-equiv burst of *p*-nitrophenol is obtained. The enzyme was found to be inhibited toward specific or nonspecific ester substrates (see Results). The return of enzyme activity was followed with proflavin which has been demonstrated to form a 1:1 complex with the active site of α-chymotrypsin[12] resulting in a shift in the visible spectrum of the dye. The absorption difference has a maximum at 465 nm. Acylation causes proflavin to be displaced from the enzyme, thereby allowing study of reactions which by themselves might exhibit no spectral changes.[13]

In a typical experiment a solution containing 7.57×10^{-5} *M* proflavin at pH 7.84 in a 1-cm pathlength cell had an initial absorbance at 465 nm of 1.096. To this was added aliquots of an α-chymotrypsin solution until a concentration of 1.87×10^{-5} *N* was reached. A plot of absorbance at 465 nm *vs.* enzyme concentration gave a straight line, and a total absorbance change of 0.229 was obtained. To 3.1 ml of a solution containing 1.87×10^{-5} *N* α-chymotrypsin and 7.57×10^{-5} *M* proflavin at pH 7.68 was added 3.64×10^{-5} *M* bis(4-nitrophenyl) carbonate, introduced by Hamilton syringe as a solution in 70 μl of acetonitrile. The absorbance of the solution at 465 nm decreased very rapidly by 0.178, indicating that 91% of the enzyme had reacted with substrate. After a brief period the absorbance started to increase in a first-order fashion. In separate experiments proflavin was added to the reaction after all the *p*-nitrophenol had been liberated, giving identical rate constants.

The spectrum of α-chymotrypsin in transparent buffers shows an absorbance minimum at 248 nm, such that absorbance changes in this region might be observable. Because the absorbance difference was small, the concentration of enzyme and substrate employed was twice that in the proflavin experiments. At pH 7.68, liberation of *p*-nitrophenol was over in 3 min (measured at 400 nm) and thereafter the reaction was followed at 245 nm. The initial absorbance was 1.376, and the change in absorbance was 0.083. The first-order rate constant obtained from these data was essentially in agreement with previously obtained values.

Kinetic data obtained in the proflavin experiments and by direct measurement at 245 nm were treated by a rigorous least-squares procedure with an IBM 360-40 computer which calculated both rate constants and infinity points. The reactions were followed to completion and were nicely first order. Calculated infinity points agreed generally to better than 2% with observed infinity points.

Upon addition of *o*-(4-nitrophenylene) carbonate to the reaction cuvette containing enzyme ($S_0 = E_0$) a rapid increase ("burst") in absorbance was observed which was nearly complete upon completion of stirring. The absorption maximum was at 379 nm. In Figure 1 is shown the uv spectrum of the acyl enzyme intermediate at pH 6.96, determined on a Cary 15 recording spectrophotometer. Deacylation of the acyl enzyme was followed by measuring the decrease of this absorbance at 379 nm as a function of time. A typical plot of absorbance *vs.* time is shown in Figure 2. The spectrum of the solution after completion of the reaction was that of 4-nitrocatechol. The rate constants were calculated with an Olivetti Underwood Programma 101, programmed to calculate a least-squares evaluation of the slope and intercept of a plot of $\ln [(OD_0 - OD_\infty)/(OD_t - OD_\infty)]$ *vs.* time. Excellent first-order kinetics were observed. Correlation coefficients were invariably in the range 0.999 to 0.9999. That the kinetic measurements are truly first order is shown by the fact that variation of either substrate or enzyme concentration did not produce a significant change in the rate constant. Rate constants were measured in at least triplicate at each pH value. First-order rate constants for appearance of 4-nitrocatechol measured at 440 nm were identical with those measured at 379 nm for disappearance of the intermediate.

In solutions containing urea, rate constants were determined by preparing a stock solution of acyl enzyme at pH 6.51, and injecting an aliquot into the buffer–urea solution. This procedure was also followed to determine the absorbance of the acyl enzyme at pH 5.05 since at that pH the absorbance changes are fairly small, and the acylation process is slower than at higher pH. To determine the rate of deacylation at pH 5.51 the enzyme was acylated at pH 6.12 and the pH of the solution was then rapidly adjusted by addition of 0.1 *M* HCl from a calibrated pipet.

Specific substrate rate assays were carried out titrimetrically employing a Radiometer TTT-1 Autotitrator and Radiometer

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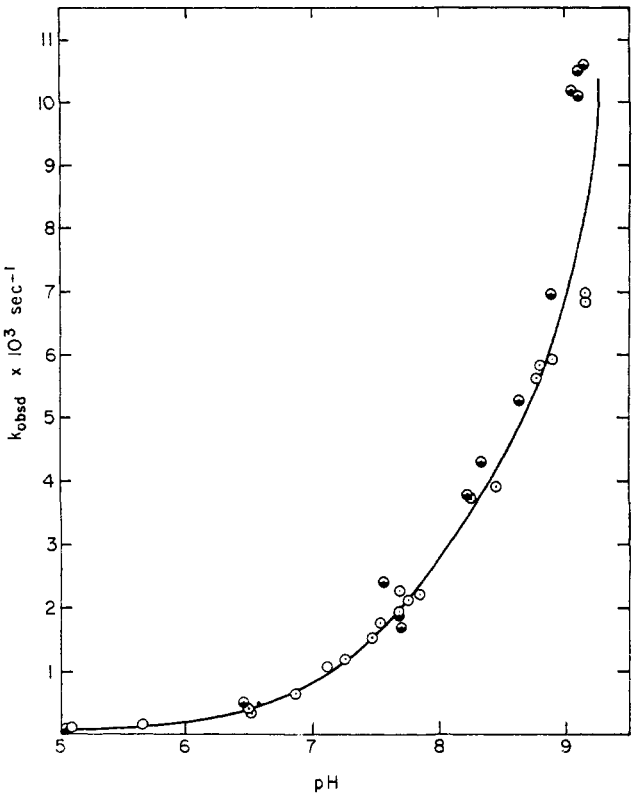


Figure 3. Plot of k_{obsd} *vs.* pH for return of activity to α-chymotrypsin after reaction with bis(4-nitrophenyl) carbonate at 25° (all *p*-nitrophenol has been liberated). Rate constants were determined from data obtained by measurements with proflavin ⊙ and by measuring the decrease of absorbance with time at 245 nm ◒.

Titrigraph utilizing a Metrohm EA 115 X electrode in a three-necked Metrohm microtitration cell. Solutions were titrated by addition of base from a syringe (0.5-ml capacity) by way of a glass capillary which extended into the cell to just above a small bar magnet stirrer. Constant temperature was maintained by circulating water at 25 ± 0.1° from a Haake Model F constant temperature bath through the water jacket of the microtitration cell. Bis(4-nitrophenyl) carbonate was introduced into a buffer solution at pH 7.8 containing enzyme, and 155-μl aliquots of this mixture were added at different times to 3 ml of water and 100 μl of 0.304 *M* *N*-acetylphenylalanine ethyl ester in acetonitrile contained in the thermostated microcell. The initial carbonate and enzyme concentrations were identical with those used in the proflavin experiments. The rate of ester hydrolysis was measured by the recorded addition of KOH from the syringe. When an aliquot was added immediately after mixing carbonate and enzyme, the rate of addition of KOH to maintain pH 7.61 was zero. The addition rate increased at first, then decreased when a significant fraction of the specific ester was consumed. A measure of the return of enzyme activity was obtained by measuring initial slopes on the titrator, as a function of time elapsed after carbonate–enzyme mixing. A first-order rate constant of 1.7×10^{-3} sec^{-1} was obtained for the return of enzyme activity. The activity returned to a maximum of 71% when allowance was made for the inhibition due to liberated *p*-nitrophenol.

The rate assay technique was also employed to measure return of activity to α-chymotrypsin after acylation with *o*-(4-nitrophenylene) carbonate. Acylation of the enzyme was achieved by the addition of *o*-(4-nitrophenylene) carbonate to 3 ml of phosphate buffer containing enzyme such that $E_0 = 2.8 \times 10^{-5}$ *N* and $S_0 = 4.8 \times 10^{-5}$ *M*. The deacylation reaction was followed spectrophotometrically at 379 nm. After a measured time interval a 62-μl aliquot was transferred to the microtitration cell which contained 3 ml of distilled water and to which had been added 100 μl of 0.308 *M* *N*-acetylphenylalanine ethyl ester in acetonitrile. The cell was thermostated at 25° and the ester hydrolysis monitored by the addition of KOH necessary to maintain a pH of 7.61. Initial rates were measured and a first-order rate constant of 0.00217 sec^{-1} was obtained.

Results

In an initial experiment with bis(4-nitrophenyl) carbonate at pH 7.68 (phosphate buffer, $\mu = 0.1$) a very rapid reaction occurred between substrate ($S_0 = 1.71 \times 10^{-5}$ *M*) and α-chymotrypsin ($E_0 = 1.87 \times 10^{-5}$ *N*) liberating the stoichiometrically required amount of *p*-nitrophenol. Immediately after, the enzyme was estimated to be about 60% active, as indicated by the "burst" and "turnover" reactions produced by the addition of excess *p*-nitrophenyl acetate [$(NPA)_0 = 9.45 \times 10^{-5}$ *M*], compared to those obtained when NPA was added to enzyme alone, under the same conditions. *p*-Nitrophenol itself had no effect on the NPA–enzyme reaction. On reacting similar quantities of substrate and enzyme, and subsequently adding NPA after 60 min, the activity rose to 85%. This remained constant up to 90 min after initiating the reaction. At the same pH, when $S_0 = 3.64 \times 10^{-5}$ *M* and $E_0 = 1.87 \times 10^{-5}$ *N*, liberation of *p*-nitrophenol was completely over in 2 min (compared with which the half-life of aqueous hydrolysis under these conditions is about 8 min). Addition of NPA at that time indicated that the enzyme was about 18% active by the "burst" reaction. Furthermore, "turnover" was noticed to increase considerably with time, indicating that enzyme activity was returning by some slow, secondary process. A measure of this process was obtained by adding NPA to substrate–enzyme mixtures at different times after mixing. After 42 min no further return of activity by "burst" or "turnover" reactions was noted. While the data were insufficient to demonstrate a first-order process, rate constants which compare favorably with those obtained by other methods (*vide infra*) could be estimated ($k = 2 \times 10^{-3}$ sec^{-1}). Similar experiments were performed at pH 5.05 using *trans*-cinnamoyl imidazole to titrate the enzyme. Using $S_0 = 7.92 \times 10^{-5}$ *M* and $E_0 = 3.59 \times 10^{-5}$ *N*, enzyme activity after total liberation of *p*-nitrophenol was 27%. This increased to 89% over a 24-hr period.

An attempt was made to determine the exact stoichiometry of the rapid enzyme–substrate reaction by examination of the burst reactions under conditions $S_0 > E_0$. When a 50% to 100% excess of substrate was employed, approximately 2.5 equiv of *p*-nitrophenol was liberated per equivalent of enzyme. A maximum of about 2.9 equiv of *p*-nitrophenol per equivalent of enzyme was obtained when S_0/E_0 varied between 4 and 9. Consequently, a twofold excess of substrate was employed in subsequent experiments to measure the return of enzyme activity.

Although more than a stoichiometric amount of *p*-nitrophenol is rapidly released when $S_0 > E_0$, there is evidence against acylation of more than one group in the active site. Prior acylation of the enzyme with *p*-nitrophenyl trimethylacetate prevented a burst reaction on treatment with substrate. Likewise, when the enzyme was given prior treatment with NPA there was no burst reaction, but merely a rapid "turnover" reaction as newly deacylated enzyme reacted preferentially with the more reactive carbonate substrate. Hydrolysis of bis(4-nitrophenyl) carbonate in 8 *M* urea at both pH 6.42 and pH 8.22 gave identical first-order rate constants both in the presence or absence of enzyme ($E_0 = S_0$) indicating that under these conditions the enzyme is not being acylated.

In Figure 3 is shown a plot of k_{obsd} *vs.* pH for the return of activity to α-chymotrypsin after reaction with bis(4-nitrophenyl) carbonate (all *p*-nitrophenol has been liberated). This reaction was followed by addition of proflavin and subsequent observation of the absorbance at 465 nm. The return of activity followed good pseudo-first-order kinetics. During the reactivation process a decrease in absorbance takes place at 245 nm. Rate constants calculated from these data were nearly identical with those obtained in the proflavin experiments, as seen in Figure 3. The pH–rate constant profile for the return of enzyme activity shows a slower increase in rate with increasing pH at high pH than would be the case if a simple hydroxide ion catalyzed reaction was taking place. A plot of log k_{obsd} *vs.* pH in the range 5–9 was linear with a slope of 0.49. A much faster increase in the return of activity was observed at pH > 10 as measured by the decrease in absorbance at 245 nm.

p-Nitrophenolate anion is released rapidly from β-(4,5-imidazole)ethyl 4-nitrophenyl carbonate at 15°. Pseudo-first-order rate constants were measured from pH 5.05 to 7.46 in phosphate or acetate buffer (μ = 0.1). Values of k_{Im} for intramolecular imidazole participation and K_{app} were obtained from a plot of k_{obsd} *vs.* $k_{obsd}a_H$ (k_{Im} = 0.115 sec^{-1} and pK_{app} = 6.47). The shape of the pH–rate constant profile and the very rapid reaction in contrast to the relatively slow rate of spontaneous hydrolysis at these pH values of other nitrophenyl carbonate esters, such as ethyl *m*-nitrophenyl carbonate,[9] indicates that the imidazole ring is participating in the reaction.

Formation of an intermediate with strong absorbance at 245 nm could be detected in the hydrolysis of β-(4,5-imidazole)ethyl 4-nitrophenyl carbonate. After complete liberation of *p*-nitrophenol, this absorbance decreased very slowly and it was therefore measured at 50°. The reaction followed first-order kinetics, and in Figure 4 a plot is shown of k_{obsd} *vs.* pH. The rate constants are pH independent from pH 5 to 7 with k_0 = 3.3 × 10^{-4} sec^{-1}.

When *o*-(4-nitrophenylene) carbonate and α-chymotrypsin are mixed at 25° a rapid increase in absorbance at 379 nm is produced. The rate of this increase is too fast to measure with conventional equipment. The absorbance at 379 nm then decreases slowly with time in a first-order process. The first-order rate constants (k_{obsd}) are given in Table I. Decreasing the buffer concentration at 3 pH values produced no effect on the rate constants. These rate constants are independent of pH from pH 6.12 to 8.78. As seen in Table II variation of either enzyme concentration or substrate concentration has no significant effect on the observed rate constant. Thus, an acyl enzyme intermediate is being produced which hydrolyzes slowly in a first-order reaction. Upon completion of the reaction the enzyme was fully active. The magnitude of the observed rate constants is considerably less than for spontaneous hydrolysis of *o*-(4-nitrophenylene) carbonate at the same pH values (k_0 = 0.011 sec^{-1} at 30° for pH-independent hydrolysis, determined by extrapolation to zero buffer concentration).[8] At pH values greater than 7 the nonenzymatic reaction is hydroxide ion catalyzed.

The acyl enzyme is inhibited toward reaction with *p*-nitrophenyl acetate. In Figure 5, a plot of absorbance *vs.* time is presented illustrating this inhibition. Addition of *p*-nitrophenyl acetate after formation of acyl enzyme produces only a very small burst of 0.02 absorbance unit, whereas addition employing the same concentrations of ester and enzyme without prior addition of *o*-(4-nitrophenylene) carbonate produced a burst of 0.20 absorbance unit.

Acylation of the enzyme at pH 6.95 with *p*-nitrophenyl trimethylacetate gives an acyl enzyme derivative that is completely unreactive toward *o*-(4-nitrophenylene) carbonate. At 379 nm there is in that case a first-order increase in absorbance with a rate constant identical with that for spontaneous hydrolysis of II in the buffer solution without enzyme.

In Table I is also reported the rate constant for deacylation of the acyl enzyme from *o*-(4-nitrophenylene) carbonate in the presence of 8 *M* urea. The rate constant is actually slightly greater in the presence of urea.

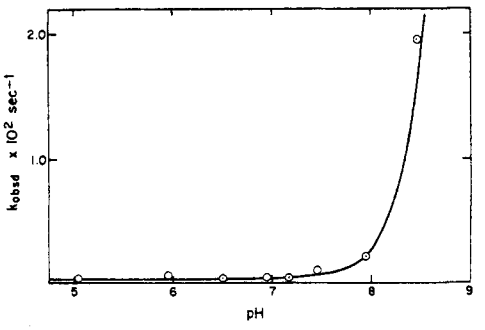


Figure 4. Plot of k_{obsd} *vs.* pH for hydrolysis at 50° and μ = 0.1 (with KCl) of the intermediate formed in the hydrolysis of β-(4,5-imidazole)ethyl 4-nitrophenyl carbonate.

Table II. The Effect of Varying Initial Substrate or Enzyme Concentration on the Rate Constant for Deacylation of the Acyl Enzyme Obtained in Reaction of α-Chymotrypsin with *o*-(4-Nitrophenylene) Carbonate at 25° and pH 6.51 in 3.13% Acetonitrile–H_2O

$E_0 \times 10^5$, *N*	$S_0 \times 10^5$, *M*	$k_3 \times 10^2$, sec^{-1}
3.53	3.66	0.212
3.53	8.16	0.195
3.53	12.24	0.195
5.45	3.56	0.212
8.11	3.56	0.207

Table I. First-Order Rate Constants for Deacylation of the Acyl Enzyme Produced by Reaction of α-Chymotrypsin and *o*-(4-Nitrophenylene) Carbonate at 25° [a]

Buffer	μ	pH or pD	$k_3 \times 10^2$, sec^{-1}
Barbital	0.1	8.78	0.220
Phosphate	0.09	7.95	0.223
Phosphate (D_2O)	0.09	7.55	0.138
Phosphate	0.09	7.46	0.212
Phosphate	0.09	6.96	0.202
Phosphate (8 *M* urea)	0.09	6.83	0.398
Phosphate	0.09	6.51	0.212
Phosphate	0.09	6.12	0.203
Phosphate	0.09	5.96	0.182
Acetate	0.10	5.51	0.185

[a] S_0 = 2.36 × 10^{-5} *M*; E_0 = 2.43 × 10^{-5} *N*.

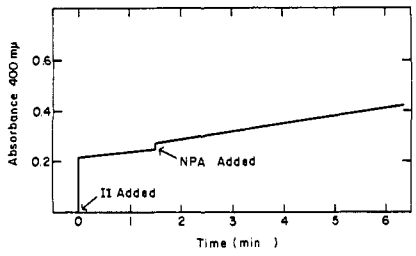


Figure 5. Plot of absorbance at 400 nm *vs.* time at 25°, pH 6.96, and $\mu = 0.09$. At zero time 100 μl of a 9.61×10^{-4} *M* solution of *o*-(4-nitrophenylene) carbonate in acetonitrile was added to a solution of 100 μl of 9.9×10^{-4} *N* α-chymotrypsin in 3.0 ml of buffer. Subsequently 100 μl of a 9.93×10^{-3} *M* solution of *p*-nitrophenyl acetate (NPA) in acetonitrile was added.

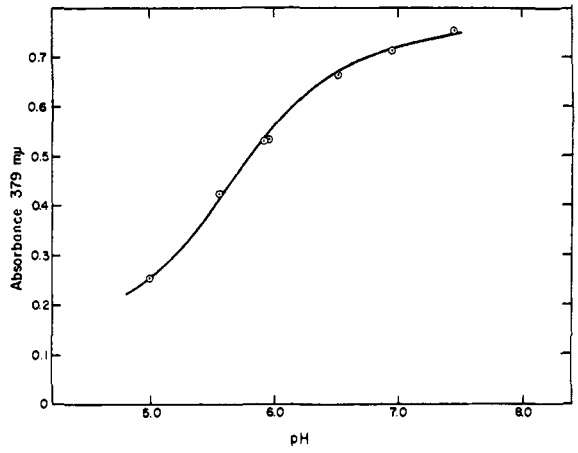


Figure 6. Plot of absorbance at 379 nm *vs.* pH at 25° (chart scale 0–0.3 OD full scale); 50 μl of a 1.24×10^{-3} *M* solution of *o*-(4-nitrophenylene) carbonate in acetonitrile was added to a solution of 200 μl of 9.0×10^{-4} *N* α-chymotrypsin in 3.0 ml of buffer. The maximum absorbance was recorded.

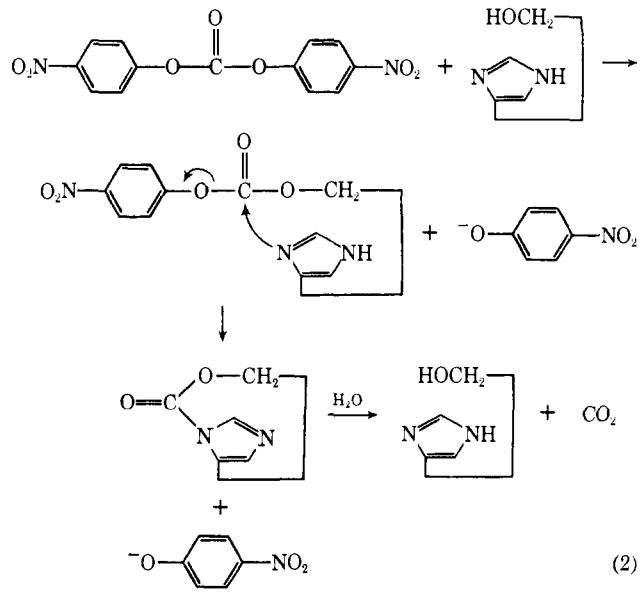
The rate constant in D_2O as the solvent was measured at pD 7.55. Since in this region pH (or pD) has no effect on the reaction, a D_2O solvent isotope effect can be calculated which is $k_{H_2O}/k_{D_2O} = 1.6$.

The pK_a value of the phenolic hydroxyl group in the acyl enzyme was determined by measuring the absorbance at 379 nm of buffer–enzyme solutions at various pH values to which constant concentrations of *o*-(4-nitrophenylene) carbonate were added. A plot of absorbance *vs.* pH is shown in Figure 6. The absorbance value refers to the maximum absorbance reading obtained. A small error will, of course, be present because of hydrolysis of the acyl enzyme, but the data are certainly of reasonable accuracy. From the plot of Figure 6 a pK_a value of 5.6 can be calculated. This is nearly identical with the pK_a of ethyl (2-hydroxy-5-nitrophenyl) carbonate determined in the same manner in the same set of buffers (λ_{max} 392 nm).

Discussion

Bis(4-nitrophenyl) Carbonate. The inhibited enzyme after complete release of *p*-nitrophenol most likely indicates that an acyl derivative of the enzyme is still present. Formation of a stable carbonate monoester intermediate could result in an inhibited enzyme. The decomposition of potassium ethyl carbonate[14] is almost instantaneous at lower pH, but in alkaline solution the compound is considerably more stable ($k_2 = 0.05$ min^{-1} at 25°). The reaction at high pH is a spontaneous first-order reaction. Thus, the observed pH dependence for the return of activity to α-chymotrypsin is not in accord with slow hydrolysis of a serine carbonate monoester anion. Furthermore, if hydrolysis of an acyl enzyme intermediate to give *p*-nitrophenol and a slowly hydrolyzing carbonate monoester derivative was occurring, then an inhibited enzyme should also result from hydrolysis of the acyl enzyme derived from *o*-(4-nitrophenylene) carbonate. In that case, however, a further intermediate was not detected, the enzyme being fully active upon complete release of 4-nitrocatechol. Thus, if a monoester intermediate is formed, it must decompose at a relatively rapid rate.

It would be expected that formation of an acyl serine derivative would proceed normally, liberating 1 equiv of *p*-nitrophenol. Liberation of the second equivalent of *p*-nitrophenol with formation of an inactive acylated enzyme could result from intramolecular nucleophilic attack by a functional group in the enzyme on the acylated serine. Nucleophilic attack would be likely because of the good leaving group in the reaction. The absorbance at 245 nm which slowly decreases as the enzyme reactivates provides suggestive evidence that the nucleophilic group is histidine, since absorbance at that wavelength is characteristic of acyl imidazoles.[15,16] The formation and decomposition of an acyl imidazole in the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate has been followed by the increase and decline of the absorbance at 245 nm.[17] The reaction can then be depicted as proceeding according to the scheme in eq 2.



(2)

In addition to eq 2, in which histidine acts as a nucleophile in deacylation, some deacylation by a general base mechanism might also take place. This would explain why complete inhibition is not obtained when equivalent

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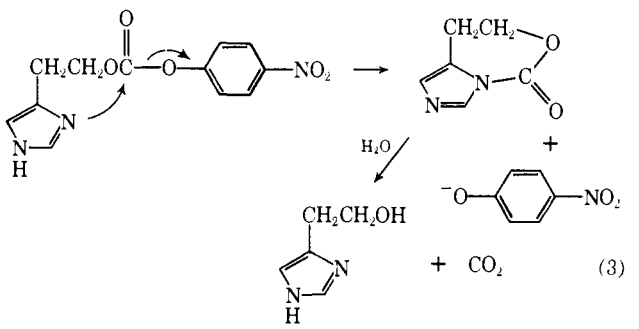
amounts of enzyme and substrate are reacted even though all *p*-nitrophenol is liberated, why more than 2 equiv of *p*-nitrophenol are released at high S_0/E_0 ratios, and why the extent of inhibition increases as relatively larger amounts of substrate are employed. Also, nonspecific acylation of other functional groups in the enzyme could result in a conformation change that inactivates the enzyme. That some nonspecific acylation does occur under the conditions of the experiments is perhaps indicated by the fact that generally only 80–85% of activity toward *p*-nitrophenyl acetate is regained. Some nonspecific acylation probably also takes place with *p*-nitrophenyl acetate and ethyl *p*-nitrophenyl carbonate.[18] However, it is unlikely that a conformation change due to nonspecific acylation is the cause of the major inhibition resulting after release of *p*-nitrophenol in view of the absorbance change resulting at 245 nm and since prior acylation of serine with *p*-nitrophenyl trimethylacetate completely abolishes reaction of bis-(4-nitrophenyl) carbonate with the enzyme. A nonspecific reaction should not be affected profoundly by acylation of the active site.

A chemical model reaction analogous to eq 2 is provided by the hydrolysis of β-(4,5-imidazole)ethyl 4-nitrophenyl carbonate. The spectrophotometric observation of an intermediate in the reaction shows con-



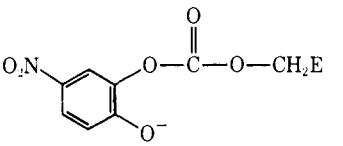
(3)

clusively that nucleophilic participation by the imidazole ring is occurring. Facile intramolecular participation by imidazole has previously been observed in the hydrolysis of esters of γ-(4-imidazole)butyric acid.[19] In the present study the ring opening reaction was studied as a function of pH (Figure 4) and it will be noted that a close correspondence exists with the pH–rate constant profile for regeneration of activity in the α-chymotrypsin reaction (Figure 3) in regard to the shape of the profile in the pH range 5–8. The rate constants at higher pH increase more slowly with increasing pH in the enzymatic reaction than would be predicted by a first-order dependence on hydroxide ion concentration, but conformational effects could be important at high pH. The observed rate constants for the two intermediates are comparable in the pH range 5–8, but since the temperature at which the rate constants were obtained differs by 25°, the intermediate derived from the model compound must hydrolyze considerably more slowly than the enzyme intermediate.

If the inhibited enzyme after complete release of *p*-nitrophenol is indeed a cyclic acyl derivative of histidine-57 and serine-195, then in normal acyl serine derivatives of the enzyme it is likely that histidine is sterically close enough for nucleophilic attack to take place. The reason for a general base mechanism in those cases, which is most likely on the basis of present evidence,[4,5,20] must be that serine is too poor a leaving group to permit nucleophilic catalysis by histidine. The pK_a of the serine hydroxyl in *N*-acetylserinamide[21] is 13.6, whereas the pK_a of histidine is close to 7. The much more weakly basic histidine cannot then displace the stronger base serine. The present data indicate, however, that the mechanism of the enzymatic reaction is dependent on the nature of the compound undergoing reaction as is the case in simple chemical reactions. The mechanism of the deacylation reaction will change from one that is presumably general base to nucleophilic when the leaving group is sufficiently good.

o-(4-Nitrophenylene) Carbonate. The evidence clearly shows that α-chymotrypsin is being acylated quantitatively by *o*-(4-nitrophenylene) carbonate at pH values from 5.96 to 8.78. It is very probable that the active site is being acylated since the resulting acyl enzyme intermediate is unreactive toward *p*-nitrophenyl acetate and *N*-acetylphenylalanine ethyl ester. This acyl enzyme is most likely a serine ester in accord with the evidence that both specific and nonspecific ester substrates give rise to serine ester intermediates.[4,5] Previous investigations of acyl and phosphoryl chymotrypsins formed by stoichiometric reaction of an appropriate reagent with the enzyme have shown that they are derivatives of serine-195.[5] The possibility of a nonspecific reaction with another functional group, which results in a conformation change deactivating the enzyme, is quite unlikely since prior acylation of serine with *p*-nitrophenyl trimethylacetate abolishes reaction of the enzyme with *o*-(4-nitrophenylene) carbonate. A previous study of the α-chymotrypsin catalyzed hydrolysis of several carbonate diesters indicated that Michaelis–Menten kinetics are followed with these substrates.[22] The enzymatic reactions of these compounds are therefore generally normal.

The acyl enzyme derived from *o*-(4-nitrophenylene) carbonate can be considered to be represented by structure III in which the cyclic carbonate ester has under-



III

gone ring opening to give a structure having the nitro group and the phenoxide ion para to each other since the best leaving group in the reaction would be that oxygen. As an illustration of this difference, the pK_a of *m*-nitrophenol is 8.28 in comparison to 7.15 for *p*-nitrophenol. It should be recognized that either C–O bond might break in the reaction, but the good fit of points to the theoretical curve in Figure 6 for one group of pK_a 5.6 is evidence that only one isomer is being obtained, as is the fact that excellent first-order kinetics are observed in the subsequent hydrolysis reaction.

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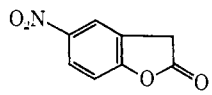
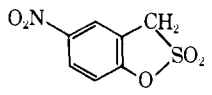
The lactone (IV) and the cyclic sultone 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (V) react with α-



IV



V

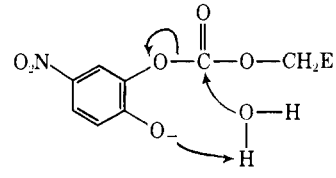
chymotrypsin to give acyl enzymes possessing neighboring phenoxide ions.[23-25] The acyl enzyme formed from *o*-(4-nitrophenylene) carbonate, however, displays different behavior in the pH dependence of deacylation, the sultone displaying a bell-shaped dependence.[24,25] It is not apparent why these acyl enzymes react with such pronounced differences although the steric situation is different with these compounds, and the phenoxide ions would be in different positions in the active site.

The release of 4-nitrocatechol gives the appearance of being a nonenzymatic hydrolysis reaction. Thus, the rate constants are essentially pH independent from pH 6.12 to 8.78, showing no dependence on ionization of a group with a pK_a of about 7, which is usually seen in deacylation reactions.[4,5] The pH–rate constant profile is strikingly similar in this pH region to that observed in nonenzymatic hydrolysis of ethyl (2-hydroxy-5-nitrophenyl) carbonate in which case there is a pH-independent reaction from pH 6.5 to pH 10.[9] A plateau region in the pH–rate constant profile is also seen in the nonenzymatic hydrolysis of both bis(4-nitrophenyl) carbonate[7] and *o*-(4-nitrophenylene) carbonate from pH 1 to 7.[8] Attack of water at the ester carbonyl is most likely taking place in these reactions and would appear to be a general reaction for nitrophenyl carbonate esters. On the other hand, the hydrolysis of nitrophenyl carbamate esters, which would result from a nonspecific reaction of II with an amino group, is hydroxide ion catalyzed at pH values as low as 5.5, probably proceeding with formation of an isocyanate intermediate.[26]

Similar rate constants have been observed for hydrolysis of *trans*-cinnamoyl-α-chymotrypsin and *O*-(*trans*-cinnamoyl)-*N*-acetylserinamide in the presence of 7.74 *M* urea,[27] whereas when urea was not present the enzymatic deacylation reaction was much faster. It was considered that high concentrations of urea produced unfolding of the protein so that the resulting deacylation reaction was chemically comparable to the nonenzymatic hydrolysis reaction of the model serine ester. In the present case, the rate of hydrolysis of the acyl enzyme derived from *o*-(4-nitrophenylene) carbonate is little affected by the presence of high concentrations of urea, the rate actually being slightly faster. Therefore, maintenance of the active site by proper folding of the protein is not a factor of importance.

Mechanisms have been suggested for deacylation of acyl-α-chymotrypsins involving histidine-57,[4,5] but effective participation by histidine either as a general base or as a nucleophile does not occur in hydrolysis of the acyl enzyme derived from *o*-(4-nitrophenylene) carbonate. In contrast, deacylation of ethoxyformyl-chymotrypsin gives a normal sigmoidal pH–rate constant profile showing dependence of the rate constants on ionization of a group in the active site with a pK_a of 7.4.[28] A deacylation reaction proceeding without apparent catalysis by histidine would also result if the acyl enzyme was in fact an acyl histidine. However, during the observed deacylation reaction the absorbance at 245 nm increased slightly. Since the intermediate is at high concentration and an acyl histidine should have strong absorbance at 245 nm, a large decrease in absorbance would be expected if the intermediate was such a compound. An additional argument against acylation of histidine is that in the nonenzymatic hydrolysis of *o*-(4-nitrophenylene) carbonate, imidazole most likely functions as a general base, not a nucleophile.[8] It thus appears likely that the observed pH-independent deacylation reaction is due to the inability of the histidine at the active site to participate in the reaction because it is sterically not possible.

Hydrolysis is probably occurring by attack of water on the acyl enzyme. Possible mechanisms might involve catalysis by the phenolic group, as in mechanism VI or a kinetic equivalent. Phenoxide ion catalysis of



VI

the attack of water at the ester carbonyl, analogous to VI, has been shown to be a mechanism of hydrolysis of esters bearing a neighboring phenolic OH group such as *p*-nitrophenyl 5-nitrosalicylate[29] and also ethyl (2-hydroxy-5-nitrophenyl) carbonate.[9] However, the lack of pronounced pH dependence of the rate constants close to the pK_a of the phenolic group indicates that either internal catalysis is not occurring in the present reaction or that a compensating effect is involved which is favored by repression of ionization.

Even a small movement of groups could remove histidine from proximity to the acyl serine intermediate and thus give rise to a reaction not involving that amino acid residue. Such movement might result from interaction of the negatively charged acyl enzyme with charged groups in or near the active site or from closeness to a hydrophobic region. The spectrophotometrically determined pK_a value of 5.6 for the phenolic hydroxyl group in the acyl enzyme is nearly identical with that of the model ester ethyl (2-hydroxy-5-nitrophenyl) carbonate.[9] Therefore, there is no evidence that ionization of this group is being perturbed by proximity to another charged group. However, a conformation change would be expected to greatly minimize any unfavorable charge interactions.

It has been suggested that the decrease in the pH-dependent rate constants for acylation of α-chymotrypsin at high pH is due to repression of ionization of the N-terminal isoleucine group,[30,31] and it has been

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shown that the conformation of the enzyme at pH 10.5 is greatly different from that at neutrality.[32] The protonated isoleucine amino group is thought to form a salt linkage with aspartic acid-194,[33] thereby influencing the configuration of the active site. Thus, interactions between charged groups have an important influence upon conformation. Unfavorable charge interactions or necessity for movement of the acyl group toward the solvent in the present case could move the acyl group away from histidine-57, and explain the relatively slow first-order rates, the pH independence of the reaction, and the lack of effect of urea.

Conclusions

It is clear that the acyl chymotrypsin derivatives prepared from I and II differ qualitatively in their hydrolytic behavior, and that both show striking mechanistic differences in comparison with normal ester acyl enzymes. The nucleophilic attack by a functional group in the protein, releasing *p*-nitrophenol and giving an inhibited enzyme, that most likely takes place with the initial acyl enzyme formed from bis(4-nitrophenyl) carbonate, cannot be detected in hydrolysis of the acyl enzyme derived from *o*-(4-nitrophenylene) carbonate even though both compounds are reactive nitrophenyl esters. With the acyl enzyme from II, the pH-independent release of 4-nitrocatechol to give an active enyzme must then result from the presence of a neighboring phenoxide ion. Thus, in both cases, the mechanism of the deacylation reaction is dependent on the chemical nature of the acyl enzyme compound.

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Kinetics and Mechanism of Decarboxylation of *N*-Arylcarbamates. Evidence for Kinetically Important Zwitterionic Carbamic Acid Species of Short Lifetime

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Abstract: The rates of decarboxylation of a series of substituted *N*-arylcarbamates are examined as a function of pH and buffer concentration. Specific and general acid catalysis, a water reaction, and an alkaline inhibition, which is not accountable for in terms of the ionic state of the reactants, are found. The latter reaction is explainable only in terms of an unstable zwitterionic form of the carbamic acid as a reaction intermediate. The lifetime of this intermediate is estimated to be in the range of 10^{-8}–10^{-10} sec. The high pH inhibition of decarboxylation shows N–CO_2 cleavage to be rate limiting for carbamates derived from weakly basic amines and proton transfer to the nitrogen atom to be rate limiting for carbamates derived from strongly basic amines. The transition state for decarboxylation has a large amount of unimolecular character with little or no participation of nucleophilic agents. The pK_a of *p*-nitrophenylcarbamic acid is 4.2 and the pK_a for nitrogen protonation of carbamates to form the zwitterionic species is estimated to be about −4.

Only recently has the mechanism of CO_2 transfer reactions been studied with the view of understanding the nature of the transition state(s) involved. Early studies of Faurholt and coworkers[1] have demonstrated the phenomenon of carbamate formation from CO_2 and amines, and over a limited pH range the rate of approach to equilibrium in aqueous solutions containing amines, CO_2, and carbonate has been measured. More recent interest in CO_2 transfer reactions has centered around the nature of the enzyme-bound *N*-carboxybiotin I, which is involved in CO_2 transfer from carbonate to appropriate substrates such as acetylCoA, pyruvate, or urea.[2] The investigations of Caplow[3,4] and of Caplow and Yager[5] have established that carbamates undergo acid-catalyzed decomposition and that carbamates derived from amines with pK_a values less than 5 are dependent upon the pK_a of the parent amine with a β value of +0.77. Carbamates derived from more strongly basic amines decarboxylate at nearly the same rate with a rate constant of $\sim 10^8$ M^{-1} sec^{-1}. In addition, general acid catalysis was found for the decarboxylation of the biotin model *N*-carboxyimidazolidone (α = 0.9) and a striking stabilization of this carbamate was observed with metal ions such as cupric and manganous ions.

In the present investigation we more fully investigate the pH dependence of carbamate decomposition over a very wide pH range, and establish free-energy relationships with a series of compounds more closely allied to each other in structure than those used by Caplow.[4] For this purpose *N*-arylcarbamates were used. Be-

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