Deacylation of Acyl Chymotrypsins Formed from Aromatic Carbonate Diesters. Evidence for Hindered Nucleophilic Attack by Histidine-57

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Received March 3, 1972

Abstract: The reaction of α-chymotrypsin with bis(4-nitrophenyl) carbonate proceeds *via* the expected acyl enzyme intermediate. Rate constants for the release of *p*-nitrophenol from this intermediate show a sigmoidal pH dependence, indicating participation by a group of $pK_a = 7.0$. The partial return of enzymatic activity concurrent with this process occurs with identical rate constants, but liberation of the remainder of the free enzyme occurs much more slowly. In addition to deacylation involving His-57 as a general base, nucleophilic attack by some group in the active site must also take place, forming a temporarily inactive enzyme species. Such a reaction is expected on the basis of the low pK_a (7.1) of the 4-nitrophenolate ion leaving group. The fraction of inactive enzyme produced appears to be constant in the pH range 5–8. Thus, both general base and nucleophilic pathways must show similar pH dependence, indicating participation by His-57 in both reactions. Comparison with the activity of imidazole as a nucleophile in the reaction with ethyl 4-nitrophenyl carbonate and 4-nitrophenyl 2,2,2-trifluoroethyl carbonate shows, however, that the proposed nucleophilic attack by histidine is markedly hindered. The isotope effect k_{H_2O}/k_{D_2O} observed for the release of *p*-nitrophenol from 4-nitrophenylcarbonyl α-chymotrypsin is 1.5, much lower than for deacylation of ethoxycarbonyl α-chymotrypsin (3.4), a reaction occurring exclusively by the general base catalyzed process. The lower value is in good agreement with the finding that approximately half the reaction in that case occurs by a nucleophilic attack which does not involve rate-determining proton transfer. 4-Nitrophenyl 2,2,2-trifluoroethyl carbonate acts as a normal substrate for the enzyme, obeying Michaelis–Menten kinetics (K_m(apparent) = 1.51×10^{-6} *M*, $k_{cat} = 0.15$ sec^{-1} at pH 8.45).

The generally accepted mechanism[1] for the α-chymotrypsin-catalyzed hydrolysis of specific and nonspecific ester substrates involves histidine-57 as a classical general base catalyst of the acylation of the hydroxyl group of serine-195, even with a substrate like *p*-nitrophenyl acetate which contains a good leaving group. In contrast, imidazole has been shown to assist in the decomposition of this substrate by a process of direct nucleophilic displacement.[2] Extensive studies[3,4] have shown this behavior to be the rule unless the pK_a of the leaving group is more than 2–3 units higher than that of the attacking base. It is important to determine whether the lack of nucleophilic activity in the enzymatic reaction is primarily due to steric factors, normal binding leading to an orientation of substrate and catalytic site favorable only to general base catalysis, or whether the conversion of the reaction to an intramolecular alcoholysis leads to a faster reaction than if histidine acts alone as a nucleophile. Possibly both factors and/or others are important.

Deacylation reactions are advantageous for study because binding is not a complicating factor as in acylation, but since both reactions have been shown to proceed through similar critical transition states,[1,5] any mechanistic conclusions should be applicable to both steps. In deacylation reactions, however, the leaving group is normally serine which has a relatively high pK_a (the pK_a of the hydroxyl group in *N*-acetylserinamide is 13.6).[6] It has been shown that O esters of *N*-acetylserinamide undergo general base catalyzed hydrolysis by imidazole[7] ($pK_a = 7$) which is unable to directly displace the stronger base serine in a nucleophilic reaction. The use of carbonate diester substrates can, however, give rise to acyl enzymes with leaving groups of much lower pK_a. Deacylation was studied with bis(4-nitrophenyl) carbonate as the substrate, since in that case the acyl enzyme intermediate possesses a leaving group of low basicity.[8] Evidence was found for nucleophilic attack on the acyl enzyme as in eq 1.[8] In the present work, evidence is presented that indicates both nucleophilic displacement and general base deacylation occur concurrently, with the latter reaction accounting for approximately 60% of the total. Separation of the rate constants for the combined process into their components has been made both on the basis of the reaction products and on the extent to which limited "turnover" occurs in the presence of excess substrate. Rate constants for the nucleophilic process, leading to a temporarily inactive enzyme, have been compared with bimolecular rate constants for the nucleophilic reaction between imidazole and analogous carbonate diesters to determine whether histidine behaves as a normal intramolecular nucleophile.[1b]

Important evidence for a general base mechanism in deacylation reactions is the solvent isotope effect $k_{H_2O}/$

(1) (a) M. L. Bender and F. J. Kezdy, *J. Amer. Chem. Soc.*, **86**, 3704 (1964); M. L. Bender, F. J. Kezdy, and C. R. Gunter, *ibid.*, **86**, 3714 (1964); (b) T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," Vol. I, W. A. Benjamin, New York, N. Y., 1966, p 212.

(2) M. L. Bender and B. W. Turnquest, *J. Amer. Chem. Soc.*, **79**, 1656 (1957); T. C. Bruice and G. L. Schmir, *ibid.*, **79**, 1663 (1957).

(3) J. F. Kirsch and W. P. Jencks, *ibid.*, **86**, 833 (1964).

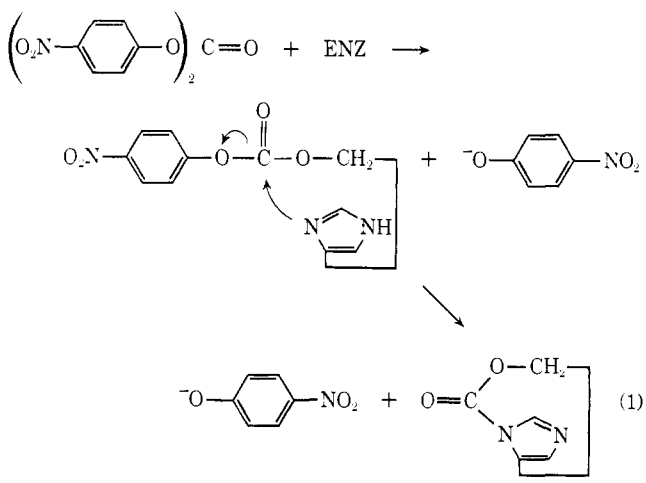
(4) See ref 1b, Chapter 1.

(5) M. L. Bender and W. A. Glasson, *J. Amer. Chem. Soc.*, **82**, 3336 (1960).

(6) T. C. Bruice, T. H. Fife, J. J. Bruno, and N. E. Brandon, *Biochemistry*, **1**, 7 (1962).

(7) J. B. Milstien and T. H. Fife, *J. Amer. Chem. Soc.*, **90**, 2164 (1968); B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.*, **236**, 455 (1961).

(8) T. H. Fife, J. E. C. Hutchins, and D. M. McMahon, *J. Amer. Chem. Soc.*, **94**, 1316 (1972).



k_{D_2O} of about 2–3,[9] this value being in accord with proton transfer in the rate-determining step.[10] The use of solvent isotope effects in enzymatic reactions as a mechanistic criterion has been subject to criticism.[11] Existence of a partial or total nucleophilic deacylation as in eq 1 should lead to a k_{H_2O}/k_{D_2O} nearer unity, which would nicely confirm the applicability of this mechanistic criterion to chymotrypsin deacylation. This has been found to be the case.

Experimental Section

Materials. α-Chymotrypsin (three times crystallized, Worthington Biochemical Corp.) was used without further purification. Stock solutions (approximately 7×10^{-4} *M*) were prepared in pH 5.05 buffer (0.1 *M* acetate) and concentration determined by the method of Schonbaum, *et al.*[12] Bis(4-nitrophenyl) carbonate (Sigma Chemicals) was recrystallized from benzene–hexane (mp 142–143°). The solubility of this compound in water is approximately 2×10^{-5} *M*, but in the presence of 2×10^{-5} *M* enzyme, this is increased to at least 4×10^{-5} *M*. Ethyl 4-nitrophenyl carbonate was prepared as described previously[13] and recrystallized from cyclohexane (mp 66–67°, lit. 68°). 4-Nitrophenyl 2,2,2-trifluoroethyl carbonate was prepared in an identical manner using 2,2,2-trifluoroethanol (Matheson Coleman and Bell) and 4-nitrophenyl chloroformate (Pierce Chemical Co.). The pale yellow liquid was distilled at bp 115–116.5° (0.6 mm). *Anal.* Calcd for $C_9H_6F_3NO_5$: C, 40.77; H, 2.28. Found: C, 41.14; H, 2.47.

Proflavin dihydrochloride (Mann assayed material, 70.9% free base) was used without further purification. Aqueous solutions were prepared ($\sim 2 \times 10^{-2}$ *M*) containing approximately 10^{-4} *M* EDTA, and were stored in a refrigerator and discarded after 2–3 weeks. Acetonitrile (Matheson Coleman and Bell spectroquality) was dried over 4A molecular sieves and was used for preparing substrate stock solutions. Buffers were made from analytical quality reagents. Deuterium oxide (99.8 mol % D_2O) was obtained from Biorad Laboratories. Imidazole (K&K Laboratories) was recrystallized from benzene and sublimed just below the melting point under vacuum (mp 88–90°).

Kinetic Measurements. The release of *p*-nitrophenol from 4-nitrophenylcarbonyl α-chymotrypsin was measured below pH 6.5 by conventional spectrophotometry using a Gilford 2000 recording spectrophotometer and 1-cm cuvettes. The cell compartment was thermostated at 25 ± 0.1° by circulating water from a temperature-controlled bath through the cell block. At pH 5.05 the reaction of bis(4-nitrophenyl) carbonate with a 10% excess of enzyme resulted in the release of *p*-nitrophenol in two separable reactions. By following the release of the second mole of *p*-nitrophenol at 330 nm, a first-order rate constant for the reaction at this pH could be obtained (Table I). From pH 5 to 6.5 the reaction was followed by similar means. Below pH 5, rates were measured by rapidly lowering the pH of reaction solutions which had been initiated at pH 5.05 and allowed to stand for sufficient time (~30 sec) for acylation to be completed. Stabilized acyl enzyme solutions at pH 2 were prepared in this manner and used in the stopped-flow experiments.

Table I. Rate Constants for the First-Order Release of *p*-Nitrophenol from 4-Nitrophenylcarbonyl Chymotrypsin at pH 5.05 (Acetate Buffer, $\mu = 0.1$)

$[E_0] \times 10^5$ *N*	$[S_0] \times 10^5$ *M*	10^2k, sec^{-1}
2.26	2.04	1.05
2.24	2.04	1.05
4.48	2.04	0.92
11.2	2.04	1.00
11.2	4.08	1.08
2.26	2.04	1.12[a]

[a] Rate constant obtained for the rapid first-order return of part of the enzymatic activity, measured at 465 nm in the presence of 8.03×10^{-5} *M* proflavin.

Above pH 6, release of *p*-nitrophenol from the acyl enzyme was measured using a Durrum-Gibson stopped-flow apparatus (Model D 110). This comprised two drive syringes immersed in a water trough whose temperature was controlled to 25 ± 0.1° by circulating the water through a coil immersed in a water bath. One of the drive syringes contained a solution at high pH; in the other was placed stabilized acyl enzyme solution at pH 2. The basic solution was varied, so that on activating the compressed air-driven ram, equal volumes from the two syringes were rapidly mixed, resulting in a buffered reaction solution at the required pH. In a typical experiment 0.7 ml of 6.77×10^{-4} *N* chymotrypsin was added to 10 ml of pH 5.05 buffer (0.1 *M* acetate). Bis(4-nitrophenyl) carbonate (100 μl, 4.27×10^{-3} *M*) was added by syringe and the solution well mixed. Either 30 or 60 sec later, 0.8 ml of 1 *M* HCl was added (the different times gave identical results). The entire solution was rapidly transferred to one of the stopped-flow syringes. The other syringe contained 0.1 *M* phosphate buffer, giving a reaction solution of pH 7.60 on mixing. Initially, OD changes at 400 nm after mixing were ~0.2 unit. Eight runs were followed. After 20 min, corresponding to reaction of roughly one-half of the acyl enzyme at pH 2, measurements were discontinued. A mean value of the rate constant 0.316 ± 0.01 sec^{-1} was obtained. The standard deviation, σ, was first obtained for a reaction series, and those values of *k* rejected which fell outside 2σ. The standard error, α,[14] was then calculated, and an average value for the limits ±2α (corresponding to 95% probability limits) was found to be 5% of the mean rate constants. Ionic strengths of the reaction mixtures tended to vary slightly ($\mu = 0.13 \pm 0.02$). However, at pH 9.6 two series of runs with ionic strengths differing by 0.2 showed discrepancies between the average of each series amounting to about 15%, not much greater than the experimental reproducibility. At pH 9.8, the reaction rate was measured at varying enzyme–substrate ratios. Increasing $[E_0]/[S_0]$ from one to two appeared to raise the rate constant by 20%, but increasing $[E_0]/[S_0]$ further to four had no effect. It was concluded that within the experimental limitations the observed rate constant was independent of $[E_0]$ at this pH as at pH 5.05 (see Results). An isotope effect was obtained for the pH-independent region at high pH by working in D_2O.

The return of enzymatic activity accompanying the release of *p*-nitrophenol was measured using the competitive inhibitor, proflavin. The dye forms a 1:1 complex with the active site of the free enzyme,[15] which results in a spectral shift. The maximum absorbance difference between complexed and uncomplexed dye occurs at 465 nm, thus allowing reactions to be followed spectrophotometrically, which by themselves exhibit no color changes.[16] Details of

(9) M. L. Bender and G. A. Hamilton, *J. Amer. Chem. Soc.*, **84**, 2570 (1962).
(10) J. M. Williams, Jr., and M. M. Kreevoy, *Advan. Phys. Org. Chem.*, **6**, 63 (1968).
(11) W. P. Jencks, *Annu. Rev. Biochem.*, **32**, 603 (1963).
(12) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).
(13) G. G. Smith, D. A. K. Jones, and R. Taylor, *J. Org. Chem.*, **28**, 3547 (1963).
(14) J. Topping, "Errors of Observation and Their Treatment," Chapman and Hall, Ltd., London, 1966, p 62.
(15) S. A. Bernhard, B. F. Lee, and Z. H. Tashjian, *J. Mol. Biol.*, **18**, 405 (1966).
(16) For examples, see: K. G. Brandt, A. Himoe, and G. P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967); F. Guillain and D. Thusius, *J. Amer. Chem. Soc.*, **92**, 5534 (1970).

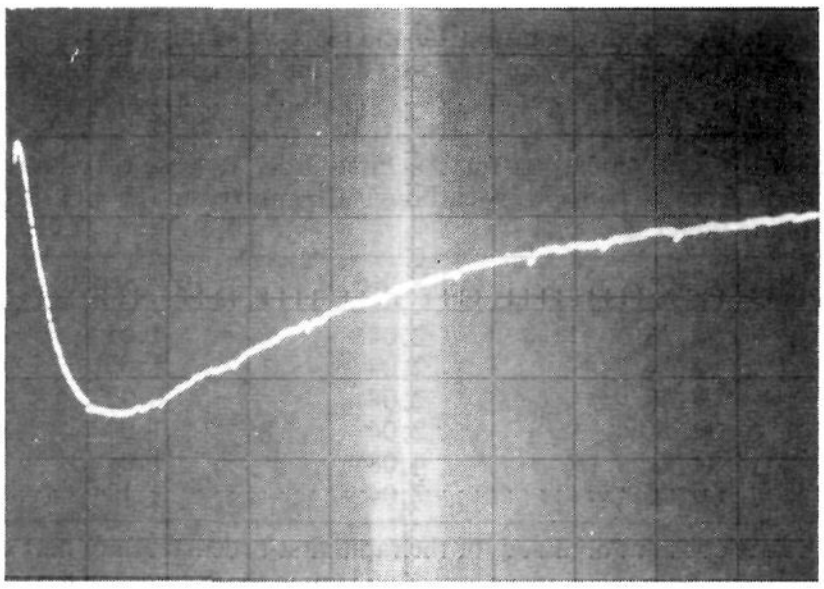


Figure 1. Photograph of oscilloscope trace from stopped flow showing absorbance changes at 465 nm, accompanying acylation and partial deacylation of α-chymotrypsin with bis(4-nitrophenyl) carbonate in the presence of proflavin at pH 7.40. Scale: horizontal division = 1 sec; vertical division = 0.01 Å; $[S_0] = 1.02 \times 10^{-5}$ *M*; $[E_0] = 1.16 \times 10^{-5}$ *N*; [proflavin] = 3.9×10^{-5} *M*.

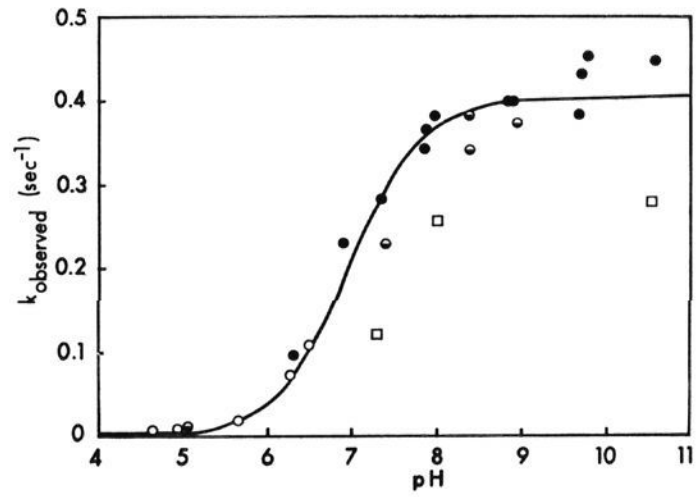


Figure 2. pH–rate constant profile for the release of *p*-nitrophenol from 4-nitrophenoxycarbonyl α-chymotrypsin; ionic strength = 0.13 ± 0.02; temp = 25°. Rate constants for release of second equivalent of 4-nitrophenol from bis(4-nitrophenyl) carbonate using conventional spectrophotometry, ○; from stopped-flow, using stabilized acyl enzyme, ●; from stopped-flow, using proflavin, ◒; D_2O results, □ (pD). The line drawn is a computer-calculated best fit for results in H_2O, using 2% weighting for ○ and 6% for ●; pK_a (apparent) = 7.02.

such an experiment at pH 5.05 are given in the Results. A limited number of experiments with proflavin were performed with the stopped flow. One syringe contained substrate dissolved in 10^{-2} *M* HCl: the other contained buffer, enzyme, and proflavin.

Results

The reaction between bis(4-nitrophenyl) carbonate and α-chymotrypsin under conditions of excess enzyme was carefully examined at pH 5.05 by monitoring the release of *p*-nitrophenol spectrophotometrically at 330 nm. With the concentrations $[E_0] = 2.24 \times 10^{-5}$ *N* and $[S_0] = 2.04 \times 10^{-5}$ *M* a rapid reaction was observed, followed by a slower process presumably representing subsequent decomposition of the acyl enzyme. Data from the last 50% of the reaction were analyzed by a rigorous least-squares computer procedure[17] and found to conform well to first-order kinetics. Actual and calculated infinity points agreed to within 3%. Raising the initial enzyme concentration (up to five times) progressively increased the rate of the first step, as expected for a bimolecular acylation process, without altering the rate of the second step. With the concentrations $[E_0] = 1.12 \times 10^{-4}$ *N* and $[S_0] = 2.04 \times 10^{-5}$ *M*, the two processes were distinctly separated. A 1 equiv "burst" of *p*-nitrophenol was obtained followed by the slower release of a second equivalent of *p*-nitrophenol. Doubling the substrate concentration at this enzyme concentration likewise had no effect on the rate of the second step. The results are tabulated in Table I.

The return of enzymatic activity accompanying decomposition of the acyl enzyme was also measured at pH 5.05 in the presence of proflavin. When $[E_0] = 2.26 \times 10^{-5}$ *N*, $[S_0] = 2.04 \times 10^{-5}$ *M* and [proflavin] = 8.03×10^{-5} *M*, a rapid drop in OD at 465 nm occurred (0.115 absorbance unit) corresponding to 96% of the absorbance rise previously obtained on adding enzyme to the buffer containing proflavin. After 45 sec the absorbance began to rise rapidly by 0.04 unit in a first-order manner with a rate constant identical with that obtained for release of the second mole of *p*-nitrophenol (see Table I). A further, much slower, absorbance rise was then observed. When the initial substrate concentration was doubled ($[S_0] = 4.08 \times 10^{-5}$ *M*), the enzyme and proflavin concentrations remaining constant, an absorbance drop of 0.110 was observed. For the next 32 min no absorbance change took place, then a gradual rise of 0.074 unit (approximately twice the slow rise in the previous experiment) occurred. The rate constant for the second process ($k = 1.6 \times 10^{-4}$ sec^{-1}) was in agreement with the previously reported value under similar conditions.[8]

The partial return of enzyme activity accompanying production of the second equivalent of *p*-nitrophenol was followed at pH >5 by the proflavin method. A photograph of the oscilloscope trace obtained at pH 7.40 is reproduced in Figure 1. At this pH, roughly 70% of the initial absorbance loss at 465 nm was rapidly regained, comparable to what was observed at pH 5.05. A few rate constants were measured at different pH values by this method and are plotted in Figure 2. Sufficient experiments were performed to show the identity of rate constants obtained by this means with those measured by the release of *p*-nitrophenol, as was the case at pH 5.05.

A pH–rate constant profile for both the release of *p*-nitrophenol from the acyl enzyme and the rapid return of part of the enzymatic activity accompanying this process is shown in Figure 2. Results were obtained as described in the Experimental Section. Results in D_2O are also plotted, enabling an isotope effect $k_{H_2O}/k_{D_2O} = 1.5$ to be calculated for the pH-independent region at high pH.

It was important to establish as accurately as possible the extent to which the enzyme could react with bis(4-nitrophenyl) carbonate when the substrate was in excess, before becoming temporarily totally inactivated. At pH 7.95, both acylation and loss of *p*-nitrophenol from the acyl enzyme were rapid by comparison with the slow return of activity from the inactive enzyme. In a series of experiments substrate concentration was maintained constant at 2.01×10^{-5} *M*, while enzyme concentration was varied between 1.10×10^{-6} *N* and 9.93×10^{-6} *N*. The release of 4-nitrophenol was observed spectrophotometrically at 400 nm. The reactions were characterized by a rapid reaction, over in 45 sec (at the most), followed

(17) Computer programs in this paper were devised by Dr. E. Anderson of this address.

by a slower process taking up to 16 min to reach completion. Exact separation of the two processes was somewhat difficult to achieve, but an estimate was made by moving a straight edge along the curve up to the point where it first assumed apparent linearity over a short region. From the absorbance change in the rapid reaction the ratio N could be obtained, representing the number of equivalents of substrate reacting with 1 equiv of enzyme before the latter became totally inactivated. The experimental results are given in Table II. The first three values are most subject to error in

Table II. Stoichiometry of the Rapid Reaction between Bis(4-nitrophenyl) Carbonate and α-Chymotrypsin at pH 7.95, $[S_0] = 2.01 \times 10^{-5}$ M

$[E_0] \times 10^6$	N[a]	$[E_0] \times 10^6$	N[a]
1.08	1.93	6.50	2.32
2.17	2.14	7.58	2.37
3.25	2.05	7.58	2.38
4.33	2.30	8.66	2.32
5.42	2.21	9.75	2.02

[a] N, the number of equivalents reacting per mole of enzyme, was corrected for the apparent burst obtained on adding substrate to solution containing no enzyme.

view of the small OD changes; the last two values are limited by lack of sufficient excess of enzyme. The average for the intervening five values is 2.32.

Attempts to repeat this experiment at widely different pH values were not possible. At pH 5.05, the acylation and subsequent loss of *p*-nitrophenol from the acyl enzyme were too slow compared with spontaneous hydrolysis. At higher pH values the return of enzymatic activity from the inactive enzyme became too rapid for effective separation of the two processes.

For comparison purposes, a partial deacylation study was undertaken with the acyl enzymes formed from ethyl 4-nitrophenyl carbonate and 4-nitrophenyl 2,2,2-trifluoroethyl carbonate. In both cases acylation proceeds by displacement of the most weakly basic leaving group (*i.e.*, 4-nitrophenolate). Deacylation of ethoxycarbonyl chymotrypsin was measured directly by following the first-order return of enzyme activity as measured by complex formation with proflavin ($[E_0] > [S_0]$). At pH 8.40, the observed rate constant was 1.94×10^{-3} sec^{-1}, in excellent agreement with the rate constant obtained from turnover measurements ($k_3 = 0.2 \times 10^{-2}$ sec^{-1}).[18] From measurements in D_2O, an isotope effect $k_{H_2O}/k_{D_2O} = 3.4$ was calculated, identical buffer concentrations being employed. It was assumed that at pD 8.92, the pD-independent portion of the rate profile had been reached as had been shown in water for pH 8.40.[19] Deacylation of 2,2,2-trifluoroethylcarbonyl chymotrypsin at pH >7 was too fast to measure in the same manner. Accordingly, k_3 was estimated from turnover experiments at pH 8.43. The plot obtained for V *vs.* $[S_0]$ when $[S_0] \gg [E_0]$ is shown in Figure 3. The value of k_3 obtained by a rigorous least-squares computer fit[17] to the V *vs.* [S] hyperbola is 0.15 sec^{-1}. Deacylation rate constants for various carbonyl chymotrypsins, including those measured previously, are given in Table III.

(18) A. A. Shah and K. A. Connors, *J. Pharm. Sci.*, 57, 282 (1968).
(19) W. B. Melchior and D. Fahrney, *Biochemistry*, 9, 251 (1970).

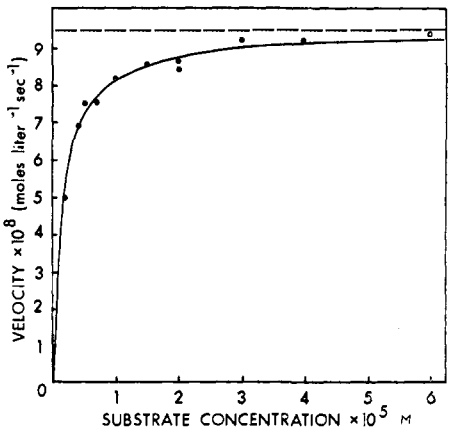


Figure 3. Plot of initial velocity for release of 4-nitrophenol *vs.* 4-nitrophenyl 2,2,2-trifluoroethyl carbonate concentration in the presence of 6.4×10^{-7} N α-chymotrypsin: pH 8.43; temp = 25°. Circles are experimental points, solid line is a computer calculated best fit. Dashed line shows V_{max}. Calculated parameters: $k_{cat} = 0.15$ sec^{-1}, K_m(apparent) $= 1.5 \times 10^{-6}$ M.

Table III. Deacylation Rate Constants for EOC(=O)OR at High pH

OR	pK_a	pH	k_3, sec^{-1}
$NO_2C_6H_4O$	7.1	8.5–10	4.02×10^{-1} [a]
CF_3CH_2O	12.36[b]	8.43	1.48×10^{-1}
CCl_3CH_2O	12.24[c]	8–9	8.2×10^{-2} [g]
EtO	16[d]	8.40	2.03×10^{-3} [e]
		8.40	2.0×10^{-3} [g]
		8.92 (D_2O)[f]	5.90×10^{-4} [e]

[a] Rate constant for loss of *p*-nitrophenol from the acyl enzyme. This may be broken down as follows: $k_n = 0.17$ sec^{-1}, $k_{gb} = 0.24$ sec^{-1} (see eq 3). [b] P. Ballinger and F. A. Long, *J. Amer. Chem. Soc.*, **81**, 1050 (1959). [c] F. A. Long and P. Ballinger, *Proc. Int. Symp., Trieste*, 152 (1959); *Chem. Abstr.*, **61**, 10098*h* (1959). [d] P. Ballinger and F. A. Long, *J. Amer. Chem. Soc.*, **82**, 795 (1960). [e] Average of two runs. [f] pD obtained as pH reading + 0.4; P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960). [g] Reference 18.

The reactions between imidazole and both ethyl 4-nitrophenyl carbonate and 4-nitrophenyl 2,2,2-trifluoroethyl carbonate, leading to a rapid release of *p*-nitrophenol in each case, were measured in half-neutralized buffers and at pH 7.07 (phosphate buffer), respectively. Ionic strengths were maintained at 0.1 M. An excellent linear relationship between observed rate constants and imidazole concentration was observed in both cases (Figure 4). Second-order rate constants, $k_2 = 0.171$ M^{-1} sec^{-1} (correlation coefficient 0.999), for the ethyl ester, and $k_2 = 3.36$ M^{-1} sec^{-1} (correlation coefficient 0.996), for the trifluoroethyl ester, were obtained using a computerized weighted linear regression technique.[16] The former reaction was also followed at 245 nm. A rapid first-order rise in absorbance was observed at this wavelength, with a rate constant identical with that obtained for *p*-nitrophenol release at the same imidazole concentration. An absorption at this wavelength is as expected for the formation of an acyl imidazole.[2] A much slower and equal loss in absorbance at 245 nm was then noted, corresponding to subsequent hydrolysis of the carbamate intermediate[19] when measured in the same buffer.

With α-chymotrypsin and bis(4-nitrophenyl) carbonate as substrate, a similar absorbance change at 245

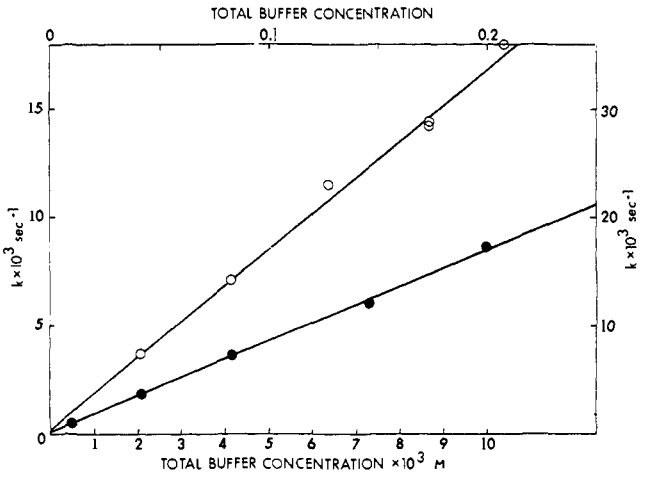


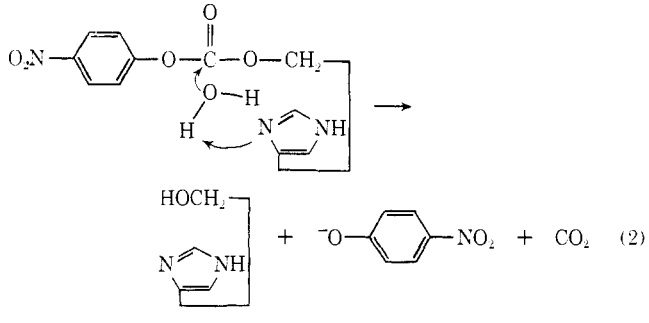
Figure 4. Rate constants for reaction of ethyl 4-nitrophenyl carbonate (closed circles, right-hand and upper scales) and 4-nitrophenyl 2,2,2-trifluoroethyl carbonate (open circles, left-hand and lower scales) with aqueous imidazole buffers at pH 7.05. Ionic strength, 0.1. Substrates introduced in acetonitrile (CH_3CN) = 0.7% v/v; temp = 25°; solid lines are those calculated by computer.

nm was noted after all *p*-nitrophenol had been released.[8] The rate constant obtained for this process was identical with the rate constant for return of free enzyme found using the proflavin method. With ethyl 4-nitrophenyl carbonate and α-chymotrypsin at similar equivalent concentrations, no absorbance changes at 245 nm were noted throughout the entire deacylation process.

Discussion

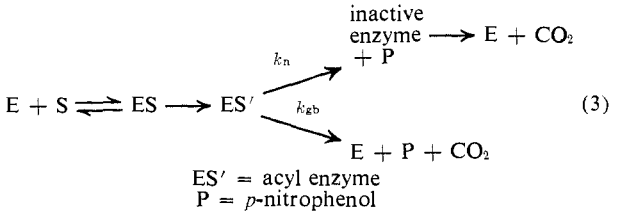
The reaction between α-chymotrypsin and bis(4-nitrophenyl) carbonate in equal concentrations at pH 7.7 resulted in the rapid release of both equivalents of *p*-nitrophenol and an enzyme still roughly 50% inactivated.[8] This reaction most likely occurs *via* a normal acyl enzyme in which the acyl group is attached to serine-195. The inactivated enzyme was considered to most likely be a cyclic acyl derivative of His-57 and Ser-195, formed by direct nucleophilic attack by histidine at the carbonyl group (eq 1).

It was suggested that part of the deacylation process might be occurring by a general base catalyzed reaction involving a water molecule (eq 2). Evidence for this



(2)

reaction has now been found. The rapid return of part of enzymatic activity with rate constants identical with those observed for the release of the second equivalent of *p*-nitrophenol and the observation of a very limited turnover reaction (results shown in Table II) are best explained in terms of the following reaction scheme.



(3)

With conditions $[E_0] = [S_0]$ the disappearance of acyl enzyme is given by eq 4. The release of the second

$$[ES']_t = [ES']_0 e^{-[k_n + k_{gb}]t} \quad (4)$$

equivalent of *p*-nitrophenol is given by

$$dP/dt = [k_n + k_{gb}][ES']_t = [k_n + k_{gb}][ES']_0 e^{-[k_n + k_{gb}]t} \quad (5)$$

whereas the rapid return of part of the enzymatic activity measured by the proflavin experiments is given by

$$dE/dt = k_{gb}[ES']_t = k_{gb}[ES']_0 e^{-[k_n + k_{gb}]t} \quad (6)$$

The exponential parts of eq 5 and 6 are identical, hence the measured rate constants are the same.[20]

With the scheme shown in eq 3 and under conditions of excess substrate, a fraction (x) of the enzyme will be temporarily inactivated by the nucleophilic pathway each time the enzyme is acylated. The corresponding equivalents of substrate destroyed enzymatically per equivalent of enzyme in each reaction cycle form a series whose sum is given by

$$N = 1 + [1 - x] + [1 - x]^2 + \ldots [1 - x]^{n-1} \quad (7)$$

Hence

$$N = (1 - [1 - x]^n)/x \quad (8)$$

and

$$\lim_{n \to \infty} N = 1/x \quad (9)$$

By knowing the amount of bis(4-nitrophenyl) carbonate rapidly destroyed under conditions of sufficient excess, a value for x can be obtained. At pH 7.95, a value of $N = 2.3$ was found. Accordingly, $x = 0.44$. This means that 44% of deacylation occurs *via* the nucleophilic pathway and 56% *via* the normal, general base catalyzed route. These ratios should be true at any pH for eq 1 and 2, and such has been indicated by the proportion of free enzyme being rapidly returned at pH 5.05 and 7.40 (see Experimental Section, proflavin experiment, and Figure 1). The phenol release method is probably more accurate because (a) the proflavin–enzyme complex is possibly unstable, and (b) *p*-nitrophenol is a competitive inhibitor for the enzyme and its presence as a reaction product will tend to somewhat diminish the apparent return of activity measured using proflavin. Some nonspecific acylation[21] might also be occurring.

Evidence for the process $[k_n + k_{gb}]$ involving histidine as in normal deacylation is provided by the pH–rate constant profile (Figure 2). To obtain good fit it was necessary to allow for a small spontaneous hydrolysis rate for the acyl enzyme [$k_0 = 4.2 \times 10^{-3}$ sec^{-1}]. Such behavior is in accord with previously observed

(20) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," Wiley, New York, N. Y., 1965, p 160.
(21) B. S. Hartley and B. A. Kilby, *Biochem. J.*, 56, 288 (1954).

water catalysis for reactive carbonate diesters.[22] Since the nucleophilic process appears to constitute a constant fraction of the total reaction [$k_n + k_{gb}$] in the pH range 5–8 (which covers the greater part of the pH–rate profile shown in Figure 2), both k_n and k_{gb} must show identical dependencies on a group or process of pK_{app} = 7.0. It is therefore most likely that the functional group involved in the k_n reaction is the same as that postulated for the k_{gb} step, namely, histidine. A group of lower pK_a such as a carboxyl group, if participating as a nucleophile, would be expected to lead to much higher rate constants in the pH range 4–7. Also, the pH dependence for reactivation of the enzyme[8] is not consistent with the stable intermediate being either a carbonate monoester[23] or a carbamate ion.[24] The fact that the enzyme reactivates spontaneously is evidence that the nucleophilic group is not an α or ϵ amino group since carbamate esters with aliphatic leaving groups are extremely stable at pH values close to neutrality.[25] Carbamate esters of imidazole are, however, reasonably reactive.[8,19,22] More definitive evidence for identity of the nucleophilic group attacking the acyl enzyme must, of course, await further experimental work such as derivatizing the inactive enzyme species.

The observed solvent isotope effect [k_{H_2O}/k_{D_2O} = 1.5] for the release of *p*-nitrophenol from 4-nitrophenylcarbonyl α-chymotrypsin may be taken as additional evidence for a partially nucleophilic process. The general applicability of solvent isotope effects in enzymatic reactions is subject to some controversy,[11] but the other evidence for partial nucleophilic attack in the subsequent reaction of the acyl enzyme is sufficient to stand alone. Therefore, use of the solvent isotope effect as a criterion for general base catalyzed deacylation seems to be vindicated. The solvent isotope effect is much smaller than previously measured effects for deacylation,[26] and is as expected since the rate of the nucleophilic reaction should not be greatly affected by change of solvent.[27] If the solvent isotope effect is given by

$$k_{H_2O}/k_{D_2O} = \frac{k_n + k_{gb}(H)}{k_n + k_{gb}(D)} = 1.51 \qquad (10)$$

and if $k_n/k_{gb}(H)$ has the value 0.79, substitution enables the ratio, $k_{gb}(H)/k_{gb}(D)$ = 2.53, to be calculated in excellent agreement with previous values and similar to that obtained for deacylation of ethoxycarbonyl α-chymotrypsin at pH 8.4 [k_{H_2O}/k_{D_2O} = 3.4]. Ethyl 4-nitrophenyl carbonate behaves typically as a substrate, and the acyl enzyme presumably undergoes deacylation by the general base mechanism.[18]

Values obtained for the second-order rate constants for imidazole displacing *p*-nitrophenol from ethyl 4-nitrophenyl carbonate and 4-nitrophenyl 2,2,2-trifluoroethyl carbonate (0.17 and 3.36 M^{-1} sec^{-1}) are of great interest. The reaction between imidazole and highly activated esters has been shown to occur *via* a nucleophilic displacement,[2] and in the present case an intermediate has been observed. From the two rate constants given, an estimate of the rate constant that might be expected for bimolecular nucleophilic attack by imidazole on 4-nitrophenylcarbonyl α-chymotrypsin can be made, assuming a linear dependence of log k_2 on the pK_a of the acyl activating group, and assuming the pK_a for the serine hydroxyl to be 13.6, as was found in *N*-acetylserinamide.[6] The estimated bimolecular rate constant obtained by this means is 1.0 M^{-1} sec^{-1}. This may be compared with the observed unimolecular rate constant for intramolecular nucleophilic attack on the acyl enzyme at high pH (0.17 sec^{-1}). If it is assumed that the nucleophilic group in the acyl enzyme is histidine, then from the ratio (k_{intra}/k_{inter}) the effective molarity of histidine at the reaction center is only 0.2 *M*, a value much smaller than normally obtained when intermolecular and intramolecular reactions of similar compounds reacting by identical mechanisms are compared. For example, with 3-dimethylamino(substituted phenyl) butyrates effective molarities of between 10^3 and 5×10^3 *M* were obtained,[28] depending on the substituent, when the intramolecular reaction was compared with the intermolecular reaction in which trimethylamine was the catalyst. When the release of *p*-nitrophenol from 4-nitrophenyl-γ-(4′-imidazolyl) butyrate in 50% aqueous ethanol is compared with the rate of *p*-nitrophenol release in the bimolecular reaction between imidazole and *p*-nitrophenyl acetate in water,[2,29] an effective molarity of 9.4 *M* is obtained for imidazole participating in the intramolecular reaction. In this laboratory, the intramolecular nucleophilic reactions of 4-substituted phenyl 2-pyridylethyl carbonate have been studied in water, and effective concentrations for the intramolecular pyridine catalyst of between 30 and 50 *M* have been found.[30] Furthermore, 4-methoxyphenyl 2-pyridylethyl carbonate appears to react by a nucleophilic displacement mechanism, even though the pK_a values of attacking base and leaving group are five units apart. Thus, not only does the nucleophilic displacement mechanism in 4-nitrophenoxycarbonyl α-chymotrypsin show a very low effective molarity for the attacking histidine, but this occurs in a system which model studies show should be unusually favorable for nucleophilic displacement. Thus, it can be concluded from the present study that strong steric hindrance exists toward histidine acting as a nucleophile.

Addendum

Since submission of this paper, another paper on the same subject has appeared.[31] The authors show evidence for a nucleophilic displacement by histidine-57 in the acyl enzyme formed from tris(*p*-nitrophenyl) phosphate, but claim that attack by some unidentified nucleophile other than histidine occurs on the acyl enzyme formed from bis(4-nitrophenyl) carbonate. Furthermore, they propose that reactivation of the enzyme from the resulting inactive species, designated E–OCO–X, occurs through a general base pathway, assisted by histidine. However, our results give no

(22) T. H. Fife and D. M. McMahon, *J. Org. Chem.*, **35**, 3699 (1970); *J. Amer. Chem. Soc.*, **91**, 7481 (1969); T. H. Fife and J. E. C. Hutchins, *ibid.*, **94**, 2837 (1972).
(23) N. F. Miller and L. O. Case, *ibid.*, **57**, 810 (1935).
(24) S. L. Johnson and D. L. Morrison, *ibid.*, **94**, 1323 (1972).
(25) L. W. Dittert and T. Higuchi, *J. Pharm. Sci.*, **52**, 852 (1963).
(26) For *trans*-cinnamoyl α-chymotrypsin, k_{H_2O}/k_{D_2O} = 2.5; for trimethylacetyl α-chymotrypsin, k_{H_2O}/k_{D_2O} = 3.0; and with *N*-acetyl-L-tryptophan methyl ester deacylation is 2.83 times faster in H_2O than D_2O (ref 1 and 9).
(27) M. L. Bender, E. J. Pollock, and M. C. Neveu, *J. Amer. Chem. Soc.*, **84**, 595 (1962).
(28) T. C. Bruice and S. J. Benkovic, *ibid.*, **85**, 1 (1963).
(29) T. C. Bruice and J. M. Sturtevant, *ibid.*, **81**, 2860 (1959).
(30) J. E. C. Hutchins and T. H. Fife, unpublished results.
(31) M. L. Bender and F. C. Wedler, *J. Amer. Chem. Soc.*, **94**, 2101 (1972).

indication of any sigmoidal pH dependency (Figure 3, ref 8) for the slow reactivation process and hence do not require the participation of His-57 as a general base. The sigmoidal pH–rate profile claimed by Bender and Wedler[31] relies heavily on data obtained for the deacylation of methoxycarbonyl chymotrypsin obtained either using methyl *p*-nitrophenyl carbonate as a substrate, or by transesterification of 4-nitrophenylcarbonyl chymotrypsin with methanol. Ethoxycarbonyl,[18,19,21] 2,2,2-trichloroethylcarbonyl,[18] and 2,2,2-trifluoroethylcarbonyl chymotrypsins (this paper) all behave normally, the respective substrates giving true "turnover" reactions with dissimilar rate constants. Hence, it seems highly probable that methoxycarbonyl chymotrypsin deacylates by the normal general base catalyzed process, and not by that shown by Bender and Wedler (k_3 step in their Scheme II). Similarities in rate constants obtained for deacylation of this species and for reactivation of E–OCO–X in the pH range 5–9 are most likely coincidental, a possibility admitted by these authors. No evidence is presented for an inhibited enzyme resulting from the reaction of methyl *p*-nitrophenyl carbonate and the enzyme in equivalent amounts after both *p*-nitrophenolate ion and methanol are released.

Results obtained by us in ref 8 and in this paper regarding the return of enzymatic activity from the acyl enzyme in two separable stages and the very limited "turnover" found with bis(4-nitrophenyl) carbonate cannot be explained by Scheme II of Bender and Wedler. We are, however, in agreement over the existence of a species E–OCO–X, but derived only from bis(4-nitrophenyl) carbonate. Bender and Wedler suggest that His-57 participates in the rate-determining step of "aging" but do not give any explanation as to how nucleophilic attack occurs in a subsequent product-determining step. Attack by X on the carbonate monoester or its anion, or carboxyl group attack assisted in a general base manner by His-57, are unattractive possibilities. The sigmoidal pH–rate profile ($pK_{app} = 7$) obtained for the combined $k_n + k_{gb}$ process and hence for its component parts (the ratio $(k_n/k_n + k_{gb})$ is pH independent in the range 5–8), plus the absorbance at 245 nm for E–OCO–X, indicate that the unknown "X" is most likely the closest and most powerful nucleophile, His-57. The presence of general base catalyzed deacylation concurrent with the process leading to the inactive enzyme species is demanded by the rapid return of part of the enzymatic activity from the acyl enzyme, by the limited turnover observed under conditions of excess substrate, and by the experiments with methanol described by Bender and Wedler.[31]

Acknowledgment. This work was supported by a research grant from the National Institutes of Health.

Directions of the Dipole Moments of Aromatic Heterocyclopentadienes

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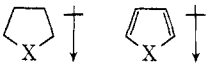
Abstract: The orientational influence of dipolar solutes on the aromatic solvents, benzene and hexafluorobenzene, as reflected by nmr chemical shifts, has been used to determine the direction of the dipole moments of thiophene, furan, pyrrole, and *N*-methylpyrrole. The direction for pyrroles with nitrogen at the positive end was confirmed. Contrary to popular belief, the direction of the dipole moments of the chalcogen heterocycles was found not to be reversed from their saturated analogs but to maintain the direction, placing the heteroatom at the negative end.

Among the major evidence for aromaticity in heterocyclopentadienes is the reversal of dipole moment direction in going from the saturated to the totally unsaturated systems. It is generally accepted[1] that the dipole moments of furan, pyrrole, and thiophene are such that the heteroatom is at the *positive* end.



Since the experimental measurement of dipole moments provides only an absolute number and *not* the direction, we became curious as to the source of this idea. The most often quoted references are those of Brown[2] and Lumbroso.[3] However, Brown's papers clearly indicate the opposite dipole direction for pyrrole[2a] (*i.e.*, with nitrogen at the negative end as in pyrrolidine) and while he originally placed oxygen at the negative pole of furan,[2a] this was later reversed.[4] Indeed, the direction of dipole moment is not a subject of Lumbroso's paper, which is devoted to a study of various solvent interactions with pyrroles using dipole moments as a probe. The definitive work on this subject is found in a report[5] by Kofod, *et al.* (1952). These

(1) (a) L. A. Paquette, "Principles of Modern Heterocyclic Chemistry," W. A. Benjamin, New York, N. Y., 1968, p 104; (b) R. M. Acheson, "An Introduction to the Chemistry of Heterocyclic Compounds," 2nd ed, Interscience, New York, N. Y., 1967, pp 65, 95, 143; (c) M. H. Palmer, "The Structure and Reactions of Heterocyclic Compounds," Edward Arnold, London, 1967, p 254.

(2) (a) I. M. Bassett, R. D. Brown, and A. Penfold, *Chem. Ind. (London)*, 892 (1956); (b) R. D. Brown and B. A. W. Coller, *Aust. J. Chem.*, **12**, 152 (1959); (c) R. D. Brown and M. L. Heffernan, *ibid.*, **12**, 319 (1959); (d) *ibid.*, **12**, 330 (1959); (e) R. D. Brown and B. A. W. Coller, *Theor. Chim. Acta*, **7**, 259 (1967).

(3) M. Gomel and H. Lumbroso, *Bull. Soc. Chim. Fr.*, 2200 (1962).

(4) Reference 2b, footnote on p 155.

(5) H. Kofod, L. E. Sutton, and J. Jackson, *J. Chem. Soc.*, 1467 (1952).