Phosphate and Carbonate Ester "Aging" Reactions with α-Chymotrypsin. Kinetics and Mechanism

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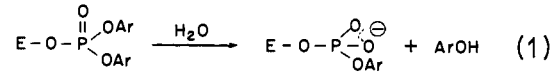
Abstract: Either tris(*p*-nitrophenyl) phosphate or bis(*p*-nitrophenyl) carbonate reacts rapidly at the active site of α-chymotrypsin to release a stoichiometric amount of *p*-nitrophenol. The resulting phosphorylated or acylated enzyme then releases a second mole of *p*-nitrophenol in a process which exhibits first-order kinetics. The pH dependence of the kinetics of this second reaction, called "aging," reveals the participation of an ionizable group with a pK_a near 7. This implicates the imidazole of histidine-57, in analogy to deacylation reactions. Added methanol does not appreciably accelerate phosphorylated enzyme aging reactions, but markedly does so with carbonate enzyme. Both reactions exhibit kinetic isotope effects, k_H/k_D, of 2.3–2.4, however. Phosphorylated enzyme incubated in 5 *M* aqueous methanol is observed to undergo a second "aging" reaction, releasing the third mole of *p*-nitrophenol, after which it can be reactivated by strong nucleophiles, contrary to its behavior upon incubation in the absence of methanol. Surprisingly, aged carbonate enzyme is inactive immediately after completion of the aging reaction but spontaneously reactivates thereafter in a much slower reaction step for which pH–rate studies indicate the participation of His-57. These observations suggest that carbonate ester acts as a bifunctional reagent in the active site of α-chymotrypsin. The most probable mechanisms for the chymotrypsin-catalyzed aging reactions of these esters involve nucleophilic participation of the His-57 side chain with the phosphate compound, but a general base role for this group with the carbonate ester.

Labile phosphate esters, as well as esters of carboxylic and carbonic acids, react readily and specifically at the active site of chymotrypsin and other serine proteases. The earliest definitive experiments to identify serine residues as catalytic participants in hydrolyses involved serine derivatization[1a] with ^{32}P-labeled DFP.[1b] Also some of the early kinetic "burst" experiments with neutral *p*-nitrophenyl esters of phosphate, acetate, and carbonate implied formation of acetyl- or phosphoryl–enzyme intermediates.[2,3] The phosphate ester group can usually be removed from serine by strong nucleophiles, thus reactivating the enzyme.[4–8] Certain phosphate compounds, after a period of incubation following enzyme phosphorylation, are no longer removable, however.[4]

The chemical basis for this "aging" reaction with pseudocholinesterase was shown by Berends, *et al.*,[9] to involve conversion of phosphorylated enzyme, a tertiary phosphate ester, into the secondary ester, with concomitant loss of one alcohol group, isopropyl alcohol in the case of DFP (reaction 1). These observations



(1)

invalidated previous suggestions[4,10] that "aging" involved migration or transfer of the phosphate group to a more stable position on the enzyme, *e.g.*, an N–O transfer from the imidazole of histidine to serine. Further experiments indicated similar mechanisms with other enzymes containing serine in their active sites.[11–14] Experiments with chymotrypsin inactivated with diphenyl phosphorochloridate[15,16] indicated that phenol is liberated from the phosphorylated enzyme. No studies on the catalytic groups or mechanism of this process have been carried out, however. The aging in chymotrypsin and acetylcholinesterase[17,18] may be different since in the latter pH and structural data point to a carbonium ion pathway. In any case aging is an important physiological process.

Based on earlier work with elastase,[19] chymotrypsin,[2,3] and trypsin[8] which indicated that the mechanism of phosphorylation is analogous to acylation with carbonyl substrates, one might propose that aging involves a mechanism similar to that involved in deacylation. Indeed, nucleophile-enhanced dephosphorylations[4,8] are observed to share certain similarities of behavior with respect to pH dependence with deacylations.[20] This research investigates these mechanis-

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(1) (a) A. K. Balls and E. F. Jansen, *Advan. Enzymol.*, 13, 321 (1952); (b) abbreviations: DFP, diisopropyl fluorophosphate; NP, *p*-nitrophenyl; NPOH, *p*-nitrophenol; $(NPO)_2CO$, bis(*p*-nitrophenyl) carbonate; $(NPO)_3PO$, tris(*p*-nitrophenyl) phosphate; MINA, monoisonitrosoacetone.

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tic analogies and seeks to elucidate details of chymotrypsin aging reactions[21] with selected phosphate esters and with the analogous carbon esters.

Experimental Section

Materials. α-Chymotrypsin was a Worthington Biochemicals product, lots no. 6114-5 and 6140-1. Tris(*p*-nitrophenyl) phosphate was synthesized according to the procedures of Rapp[22] and Hoeflake[23] by reaction of phosphorous oxychloride with anhydrous sodium *p*-nitrophenoxide in benzene. After filtration of precipitated NaCl and removal of solvent *in vacuo*, two recrystallizations from glacial acetic acid gave white crystals, mp 154–156°, lit.[23] mp 148–155°. Bis(*p*-nitrophenyl) carbonate and methyl *p*-nitrophenyl carbonate were prepared by similar procedures from phosgene and methyl chloroformate, with mp 139–141° (lit. mp 141–142°) and mp 109–111° (lit. mp 114°), respectively.[23] Diphenyl phosphorochloridate was an Aldrich Co. product, used without further purification. Dimethyl carbonate, prepared from phosgene reacted in excess methanol, was purified by distillation, bp 90°.

p-Nitrophenyl *N*-carbobenzyloxy-L-tyrosinate was a Mann Research Labs product, mp 156° (lit.[24] mp 157°). *p*-Nitrophenyl *N*-carbobenzyloxyglycinate was a Cyclo Chemical Co. product, mp 126–127°, lit.[25] mp 128°. *N-trans*-Cinnamoylimidazole was a Mann product, recrystallized from dry hexane, mp 133–134°. Diazotized *p*-nitroaniline was freshly prepared immediately prior to use according to the procedures of Lee and Turnbull.[26] Phenol, a Baker Analyzed reagent, was sublimed and dried *in vacuo*. Proflavin (3,6-diaminoacridinium monohydrogen sulfate), an Aldrich product, was recrystallized in the dark from water. Crystals were subsequently kept from air and light. Stock solutions were remade weekly, or as soon as any turbidity could be detected. 1,2-Propanedione-1-oxime was prepared according to the procedures of Freon,[27] mp 66–67°, lit. mp 67–69°. Formohydroxamate was prepared according to the procedures of Hickinbottom,[28] mp 73°, lit. mp 72–74°.

"Spectrograde" methanol was an Eastman product. Acetonitrile used for preparation of substrate stock solutions was a Mallinckrodt "Nanograde" reagent. Deuterium oxide was a Volk Chemicals product, lot 20280, rated at 99.64% enrichment. These solvents were used as delivered. All buffers were prepared from Baker reagent grade chemicals in water doubly distilled from glass according to published procedures.[29,30]

Enzyme Concentration and Activities. Spectrophotometric titrations and turnover kinetic assays[31] were carried out with cinnamoylimidazole ($\Delta\epsilon_{335} = 8.95 \times 10^3$, pH 5) and *p*-nitrophenyl *N*-CBZ-L-tyrosinate or *N*-CBZ-L-glycinate, respectively. Stock enzyme solutions, generally quite concentrated ($\geq 10^{-3}$ *M*), were prepared in pH 5 acetate buffer and stored at 5°.

Instruments and Techniques. pH measurements were made to ±0.002 unit with either a Radiometer Model 4c meter with G-200B and K-100 electrodes, or a Model 26 meter with a Sargent S-30070-10 combination electrode, standardized against phthalate and other standard buffers. Calculations of pH with added methanol[32] and in D_2O solutions[33] were made by published methods. In the latter pD = pH + 0.40 was used. Temperature dependence of pH readings was predicted from published data.[34] All weighings were made to ±0.1 mg with a Mettler B6H26 analytical balance. All melting points are uncorrected.

Kinetic measurements on the seconds time scale were made with Cary 14 or Beckman DB instruments, the latter with a Sargent SRLG recorder. Cell compartments and cuvette holders were thermostated to ±0.1° of the desired value (usually 25°). A stopped flow spectrophotometer and an oscillograph recorder[35] were used to observe kinetics on the millisecond time scale. Kinetics of pH changes were observed with the recording pH meter described elsewhere.[36]

Maximum solubilities of *p*-nitrophenyl phosphate and carbonate esters were determined by turbidity measurements at 600 nm to be *ca.* 4×10^{-6} *M* and *ca.* 10^{-5}–10^{-4} *M*, respectively. Excess enzyme (25–250×) was used in phosphate aging reaction experiments to assure completion of phosphorylation prior to aging, but equal levels of enzyme and the carbonate ester were used. The presence of free enzyme active sites was monitored with proflavin dye[37] at 465 nm by difference spectrophotometry. A Beer's law plot for phenol coupled to diazotized *p*-nitroaniline was determined according to Lee and Turnbull.[26]

Other techniques are described in the text with the experimental results. All kinetic constants are reported in time units of seconds or reciprocal seconds.

Results

A. Phosphate Ester Reactions. Aging with Diphenyl Phosphochloridate Inhibited Chymotrypsin. The results reported by Lee and Turnbull[15,16] were first confirmed. α-Chymotrypsin (4×10^{-5} *M*) was allowed to react with diphenyl phosphorochloridate (6×10^{-5} *M*) at pH 8.0 and 25°. The hydrolase activity of the enzyme, assayed with *p*-nitrophenyl *N*-CBZ-L-tyrosinate, was inhibited 100% quite rapidly. The phosphorylated enzyme then released 1 mol of phenol in a reaction with first-order kinetics. The phenol-releasing reaction was detected by sampling and coupling the phenol product with 0.10 m*M* diazotized *p*-nitroaniline and observing the product at 480 nm. Appropriate blanks and corrections for side reactions of the coupling reagent were carried out. The $t_{1/2}$ of the reaction at pH 8.0, 25°, was *ca.* 200 sec, in reasonable agreement with Lee and Turnbull's data. The "aged" enzyme could not be reactivated by strong nucleophiles.

Rates of Spontaneous (Nonenzymatic) Hydrolysis. If one postulates that the observed rate of hydrolysis, k_0, is the sum of three rates, $k_0 = k_W[H_2O] + k_B[OH^-] + k_A[H_3O^+]$, then in pure aqueous solution with catalysis by buffer ion negligible, for tris(*p*-nitrophenyl) phosphate in the range of pH 3–7, it was observed that $k_W[H_2O] \simeq 10^{-3}$ sec^{-1}, but $k_A[H_3O^+]$ and $k_B[OH^-]$ were negligibly small. Above pH 8, $k_B = 10^3$ M^{-1} sec^{-1}, so $k_B[OH^-]$ predominated and $k_A[H_3O^+]$ and $k_W[H_2O]$ were negligibly small. It was important to determine these rates at various pH values so as to select conditions for the enzymic aging reactions at which spontaneous hydrolysis could be neglected or prevented from interfering. Buffer effects were negligible.

Rates of Phosphorylation and Aging. The reaction of tris(*p*-nitrophenyl) phosphate with chymotrypsin is biphasic, as shown by the spectrophotometer trace shown in Figure 1. Enzyme was considerably in excess of phosphate ester in these experiments to ensure that phosphorylation was several-fold faster than and

(21) Although the classical definition of "aging" is conversion of phosphorylated enzyme to a form which resists reactivation by nucleophiles or other reagents, the term is used in this paper with a more general meaning to avoid verbosity and for clarity in certain situations. With the compounds used here, we use "aging" to mean a dearylation reaction of the phosphorylated or carbonate-derivatized enzyme. The reader should keep this special usage in mind.

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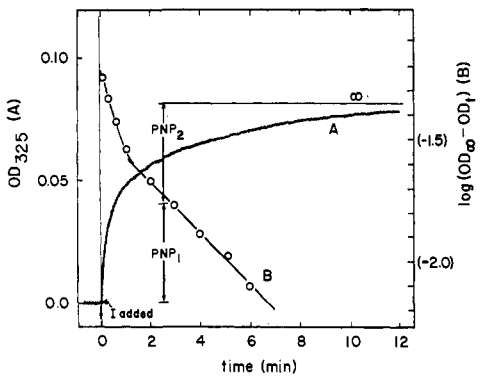


Figure 1. A typical reaction profile (curve A) for the phosphorylation and aging reactions with α-chymotrypsin (4.45×10^{-6} *M*) and tris(*p*-nitrophenyl) phosphate, demonstrating their kinetic separability (curve B). Reaction conditions: 3.0 ml, pH 3.80, of citrate buffer, 0.05 *M*, ionic strength μ = 0.20 *M*. The right-hand axis refers to the first-order plot of the kinetic data (circles, curve B).

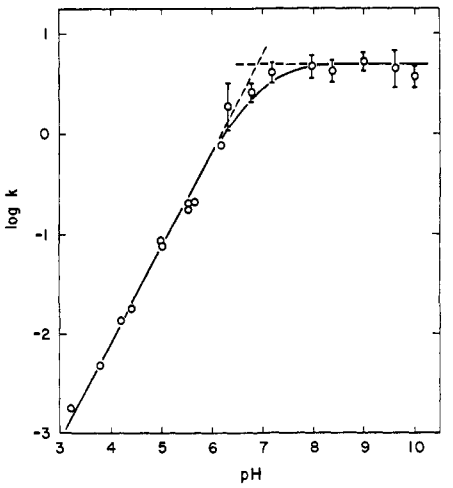


Figure 2. The pH–log rate dependence for the first-order aging reaction of tris(*p*-nitrophenyl) phosphate inhibited α-chymotrypsin, 25°. All buffers were 0.05 *M*, μ = 0.20 *M*. *k* is in sec^{-1}. The solid line is a theoretical curve derived from eq 2 with pK_a = 6.80.

therefore kinetically separable from the aging reaction which follows it. The first-order plot of the kinetic data in Figure 1 indicates this was accomplished and that each reaction was first order and involved stoichiometric release of 1 mol of *p*-nitrophenol/mol of phosphoryl–enzyme formed. This kinetic separation of phosphorylation and aging could be accomplished at all pH values in the range 3–10. The aging rate is independent of enzyme concentration when $[E_0] \gg [I_0]$, where I is phosphate ester, indicating complete formation of the E·I complex.

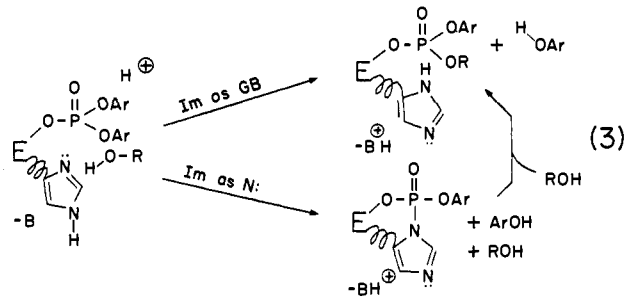
Because phosphorylation is somewhat analogous to acylation with ester substrates, this reaction was studied with two phosphate esters which do not undergo aging, diethyl *p*-nitrophenyl phosphate (Paraoxon) and its thiophosphate analog, called Parathion. With Paraoxon and chymotrypsin each at 10^{-4} *M*, the pH dependence of phosphorylation indicated a sigmoidal curve with $pK_a \approx 6.4$–6.5 and a limiting or maximal rate at pH 9.55 of 4.62×10^{-3} sec^{-1}. Parathion behaved in a similar manner but was some 70-fold less reactive than Paraoxon under the same conditions.

pH Dependence of Aging. The data in Figure 2 indicate catalysis of the aging reaction in tris(*p*-nitrophenyl) phosphate inhibited α-chymotrypsin by a single ionizable group with pK_a near 6.9, active in its deprotonated state. At 25°, the average maximal first-order kinetic constant k_{lim} = 4.5 sec^{-1}. The solid curve in this and other figures was predicted using eq 2.

$$\log\left(\frac{k_{lim}}{k_{obsd}} - 1\right) = pK_a - pH \qquad (2)$$

The resemblance of this pH–rate profile to those already determined for substrate deacylation and turnover reactions of α-chymotrypsin is striking. If one assumes as a working hypothesis that the same catalytic group, namely the imidazole of histidine-57, is operative in both reaction types, one may then inquire further into its exact role in the reaction.

Effects of D_2O and CH_3OH. To determine whether a proton is directly transferred in the rate-limiting step, and also to prove whether the imidazole is acting as a general base with water or directly attacking the tertiary phosphate ester as a nucleophile, aging reactions were carried out in pure (>99.6%) D_2O solutions and in methanol–water mixtures. If water is not directly involved in the step which releases *p*-nitrophenol and imidazole acts as a nucleophile toward the phosphate ester, then added methanol should cause no rate enhancement. However, if imidazole does act as a general base, removing a proton from water (or methanol) which in turn attacks the phosphoryl group, such rate enhancement is expected with added methanol (a better nucleophile than water). Reaction 3 outlines these alternatives.



(3)

Complete pH– and pD–rate profiles of aging with tris(*p*-nitrophenyl) phosphate inhibited chymotrypsin were determined at 10° with a Cary 14 spectrophotometer. These data are presented in Table I. Compari-

Table I. pH– and pD–Rate Dependence for the Aging Reaction of Tris(*p*-nitrophenyl) Phosphate Inhibited α-Chymotrypsin, 10°

Buffer[a]	pH	10^3k, sec^{-1}
Acetate	5.36	1.1
Phosphate	6.42	8.7
Phosphate	7.02	14.0
Phosphate	7.44	15.4
Borate	8.45	28.0
Borate	9.50	18.0
Phosphate	6.45[b]	2.9
Phosphate	7.83[b]	7.7
Borate	8.66[b]	7.9
Borate	8.90[b]	9.0

[a] 0.05 *M*, *I* = 0.1–0.2 *M*. [b] pD = pH_{obsd} + 0.40.

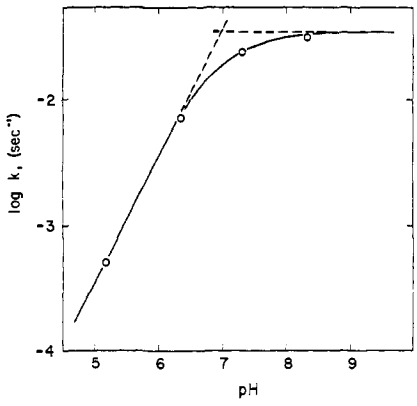


Figure 3. The pH–log rate dependence of the first-order release of the third mole (per mole of phosphorylated enzyme) from tris(*p*-nitrophenyl) phosphate inhibited α-chymotrypsin, aged and observed in 20% CH_3OH–H_2O, 25°. This step is referred to as the "second aging." The solid line is a theoretical curve derived from eq 2 with $pK_a = 7.00$.

son of the average maximal rates at high pH, determined by plots of pH dependence of k, indicates a $k_H/k_D = 2.4 \pm 0.2$.

Variation of added methanol concentration up to 4.0 *M* at pH 4.00, 25°, resulted in only a 1.6-fold overall enhancement of the aging rate. This enhancement is in contrast to deacylation rate enhancements of up to tenfold[38] normally observed with 4–5 *M* methanol.

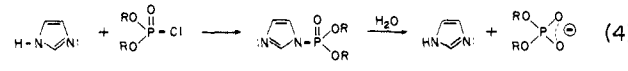
The Second Aging Reaction. Although added methanol enhances the aging rate very little, its presence in the reaction mixture was observed to have a mechanistically important consequence: tris(*p*-nitrophenyl) phosphate inhibited chymotrypsin, aged in 5 *M* methanol and allowed to incubate in that solution, is observed to release, stoichiometrically, the *third* mole of *p*-nitrophenol, *i.e.*, undergo a second aging step. Although the rate of this reaction is much slower than the rate of the first aging, enzyme aged in pure water does not undergo this reaction at all, even upon addition of 5 *M* methanol after the first aging step. This observation implies that eventual methanolysis of the phosphoryl group does occur and that the tertiary neutral phosphate ester is preserved, in contrast to formation of a monoanion (reaction 1).

The pH–rate dependence of this second aging step is essentially identical in shape with that of the first, as a comparison of Figures 2 and 3 indicates, except that the maximal rate constant k_{lim} is 0.05 sec^{-1}, about 100 times slower than that for the first aging step, and that the p*K* of the profile is near 7.0. These data imply that the same catalytic group is free and active in both reactions.

Reactivation of Methanol-Incubated Enzyme. If a second aging step is possible, methanolysis must occur so as to free imidazole and preserve the tertiary ester, $EOPO(OCH_3)(ONP)$. Aging of this compound then logically should produce $EOPO(OCH_3)_2$ in 5 *M* methanol. To test this prediction, reactivation experiments were carried out on phosphorylated enzyme aged and incubated in methanol. The strong nucleophiles chosen for this study were monoisonitrosoacetone (MINA) and formylhydroxamate. After aging and incubation, methanol was removed with a Sephadex G-25 column and enzyme activity assayed. Then 1 *M* nucleophile was added, the pH was adjusted to 7.8, and phosphorylated enzyme was incubated for 48 hr at 25°. Nucleophile was removed on Sephadex and enzyme activity again assayed.

Incubation with MINA produced little or no reactivated enzyme under these conditions, but formylhydroxamate had a considerable effect. The overall procedure yielded 16.5% of the total initial activity, which is at least half of the expected total, as will be discussed.

Model Reactions. Since the above data indicate a nucleophilic role for imidazole toward the serine-bound tertiary phosphate ester group, an attempt was made *via* a model reaction to observe spectrophotometrically the formation and hydrolysis of an intermediate in the reaction between 0.1 *M* imidazole and diethyl phosphochloridate (0.01 *M*). At 236 nm, at pH 7.8, 0.05 *M* phosphate buffer, 25°, an intermediate, presumably that shown in reaction 4, is readily observable, with



spectral properties quite distinct from either products or reactants determined in separate experiments. Kinetics, *via* difference spectrophotometry as a function of pH, reveal that the rate of formation is dependent upon deprotonated (neutral) imidazole, and exhibits a sigmoidal dependence profile with p*K ca.* 7. The rate of formation of the intermediate is quite kinetically distinct and separable from the rate of subsequent hydrolysis.[39]

Model reactions were carried out at pH (or pD) 8.00, 25°, with diethyl *p*-nitrophenyl phosphate and 1 *M* imidazole in either H_2O or D_2O (>99%), each of these in either the presence or absence of 5 *M* methanol. It was found that the added methanol did not cause any discernible rate enhancement in either case. It is significant, however, that an effect of $k_{H_2O}/k_{D_2O} = 1.80$ was observable.

B. Carbonate Ester Reactions. Rates of Spontaneous Hydrolysis. Above pH 2 no significant contribution of the $k_A[H_3O^+]$ term for bis(*p*-nitrophenyl) carbonate was observable, in analogy to the behavior of the phosphate ester analog. Hydrolysis at 25° releases 2 mol of *p*-nitrophenol in a single step, monophasic to at least 3 half-lives in the range of pH 2–8, with $k_W[H_2O] \simeq 5 \times 10^{-4}$ sec^{-1}. Similar behavior at 50° and acid pH values was reported by Fife and McMahon,[40] in addition to a kinetic isotope effect of $k_H/k_D = 2.88$ in 0.1 *M* acid. Interestingly, further experiments[41] indicated that imidazole catalyzes overall hydrolysis of the carbonate ester *via* a nucleophilic mechanism with formation of a detectable imidazole *p*-nitrophenyl carbamate intermediate.

Rates of Acylation and Carbonate Aging. The acylation reaction between α-chymotrypsin and bis(*p*-nitrophenyl) carbonate is quite rapid with $[E_0] = [S_0] = 10^{-4}$ *M*, and clearly is kinetically separable from spontaneous processes or the subsequent carbonate aging reaction. As observed for the phosphate ester, the

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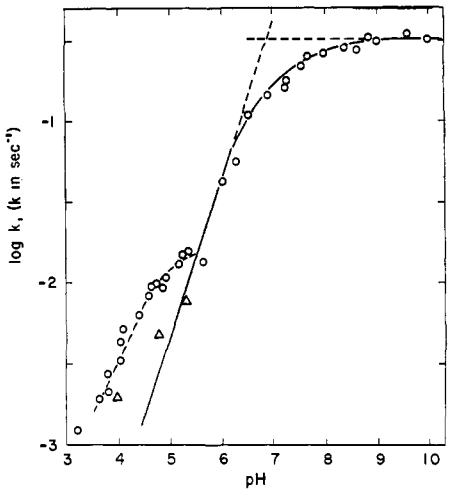


Figure 4. The pH–log rate dependence for the first-order carbonate-aging reaction of bis(*p*-nitrophenyl) carbonate inhibited α-chymotrypsin, 25°. Buffer concentrations were 0.05 *M*, with μ = 0.20 *M* (circles) and μ = 1.00 *M* (triangles). The solid line is a theoretical curve derived from eq 2 with pK_a = 6.90.

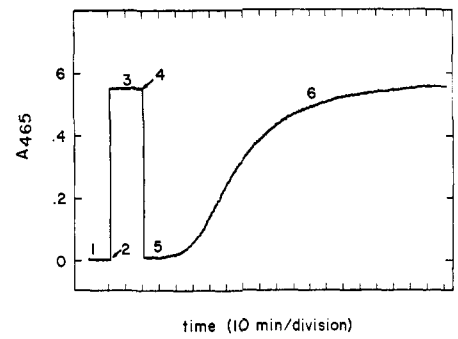


Figure 5. A reaction profile for α-chymotrypsin and bis(*p*-nitrophenyl) carbonate, as monitored by proflavin dye binding, 25°, pH 6.40, phosphate buffer, 0.05 *M*, μ = 0.2 *M*. Spectral shifts in dye *vs.* dye plus enzyme were observed at 465 nm: region 1, E + buffer *vs.* buffer; region 2, dye added to enzyme and reference cuvettes; region 3, E–dye complex *vs.* dye; region 4, bis(*p*-nitrophenyl) carbonate added, equal to [E]; region 5, E–dye complex abolished, and carbonate aging reaction complete within 5 min without restoration of free active enzyme capable of binding proflavin; and region 6, after a lag, reappearance of E–dye complex and enzyme activity in a first-order process.

observed biphasic spectrophotometric traces are quite analogous to that shown in Figure 1. Acylation stoichiometrically releases 1 mol of *p*-nitrophenol/mol of enzyme. The pH dependence of the kinetics of acylation was not studied further.

The carbonate aging reaction is observed as the release of the second mole of *p*-nitrophenol from bis(*p*-nitrophenyl) carbonate inhibited chymotrypsin, with first-order kinetics. These kinetics depend upon pH in the manner shown in Figure 4. The kinetically observed pK_a = 6.9 for the sigmoidal pH–rate profile again implies, as a working hypothesis, catalysis by the imidazole of histidine-57.

Table II. pH- and pD-Rate Dependence for the Aging Reaction of Bis(*p*-nitrophenyl) Carbonate Inhibited α-Chymotrypsin, 25°

Buffer[a]	pH	10^3k, sec^{-1}
Citrate	4.60	9.30
Acetate	5.60	19.0
Phosphate	6.23	57.0
Phosphate	6.88	144
Phosphate	7.22	178
Phosphate	7.70	250
Borate	8.67	275
Borate	10.00	320
Acetate	5.36[c]	3.50
Acetate	6.13[c]	15.6
Phosphate	6.58[c]	32.2
Phosphate	7.21[c]	84.7
Phosphate	7.86[c]	128
Borate	8.27[c]	128
Borate	8.53[c]	141
Borate	8.98[c]	155

[a] 0.05 *M*, I = 0.2 *M* unless otherwise noted. [b] 0.50 *M*, I = 0.5 *M*. [c] pD = pH + 0.40.

Below pH 5 the kinetic data of Figure 4 show a marked dependence upon ionic strength, with large positive deviations from the solid line of slope 1.0 at low (0.2 *M*) ionic strengths. Partial restoration to the theoretical values at higher ionic strength is observed.

Effects of D_2O and CH_3OH. Information about the role that the group of pK = 6.9 plays in catalysis and the nature of the rate-determining step was sought by substitution of D_2O for H_2O and addition of methanol to the reaction mixtures. The effect of D_2O is shown by the data of Table II: comparison of average k_{lim} values at high pH reveals a k_H/k_D of 2.3 ± 0.2.

In contrast to the kinetics of phosphate ester aging, bis(*p*-nitrophenyl) carbonate inhibited chymotrypsin aging rates are enhanced about sevenfold at 5.0 *M* methanol at pH 6.00, 25°. These data, presented in Table III, imply that water is involved in the rate-determining step of carbonate aging. Further, the k_H/k_D effect implies that proton transfer is also involved.

Table III. Kinetics of the Aging Reaction of Bis(*p*-nitrophenyl) Carbonate Inhibited α-Chymotrypsin with Various Concentrations of Added Methanol[a]

MeOH	$k_{obsd} \times 10^{-1}$, sec^{-1}	$k_4(N) \times 10^{-1}$, sec^{-1} [b]
0.00	0.46 = k_3	0.00
1.26	1.17	0.71
2.52	1.91	1.45
3.78	2.46	2.00
5.04	3.16	2.70

[a] 25°, pH 6.00, 0.5 *M* phosphate buffer, μ = 0.2 *M*. [b] $k_{obsd} = k_3 + k_4(N)$, $k_4(N) = k_{obsd} - k_3$.

Kinetics of Activity and Binding Capacity after Aging. Proflavin dye, which binds only to free chymotrypsin active sites,[37] was used to monitor inhibition and recovery of active sites upon addition of bis(*p*-nitrophenyl) carbonate to enzyme. This led to a somewhat surprising observation (Figure 5). Acylation with carbonate ester abolishes the enzyme–dye complex as expected, but when aging has been completed, essentially no free, active enzyme has reappeared. Thus, reactivation does not parallel aging, but occurs after aging in a process some 50-fold slower, designated region 6 in Figure 5. The rate of recovery of proflavin binding exactly paralleled the rate of recovery of enzyme activity, determined by means of sampling and specific substrate assay procedures with *N*-CBZ-L-tyrosine *p*-nitrophenyl ester, or by *in situ* turnover assays using *N*-CBZ-glycine *p*-nitrophenyl ester.

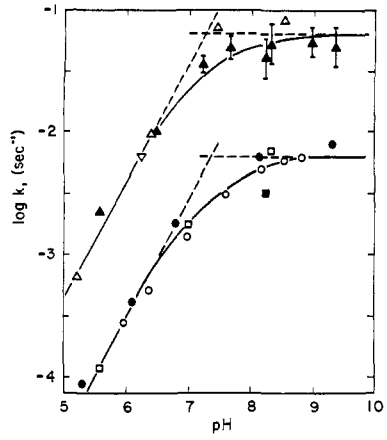


Figure 6. The pH–log rate dependence for the first-order reappearance of active, proflavin-binding α-chymotrypsin after acylation and aging with carbonate substrates, assayed by the techniques outlined below, 25°. Lower curve (H_2O alone): open circles, restoration of proflavin binding (*cf.* Figure 5, region 6) after reaction with bis(*p*-nitrophenyl) carbonate; closed circles, turnover of methyl *p*-nitrophenyl carbonate; open squares, time-dependent samplings and activity assays with specific substrates; closed square, turnover of bis(*p*-nitrophenyl) carbonate. Upper curve (20% CH_3OH–H_2O, v/v): open triangles, restoration of proflavin binding (*cf.* Figure 5, region 6) after reaction with bis(*p*-nitrophenyl) carbonate; closed triangles, turnover of methyl *p*-nitrophenyl carbonate; inverted open triangle, time-dependent sampling and activity assays with specific substrates. The solid lines are theoretical curves calculated from eq 2 with pK's $\cong$ 7.3 and 7.4 for the upper and lower curves, respectively.

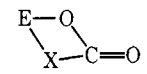
pH Dependence of Activity Recovery and Turnover Kinetics. A series of different assay techniques was employed to probe the nature of the intermediate formed in the carbonate aging reaction. Variations in pH, in added methanol concentration, and in type of carbonate ester substrate were carried out (Figure 6).

The pH–rate profiles for reactivation (Figure 6) imply that an ionizable group of pK = 7.2–7.3 is involved catalytically, as with carbonate aging, and that 5 *M* methanol enhances the rate observed in pure water (lower curve) by some 15-fold (upper curve). These data argue for general base catalysis by a histidine side chain.

Rate enhancement by added methanol implies formation of methyl carbonate enzyme; therefore, acylation and turnover studied with methyl *p*-nitrophenyl carbonate as substrate were carried out in the absence (closed circles) and presence (closed triangles) of 5 *M* methanol. Interestingly these rates are identical with those observed for *turnover* of bis(*p*-nitrophenyl) carbonate and recovery of activity or of proflavin binding under the same conditions.

Isolation and Identification of the Dimethyl Carbonate Product. One logical extension of the data in Figure 6 is that turnover in 5 *M* methanol of either bis(*p*-nitrophenyl) carbonate or methyl *p*-nitrophenyl carbonate inhibited chymotrypsin should produce dimethyl carbonate. This prediction was tested at pH 7.8 by carrying out extensive turnover of 10^{-3} *M* chymotrypsin in 5 *M* methanol with successive aliquots of bis(*p*-nitrophenyl) carbonate added to the limit of solubility. Extraction of the 5-ml reaction solution with three 5-ml portions of ether was then carried out. Evaporation of the ether left a relatively less volatile residue which was distilled *in vacuo* and inspected by vapor phase chromatography and infrared spectroscopy. Comparison of the vpc behavior with authentic dimethyl carbonate synthesized from methanol and phosgene gave identical retention times of the major (>95%) component under several different conditions of column temperature and gas flow rate. The infrared spectra were also identical and exhibited characteristic absorption bands for dimethyl carbonate.

Attempts to Observe or Trap Intermediates. As will be discussed below, one interpretation of the above results is that bis(*p*-nitrophenyl) carbonate acts as a bifunctional reagent in the active site of chymotrypsin. Thus, following acylation, the aging reaction may involve nucleophilic participation of a protein side-chain group X to form a compound denoted

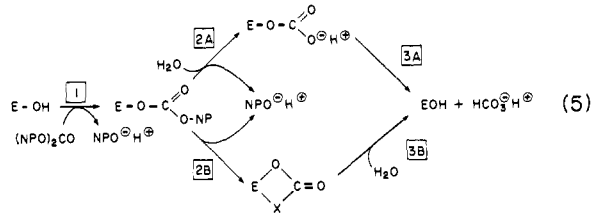


Several different types of experiments were carried out to try to identify group X.

Reaction of α-chymotrypsin with methyl chloroformate transiently abolishes its ability to bind proflavin dye, just as was observed for the *p*-nitrophenyl carbonate esters. However, difference spectroscopy at 230 nm revealed no significant spectral changes which might give clues to the nature of group X.

In another experiment, chymotrypsin actively turning over bis(*p*-nitrophenyl) carbonate at pH 7.8 in the presence of 0.1 *M* hydroxylamine failed to produce any colorimetrically detectable levels of hydroxamate. Acidic ferric chloride solution was added to stop reaction, and should have detected $\gtrsim$0.1 m*M* hydroxamate, either free in solution or enzyme bound. Under the acidic conditions, however, HONHCOOH, if formed, might be too unstable for detection. Enzyme precipitation might have prevented detection of ECONHOH.

Proton Release Studies. Consideration of the different pathways possible for acylation, aging, and reactivation with bis(*p*-nitrophenyl) carbonate and chymotrypsin is presented in reaction 5. In addition, one



(5)

must consider whether acylation itself produces proton uptake due to enzyme conformational changes at pH 9.3 as is known to occur with other substrates and inhibitors.[36] At a carefully selected pH, the number and kinetics of protons released in each step can serve to distinguish between the two major pathways. Table IV presents the results of such comparisons, with the X group in various states of protonation or ionization, at pH 9.3.

For example, in path 1-2A-3A in reaction 5, acylation with $(NPO)_2CO$ and release of NPOH above its pK_a results in release of one proton from the NPOH (step 1, reaction 5); aging results in release of another NPOH and proton, plus ionization of the resulting carbonate monoester and hence two protons overall (step

Table IV. Comparison of the Number of Protons Released in These Kinetics with Spectrophotometric Assay Kinetics[a] for the Reaction of Bis(*p*-nitrophenyl) Carbonate with α-Chymotrypsin[b]

	No. of protons (pH 9.3)		
	Acylation	Aging	Reactivation
Models			
(a) path 1-2A-3A	1	2	0
(b) path 1-2A-3A with high pH Δ conformation[c]	0	2	1
(c) path 1-2B-3B			
X = X^-	1	0	2
X = XH	1	1	1
X = XH_2^+	1	2	0
(d) Path 1-2B-3B with high pH Δ conformation[c]			
X = X^-	0	0	3
X = XH	0	1	2
X = XH_2^+	0	2	1
Observed			
release of NPOH in aging[d]	k = >1.0 sec^{-1}	0.32 sec^{-1}	0.0065 sec^{-1}
kinetics of proton release, sec^{-1}			0.0058 sec^{-1}
no. of protons released	0	0	2.8

[a] See Figure 6. [b] pH 9.30, 25°. See reaction 4 in text. [c] Assuming protonation of the pK = 8.8 group is maintained until step 3. [d] *Cf.* Figures 4 and 6.

2A, reaction 5); hydrolysis of the carbonate enzyme monoester (step 3A) results in no change in the ionization state of any groups. If, however, for this same pathway a conformational change in the enzyme occurs upon acylation, such that proton uptake by a pK = 8.8 group occurs in step 1, and is reversed in step 3A, the proton release pattern is altered: the proton released by acylation (step 1) upon release of the first NPOH is taken up, so net proton release is zero. The pattern in step 2A is the same, but the reversal of the conformational change in step 3A upon formation of free enzyme results in release of the third proton. Similar logic was used to derive the proton release pattern for pathway 1-2B-3B.

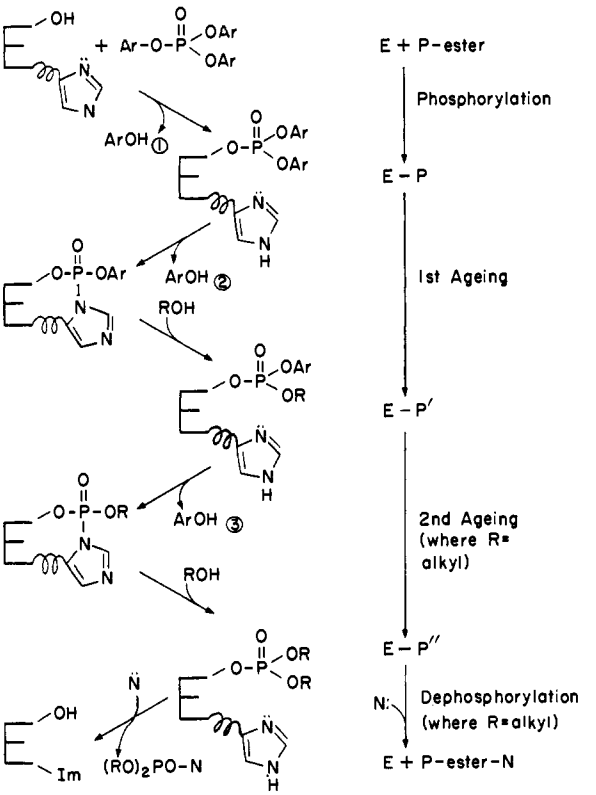
The experimentally observed proton release pattern and the kinetics thereof, determined with a recording pH meter, seem to imply that a group X is involved, that enzyme–monocarbonate is not a likely intermediate, that X exists as an anion at pH 9.3, and that acylation results in proton uptake which is maintained until reactivation occurs.

Discussion

There are several rather striking differences in the mechanisms of aging between the phosphate and carbonate esters, and in comparison of these processes to the spontaneous or model reactions. In both enzymic reactions, the pK = 7 group, presumably the imidazole moiety of histidine-57, is involved. However, the data of Figures 2 and 4, and Tables I–III and V, indicate that the imidazole acts as a nucleophile toward the tertiary enzyme–phosphate ester, as it does in model reactions. The role of imidazole toward enzyme–carbonate ester, however, is apparently as a general base, whereas imidazole in solution has been shown to attack bis(*p*-nitrophenyl) carbonate directly as a nucleophile.[39,40]

These differences in role for imidazole have implications for the types of reaction intermediates one may propose. Reaction Scheme I presents the pathway

Scheme I



which seems most consistent with the data for the reaction of chymotrypsin with tris(*p*-nitrophenyl) phosphate.

Phosphorylation, as proven for DFP and similar phosphate esters, will derivatize the β-OH of serine-

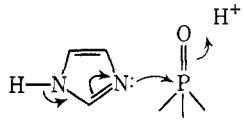
Table V. Effect of Increasing Methanol Concentration on the Rate of Aging with Tris(*p*-nitrophenyl) Phosphate Inhibited Chymotrypsin at pH 4.00[a]

[CH_3OH], M	k/k_0
0	1.00
1.25	1.20
2.50	1.45
3.75	1.55

[a] 0.5 M citrate; μ = 0.5 M at 25°. k_0 is the rate in the absence of methanol, k the observed rate with added methanol.

195.[42] Not surprisingly the rate of phosphorylation is markedly slowed upon substitution of less electron-withdrawing sulfur for oxygen, as shown by comparison of the phosphorylation rates with Paraoxon and Parathion. The pH–rate dependence of phosphorylation indicates the participation of imidazole, but its exact role is undefined at this time.

The model aging reaction between imidazole and diethyl phosphochloridate, observed by ultraviolet difference spectroscopy, indicates that diethyl phosphorylimidazole is a kinetically isolable and stable intermediate, implying that imidazole acts preferentially as nucleophile and leaving group in such reactions. In the enzymic case, this is the first proven instance of such a role for this crucial side-chain functional group in the active site of chymotrypsin. Heterocyclic amines, in particular imidazole, possess unique nucleophilicity toward phosphoryl phosphorus as Cox and Ramsey[43a] and others[43b-d] have pointed out. Furthermore, the product of such a nucleophilic displacement shown in reactions 3 or 4, for example, is particularly reactive and resembles creatine phosphate.[44] In contrast to this behavior for imidazole and tertiary amines, recent studies indicate that primary amines behave as general bases toward DFP.[45] The kinetic effect of substituting D_2O for H_2O in the reaction of Paraoxon with imidazole is appreciable but of such magnitude (1.80) that it is unclear whether this arises from solvent interactions or transfer of a proton from imidazole, as in the concerted reaction



In either case the nucleophilic role of imidazole in phosphate aging is still consistent with a k_H/k_D of 1.5–2.5.

The second phosphate aging step, observed only in the presence of added (5 *M*) methanol, implies that eventual methanolysis does occur. The extent of release of the third mole of *p*-nitrophenol ($\geq 90\%$) implies that the cyclic imidazole intermediate from $EOPO(ONP)_2$ is kinetically partitioned $\geq 9:1$ between methanol and water, respectively. Whether this ratio is still maintained with the cyclic intermediate derived from $EOPO(OCH_3)(ONP)$ is uncertain. The extent of reactivation of $EOPO(OCH_3)_2$ with nucleophiles implies that it must be $\geq 1:1$, as discussed below.

As Cunningham[46] and Erlanger[47] report, strong nucleophiles can lead to "induced aging," depending upon the relative lability of the alcohol groups on the tertiary enzyme phosphate ester. In $EOPO(OCH_3)_2$ the enzyme serine-195 group and methoxy groups are estimated to have approximately equal leaving group capacities,[48] other factors being equal. Thus, one would expect added nucleophile to yield one-third free enzyme and two-thirds secondary (anionic) enzyme phosphate ester, the latter *via* an "induced aging." Therefore, one would expect to get only 33% recovery of total original enzyme activity *via* nucleophilic reactivation. The observed extent of reactivation was 16.5%, or half of "theoretical" (if 33% is an accurate value). It is not unreasonable to expect that half the enzymic activity could be lost through spontaneous denaturation or autolysis upon exposure to high levels of organic solvents and strong nucleophiles at pH 8 for 48 hr at 25°. The fact that the second phosphate aging step is some 100-fold slower than the first is most likely due to substitution of the less electron-withdrawing methyl group for *p*-nitrophenyl. The formation of an unreactive stereoisomer in $EOPO(OCH_3)(ONP)$ is also a possibility.

In conclusion, the kinetic and extrakinetic data are consistent with the mechanism and intermediates proposed in Scheme I.

The kinetic data for the carbonate aging reaction are most consistent with imidazole of histidine-57 acting as a general base, with a proton transfer directly involved in the rate-determining step of this reaction. Most surprising was the observation that aged carbonate-inhibited enzyme regained its activity and binding ability at a rate some 100-fold slower than the rate of aging at a given pH. This raises the question of whether water is involved directly in carbonate aging, and whether the rate enhancements with added methanol can be explained alternatively.

If one proposed that carbonate aging in water produces an enzyme monocarbonate species, $EOCOO^-$, one must argue that rather unusual conditions are maintained in the active site to explain the stability of this species, especially at low pH. This seems unlikely since the active site of α-chymotrypsin is known to be rather open and accessible to solvent.[49,50] Both Faurholt[51] and Miller and Case[52] have shown that protonation of a monoalkyl carbonate ester ($pK \sim 6.3$–6.5) renders it quite unstable and enhances its rate of hydrolysis markedly. In contrast to this, the data of Figures 5 and 6 vividly demonstrate the low pH stability of the inactive carbonate-aged enzyme species.

The kinetically derived $pK = 7.2$–7.4 of Figure 6 for the rate of reactivation argues that the imidazole of histidine-57 is free to act in the catalysis and is not involved in an imidazole–carbonate intermediate. The kinetic data of Figure 6 also appear to imply that the reactivation rate for bis(*p*-nitrophenyl) carbonate inhibited enzyme is identical with that for methyl *p*-nitrophenyl carbonate inhibited enzyme, either in H_2O or in 5 *M* methanol–H_2O solution. This identity of rates could be explained either by (a) EOCOONP and $EOCOOCH_3$, leading to formation of a common intermediate, or (b) formation of different intermediates, EOCOX and $EOCOOCH_3$, respectively, with nearly identical rates of breakdown. Interestingly, the turnover rate

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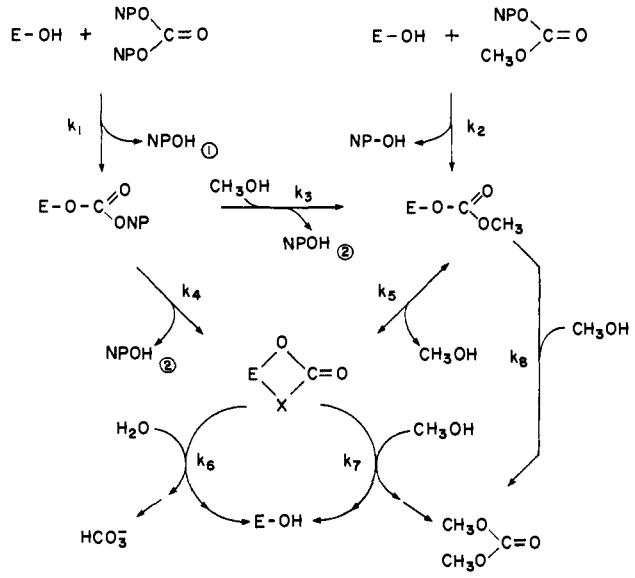
for methyl *p*-nitrophenyl carbonate inhibited α-chymotrypsin, $EOCOOCH_3$, is almost identical with that reported[53] for the deacylation rate of the acyl enzyme, propionyl-α-chymotrypsin, and $EOCOCH_2CH_3$, which are sterically somewhat similar. The various reaction intermediates most consistent with these data and hypotheses are presented in Scheme II.

Scheme II



That overall aging and reactivation in 5 *M* methanol are observed to produce isolable amounts of dimethyl carbonate lends support to steps k_3, k_7, and k_8 of Scheme II.

Although the identity of the protein group X proposed to participate in the reactions of Scheme II remains to be determined, the data at hand can be used to argue against several of the possible types of groups. Chemical and X-ray crystallographic studies have shown that chymotrypsin contains no free sulfhydryl groups. Lack of ultraviolet spectral perturbations upon aging argues against side-chain groups of tyrosine, cysteine, methionine, or histidine.

α- or ε-amino groups are not likely candidates, based on previous observations that carbamylated enzyme is essentially nonreactivatable without strong nucleophiles.[54] From our data, the reactivation process is twice as sensitive to added methanol as aging. This is inconsistent with a carbamate–enzyme intermediate, which would be expected to be less sensitive.

Other possible side-chain groups include the carboxylates of aspartate and glutamate and the hydroxyls of serine and threonine. The proton release experiments of Table IV suggest the former type of group. Participation of a carboxylate may explain the strong positive deviations in aging rate, found to be sensitive to ionic strength, observed in Figure 4. The resulting mixed acyl–carbonate anhydride EOCOOCO is expected to be at least as sensitive to added methanol as its precursor EOCOONP. The shift in p*K* from 6.9 to 7.4 observed kinetically (Figures 4 and 6), comparing aging and reactivation processes, might also be explained by the altered electronics of such an intermediate.[55] Alternatively, change in polarity and solvation of the active site upon aging are also viable explanations for this p*K* shift.

Based on crystallographic models of chymotrypsin, it is not at all obvious which anionic, presumable carboxylate, side-chain groups might be involved.[50] Those of aspartate-102 or -194 appear either too far away or to require major alterations in active-site stereochemistry to participate.

Elucidation and proof of the identity of the –X species in the aged carbonate–inhibited enzyme obviously await further careful experimentation, including model kinetic systems and methods of trapping or derivatizing and identifying the moieties involved.

In summary, the data for the phosphate compound are rather definitive and strongly suggest nucleophilic participation by the side chain of His-57 as shown in Scheme I. In contrast, the results with the carbonate compound are rather less definitive, particularly in regard to the active-site groups involved in the overall aging and reactivation processes. In the rate-limiting step of aging, nonetheless, it seems clear that the side-chain group of His-57 acts as a general base. Defining the other groups involved is an area which clearly requires additional research.

Acknowledgments. The authors are pleased to acknowledge support for this research from Grant No. HEO 5726 of the National Institutes of Health. F. C. W. wishes to acknowledge support as an NIH Predoctoral Fellow, 1965–1968, and through a departmental grant to Rensselaer from the National Science Foundation (Grant No. GU 3182).

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