High Pressure Studies. XVI. Hydrolysis of *p*-Nitrophenyl Esters Catalyzed by α-Chymotrypsin[1,2]

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Abstract: The hydrolysis rates of the *p*-nitrophenyl esters of acetic, isobutyric, and trimethylacetic acids are accelerated by pressure using 0.05 *M* Tris buffer. Under these conditions, the first two esters react to a measurable extent with Tris as well as hydroxide. Apparent activation volumes for hydroxide and Tris catalysis are: (ester, ΔV^*_{OH}, ΔV^*_{Tris}) acetate, −3 cc/mol, −17 cc/mol; isobutyrate, −14, −21; trimethylacetate, −11, unknown. The values of ΔV^*_{OH} parallel volume changes for ionization of the corresponding carboxylic acids and hydration of the homologous aldehydes. These results are compared and discussed. The values of ΔV^*_{OH} are contrasted with similar results for a series of alkyl acetates. While the tetrahedral intermediates from this latter group of esters may return to starting ester, this is unlikely with the *p*-nitrophenyl esters, suggesting ambiguities in the interpretation of values of ΔV^*_{OH} for ester hydrolysis. These data are used to determine the effects of pressure on α-chymotrypsin-catalyzed hydrolysis of the same esters. The enzymatic rates are slightly pressure accelerated but in a different order than found for base-catalyzed hydrolysis: (ester, ΔV^*_{cat}) acetate, −6 cc/mol; isobutyrate, −3; trimethylacetate, −2. The values of ΔV^*_{cat} are proposed to correspond to the volume changes for deacylation of the corresponding acyl-enzymes. Direct measurement of the pressure effect on the rate of deacylation of indoleacryloyl-α-chymotrypsin is −7 cc/mol. The result are discussed in terms of models for deacylation suggested by steady-state kinetic studies and X-ray studies.

Pressure effects on solution phase reactions reflect differences in apparent volumes of reactants, transition states, and products. These volume changes in turn arise from the breaking and making of chemical bonds, changes in molecular conformation, and changes in solvation.[3] Recently, we became interested in studying pressure effects on reactions of proteins in the hope of learning more about conformation changes involved in enzyme reactions and protein denaturation. As a prelude to these studies, we determined the effects of pressure on the pK_a of several common buffers used in studies of protein reactions.[4] These data have already been utilized in a study of protein denaturation,[5] and the need to recognize that pressure can markedly affect the pK_a of buffers has been demonstrated.[6]

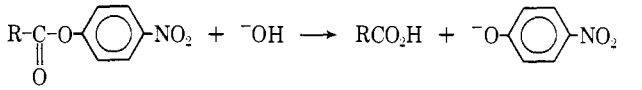
This report describes our initial investigations of enzyme-catalyzed reactions. We have examined the effect of pressure on the base-catalyzed hydrolyses of the *p*-nitrophenyl esters of acetic, isobutyric, and trimethylacetic acids and used these data to extract the pressure dependences of the enzymatic hydrolyses of these esters by α-chymotrypsin under comparable conditions.

The nonenzymatic hydrolysis rates of these esters are increased by pressure, and the activation volumes parallel reaction volumes recently determined for hydration of the homologous aldehydes.[7] These data for ester hydrolysis are contrasted with those from other pressure studies of ester hydrolysis and indicate that certain mechanistic complexities have previously been ignored. Pressure also increases the enzymatic hydrolysis rates. However, the accelerations are small and the substituent effect on the catalysis activation volumes is opposite to that observed in the nonenzymic hydrolyses.

Pressure studies of other enzyme-substrate reactions have been reported,[6b,8] but this study is unique in that a series of similar substrates have been examined under the same conditions. Care has also been taken to try to control or assess the full range of special problems associated with pressure studies of enzymatic reactions.[9]

Results and Discussion

Determination of ΔV^*_{cat}. The hydrolyses of *p*-nitrophenyl acetate (**1a**), *p*-nitrophenyl isobutyrate (**1b**), and *p*-nitrophenyl trimethylacetate (**1c**) were followed by continuously monitoring the appearance of *p*-nitrophenoxide ion at 400 nm while hydrolysis was progressing under pressure. The reactions were run using Tris buffer since the ionization constant K_a (eq 2), while sensitive to temperature variation, does not change significantly with pressure.[4] The molar absorptivity (ε) of *p*-nitrophenoxide decreases slightly with increasing pressure, and corrections for this as well as for compression of the medium were included in the calculations of the hydrolysis rate constants.[4]



1a, R = CH_3
b, R = $(CH_3)_2CH$
c, R = $(CH_3)_3C$ (1)

$$(HOCH_2)_3CNH_3^+ + H_2O \overset{K_a}{\rightleftharpoons} (HOCH_2)_3CNH_2 + H_3O^+ \quad (2)$$

Pseudo-first-order rate constants k_1 obtained from nonenzymatic ester hydrolyses were used to extract values of k_{cat} (eq 3) for hydrolysis of the same esters by α-chymotrypsin (Table I). Values of $k_{cat}(E)_0$ were obtained as the difference between the measured initial rates of product formation $(dP/dt)_0$ and the calculated quantities $k_1(ester)_0$. In all cases, the latter term was small compared to $k_{cat}(E)_0$.

$$-d(ester)/dt = k_1(ester) + k_{cat}(E) \quad (3)$$

While k_1 increases substantially with pressure, the increases in k_{cat} (Figure 1) are less dramatic. The apparent volume changes associated with enzymatic ester hydrolysis (ΔV^*_{cat}) are rather small (Table II), and they also vary with R in a rather different way than the values for nonenzymatic hydroxide catalyzed hydrolysis (ΔV^*_{OH}) of the same esters (Table II).

Base-Catalyzed Ester Hydrolysis. The values of k_1 do not include just the hydroxide ion catalyzed hydrolysis of the esters. In the case of *p*-nitrophenyl acetate, it has been shown that the free amine Tris reacts with the ester, and k_1 is described by eq 4.[10] The value of k_{Tris} is substantially

$$k_1 = k_{OH}(OH) + k_{Tris}(Tris) \quad (4)$$

less than k_{OH}, but under the conditions of our experiments

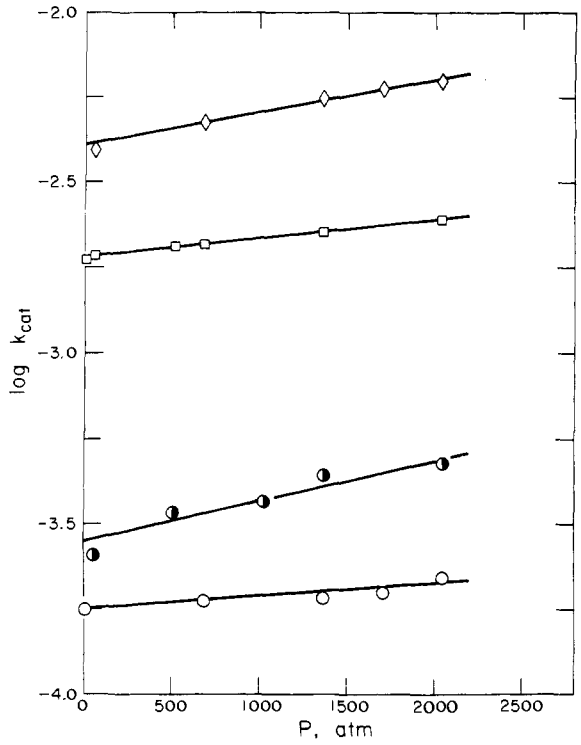


Figure 1. Pressure dependence of log k_{cat} for enzymatic hydrolysis of *p*-nitrophenyl acetate (◇), isobutyrate (□), and trimethylacetate (○) and for deacylation of indoleacryloyl-α-chymotrypsin (◑).

where (Tris) ≫ (OH), it turns out that the two terms contribute about equally to k_1 (Table III).

We determined low- and high-pressure values of k_1 for the acetate and isobutyrate using 0.005 *M* Tris buffer so that separate values of k_{OH} and k_{Tris} could be estimated at each pressure for which k_1 values were available using 0.05 *M* Tris.[11] Values of k_{OH}/k_{Tris} were calculated from the data in Table III assuming that log k_{OH}/k_{Tris} varies linearly with pressure. This is equivalent to making the relatively conservative assumption that the *difference* in activation volumes for Tris and hydroxide catalysis is pressure invariant.

In order to calculate the values of k_{OH} and k_{Tris}, it was necessary to correct both the concentration of hydroxide and Tris for compression of the solution. Additionally, in the case of hydroxide the increase in the ionization constant of water (eq 5) with increasing pressure had to be consid-

$$H_2O \overset{K_W}{\rightleftharpoons} H^+ + \bar{O}H \qquad (5)$$

ered.[12] The hydroxide ion concentration depends both on K_W and K_a (eq 6), and since the pressure dependence of K_a

$$(OH^-) = (K_W/K_a)((B)/(BH)) \qquad (6)$$

for Tris is nil,[4] the pressure variation of (OH) is almost entirely determined by $\partial(K_W)/\partial P$.

While Tris catalysis is an important contributor to k_1 for *p*-nitrophenyl acetate, it is substantially less important for the isobutyrate (Table III). This must reflect steric hindrance of the isopropyl group toward attack at carbonyl by the bulky neutral amine Tris (see eq 2). Although data are not available, it seems unlikely that Tris catalysis is significant in hydrolysis of *p*-nitrophenyl trimethylacetate, and it has been assumed in this case that $k_{OH} = k_1/(OH)$.

The activation volumes ΔV^*_{OH} and ΔV^*_{Tris} (Table II) were obtained from plots of k_{OH} and k_{Tris} (Figures 2 and 3). Values of ΔV^*_{Tris} are larger in magnitude than those of ΔV^*_{OH} for both esters for which data are available. It has generally been observed that when two processes compete with each other, the more sterically unfavorable process gets the biggest assist by application of hydrostatic pressure,[3c] and the relative values of ΔV^*_{Tris} and ΔV^*_{OH} are consistent with this.

The trend in values of ΔV^*_{OH} for the different esters parallels data for the hydration of homologous aldehydes (eq 7)[7] and the ionization constants of the corresponding carboxylic acids (eq 8)[13] (Table IV). Wolfenden suggested

$$RCHO + H_2O \rightleftharpoons RCH(OH)_2 \qquad (7)$$

$$RCO_2H \rightleftharpoons RCO_2^- + H^+ \qquad (8)$$

that aldehyde hydration might be an appropriate model for ester hydrolysis, and this correspondence seems to bear out his prediction. In all three sets of data a "low" value of ΔV for R = CH_3 is evident. Both Wolfenden and Kauzmann

Table I. Rate Constants for Hydrolysis of *p*-Nitrophenyl Esters (RCO_2-*p*-NO_2Ph) with and without α-Chymotrypsin in 0.05 *M* Tris Buffer[a]

Pressure, atm	R[b]	*T*, °C	pH[c]	10^5k_1, sec^{-1}	10^5k_{cat}, sec^{-1}
51	CH_3	20	7.84	2.58	397
681				4.44	479
1021				5.62	
1362				7.25	563
1702				9.64	596
2042				12.4	629
1	$(CH_3)_2CH$	20	7.84	0.747	188
51				0.665	192
511				1.54	206
681				2.02	208
1021				2.70	
1361				3.71	226
1702				4.88	
2042				7.72	245
1	$(CH_3)_3C$	25	7.70[d]	1.97[d]	17.9
681				4.64	18.8
851				6.28	
1021				6.38	
1362				10.5	19.2
1702				14.2	20.0
2042				18.3	22.2
1	IA-α-ChT[e]	20	7.29		20.6[f]
51					25.6
511					34.3
1021					37.2
1361					44.3
2042					47.9

[a] Errors in values of k_1 and k_{cat} are estimated to be about ±5 and ±10%, respectively. [b] $(Acetate)_0 = 2.53 \times 10^{-5}$ *M*, $E_0 = 3.7$ or 7.2×10^{-7} *M*, $K_m(app) = 1.59 \times 10^{-6}$ *M* (25°, pH 7.8);[g] $(isobutyrate)_0 = 3.00 \times 10^{-5}$ *M*, $E_0 = 1.8 \times 10^{-6}$ *M*, $K_m(app)$ not available; $(trimethylacetate)_0 = 2.02 \times 10^{-5}$ *M*, $E_0 = 1.9$ or 3.1×10^{-6} *M*, $K_m(app) \simeq 8 \times 10^{-7}$ *M* (25°, pH 8.2).[h] [c] Measured at the temperature *T*. [d] Nonenzymic hydrolysis of *p*-nitrophenyl trimethylacetate was studied at pH 8.86; values of k_1 reported here were calculated from values obtained at pH 8.86 assuming $k_1 = k_{OH}(OH)$ (see text). [e] Indoleacryloylimidazole. [f] First-order rate constants for deacylation of IA-α-ChT; errors *ca.* ±5%. [g] F. J. Kezdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962). [h] G. A. Hamilton and M. L. Bender, *J. Amer. Chem. Soc.*, **84**, 2570 (1962).

Table II. Activation Volumes for Enzymic and Nonenzymic Hydrolysis of *p*-Nitrophenyl Esters in 0.05 *M* Tris Buffer[a,b]

R	*T*, °C	ΔV^*_{cat}[c]	ΔV^*_{OH}[c]	ΔV^*_{Tris}[c]
CH_3	20	−6	−3	−17
$(CH_3)_2CH$	20	−3	−14	−21
$(CH_3)_3C$	25	−2	−11	
IA-α-ChT	20	−7		

[a] For conditions, see Table I. [b] Activation volumes were calculated from the slopes of the straight lines (solid or dashed) in Figures 1 and 2. [c] Units of cc/mol.

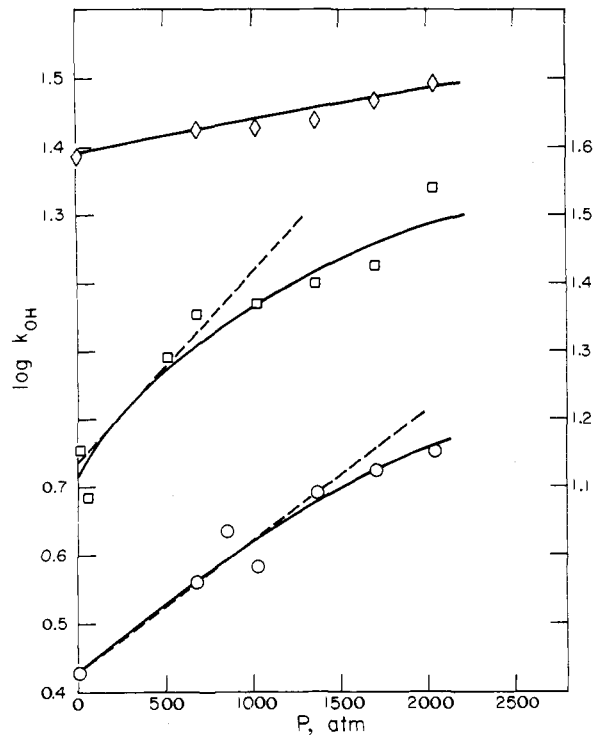


Figure 2. Pressure dependence of log k_{OH} for nonenzymatic hydrolysis of *p*-nitrophenyl acetate (◇, left scale), isobutyrate (□, right scale), and trimethylacetate (○, left scale).

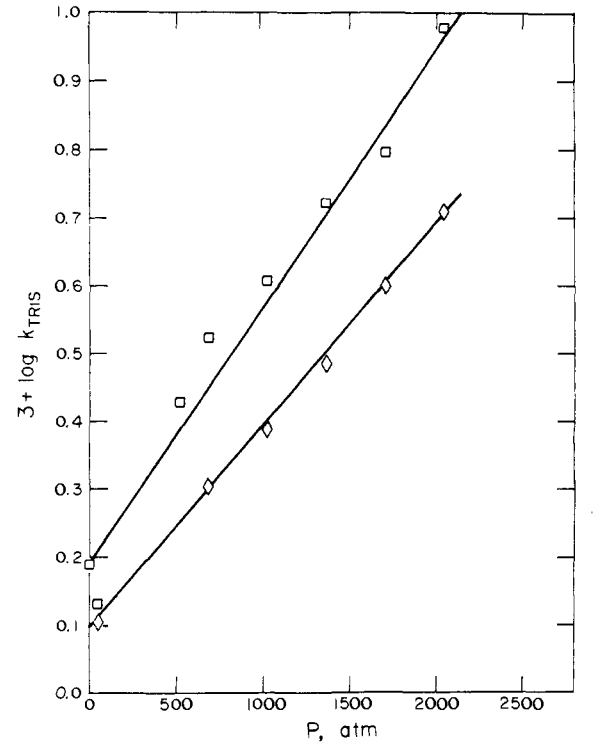


Figure 3. Pressure dependence of log k_{Tris} for *p*-nitrophenyl acetate (◇) and isobutyrate (□, points displaced upward by 1.35 log units).

Table III. Relative Rate Constants for Reaction of *p*-Nitrophenyl Esters (RCO_2-*p*-NO_2Ph) with Hydroxide and with Tris (20°)

Pressure, atm	R	k_{OH}/k_{Tris}[a] × 10^{-4}	k_{OH} (OH)/k_{Tris} (Tris)[c]
1	CH_3	1.98	0.86
2042		0.61	1.1
1	$(CH_3)_2CH$	(20.6)[b]	(9.0)[b]
681		15.2	11.2
2042		8.1	14.5

[a] Calculated from k_1 data using both 0.05 and 0.005 Tris buffer solutions. [b] Extrapolated from data at 681 and 2042 atm assuming log (k_{OH}/k_{Tris}) varies linearly with pressure (see text). [c] 0.05 *M* Tris, pH 7.8.

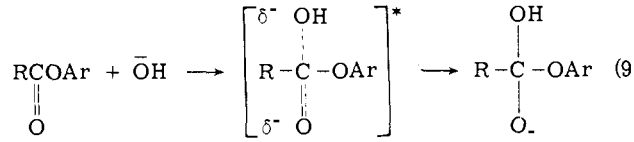
Table IV. Comparison of Activation Volumes for Ester Hydrolysis with Reaction Volumes for Aldehyde Hydration and Carboxylic Acid Ionization

R	RCO_2Ar[a] ΔV^*_{OH}[d]	RCHO[b] $\Delta V_{hydration}$[d]	RCO_2H[c] $\Delta V_{Ionization}$[d]
CH_3	−3	−8	−11
CH_3CH_2		−12	−13
$(CH_3)_2CH$	−14	−13	−14
$(CH_3)_3C$	−11	−12	(−18)[e]

[a] From Table II. [b] Reference 7. [c] Reference 13 [d] Units of cc/mol. [e] This number may be a little less negative.[13]

have noted this trend and generally ascribed it to effects of variation of R on the structure of water. In the case of ester hydrolysis, we suggest that it primarily reflects steric hindrance to solvation of the *p*-nitrophenyl isobutyrate and trimethylacetate compared with *p*-nitrophenyl acetate.

Values of ΔV^*_{OH} correspond to the volume change associated with the formation of the transition state leading to the tetrahedral intermediate from the esters and hydroxide ion (eq 9). In the absence of solvation considerations, bimolecular reactions are expected to have negative ΔV's because the molar volume of the transition state should be less

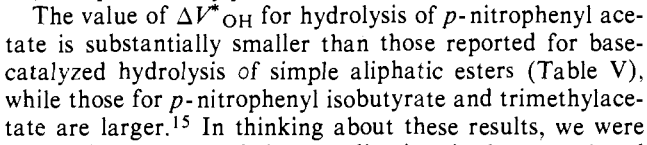


(9)

than the combined volumes of the reactants.[3] In ester hydrolysis, the decrease in volume could be offset by a volume increase associated with solvation. The hydroxide ion is strongly solvated, and some solvent release might be expected due to charge dispersal in the transition state (eq 9). This is consistent with a relatively "small" value of ΔV^*_{OH} for *p*-nitrophenyl acetate. If the steric bulk of the R group has a greater effect on solvation of the neutral ester than on solvation of the polar transition state, the more negative values of ΔV^*_{OH} for the isobutyrate and trimethylacetate (Table IV) are qualitatively predicted.[14]

The value of ΔV^*_{OH} for hydrolysis of *p*-nitrophenyl acetate is substantially smaller than those reported for base-catalyzed hydrolysis of simple aliphatic esters (Table V), while those for *p*-nitrophenyl isobutyrate and trimethylacetate are larger.[15] In thinking about these results, we were struck by a mechanistic complication in base-catalyzed ester hydrolysis which has not been fully probed in connection with pressure studies.

Oxygen labeling studies suggest that the tetrahedral intermediate (eq 10) in certain esters can revert to starting



(10)

ester (k_{-1}) as well as decompose to products (k_2).[16] Thus, the observed rate constant for ester hydrolysis is not necessarily k_1, but the more complex expression shown in eq 11.

$$k_{obsd} = k_1(k_2/(k_{-1} + k_2)) \quad (11)$$

If so, the pressure dependence of ester hydrolysis (ΔV^*_{obsd}) depends on the effect of pressure on k_{-1}/k_2 (eq 12). It is

Table V. Activation Volumes for Hydroxide Catalysis of Alkyl Acetate (CH_3CO_2R) Hydrolysis[a]

R	T, °C	ΔV^*_{OH}, cc/mol	Ref
CH_3	25	−9.9	15a
CH_3CH_2	25	−8.8	15a
	20	−5.6	15b
$(CH_3)_2CH$	20	−5.6	15b
$CH_3(CH_2)_3$	20	−5.6	15b
$(CH_3)_2CHCH_2$	20	−6.3	15b
$CH_3(CH_2)_4$	20	−5.8	15b

[a] These data taken from ref 7.

$$\Delta V_{obsd}^* = \Delta V_1^* + RT\partial \ln (1 + k_{-1}/k_2)/\partial P \quad (12)$$

possible that k_{-1}/k_2 is significantly greater than zero for the alkyl esters previously studied (Table V) and that in those cases ΔV^*_{obsd} cannot be simply interpreted as being equal to ΔV^*_1.[17] However, in the case of the *p*-nitrophenyl esters studied here, we would expect that k_{-1}/k_2 is essentially zero since $R'O^-$, the stable *p*-nitrophenoxide group, would be expected to leave the tetrahedral intermediate (eq 10) much more readily than ^-OH.[16d] A comparison of the data for the alkyl acetates (Table V) with ΔV^*_{OH} for *p*-nitrophenyl acetate would thus suggest that $\partial \ln (1 + k_{-1}/k_2)/\partial P$ is negative and that k_{-1}/k_2 gets smaller with increasing pressure. We propose to explore these questions in future studies.

Activation Volumes for Enzyme Catalysis. The values of ΔV^*_{cat} presumably reflect the pressure dependence of acyl-enzyme hydrolysis, a process *formally* equivalent to that giving rise to ΔV^*_{OH} in the absence of enzyme. Chymotrypsin-catalyzed hydrolysis of esters **1a–c** follows a pathway (eq 13) involving rapid formation of an enzyme–ester com-

$$E + S \overset{K_m}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_2} E' + P_1 \xrightarrow{k_3} E + P_2 \quad (13)$$

plex (E · S), rapid acylation with concomitant production of *p*-nitrophenol (P_1), and slow hydrolysis of acyl-enzyme (E′) giving carboxylic acid (P_2).[18] If pressure does not substantially alter the relative rates of the various steps (eq 13), $k_{cat} = k_3$ and the values of ΔV^*_{cat} reflect the volume changes associated with deacylation of the respective acyl-enzymes. To test this we prepared indoleacryloyl-α-chymotrypsin[19] and determined the effect of pressure on its deacylation in 0.05 *M* Tris buffer at pH 7.3. Its hydrolysis is pressure accelerated (Table I, Figure 1), and the apparent activation volume of −7 cc/mol is comparable to ΔV^*_{cat} for *p*-nitrophenyl acetate hydrolysis by α-chymotrypsin (Table II).

These data lead to the conclusion that deacylation of acyl-enzymes, formed from nonspecific substrates, is characterized by a small decrease in volume.[20] It has been suggested that the acyl group in such acyl-enzymes has a fair amount of conformational mobility and is not held in a position particularly favorable for attack by water at the carbonyl to form the presumed tetrahedral intermediate.[21] The negative values of ΔV^*_{cat} could thus be interpreted as reflecting the repositioning of the carbonyl oxygen toward the NH groups thought to be involved in stabilizing its developing negative charge through hydrogen bonding.[21b,c]

Deacylation reactions of the isobutyrate and trimethylacetate are significantly slower than for acetyl-chymotrypsin.[21a,22] This may be due to steric constraints imposed by the isopropyl and *tert*-butyl groups preventing full stabilization of the tetrahedral intermediate *via* hydrogen bonding. These steric constraints may be reflected by the smaller volume changes observed (Table II) for these systems.

Since the values of ΔV^*_{cat} for the three *p*-nitrophenyl esters were determined under very similar conditions, their differences cannot be ascribed to variations in the extent of ionization of basic and acidic groups of the protein. Additionally, it is unlikely that the extent of ionization of catalytically important groups has been affected by pressure. While ionization of carboxylic acid groups is increased with pressure,[4] carboxylate groups (*e.g.*, Asp-102) should be completely ionized at all pressures used in these studies given the initial buffer pH of 7.8.[23] The pK_a of the imidazole of His-57, although close to the pH of the buffer,[23] should be relatively unchanged since the pK_a of imidazole itself is virtually insensitive to pressure.[13]

Further study is required to probe the significance of the differences in values of ΔV^*_{cat}. Experiments are now aimed at directly measuring pressure effects on deacylation of the specific acyl-enzymes. In any case, these data seem to suggest that α-chymotrypsin is a rigid enzyme[24] in which little conformational change occurs during at least the deacylation step.

Experimental Section

Materials. *p*-Nitrophenyl acetate (Aldrich Chemical Co.) was recrystallized three times from chloroform–hexane, mp 78–79° (lit.[25] mp 79.5–80°). The plate-like crystals were nearly colorless. *p*-Nitrophenyl isobutyrate was prepared from isobutyryl chloride and *p*-nitrophenol in pyridine.[26] Three recrystallizations from chloroform–hexane gave colorless needles, mp 39–40° (lit.[27] mp 39–40°). *p*-Nitrophenyl trimethylacetate was prepared from trimethylacetyl chloride and *p*-nitrophenol.[26] Recrystallization from chloroform–hexane gave colorless crystals, mp 95–96° (lit.[26] mp 94–95°). The purity of the esters was checked by monitoring the absorption spectrum at 400 nm before and after basic hydrolysis in 0.1 *M* NaOH. A stoichiometric amount of *p*-nitrophenoxide ion was formed.

α-Chymotrypsin (3× crystallized, dialyzed product) obtained from Worthington Biochemical was used as received. Titration with *p*-nitrophenyl acetate indicated at least 80% initial activity.

Indoleacryloylimidazole (IA1) was prepared[19a] from indole acrylic acid (Aldrich) and imidazole (Matheson) using dicyclohexylcarbodiimide (Matheson). Recrystallization from benzene yielded a yellow powder, mp 182–183° (two melting points have been reported:[19a] 182 and 190°).

Tris buffers were prepared from "Tris · HCl" and "Tris Base" obtained from the Sigma Chemical Co.

Ester Hydrolysis. Reaction solutions for nonenzymic hydrolysis studies were prepared by diluting 100–200 μl of a stock solution of the appropriate ester (1.5–2.5 × 10^{-3} *M* in dry acetonitrile) to 10 ml with Tris buffer. For the enzymatic reactions, solutions were prepared by diluting 100–200 μl of the stock ester solutions and 10–75 μl of stock solutions of α-chymotrypsin in Tris buffer (4–6 × 10^{-4} *M*) to 10 ml with Tris buffer. The enzyme stock solutions were assayed using *p*-nitrophenyl acetate immediately before each run. Formation of *p*-nitrophenoxide ion was monitored at 400 nm using a Cary 14 uv–vis spectrophotometer. Kinetic plots were strictly apparent zero order under the conditions employed.

Deacylation of Indoleacryloyl-α-chymotrypsin. Indoleacryloyl-α-chymotrypsin was prepared by addition of 250 μl of IA1 stock solution (5 × 10^{-3} *M* in dry acetonitrile) to excess enzyme in 1.0 ml of pH 5.7 phosphate buffer (0.1 *M*). Solutions of IA-α-ChT (2 × 10^{-5} *M*) were prepared by dilution of 100 μl of the IA-α-ChT solution to 5.0 ml with Tris buffer. Deacylation of IA-α-chymotrypsin was monitored by following a decrease in absorbance at 355 nm. Kinetic plots were strictly first order.

Enzyme Stability under Pressure. Aliquots of *ca.* 2 × 10^{-4} *M* α-chymotrypsin in pH 7.8 Tris buffer (0.05 *M*) were pressurized for varying lengths of time (22°) in the high-pressure optical bomb and assayed after pressure release to determine whether change in activity occurred under the conditions of the ester hydrolysis experiments. In all cases, nonpressurized control samples taken simultaneously from the same stock solution and held for the same time at the reaction temperature were assayed for comparison. Very slow loss of activity was observed at 2000 atm such that after 5 hr under pressure the enzyme solution had lost about 15% of its initial activity compared with the control. There was no loss of ac-

tivity after 1 hr within experimental error, and data used from all enzyme hydrolysis runs were those gathered during the first 30 min of each run. Other data obtained demonstrated that pressure effects on enzyme activity decreased with decreasing pressure.

High-Pressure Optical Bomb. The high-pressure studies were performed in a high-pressure optical bomb, identical with one described previously.[28] The bomb was fitted with a thermostatted jacket and the kinetics followed by positioning the bomb in the sample compartment of a Cary 14 spectrophotometer. The atmospheric pressure studies were performed in standard cuvettes. In all cases, the temperature was controlled to ±0.1° during reaction and spectroscopic analyses.

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References and Notes

(1) (a) Part XV: R. C. Neuman, Jr., and R. P. Pankratz, *J. Amer. Chem. Soc.*, **95**, 8372 (1973). (b) Presented at the Ninth Western Regional Meeting of the American Chemistry Society, San Diego, Calif., Nov 3, 1973, Paper 232.

(2) Support by the National Science Foundation through Grant GP-36003X is gratefully acknowledged.

(3) For recent reviews, see (a) R. C. Neuman, Jr., *Accounts Chem. Res.*, **5**, 381 (1972); (b) C. Eckert, *Annu. Rev. Phys. Chem.*, **23**, 239 (1972); (c) W. J. le Noble, *Progr. Phys. Org. Chem.*, **5**, 207 (1967).

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(6) (a) Besides the use of this information as outlined in ref 5, it is conceivable that the pressure retardation of the activity of fumarase recently reported[6b] is primarily due to a pressure-induced change in pH of the phosphate buffer solutions employed in the study. Fumarase is essentially inactive below pH 6 at atmospheric pressure.[6c] While the pressure study began with pH 6.5 phosphate buffer at 1 atm, at 3000 atm, where it was observed that activity ceased, the pH of that buffer was *ca.* 5.5.[4] (b) B. Andersen and P. E. Broe, *Acta Chem. Scand.*, **26**, 3691 (1972); (c) M. Dixon and E. C. Webb, "Enzymes," 2nd ed, Academic Press, New York, N.Y., 1964, p 446.

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(8) (a) These references do not include studies of pressure effects on enzymes leading to changes in their activity at *atmospheric pressure*.[8b,c] We have shown (see Experimental Section) that this did not occur for α-chymotrypsin *under the conditions* of our kinetic experiments. Most of the references involve steady-state kinetic studies;[8d-g] however, Grieger has recently reported studies of fast reactions of proteins using temperature-jump methods.[8h] (b) For example, see A. L. Curl and E. F. Jansen, *J. Biol. Chem.*, **184**, 45 (1950). (c) For a more recent study, see K. Miyagawa and K. Suzuki, *Rev. Phys. Chem., Jap.*, **32**, 51 (1963); (d) H. Werbin and A. D. McLaren, *Arch. Biochem. Biophys.*, **31**, 285 (1951); **32**, 325 (1951); (e) J. T. Penniston, *Ibid.*, **142**, 322 (1971); (f) W. M. Neville and H. Eyring, *Proc. Nat. Acad. Sci., U.S.*, **69**, 2417 (1972); (g) R. K. Williams and C. Shen, *Arch. Biochem. Biophys.*, **152**, 606 (1972); (h) M. D. Waissbluth and R. A. Grieger, *ibid.*, **159**, 639 (1973).

(9) These include pressure dependence of the buffer pK_a, determination of pressure effects on simultaneous nonenzymic reactions of the substrate, determination of the stability of the protein under the conditions of the pressure runs, control experiments to assess effects of nonequilibrium temperature conditions after pressurization, and the determination of reaction rates by monitoring concentration changes *during* the pressure runs.

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(11) At atmospheric pressure for *p*-nitrophenyl acetate, our value of log (k_{OH}/k_{Tris}) is *ca.* 4.3 (Table III, 20°) while that calculated from data presented by Jencks[10] (25°) is 4.1.

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