Steric Effects in the Deacylation of Acyl-Chymotrypsins*

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ABSTRACT: The deacylation of acyl-α-chymotrypsin derivatives, with the exception of *n*-hexanoyl, shows a normal steric order of reactivity at 25,° increased branching decreasing the rate of enzymatic deacylation greatly. A plot of log (k_3'/k_0) *vs.* the Taft steric constants (E_s) was linear at 25° with a slope of 1.0. The rate of deacylation of *n*-hexanoyl-chymotrypsin deviated greatly from this line, however, being much faster than expected. A plot of ΔH^* *vs.* ΔS^* was linear over a range of 3.0 kcal in ΔH^* and 7.1 eu in ΔS^* for those derivatives that are not highly branched in the acyl group. Positive deviations from the isokinetic line were found for the activation parameters of the trimethylacetyl and 3,3-dimethylbutyryl derivatives, and a large negative deviation was found for deacylation of *n*-hexanoyl-chymotrypsin. The slope of this compensation plot is 435°K. Tris buffer has an accelerating effect on the rate of deacylation of those acyl-chymotrypsins with little steric bulk in the acyl group.

The Tris buffer effect is subject to steric hindrance; in those cases where a high degree of branching is present in the acyl group the effect is small. No acylation could be detected with *p*-nitrophenyl triethylacetate as the substrate.

General base catalysis, involving proton transfer in the transition state, appears generally to be only moderately affected by increasing steric bulk in the catalyzing base (Covitz and Westheimer, 1963; Feather and Gold, 1965). Nucleophilic catalysis of ester hydrolysis by imidazole, however, is strongly retarded by substitution of large groups at the 2 position of imidazole (Bruice and Schmir, 1958). Bulky substituents in the substrate also appear to affect the rate of reactions involving these two mechanisms in a different manner. Increased alkyl group branching in the acyl group has a marked effect on the nucleophile-catalyzed hydrolysis of *p*-nitrophenyl esters (Fife, 1965), producing large rate decreases. These effects parallel those found in the hydroxide ion catalyzed hydrolysis of the ethyl esters of these acids (Evans *et al.*, 1938; Davies and Evans, 1940) and undoubtedly reflect the increased difficulty of attack at the ester carbonyl by the nucleophile. In the imidazole general base catalyzed hydrolysis of *N*-acylimidazoles, however, branching at the α-carbon of the acyl group produces little effect on the rate of hydrolysis, a small acceleration actually being noted (Fife, 1965). Branching at the β-carbon causes rate decreases but to a smaller extent than found for nucleophilic catalysis of ester hydrolysis. If the different patterns of steric effects observed in the hydrolysis of *p*-nitrophenyl esters and *N*-acylimidazoles prove to have generality for reactions proceeding by nucleophilic catalysis and classical general base catalysis, then the study of acyl group branching might serve as a useful approach in the investigation of the mechanism of action of esteratic enzymes since the usual methods for distinguishing between these mechanisms, such as the D_2O solvent isotope effect, can give ambiguous results when applied to enzymatic reactions (Jencks, 1963). The rates of deacylation of a series of sterically hindered acyl-α-chymotrypsin derivatives have, therefore, been measured. The order of reactivity found in these deacylation reactions is of special interest since substrate binding to the enzyme is not a complicating factor as in acylation. If acylation and deacylation proceed *via* identical mechanisms, as proposed (Bender and Kézdy, 1964), then information obtained in the study of one of these reactions should be applicable to both. The deacylation of several acyl-α-chymotrypsins with moderate steric differences in the acyl group has been previously studied (McDonald and Balls, 1957; Bender *et al.*, 1964), and it was found that trimethylacetyl-chymotrypsin deacylates more slowly than acetyl-chymotrypsin. However, only by the study of an extensive series with large structural variations under the same experimental conditions can a quantitative assessment be made of steric influences. This has now been accomplished by employing the E_s steric effects constants (Taft, 1956) to correlate the rate constants and by measuring activation parameters for the reactions.

Experimental Section

Materials. α-Chymotrypsin (three-times crystallized) was obtained from Worthington Biochemical Corp. Acid chlorides were either commercially obtained (Matheson Coleman and Bell) or prepared from the commercially obtained carboxylic acids (K & K Laboratories) by reaction with thionyl chloride. Acetonitrile was Eastman Kodak Spectro Grade which had been twice distilled over P_2O_5 and once over K_2CO_3.

Several of the *p*-nitrophenyl esters were the same as

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previously studied (Fife, 1965). *p*-Nitrophenyl isovalerate was prepared by the same method and boiled at 123° at 1.5 mm, $n_D^{22.3}$ 1.5193, lit. (Kreisky, 1957) bp 158–160° at 6 mm. *p*-Nitrophenyl *n*-hexanoate boiled at 135–138° at 0.8 mm, $n_D^{22.5}$ 1.5178, lit. (Kreisky, 1957) bp 174–175° at 6 mm. *p*-Nitrophenyl triethylacetate was prepared by adding triethylacetyl chloride (16.6 g, 0.1 mole) to a solution of *p*-nitrophenol (14.2 g, 0.1 mole) in 37 ml of pyridine (0.46 mole). The mixture was washed with 5% sodium carbonate solution and then extracted with ether. The ether extract was dried over anhydrous sodium sulfate. The ether was flash evaporated, and the residual liquid was distilled. The product was a yellow liquid that boiled at 139–140° at 0.8 mm, $n_D^{22.5}$ 1.5190. The material solidified when placed in the cold and melted at 45–46.5°. *Anal.* Calcd for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.06; H, 7.25; N, 5.46.

Kinetic Measurements. The rates of deacylation of the acyl-chymotrypsins were obtained by following the appearance of *p*-nitrophenol on a Zeiss PMQ 11 or Gilford Model 2000 recording spectrophotometer at 330 mμ, or *p*-nitrophenolate ion at 400 mμ, after injection of an excess solution of *p*-nitrophenyl ester into the enzyme solution. The steady-state hydrolysis of ester following the initial rapid acylation of the enzyme provides a direct measure of deacylation at substrate concentrations much greater than the apparent K_m. Constant temperature (±0.1°) was maintained during the kinetic runs by circulating water from a Haake Model F circulating bath through a Zeiss constant-temperature cell holder or around the cell compartment of the Beckman DU component of the Gilford. Ester stock solutions were made in acetonitrile at a constant concentration of 3×10^{-3} M for all esters so that "substrate activation" effects (Kézdy and Bender, 1962; Faller and Sturtevant, 1966) were of minimal importance in determining relative rates. A two- to four-fold variation in substrate concentrations produced no significant change in the relative rate ratios. Enzyme solutions were made up at pH 5.05 (acetate buffer, μ = 0.1 M) with a normality of 8×10^{-4} N as determined by the titrimetric procedure of Schonbaum *et al.* (1961). Phosphate buffers were employed for the kinetic measurements at pH values from 5.88 to 7.66, with μ = 0.075 M. A Tris buffer was employed for measurements at pH 8.80. The reaction cuvet contained a final volume of 3.1 ml of solution being 1.6% acetonitrile (3.0 ml of buffer, 0.05 ml of ester solution, and 0.05 ml of enzyme solution). Appropriate corrections were made to compensate for spontaneous hydrolysis of the ester. This was negligible except at higher temperatures and pH. Rates were measured in triplicate at each pH and temperature. The deacylation rate constant was calculated from the slope of the steady-state plot of absorbance *vs.* time by converting to *p*-nitrophenol concentration units and dividing by the enzyme concentration. For the conversion of absorbance to *p*-nitrophenol concentration the extinction coefficient was determined in the same buffer solutions as employed in the measured reactions. These values are reported in Table I.

TABLE I: Extinction Coefficients of *p*-Nitrophenol or *p*-Nitrophenolate Ion in Various Buffers at μ = 0.075 M and 25°.

pH	Buffer	λ	$\epsilon \times 10^{-4}$
8.80	Tris	400	1.67
7.66	Phosphate	400	1.46
7.28	Phosphate	400	1.16
6.20	Phosphate	330	0.762
5.88	Phosphate	330	0.831

Activation energies were calculated by means of a computer program designed to carry out least-squares evaluation of ln k_3' *vs.* $1/T$°K. The output of interest consisted of the regression coefficient, the intercept value, and the correlation coefficient. The errors reported in ΔH^* and ΔS^* were calculated from the standard error of the regression coefficient.

Results

The rate constants for deacylation of acyl-chymotrypsin derivatives at 25° are given in Table II at several pH values. Values of the pH-independent rate constants (k_3) and the apparent dissociation constant of the essential group at the active site were calculated from plots of k_3' *vs.* $k_3'a_H$. These plots were linear with the intercept being k_3 and the slope having the value $-1/K_a'$. The values of pK_a' are reported in Table II. Tris buffer has a pronounced effect on the magnitude of the deacylation rate constant. As can be seen in Table II the value of k_3' for several of the acyl-enzymes at pH 8.80 (Tris buffer) is considerably greater than the value of k_3 obtained from the plot of k_3' *vs.* $k_3'a_H$ where all of the rate measurements were made in phosphate buffers. Faller and Sturtevant (1966) have previously shown that the deacylation of acetyl-α-chymotrypsin is greatly increased by increasing concentrations of Tris. It was calculated by these workers that Tris is 600 times more reactive in deacylation than water or if only the unprotonated species is participating then it is 6000 times more reactive. The effect of Tris on deacylation is subject to a marked steric effect; there is considerably smaller rate acceleration when the acyl group is highly branched as in the case of trimethylacetyl or 3,3-dimethylbutyryl. The values of k_3' obtained by extrapolation to zero Tris concentration are in most cases closely similar to the values of k_3 obtained from the phosphate buffer data and are included in Table II. A typical plot of k_3' *vs.* Tris concentration is shown in Figure 1. Plots of k_3' *vs.* pH are essentially sigmoid for all of the acyl-chymotrypsins, however, it would appear that pK_a' is slightly lower when the acyl group is highly branched as with the trimethylacetyl and 3,3-dimethylbutyryl derivatives.

The order of reactivity is normal at 25° in that increasing steric bulk in the acyl group leads to decreased

TABLE II: Rate Constants for Deacylation of Acyl-α-Chymotrypsins at Various pH Values, 25°, and μ = 0.075 M.

No.	Acyl Group	pH	$k_3' \times 10^4$ sec^{-1}	$k_3 \times 10^4$ sec^{-1}	pK_a'
1	Acetyl	7.66	34.7 ± 2.0		
2	Propionyl	5.88	4.54 ± 0.06	79.2	7.10
		7.28	48.4 ± 0.3		
		7.66	62.1 ± 3.2		
		8.80	110 ± 2.4		
		8.80[a]	71.0		
3	Butyryl	5.88	2.61 ± 0.10	59.8	7.22
		6.20	4.77 ± 0.05		
		7.28	32.3 ± 0.4		
		7.66	43.8 ± 1.3		
		8.80	94.0 ± 0.9		
		8.80[a]	56.0		
4	Hexanoyl	5.88	12.1 ± 0.4	364.0	7.35
		6.20	23.8 ± 0.5		
		7.28	161 ± 5.5		
		7.66	252 ± 13		
		8.80	525 ± 10		
5	Isobutyryl	5.88	1.29 ± 0.06	24.8	7.11
		6.20	3.08 ± 0.12		
		7.28	16.1 ± 0.7		
		7.66	18.2 ± 0.2		
		8.80	28.1 ± 0.6		
		8.80[a]	20.5		
6	Isovaleryl	5.88	0.620 ± 0.037	12.5	7.18
		7.28	6.62 ± 0.04		
		7.66	9.27 ± 0.30		
		8.80	23.8 ± 0.9		
		8.80[a]	15.0		
7	Trimethylacetyl	5.88	0.180 ± 0	1.60	6.75
		6.20	0.336 ± 0.014		
		7.28	1.19 ± 0.02		
		7.66	1.41 ± 0.02		
		8.80	1.87 ± 0.06		
		8.80[a]	1.62		
8	3,3-Dimethylbutyryl	5.88	0.246 ± 0.007	1.50	6.60
		7.28	1.25 ± 0.002		
		7.66	1.34 ± 0.01		
		8.80	1.50 ± 0.01		
		8.80[a]	1.35		

[a] Extrapolated to zero Tris buffer concentration.

rates of deacylation. The reaction can be described by the equation

$$\log \frac{k_3}{k_0} = \rho^*\sigma^* + \delta E_s \qquad (1)$$

The constants σ^* and E_s are the Taft aliphatic substituent constants and steric constants, respectively, for the various acyl groups (Taft, 1956); k_0 is the rate constant for deacylation of acetyl-chymotrypsin.

A δ of 0.96 ± 0.01 was obtained by solving eq 1 by the method of Pavelich and Taft (1957) for values of δ and ρ^* that give the best fit to the data (correlation coefficient = 0.989 excluding *n*-hexanoyl). Although a precise value of δ was obtained, the best estimate of ρ^*

TABLE III: Rate Constants for Deacylation ($k_3' \times 10^4$ sec^{-1}) of Acyl-α-Chymotrypsins at Various Temperatures, and $\mu = 0.075$ M.

Acyl Group	15°	25°	35°	40°
	pH 7.66 (phosphate buffer)			
Acetyl	13.7 ± 1.0	34.7 ± 2.0	86.7 ± 0.5	113[a] ± 4.6
Propionyl	22.3 ± 0.6	62.1 ± 3.2	137 ± 1.1	204 ± 6.9
Butyryl	16.3 ± 0.8	43.8 ± 1.3	107 ± 1.9	147 ± 5.2
Hexanoyl	96.5 ± 1.5	252 ± 13	611 ± 7.4[b]	861 ± 11[c]
Isobutyryl	5.57 ± 0.25	18.2 ± 0.2	40.6 ± 0.4	59.2 ± 1.2
Isovaleryl	2.58 ± 0.05	9.27 ± 0.30	24.4 ± 0.19	34.9 ± 1.0
Trimethylacetyl	0.38 ± 0.01	1.41 ± 0.02	3.56 ± 0.13	5.80 ± 0.08
3,3-Dimethylbutyryl	0.46 ± 0.02	1.34 ± 0.01	2.87 ± 0.15	4.71 ± 0.05
	pH 8.80 (Tris buffer, 0.075 M)			
Propionyl	60.6 ± 1.4	110 ± 2.4	219 ± 2.8	
Butyryl	46.2 ± 1.0	94.0 ± 0.9	195 ± 9.6	267[a]
Isobutyryl	14.1 ± 0.6	28.1 ± 0.6	61.1 ± 0.5	
Trimethylacetyl	0.626 ± 0.015	1.87 ± 0.06	4.28 ± 0.07	

[a] 39.5°. [b] 34.5°. [c] 39.2°.

TABLE IV: Activation Parameters for Deacylation of Acyl-α-Chymotrypsins.

		pH 7.66 (phosphate)		pH 8.80 (Tris, 0.075 M)	
No.	Acyl Group	ΔH^* (kcal/mole)	ΔS^* (eu)[a]	ΔH^* (kcal/mole)	ΔS^* (eu)[a]
1	Acetyl	15.0 ± 0.3	−19.1 ± 1.0	9.7[b]	−36.5
2	Propionyl	15.2 ± 0.3	−17.7 ± 1.0	10.8	−31.2
3	Butyryl	15.3 ± 0.3	−17.9 ± 1.0	12.2	−26.8
4	Hexanoyl	15.7 ± 0.3	−13.1 ± 1.0		
5	Isobutyryl	16.4 ± 0.5	−16.1 ± 1.6	12.5	−28.2
6	Isovaleryl	18.0 ± 0.7	−12.0 ± 2.3		
7	Trimethylacetyl	18.7 ± 0.4	−13.4 ± 1.4	16.7	−19.5
8	3,3-Dimethylbutyryl	16.0 ± 0.4	−22.5 ± 1.3		

[a] Calculated at 25° with the rate constants having the units sec^{-1}. [b] From the data of Bender *et al.* (1964). The buffer was 0.1 M total Tris.

(1.92) contained uncertainty, presumably due to the small range of σ^* constants. It can be shown that δ is fairly insensitive to any value chosen for ρ^*. For example, varying ρ^* from 1.0 to 2.48 changes δ from 1.0 to 0.9. The value of ρ^* in the alkaline hydrolysis of ethyl esters is 2.48 (Taft, 1956). For purposes of illustration, therefore, a plot of log (k_3'/k_0) *vs.* E_s is shown in Figure 2 having a δ of 1.0. The data plotted in Figure 2 were obtained at pH 7.66. A similar plot utilizing the pH-independent constants (k_3) was also linear with a slope of 1.1.

Rates were measured at four temperatures (15, 25, 35, and 40°) (Table III). The values of ΔH^* and ΔS^* are recorded in Table IV. The activation parameters obtained at pH 7.66 are uncorrected for changes in K_a' with temperature. A plot of ΔH^* *vs.* ΔS^* is shown in Figure 3. A straight-line relationship is obtained over a range of 3.0 kcal in ΔH^* and 7.1 eu in ΔS^* having a slope of 435°K. A plot of ΔH^* *vs.* ΔS^* in which the activation parameters have been corrected for changes in K_a' with temperature is shown in Figure 4 taking the ΔH of ionization to be 7 kcal/mole as determined by obtaining K_a' over a temperature range of 25° for the deacylation of acetyl-chymotrypsin (pK_a' was 6.98 at 40° and 7.40 at 15°).[1] Kézdy and Bender (1962) ob-

[1] A value of 11 kcal/mole was previously reported by Cunningham and Brown (1956) for ΔH_i over a temperature range of 11° for hydrolysis of the specific substrate *N*-acetyl-tryptophan ethyl ester.

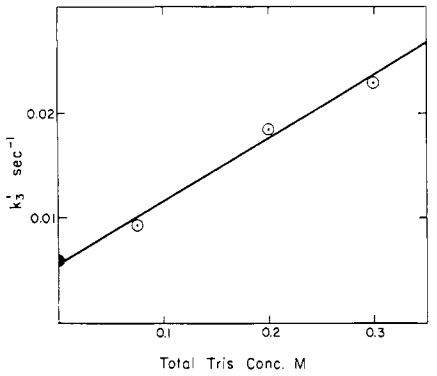

FIGURE 1: A plot of k_3' for deacylation of butyryl-α-chymotrypsin at pH 8.80 at 25° *vs.* the total concentration of Tris buffer.

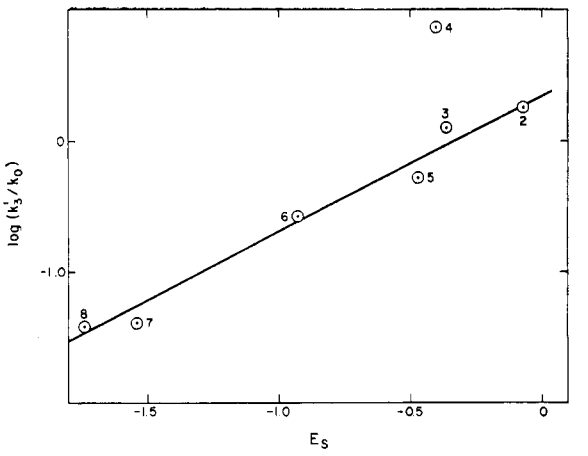

FIGURE 2: Plot of log (k_3'/k_0) *vs.* E_s for deacylation of acyl-α-chymotrypsins at 25°, pH 7.66, and μ = 0.075 M. The numbers pertain to the acyl-enzymes listed in Table II.

tained 7.28 as the value of pK_a' for deacylation of acetyl-chymotrypsin at 25° under conditions similar to those employed in the present study. The corrected activation parameters are reported in Table V. The corrected plot is very similar to Figure 3 in which uncorrected parameters were employed having a slope of 417°K. This is necessarily the case since the values of K_a' are closely similar for all of the acyl-enzymes whose rates of deacylation lie on the isokinetic line. Thus the correction due to the effect of temperature on K_a' is small and nearly the same for all. As a consequence, the slope of the compensation plot is nearly insensitive to the value of ΔH_i. This treatment assumes that there are no large variations in ΔH_i for the different acyl-enzymes, which is very likely in view of the similar K_a' values. It can be seen from Table V that for the trimethylacetyl and 3,3-dimethylbutyryl derivatives the correction is negligible (<1 kcal) while for the remainder of the series it is in the range 1–2 kcal. The points for the highly branched acyl-enzymes fit the isokinetic line considerably better in the corrected plot with that for trimethylacetyl having only a small positive deviation. The point for *n*-hexanoyl, however, still shows a large negative deviation.

Values of ΔS^* and ΔH^* measured in Tris buffer at pH 8.80 where the reaction is pH independent differ considerably from those obtained at pH 7.66 in phosphate buffer, both ΔS^* and ΔH^* being less positive. A portion of these differences can be attributed to the fact that at pH 8.80 ionization of the essential group at the active site is more nearly complete, but it would also appear that for some of the reactions the participation of Tris is affecting the activation parameters. It is of interest that the smallest difference is found for the trimethylacetyl derivative whose rate is not accelerated to any great extent by Tris. The most extensive measurements were therefore made at pH 7.66 in phosphate buffer where the values obtained are a better representation of the relative magnitudes of these parameters for the enzyma-

TABLE V: Activation Parameters for Deacylation of Acyl-α-Chymotrypsins Taking into Account the Heat of Ionization of the Essential Group in the Active Site.

No.	Acyl Group	ΔH^* (kcal/mole)	ΔS^* (eu)
1	Acetyl	13.0	−25.5
2	Propionyl	13.8	−21.8
3	Butyryl	13.5	−23.5
4	Hexanoyl	13.5	−19.8
5	Isobutyryl	14.9	−20.5
6	Isovaleryl	16.3	−17.1
7	Trimethylacetyl	18.0	−15.4
8	3,3-Dimethylbutyryl	15.5	−24.2

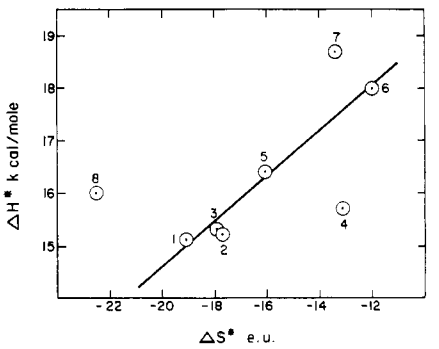

FIGURE 3: Plot of ΔH^* *vs.* ΔS^* for deacylation of acyl-α-chymotrypsins at pH 7.66 and μ = 0.075 M. The numbers pertain to the acyl-enzymes listed in Table IV.

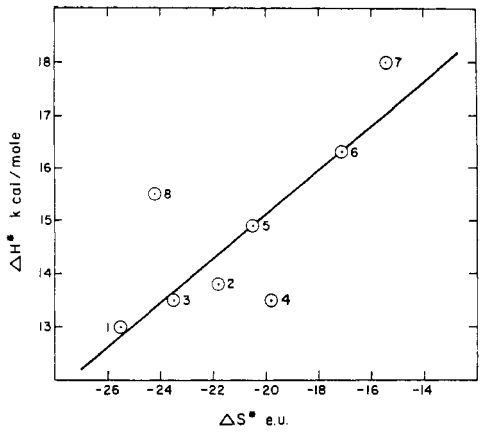

FIGURE 4: Plot of ΔH^* *vs.* ΔS^* for deacylation of acyl-α-chymotrypsins taking into account the heat of ionization of the essential group at the active site of the enzyme. The numbers pertain to the acyl-enzymes listed in Table V.

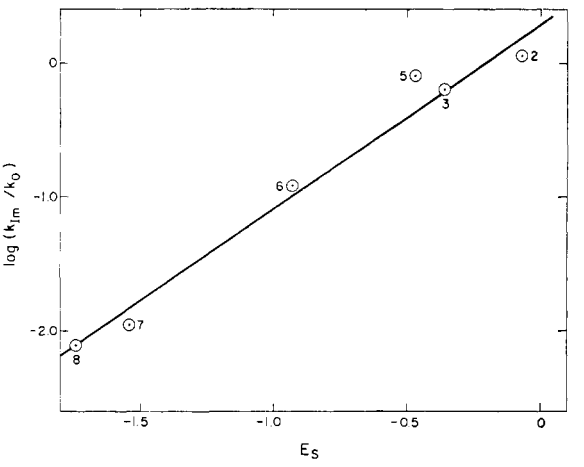

FIGURE 5: Plot of log (k_{Im}/k_0) *vs.* E_s for the imidazole-catalyzed hydrolysis of *p*-nitrophenyl esters at 30° and μ = 1.0 M. The data are those of Fife (1965) except for *p*-nitrophenyl isovalerate, k_{Im} = 6.02 l. mole^{-1} min^{-1} and *p*-nitrophenyl acetate, k_{Im} = 49.5 l. mole^{-1} min^{-1} = k_0 determined in this study. The numbers correspond to the acyl groups listed in Table II.

tic reaction, although it will be noted that the values measured in Tris show the same relative trends.

Acylation of the enzyme could not be achieved when *p*-nitrophenyl triethylacetate was employed. No reaction could be observed after the enzyme and ester solution had stood for 4 hr at 25°. Thus, the triethylacetyl group imposes steric requirements that are too severe for the reaction to occur.

Discussion

The α-chymotrypsin-catalyzed hydrolysis of esters and amides very likely involves initial binding of the substrate to the enzyme followed by acylation of the active site to form an acyl-enzyme intermediate which subsequently hydrolyzes (Gutfreund and Sturtevant, 1956; Kézdy and Bender, 1962).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2'} ES' + P_1 \xrightarrow{k_3'} E + P_2 \quad (2)$$

It has been well established that an acyl-enzyme intermediate is formed during the hydrolysis of both specific and nonspecific ester and amide substrates, and this acyl-enzyme is most likely a serine ester (Bender, 1962; Bender and Kézdy, 1964).

The rates of deacylation of the extensive series of acyl-chymotrypsins reported in Table II shows that at 25° the observed steric effects are quite normal, increased branching at both the α- and β-carbons of the acyl group decreasing the rates greatly. That these rate decreases are primarily reflecting steric hindrance to approach of a nucleophile, with the rate constants not being subject to large steric influences due to the protein environment is shown by the linearity of the plot of log (k_3'/k_0) *vs.* E_s (Figure 2) with slope of 1.0. The steric constants (E_s) were obtained from ester hydrolysis data (Taft, 1956) and can be considered a measure of potential energy and kinetic energy effects resulting from increasing hindrance to nucleophilic attack at the ester carbonyl. Additional influences such as conformational changes induced by interaction of the acyl group with the protein would, therefore, be expected to cause deviations from the observed line unless such interactions produced effects on rate that were also a linear function of the E_s constants which would seem unlikely. Supporting this viewpoint is the fact that the point for the *n*-hexanoyl derivative, which has the fastest rate of deacylation in the series, shows a marked deviation from the line. The fast rate of deacylation of this acyl-enzyme is very likely due to interaction of the long-chain acyl group with a hydrophobic region near the active site, perhaps inducing a conformational change so that unfavorable changes of free energy can be avoided in attaining the transition state. In transesterification reactions the rate has been found to increase with increasing chain length of the primary alcohol (Inward and Jencks, 1965) indicating that binding of the nucleophile occurs at a hydrophobic region adjacent to the active site.

The susceptibility of the deacylation reaction to steric hindrance is less than that shown in the imidazole-catalyzed hydrolysis of *p*-nitrophenyl esters, reactions in which imidazole is functioning as a nucleophile. A plot of log (k_{Im}/k_0) *vs.* E_s for the hydrolysis of *p*-nitrophenyl esters is shown in Figure 5. The slope of this plot is 1.4, as compared to 1.0 for the deacylation reaction. Both of these reactions, however, give normal plots of similar precision in contrast to the abnormal order of reactivity found in the hydrolysis of *N*-acylimidazoles (Fife, 1965), the only nonenzymatic example of a classical

general base catalyzed ester or amide hydrolysis reaction in which steric effects have been systematically studied.

A rate-accelerating effect producing a more favorable ΔS^* due to changes in the acyl group must also be occurring since a plot of ΔH^* *vs.* ΔS^* is linear for all acyl-enzymes except those that are highly branched (trimethylacetyl and 3,3-dimethylbutyryl) and hexanoyl. Thus, small increases in chain length and bulk are causing compensation in the activation parameters, both ΔH^* and ΔS^* becoming more positive. The slope of this isokinetic plot (Leffler, 1955; Leffler and Grunwald, 1963) is 435°K, a temperature far above any that would be experimentally feasible with the enzyme.

Compensation in the activation parameters caused by increasing steric hindrance in the acyl group is not a generally observed phenomenon in ester hydrolysis. Both ΔH^* and ΔS^* apparently become more unfavorable in hydroxide ion and hydronium ion catalyzed hydrolysis of ethyl esters (Taft, 1956), both types of reactions being examples of nucleophile-catalyzed reactions. Steric effects in general base catalyzed ester and amide hydrolysis have not been studied to an extent sufficient to permit assessment of the effect on the activation parameters, although it was found that a small compensation is observed for hydrolysis of *N*-acylimidazolium ions (Fee and Fife, 1966a,b), compounds whose hydrolysis reactions are subject to general catalysis (Jencks and Carriuolo, 1959). Pronounced steric bulk also causes a marked deviation from the isokinetic line in those reactions.

The nature of the accelerating effect that can be inferred because of the compensation in ΔH^* and ΔS^* in the deacylation reaction is not clear at the present time. Solvation effects could be very important although compensation due to solvation effects is usually marked by an isokinetic temperature in the range 300–400°K (Leffler and Grunwald, 1963). The deacylation of acyl-chymotrypsins prepared from specific substrates is characterized by ΔS^* values considerably more positive than those for acyl-chymotrypsins from certain nonspecific substrates (Bender *et al.*, 1964). It was suggested that this might be due to interaction of the specific acyl group with a binding site so that the ground-state conformation resembles the transition state. Nonspecific acyl groups were presumed not to be able to interact in this manner. If the nonspecific acyl groups of the present study were interacting with a hydrophobic region in the active site, compensation might be observed, but this explanation is made less likely by the linear Taft steric effects plot that was observed (Figure 2) and the deviation in both the Taft plot and the compensation plot of the point for the *n*-hexanoyl derivative, which probably does interact with a hydrophobic region. It is likely, therefore, that the observed compensation is a direct consequence of the mechanism of the reaction and is not caused by hydrophobic interactions between the acyl group and the protein.

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