Acylation of Chymotrypsin by Active Esters of Nonspecific Substrates. Evidence for a Transient Acylimidazole Intermediate†

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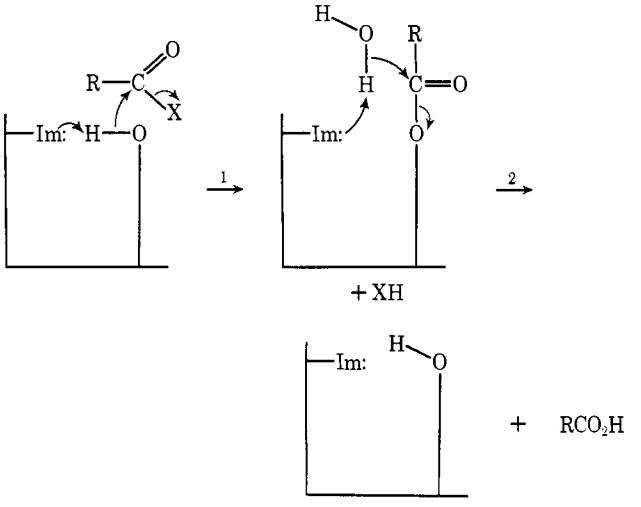
ABSTRACT: The second-order rate constants for the acylation of chymotrypsin by 15 meta- and para-substituted *p*-nitrophenyl benzoates and by 14 2,4-dinitrophenyl benzoates were determined by a stopped-flow method under the condition of [E] ≫ [S]. The Hammett ρ values obtained for the two series are 0.97 ± 0.11 and 1.6 ± 0.3, respectively. The former figure is considerably less than that reported for the deacylation of substituted benzoyl-chymotrypsins. A comparison to the results for nonenzymatic reactions indicates that the atoms bonded to the acyl carbon atom in the transition state bear a net charge of *ca.* −1 in deacylation but *ca.* 0 in acylation, and that the acylation reaction proceeds through a pathway involving the transient formation of an acylimidazole intermediate en route to the acylserine. Further support for this hypothesis comes from an analysis of previously published structure–reactivity studies of leaving group variation and from the fact that the kinetic solvent isotope effects, k_{H_2O}/k_{D_2O}, are only 1.07 and 1.64 for a typical *p*-nitrophenyl benzoate and 2,4-dinitrophenyl benzoate, respectively. All reported solvent isotope effects on the rates of deacylation are greater than 2. These results are indicative of transition states involving rate-determining general-base catalysis in deacylation but not in acylation. At present these conclusions are only applicable to the mechanism of acylation of chymotrypsin by active esters of nonspecific substrates. The Hammett ρ values for the reactions of imidazole and hydroxide ion with substituted 2,4-dinitrophenyl benzoates are 1.73 ± 0.07 and 2.20 ± 0.09, respectively, in 33% acetonitrile at 25°. The rate constant for the reaction of imidazole with *p*-nitrophenyl thiolacetate is 70.1 M^{-1} min^{-1}, a figure which is only a factor of two greater than that previously obtained for the homologous oxy ester, *p*-nitrophenyl acetate.

Cunningham (1957) first suggested that the mechanism of acylation of chymotrypsin by substrates involves general-base-catalyzed activation of the serine hydroxyl by the imidazole group of a histidine to form the acyl-enzyme, and that the deacylation reaction proceeds by an analogous general-base-catalyzed attack of water on the acyl-enzyme mediated by the same imidazole group. The elements of this mechanism, excluding the postulated tetrahedral intermediate, are shown in Scheme I.

A current version of this mechanism, which is based on the crystallographically determined structure, retains Cunningham's basic suggestion but embodies additional activation of the key imidazole of His-57 by the β-carboxylate ion of Asp-102 through a "charge-relay mechanism" and differs from the original in details of the proton-transfer steps (*e.g.*, Birktoft *et al.*, 1970).

Of the two fundamental reactions in chymotrypsin catalysis, acylation and deacylation, the latter has been subject to the most definitive mechanistic probing. Both the magnitude of the solvent deuterium isotope effect (Bender and Kézdy, 1965) and the results of structure–reactivity correlations (Inward and Jencks, 1965; Riddle and Jencks, 1971; Kirsch, 1972) support the general-base catalysis mechanism for the deacylation of nonspecific acyl-enzymes. While the kinetics of the

SCHEME I



acylation of chymotrypsin by *p*-NP[1] esters of nonspecific substrates have been examined (Faller and Sturtevant, 1966; Milstien and Fife, 1969; Frankfater and Kézdy, 1971; and references therein), much less information is available upon which to base mechanistic conclusions concerning this aspect of the reaction. Just the same it is almost universally implied in the various mechanistic proposals that acylation proceeds as shown in Scheme I. However, the very fact that substrates like *p*-nitrophenyl acetate are nonspecific, *i.e.*, they are not N-substituted derivatives of L-amino acids, implies that their

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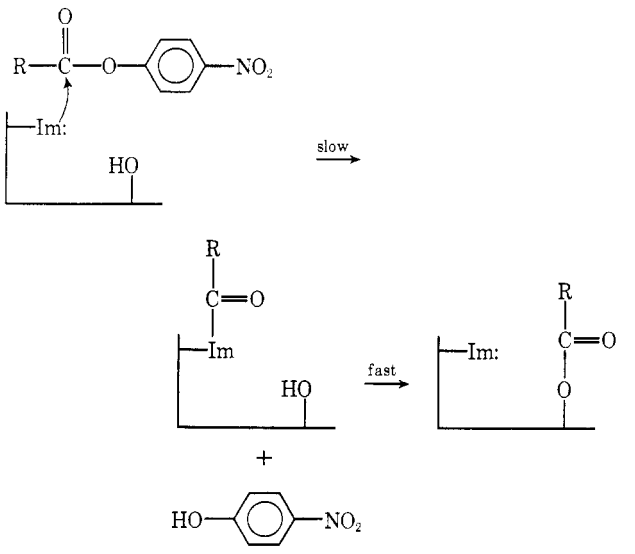
[1] Abbreviations used are: *p*-NP, *p*-nitrophenyl; and 2,4-DNP, 2,4-dinitrophenyl.

SCHEME II



geometries are not precisely oriented to the requirements of the active site, and it is possible that in certain orientations these compounds may react preferentially with an imidazole nitrogen atom of His-57 to produce an acylimidazole en route to the metastable acylserine in the acylation reaction (Scheme II).

Earlier nonenzymatic investigations of the mechanism of imidazole-catalyzed hydrolysis of esters of acetic acid showed that acetylimidazole was formed as an intermediate in the hydrolysis of reactive esters such as *p*-nitrophenyl acetate and phenyl acetate, but that imidazole acted as a classical general-base catalyst for esters with poor leaving groups, such as ethyl acetate (Kirsch and Jencks, 1964, and references therein).

The results of experiments reported herein, when considered together with those in the literature, are most reasonably interpreted in terms of a transient acylimidazole being formed in the acylation of chymotrypsin by active esters of *nonspecific* substrates.

Experimental Section

Materials. Three-times-crystallized α-chymotrypsin was obtained from Worthington Biochemical Corp.

N-trans-Cinnamoylimidazole (Aldrich) was recrystallized several times from redistilled dry cyclohexane. The purified product had mp 132.5–133.0° (lit. (Schonbaum *et al.*, 1961) mp 133–133.5°). Diphenylcarbamyl chloride was recrystallized from warm methanol, mp 85.0° (lit. (Erdmann and Huth, 1897) mp 85.0°). Acetonitrile was redistilled. Deuterium oxide, 99.8%, and deuterium chloride, a 38% solution in 99% D_2O, were supplied by Stohler Isotope Chemicals.

Esters. The *p*-nitrophenyl benzoate esters (*p*-CH_3, *p*-H, *p*-Cl, and *p*-NO_2) were those previously used (Kirsch *et al.*, 1968). All other esters were synthesized according to methods described in the reference.

The elemental analyses[2] and melting point range of those esters which are new compounds are reported in Table I. The melting points of the esters which were not available but whose syntheses have been reported are listed as follows: *p*-nitrophenyl *p*-methoxybenzoate, mp 165.0° (lit. (Barnett and Nixon, 1924) mp 166°); *p*-nitrophenyl *p*-isopropylbenzoate, mp 106.5–107.5° (lit. (Letsinger and Klaus, 1965) mp 104.5–105.0°); *p*-nitrophenyl *p-tert*-butylbenzoate, mp 124.5–125.0° (lit. (Silver, 1966) mp 123–125°); *p*-nitrophenyl *p*-fluorobenzoate, mp 127.0–128.0° (lit. (Joshi and Giri, 1962) mp 102–103°); 2,4-dinitrophenyl benzoate, mp 131.5–132.5° (lit. (Wasserman and Wharton, 1960) mp 131.0–132.0°); 2,4-dinitrophenyl *p*-isopropylbenzoate, mp 115.5–116.5° (lit. (Letsinger and Klaus, 1965) mp 116.5–117.5°).

p-Nitrophenyl thiolacetate was prepared by the method of Frankfater and Kezdy (1971), mp 82.0–83.0°, lit. mp 82.3–82.6°. All other materials used were reagent grade.

Methods. The operational normality of chymotrypsin was determined according to the method of Schonbaum *et al.* (1961). The protein concentration of each enzyme solution was determined from the absorbance at 280 nm, using a molecular weight of 25,000 and extinction coefficient of 5×10^4 M^{-1} cm^{-1} (Dixon and Neurath, 1957). A check on the constancy of the activity of chymotrypsin was made by periodically examining the rate of acylation at pH 7 of the enzyme by *p*-nitrophenyl *p*-chlorobenzoate using solutions of about 0.04 mM in enzyme and 2.5 μM in the ester. During the course of this study, the second-order rate constant representing acylation by this ester was essentially invariant. Diphenylcarbamyl chloride treated chymotrypsin (Erlanger *et al.*, 1966) did not react with the ester used in this study.

For experiments carried out in D_2O, the buffering reagents were dried and then dissolved in 99.8% D_2O and the resulting solution was then evaporated to dryness. This process was repeated. DCl was used to bring about desired changes in pH when using the pyrophosphate buffer system. pH measurements were made with a Radiometer pH meter, type PHM 4b, or with a Beckman SS-2 pH meter. The pD values of deuterium oxide solutions were estimated from the formula pD = pH + 0.40 (Glasoe and Long, 1960).

Kinetics. Acylation rates were followed in either one of two Gibson-Durrum stopped-flow spectrophotometers. Both instruments were fitted with a 2-cm path-length cuvet, the only difference between the two being that the material of the flow path of one was stainless steel while KEL-F was used for the flow path of the second. Identical results were obtained for the same reaction in both instruments. Kinetic runs were carried out under the condition of enzyme concentration much greater than substrate concentration. Solutions of enzyme and substrate each containing 0.1 M phosphate buffer and 5% acetonitrile were mixed in the stopped-flow instrument and the production of the *p*-nitrophenoxide ion or 2,4-dinitrophenoxide ion was followed at pH 6.6 and above, at 400 nm, while at pH below 6.6 wavelengths of either 330 or 340 nm were employed to monitor the absorbance characteristic of *p*-nitrophenol. At higher pH values, above 8.5 in this study, the buffer mediated hydrolysis of substrate is no longer negligible. Consequently, the enzyme was buffered, 0.1 M, and the substrate was dissolved in a 0.1 M NaCl or $NaNO_3$ solution. This salt prevents any artifacts arising in the first few milliseconds after mixing enzyme and substrate in the instrument due to a large disparity in the ionic compositions of the solutions. It is assumed that the mixed solution attains the ambient pH within the mixing time. Program TRDAT2[3] was used to convert measurements

[2] Elemental analyses carried out by A. Bernhard, Elbach über Engelskirchen, West Germany, or the Microanalytical Laboratory of the Department of Chemistry, University of California, Berkeley, or in the Department of Chemistry, University of New Hampshire.

[3] We are grateful to Mr. William Barsky for writing this program.

TABLE I: Analytical Data for *p*-Nitrophenyl and 2,4-Dinitrophenyl Benzoates.

Benzoyl Substituent	Calculated			Found			Mp (°C)
	C	H	N	C	H	N	
			p-Nitrophenyl Esters				
m-F	59.77	3.07	5.36	59.61	3.05	4.89	164.5–166.0
m-CF_3	54.01	2.57	4.50	54.22	2.62	4.70	82.0–82.5
m-Cl	56.21	2.88	5.04	56.51	2.82	5.21	132.5–133.0
m-CH_3	65.45	4.28	5.45	65.06	4.20	5.36	94.5–95.5
p-CF_3	54.01	2.57	4.50	53.93	2.81	4.75	92.5–94.0
p-CN	62.68	2.99	10.45	62.79	2.90	10.52	195.0–196.0
p-C_2H_5	66.41	4.80	5.17	66.64	4.97	5.38	72.0–73.0
			2,4-Dinitrophenyl Esters				
m-F	50.98	2.29	9.15	50.83	2.26	8.99	110.0–111.0
m-CF_3	47.20	1.97	7.87	47.18	2.20	7.99	98.0–99.0
m-Cl	48.37	2.17	8.68	48.35	2.02	8.68	123.5–124.0
m-CH_3	55.60	3.31	9.27	55.86	3.35	9.22	116.5–118.0
p-CF_3	47.20	1.97	7.87	46.96	2.07	7.87	106.5–107.5
p-CN	53.67	2.24	13.42	54.26	2.35	13.25	168.5–170.5
p-C_2H_5	56.96	3.80	8.86	56.96	3.85	8.64	124.0–125.0
p-Cl	48.37	2.17	8.68	48.60	2.47	8.91	146.5–147.5
p-NO_2	46.85	2.10	12.61	46.77	2.05	12.72	140.0–141.0
p-CH_3	55.62	3.31	9.27	55.70	3.55	9.47	160.0–161.0
p-OCH_3	52.84	3.15	8.81	53.23	3.41	8.78	136.0–136.5
p-F	50.98	2.29	9.15	50.65	2.56	9.14	99.5–102.5

of photographic recordings of oscilloscope traces into absorbance units and, since first-order kinetics were observed, to plot the logarithm of $A_\infty - A_t$ *vs.* time, where A_∞ and A_t are the absorbance values at infinite time and time t, respectively. The program calculates the weighted least-squares straight line to give the pseudo-first-order rate constant. The pH *vs.* rate profiles were fit by nonlinear regression analysis using Program NLIN from the library of the University of California at Berkeley computer center. Hammett plots were constructed by the weighted least-squares method described previously (Kirsch and Kline, 1969).

The rates of alkaline and imidazole catalyzed hydrolysis were followed by the methods described elsewhere (Jencks and Carriuolo, 1960; Kirsch and Jencks, 1964; Kirsch *et al.*, 1968). The low solubilities of some esters necessitated the use of the 0–0.1 absorbance scale of the Gilford Model 222 recording spectrophotometer.

Results

Rates of Acylation in Water. Under the pseudo-first-order conditions of [E] ≫ [S] employed here the observed first-order rate constants were independent of [S] and were directly dependent on [E] throughout the entire range of [E] used in this investigation (Figure 1). Deviations attributable to enzyme polymerization (Shiao and Sturtevant, 1969; Faller and LaFond, 1971, and references therein) or to saturation of substrate by enzyme are therefore not of experimental consequence in this work, and the rates of substrate disappearance (acylation) are adequately described by eq 1.

$$\frac{-\mathrm{d[S]}}{\mathrm{d}t} = k[\mathrm{E}][\mathrm{S}] \qquad (1)$$

$$k = k_{\mathrm{obsd}}/[\mathrm{E}]$$

The rate constants for the acylation of chymotrypsin by 15 meta- or para-substituted *p*-nitrophenyl benzoates at pH 7, by 13 of the same esters at pH 6, and by 14 2,4-dinitrophenyl benzoates at pH 7 are collected in Table II. The enzyme concentrations were chosen for their convenience in determining the rates of reaction by the stopped-flow method while the substrate concentrations were selected so as to be high enough to produce reasonably large changes in absorbance, but small enough so that [S] ≪ [E]. The concentrations of the *p*-isopropyl and *p-tert*-butyl esters were limited by their low solubility, with 2,4-dinitrophenyl *p-tert*-butylbenzoate being too

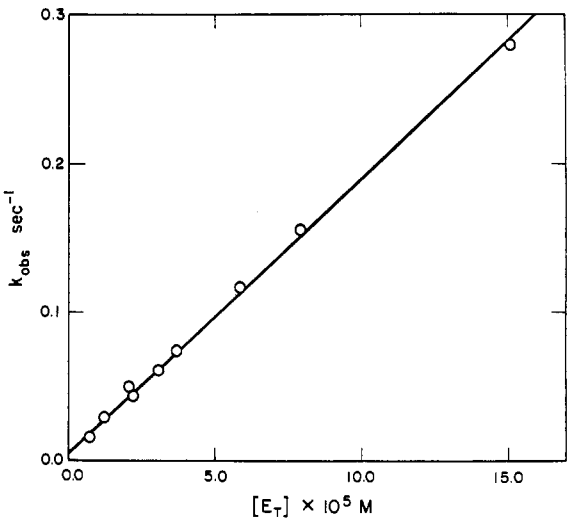


FIGURE 1: The effect of enzyme concentration on the rate of acylation of chymotrypsin by *p*-nitrophenyl *p*-toluate at pH 7.0 and 25°. [S_0] = 0.5 μM for [E] ≤ 20 μM; [S_0] = 2.5 μM for [E] > 20 μM.

TABLE II: Second-Order Rate Constants for the Reactions of Chymotrypsin with *p*-Nitrophenyl and 2,4-Dinitrophenyl Esters of Substituted Benzoic Acids at 25°.[a]

Substituent	σ Value[b]	k (Std Dev), M^{-1} sec^{-1} × 10^{-3}: *p*-Nitrophenyl Benzoates at pH 7[c]	No. of Determinations	*p*-Nitrophenyl Benzoates at pH 6[d]	No. of Determinations	2,4-Dinitrophenyl Benzoates at pH 7[e]	No. of Determinations
p-CH_3	−0.268	0.929 (0.05)	10	0.598 (0.150)	5	90.4 (8.4)	5
p-*t*-Bu	−0.197	11.8 (2.1)	9				
p-CH_3	−0.17	2.04 (0.04)	8	0.709 (0.067)	8	167 (10)	6
p-*i*-Pr	−0.151	27.5 (1.7)	5	7.47 (0.64)	7	971 (102)	7
p-C_2H_5	−0.151	9.33 (0.41)	9	3.13 (0.29)	7	339 (25)	9
m-CH_3	−0.069	1.94 (0.12)	8			55.9 (2.0)	6
p-H	0	1.44 (0.09)	9	0.442 (0.028)	6	72.7 (3.8)	6
p-F	0.062	1.29 (0.08)	6	0.545 (0.043)	6	67.6 (5.0)	9
p-Cl	0.227	3.60 (0.20)	18	1.86 (0.05)	6	355 (30)	6
m-F	0.337	3.98 (0.16)	7	1.49 (0.07)	6	177 (12)	6
m-Cl	0.373	5.85 (0.33)	11	2.69 (0.30)	14	295 (29)	5
m-CF_3	0.43	3.87 (0.08)	6	1.66 (0.06)	6	298 (24)	7
p-CF_3	0.54	9.01 (0.56)	9	2.58 (0.13)	6	2520 (460)	19
p-CN	0.66	8.67 (0.54)	8	2.25 (0.19)	7	868 (77)	6
p-NO_2	0.778	9.05 (0.22)	6	3.55 (0.06)	7	1480 (130)	6

[a] All reaction rates were determined under pseudo-first-order conditions with [E] ≥ 10[S] in 0.1 M Na phosphate buffers and 5% v/v acetonitrile. [b] Taken from Leffler and Grunwald (1963). [c] [E], 9.0–120.0 μM; [S], 0.25–2.5 μM. [d] [E], 37.0–68.0 μM; [S], 0.75–5.0 μM. [e] [E], 3.5–22.2 μM; [S], 0.13–1.0 μM.

insoluble to permit reliable kinetic measurements to be made.

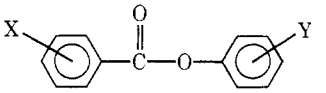
Acylation Rates as Functions of pH and pD. The rates of reaction of one *p*-NP ester, the *p*-trifluoromethyl benzoate, and one 2,4-DNP ester, the unsubstituted benzoate, were determined as functions of pH and pD (Figure 2). Phosphate buffers were employed in the pH (pD) range 6.0–8.2 and pyrophosphate buffers at higher values of pH (pD). No significant variations in rates were noted when the different buffers were used at the same pH, although the acylation rates were reduced in borate and *N*-ethylmorpholine buffers (not reported). The curves shown in Figure 2 were calculated from least-squares fitting to eq 2 (Bender *et al.*, 1964) by nonlinear regression analysis.

$$k = \frac{k^{lim}}{1 + [H^+]/K_1 + K_2/[H^+]} \quad (2)$$

The values of pK_1, pK_2, and the limiting rate constant for acylation k^{lim} are given in Table III. Because relatively fewer measurements were made over the more basic side of the pH maximum, the uncertainties in the listed values of pK_2 are considerably greater than those in pK_1. The ionization constants represented by the more acidic limbs of the pH *vs.* rate profiles are identical for both esters in H_2O and in D_2O. The magnitudes of the shifts observed for pK_1 on changing from H_2O to D_2O are consistent with those previously reported (Bender *et al.*, 1964). The limiting values of the rate constants for acylation exhibit the relatively small solvent isotope effects (k_{H_2O}/k_{D_2O}) of 1.07 and 1.64 for the *p*-NP and 2,4-DNP esters, respectively.

Reactions of Imidazole and Hydroxide Ion with Substituted 2,4-Dinitrophenyl Benzoates. The reactions of imidazole with substituted *p*-nitrophenyl benzoates have been studied by Caplow and Jencks (1962) who reported a Hammett ρ value = 1.19 for this series in 33% CH_3CN at ionic strength 1.0. They also noted a kinetically significant reaction whose rate was proportional to the second power of the imidazole free base concentration and for which a ρ = 1.57 was obtained. The rate constants for the reactions of 2,4-dinitrophenyl benzoates with imidazole, determined under the same conditions as those of Caplow and Jencks (1962), are recorded in Table IV. In all cases, plots of k_{obsd} *vs.* imidazole concentration generated straight lines within experimental error, implying the absence of terms dependent on higher powers of imidazole concentration for the 2,4-DNP esters. The ρ obtained for the reaction of imidazole with 2,4-dinitrophenyl benzoates of 1.73 ± 0.07 is significantly greater than the one of 1.19 reported for the *p*-nitrophenyl esters.

The rates of alkaline hydrolysis of several series of acyl- and alcohol-substituted phenyl benzoates have been reported previously (Kirsch *et al.*, 1968).



Unlike the reactions of these compounds with the nucleophiles imidazole or ammonia, where the ρ for a given set of variations in X is dependent on Y, *i.e.*, ρ_{NH_3} = 1.08 and 1.43 for series of *p*-chlorophenyl and *p*-nitrophenyl benzoates, respectively (Kirsch and Kline, 1969), the ρ values for acyl variation in alkaline hydrolysis reactions are independent of leaving group. For example, where Y = CH_3, ρ_{OH^-} = 2.032 ± 0.013 and where Y = NO_2, ρ_{OH^-} = 2.006 ± 0.012 (Kirsch *et al.*, 1968). The rate constants reported in Table V extend these

TABLE III: Acylation in H_2O and D_2O.[a]

Ester	pK_1	pK_2	k^{lim} (M^{-1} sec^{-1} × 10^{-3})	Solvent	k_{H_2O}/k_{D_2O}
p-Nitrophenyl benzoate	6.78	9.2	14.8	H_2O	
p-Trifluoromethyl benzoate	7.23	9.5	13.8	D_2O	1.07
2,4-Dinitrophenyl benzoate	6.76	9.0	118	H_2O	
	7.23	9.7	72	D_2O	1.64

[a] From the least-squares fits to the data shown in Figure 2.

earlier results to the 2,4-dinitrophenyl benzoates which are somewhat less than an order of magnitude more reactive toward hydroxide ion than the *p*-nitrophenyl benzoates. The $\rho_{OH^-} = 2.20 \pm 0.09$ is not significantly larger than the ρ values obtained in the earlier studies with the less reactive esters.

Since the rates of reaction of chymotrypsin with *p*-nitrophenyl thiolacetate have recently been reported (Frankfater and Kézdy, 1971), it became important for the consideration of the mechanism of the reaction to determine the rate of reaction of this thiol ester with imidazole. The rate constant is reported in Table VI. The value of 70.1 M^{-1} min^{-1} is approximately a factor of two larger than that previously obtained for the reaction of imidazole with the corresponding oxy ester, *p*-nitrophenyl acetate.

Discussion

Comparison of Acylation and Deacylation Reactions. While the deacylation of an acyl-enzyme can reasonably be considered to be a single-step reaction (step 2, Scheme I), the acylation process includes contributions attributable to both the formation of a noncovalent Michaelis complex and the rate constant for acylation by the bound substrate (eq 3). Under

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_{acyl}} AE + P_1 \qquad (3)$$

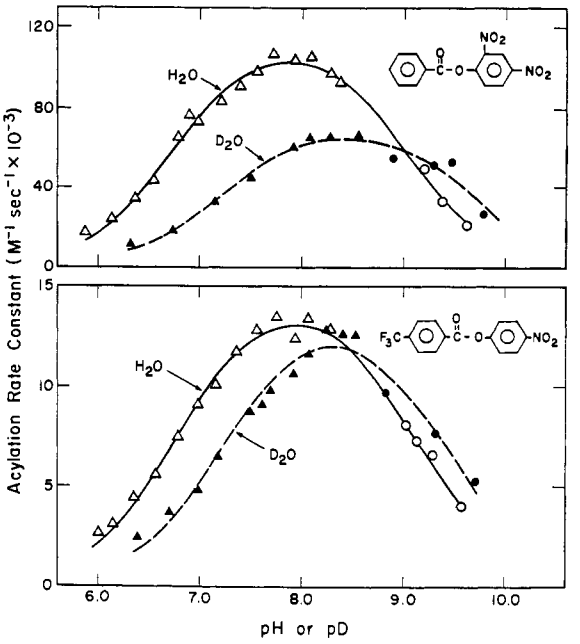


FIGURE 2: The rates of acylation of chymotrypsin by 2,4-dinitrophenyl benzoate (upper curves) and *p*-nitrophenyl *p*-trifluoromethylbenzoate (lower curves) as a function of pH (solid lines) or pD (dashed lines) at 25°. The lines represent the least-squares fits to the function given in eq 2 with the minimizing parameters listed in Table III. (△,▲) 0.1 M phosphate buffers; (○,●) 0.05 M pyrophosphate buffers. Each point generally represents an average of four to seven determinations.

conditions of [E], [S] $\ll K_s$, the second-order rate constants determined in the present study are related to the parameters shown in eq 3 by

$$k = k_{acyl}/K_s \qquad (4)$$

Thus substituent effects on k might be attributable to binding, reflected in K_s, or to reactivity, measured by k_{acyl}. The effects of substituents on orientation of the substrate within the active site could be reflected in either constant and might also be a

TABLE IV: Rate Constants for the Reactions of Imidazole with Substituted 2,4-Dinitrophenyl Benzoates.[a]

Substituent	Total Imidazole (M)	Fraction Free Base	No. of Determinations	k_2[b] (M^{-1} min^{-1})	Std Dev
p-OCH_3	0.067–0.400	0.35	6	2.59	0.07
p-CH_3	0.067–0.400	0.35	5	6.08	0.09
p-H	0.02–0.08	0.4	4		
	0.04–0.16	0.2	4	16.8	0.4
p-Cl	0.02–0.12	0.35	6	29.1	0.4
p-NO_2	0.007–0.04	0.2	6	226	3

[a] In imidazole buffers; other conditions as described in Table V, footnote *a*. [b] k_{obsd}/imidazole free-base concentration, determined from the least-squares fit to the equation $k_{obsd} = k_2$[imidazole]. Corrections for alkaline- or acid-catalyzed hydrolysis were not required under these conditions.

TABLE V: Rate Constants for the Alkaline Hydrolysis of Substituted 2,4-Dinitrophenyl Benzoates.[a]

Substituent	Hydroxide[b] Ion Concn (M × 10^5)	k_2 (Std Dev),[c] M^{-1} min^{-1}
p-OCH_3	450–2150	55.6 (1.1)
p-CH_3	500–1740	115 (2)
p-H	430–1650	289 (4)
p-Cl	2.5–5.4	790 (10)[d]
p-NO_2	2.5–5.5	12,500 (300)[d]

[a] In 33% acetonitrile ionic strength 0.3 at 25°. Initial ester concentrations were 6 × 10^{-6} M. [b] Hydroxide ion concentrations of less than 1 mM were determined from the measured pH and the relationship log [OH^-] = pH −14.58 established for the conditions of these experiments (Kirsch *et al.* (1968)). Higher concentrations were calculated directly from the known amount of added KOH. [c] k_{obsd}/[OH^-]. Each rate constant is based on six to eight determinations. [d] Determined in 0.05 M triethylamine buffers.

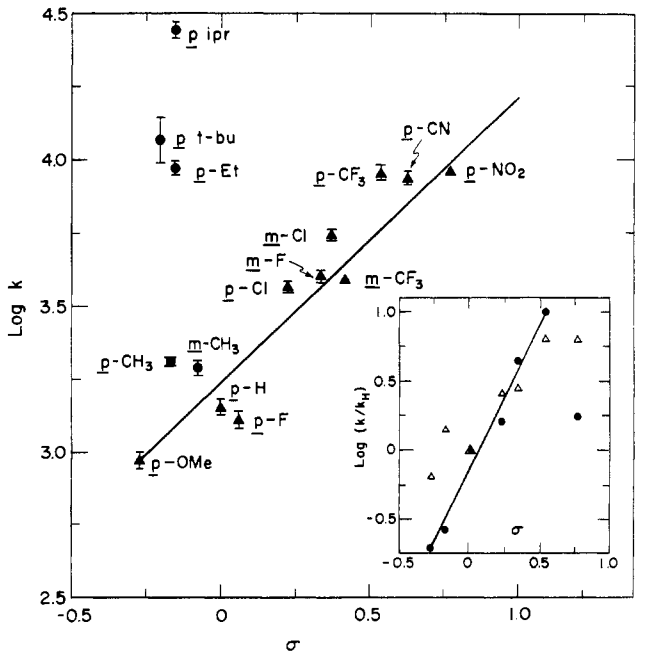


FIGURE 3: Hammett plot of the rate constants for acylation of chymotypsin by *p*-nitrophenyl benzoates at pH 7 and 25°. Data from Table II. The Hammett plot was constructed for the rate constants denoted by ▲ only, with each point weighted as described in Kirsch and Kline (1969). Insert: log k/k_H for the *deacylation* of substituted benzoyl chymotrypsins (●). The line represents the least-squares fit to all the data points except for *p*-nitrobenzoyl-chymotrypsin (from Caplow and Jencks, 1962); (△) log k/k_H for the rates of *acylation* of chymotrypsin by the corresponding *p*-nitrophenyl benzoates.

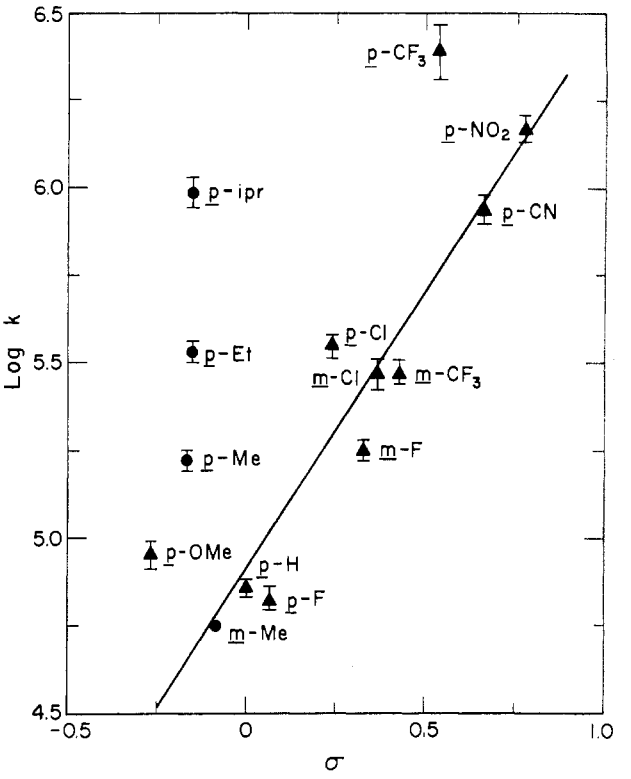


FIGURE 4: Hammett plot for the rate constants for acylation of chymotrypsin by 2,4-dinitrophenyl benzoates at pH 7 and 25°. Data from Table II. The weighted least-squares line is constructed for the nonhydrocarbon substituents (▲).

factor in influencing the rates of deacylation (Henderson, 1970; Hinkle and Kirsch, 1970, 1971). It has been well established that chymotrypsin has high affinity for nonpolar ligands (Knowles, 1965; Hymes *et al.*, 1965; Wildnauer and Canady, 1966; Berezin *et al.*, 1970; Cuppett and Canady, 1970). The nonpolar binding site has been located through crystallographic studies (Blow and Steitz, 1970).

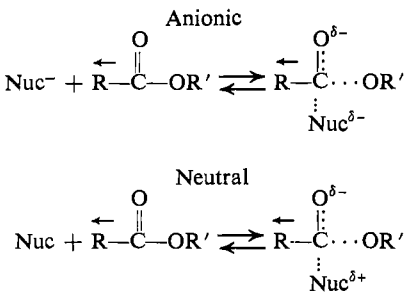
Acyl Substituent Effects. In this study, nonpolar substituents have large effects on the observed rate constants for acylation, as shown by Hammett plots of log k *vs.* σ for the substituted *p*-nitrophenyl and 2,4-dinitrophenyl benzoates (Figure 3 and Figure 4, respectively). The esters with the larger hydrocarbon substituents in the para position have rate constants which are up to 1.5 orders of magnitude enhanced over those expected from electronic effects alone. The latter effects, which presumably would only be reflected in k_{acyl}, can be satisfactorily differentiated from the nonpolar binding interactions, which should be largely confined to K_s, by measuring the rate constants for a large number of esters and examining their logarithmic dependence upon σ, which is only a measure of electronic effects (*e.g.*, Leffler and Grunwald, 1963). If the points for the pure hydrocarbon substituted esters (circles) are omitted from the regression analysis, those for the remaining esters (triangles) generate satisfactory straight lines. The ρ values are given in Table VII together with those obtained for a number of nonenzymatic reactions of these esters. If the logarithms of the rate constants for all the *p*-nitrophenyl esters are plotted against the sum of σ and π, where π is a measure of the hydrophobicity of the substituent (Fujita *et al.*, 1964), or against π alone no obvious improvement in correlation could be detected. The π constants for most substituents were taken from the list given for the phenoxyacetic acid series. The important point to be gleaned from the model studies is that reactions of anionic nucleophiles such as OH^- or N_3^- are more sensitive to electron withdrawal in the acyl moiety of the ester, as shown by the larger ρ values, than are the reactions of neutral nucleophiles such as *N*,*N*-dimethylhydroxylamine, imidazole, or ammonia. Moreover, the ρ values are not significantly dependent on the nucleophilic element itself, *i.e.*, O *vs.* N. These results can be understood in terms of electron withdrawal contributing more significantly to the stabilization

TABLE VI: Rate Constants for the Reactions of Imidazole with *p*-Nitrophenyl Acetate and *p*-Nitrophenyl Thiolacetate.[a]

Ester	Imidazole Buffer Concn (M)	Fraction Free Base	No. of Determinations	k_2[b] (M^{-1} min^{-1})
p-Nitrophenyl thiolacetate[c]	0.010–0.06	0.5	6	75
	0.015–0.06	0.25	4	65.3
				70.1 (Av)
p-Nitrophenyl acetate				34.9[d]

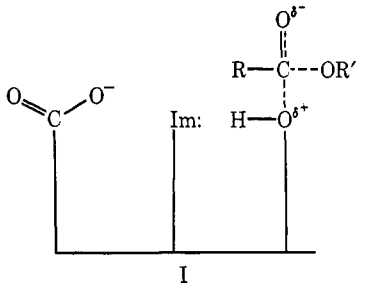
[a] In H_2O, 25°, and ionic strength 1.0. [b] k_{obsd}/imidazole free-base concentration. [c] Rate of reaction, monitored by following release of *p*-nitrobenzenethiol at 410 nm. Initial ester concentration was 3×10^{-5} M. [d] Kirsch and Jencks, 1964. This rate constant was determined under conditions identical with those employed here for the thiol ester.

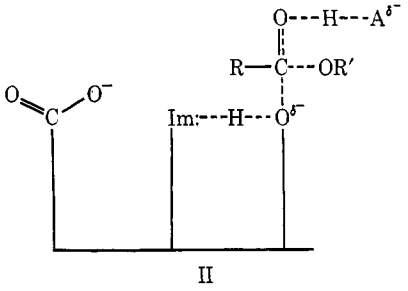
of anionic than of neutral transition states compared to the respective ground states. Similar conclusions have been

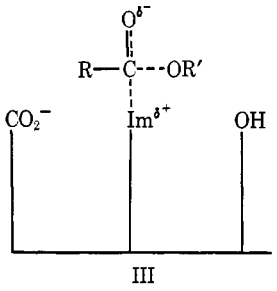


reached previously (Koehler *et al.*, 1966; Kirsch and Kline, 1969; Holmquist and Bruice, 1969; Bruice *et al.*, 1970).

The ρ values for the reactions of chymotrypsin with both series of esters are much closer to those obtained for the reactions of neutral rather than anionic nucleophiles, with the similarity to the ρ's for the reactions of imidazole being particularly noteworthy. At this point three possible transition states for the acylation reactions of chymotrypsin with these esters can be considered (I–III) which have the requirement of no net change in charge induced in the ester on proceeding from the ground state to the transition state as a common limitation. Transition state I is an expression of the charge







relay mechanism (Birktoft *et al.*, 1970) in which essentially none of the negative charge of Asp-102 has been transferred to the serine. This is equivalent to saying that the Brønsted $\alpha \approx 0$ and that there is no activation by general-base catalysis so that the serine hydroxyl group must displace R′O⁻ without such assistance. The fact that unactivated alcohols are not

TABLE VII: ρ Values for the Reactions of Nucleophiles with Substituted *p*-Nitrophenyl and 2,4-Dinitrophenyl Benzoates.

Nucleophile	ρ (Std Dev)	Correlation Coeff	No. of Compounds
p-Nitrophenyl Benzoates			
Chymotrypsin[a]	0.97 (0.11)	0.95	10
Imidazole[b,c]	1.21 (0.07)	0.997	6
NH_3[c,d]	1.43 (0.02)	0.997	5
$(CH_3)_2NOH$[c,e]	1.28		5
OH^-[c,f]	2.01 (0.01)	0.999	5
N_3^-[c,e]	1.8		5
2,4-Dinitrophenyl Benzoates			
Chymotrypsin[g]	1.6 (0.3)	0.86	10
Imidazole[c,h]	1.73 (0.07)	0.98	5
OH^-[c,i]	2.20 (0.09)		5

[a] From data of Figure 3. [b] Calculated from the data of Caplow and Jencks (1962). [c] Determined at 25° in 33% acetonitrile. [d] Kirsch and Kline (1969). [e] J. F. Kirsch and L. B. Rall, unpublished data. [f] Kirsch *et al.* (1968). [g] From data of Figure 4. [h] From data listed in Table IV. [i] From data listed in Table V.

sufficiently strong as nucleophiles to displace *p*-nitrophenolate at enzymatically significant rates (see below) and that no function is provided for the catalytically important group of $pK_a = 6$–7 argue against I. Transition state II is a representation of the charge relay mechanism in which significant negative charge has been transferred to the serine oxygen and which achieves the no net change in charge criterion by an additional unidentified functional group acting as a general-acid catalyst. Electrophilic catalysis has been postulated to be a contributing component in the reactions of chymotrypsin with carbamylating agents (Metzger and Wilson, 1964) and with phenyl esters of hippuric acid and of *N*-methanesulfonyl-L-phenylalanine (Williams, 1970). The latter author has suggested that the proton required for general-acid catalysis might be supplied by the peptide backbone of the protein. Transition state II is therefore consistent with the observed acyl variation effects, but will be considered further below. Transition state III represents the unassisted attack of the imidazole side chain of His-57 on the ester and is supported by the close similarity of the ρ values for acylation by imidazole and by chymotrypsin.

It is clear from the insert in Figure 3 that the deacylation rates ($\rho = 2.1$) are very much more sensitive to electron withdrawing groups than are the acylation rates. This result for deacylation is suggestive of a transition state in which the attacking water molecule is activated to a considerable degree by general-base catalysis, so that the reaction resembles that of alkaline hydrolysis (Caplow and Jencks, 1962). The conclusion from these experiments is therefore that the transition state charge on the atoms immediately adjacent to the acyl carbon atom is *ca.* −1 in deacylation and *ca.* 0 in acylation. This conclusion provides an argument against general-acid catalysis participating in the deacylation reaction.

Slight variations in the pK_a's controlling the rates of deacylation of different benzoyl-chymotrypsins have been reported (Bernhard *et al.*, 1966a; Vishnu and Caplow, 1969).

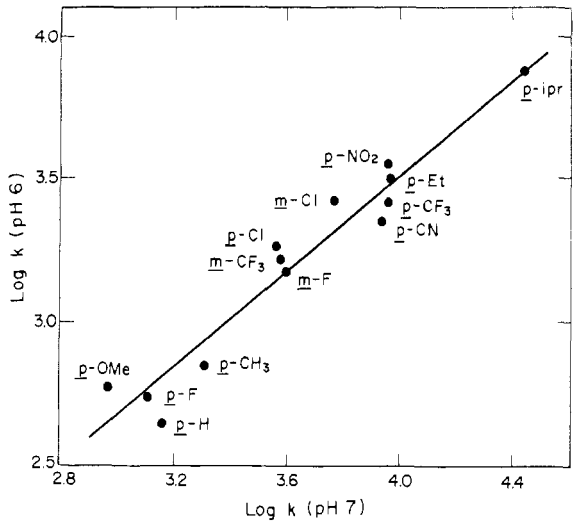


FIGURE 5: The logarithms of rate constants for acylation of chymotrypsin by *p*-nitrophenyl benzoates at pH 6 *vs.* the logarithms of those obtained at pH 7. Data from Table II. The line represents the unweighted least-squares fit to the data.

For example, the pK_a's for the deacylation of unsubstituted, *p*-nitro-, and *p*-methoxylbenzoyl-chymotrypsins are 7.36 ± 0.03, 7.22 ± 0.03, and 7.32 ± 0.02, respectively. If all the *p*-nitrophenyl esters used in this study depend on the same pK_a for acylation, a plot of the logarithms of the rate constants at pH 6 *vs.* those obtained at pH 7 should generate a straight line with slope = 1. The least-squares line for such a plot has a slope of 0.84 ± 0.06 (Figure 5) indicating the possibility of slightly substrate-dependent pK_a's or possibly enzyme oligomerization being important at the lower pH value. This point may be worth further investigation, but the deviation from the theoretical slope of 1.0 is not so great as to affect the ρ values by more than 15%. There seems to be no correlation of deviations from this straight line with pK_a of the benzoic acids, as strongly electron-withdrawing substituents are found both above and below the correlation line.

Substituent Effects in the Leaving Group. Bender and Nakamura (1962) determined the rates of acylation of chymotrypsin by seven substituted phenyl acetates including the *o*-nitrophenyl derivative and calculated a ρ value of 1.8 for this reaction, using the σ^- values of the substituents. The proper choice of substituent constants where $\sigma \neq \sigma^-$ for reactions of phenyl esters apparently depends on the specific reaction under consideration (Ryan and Humffray, 1966; Kirsch *et al.*, 1968) with values nearer to σ being appropriate for reactions having little C–O bond breaking and figures nearer to σ^- for those having extensive C–O bond rupture in the transition state (Kirsch *et al.*, 1968; Jencks and Gilchrist, 1968). For this reason, the ρ = 1.8 of Bender and Nakamura (1962) represents a lower limit of the effect of electron withdrawing substituents in the leaving group upon the rates of acylation. Further evidence that the selectivity of chymotrypsin for the leaving group is large comes from a comparison of the ratios of the rates of reaction for the 2,4-DNP and the corresponding *p*-nitrophenyl benzoates which range between 35 for the *p*-isopropyl and 270 for the *p*-CF_3 derivatives, with the average ratio equal to 50. This ratio may be compared with those observed for the reactions of imidazole and hydroxide ion with 2,4-dinitrophenyl and *p*-nitrophenyl benzoate which are equal to 19 (Caplow and Jencks, 1962, and Table IV) and 6 (Kirsch *et al.*, 1968, and Table IV), respectively. The high selectivity of chymotrypsin as indicated by the large ρ value for the reactions of substituted phenyl acetates and the high discrimination ratio of the enzyme toward *p*-NP and 2,4-DNP esters are features characteristic of the reactions of neutral amine as opposed to anionic nucleophiles and are indicative of a large amount of C–O bond breaking in the transition state (Jencks and Gilchrist, 1968). Thus the analysis of the substituent effects for the leaving group also supports transition-state III. The ρ values for the leaving group for the reactions of chymotrypsin with *N*-methanesulfonyl-L-phenylalaninate and with hippurate esters are considerably smaller than those obtained for the nonspecific phenyl acetates, and may be indicative of a different mechanism of acylation for different series of substrates (see below).

Steric Factors. In principle, the transition state for the deacylation of acyl-chymotrypsins could have identical geometry as that for acylation with water replacing the leaving group (Bender and Kézdy, 1965; Henderson, 1970). The fact that certain nucleophilic acyl acceptors appear to be bound to the acyl-enzyme through hydrophobic interactions (Inward and Jencks, 1965) suggests that differences in geometry for the two steps have to be considered. Experimental evidence for this conclusion emanates from the demonstration of Milstien and Fife (1969) that steric factors affecting the rates of deacylation of straight- and branched-chain acyl-chymotrypsins are not exactly the same as they are for the rates of acylation by the corresponding *p*-NP esters. The deviation for the trimethylacetyl group is particularly noticeable in this respect. The insert in Figure 3 suggests that the *p*-nitrobenzoyl group has a different orientation in the two steps, as the point for the rate constant for deacylation of *p*-nitrobenzoyl-chymotrypsin is about an order of magnitude below the Hammett line generated by the other benzoyl-chymotrypsins while that for acylation by *p*-nitrophenyl *p*-nitrobenzoate falls on the regression line. Finally, evidence that even the closely related *p*-nitrophenyl and 2,4-dinitrophenyl benzoates may not be identically oriented in the acylation reaction is indicated by the considerable scatter in a graph correlating the rate constants for the two derivatives (Figure 6). Different geometries in acylation and in deacylation are compatible with different mechanisms for the two processes. Stevenson and Smillie (1970) find that minor structural differences in chloromethyl ketones are important in determining whether histidine or methionine will be alkylated. The fact that some of these derivatives can alkylate both residues is excellent evidence for at least 2 allowed binding orientations at the active site.

Solvent Isotope Effects. Compelling evidence for the acylation mechanism outlined in Scheme II is provided by a comparison of the solvent deuterium isotope effects on the rates of the two steps involved in chymotrypsin catalysis. The rate of hydrolysis of benzoyl-chymotrypsin is 2.3-fold slower in D_2O than in H_2O (M. Caplow, 1964, personal communication based on work reported in Caplow and Jencks, 1962), and the rates of deacylation of trimethylacetyl- and *trans*-cinnamoyl-chymotrypsin are 3.0- and 2.5-fold slower in D_2O, respectively (Bender and Hamilton, 1962). The values of k_{cat}, representing deacylation, for the specific substrates *N*-acetyl-L-tryptophan methyl and ethyl esters are also decreased in D_2O by factors of 2.8 and 2.7, respectively (Bender *et al.*, 1964). Solvent isotope effects of greater than twofold in ester hydrolysis are indicative of a deacylation mechanism involving partially rate-determining proton transfer through general-base catalysis. (Scheme I, step 2; Johnson, 1967). Solvent isotope effects on the rate constant for acylation which is equal to k_{acyl}/K_s (eq 4) can in principle be attributed to effects on either the dis-

TABLE VIII: Rates of Reactions of Nucleophiles with *p*-Nitrophenyl Benzoate at 25°.

Nucleophile	Log k (M^{-1} sec^{-1})[a]
H_2O	$(-10)^b$
Ser-OH	$(-9$ to $-11)^c$
Imidazole	-2.7^d
NH_3	-2.2^e
$(CH_3)_2NOH$	-1.9^f
OH^-	-0.1^g
Ser-O^-	$(1.1)^h$
Chymotrypsin	3.4^i

[a] Values in parentheses have been extrapolated as described. [b] Calculated from the known values of k_{OH^-} for *p*-nitrophenyl benzoate and *p*-nitrophenyl *p*-nitrobenzoate, and the value of k_{H_2O} for the latter assuming $\rho_{OH^-} = \rho_{H_2O}$. [c] Assumed to be within an order of magnitude of the water rate. [d] Caplow and Jencks (1962). [e] Kirsch and Kline (1969). [f] J. F. Kirsch and L. B. Rall, unpublished results. [g] Kirsch *et al.* (1968). [h] Calculated from the known rates of k_{OH^-} for *p*-nitrophenyl acetate (Kirsch and Jencks, 1964) and *p*-nitrophenyl benzoate (footnote *g*) and k_{SerO^-} for *p*-nitrophenyl acetate (Bruice *et al.*, 1962) assuming the ratio k_{OH^-}/k_{SerO^-} is the same for both esters. [i] From Table II assuming the rate constant determined at pH 7 is 60% of the maximum value as in the case for *p*-nitrophenyl *p*-trifluoromethylbenzoate (Tables II and III).

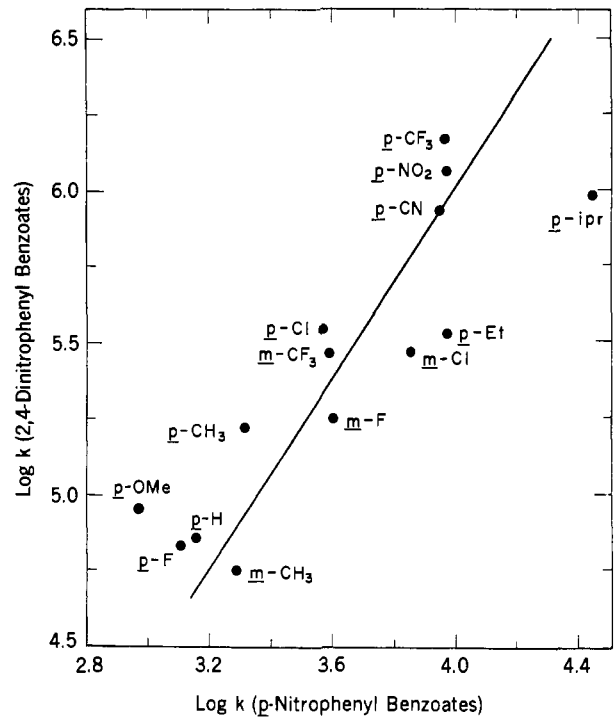


FIGURE 6: Logarithms of the rates of acylation of chymotrypsin by substituted 2,4-dinitrophenyl benzoates plotted as a function of the logarithms of the rates of acylation by the corresponding *p*-nitrophenyl benzoates. The line has a slope of 1.6 determined by the ratio of the ρ values for the two series. All rate constants were determined at pH 7.

sociation constant for the enzyme–substrate complex, K_s, or on the rate constant for acylation by the bound substrate, k_{acyl}, or both. Since the average values of K_m (= K_s, since $k_{acyl} \ll k_{deacyl}$) for the hydrolysis of *N*-acetyl-L-tryptophanamide are virtually the same in H_2O and in D_2O over the pH (pD) range 6–10, we may tentatively conclude that the binding of substrates to chymotrypsin is not subject to an appreciable solvent isotope effect (Bender *et al.*, 1964). The data in Table III show that the composite constant k_{acyl}/K_s for a typical *p*-nitrophenyl benzoate is also unaffected by the isotopic substitution in solvent and the acylation constant for a typical 2,4-DNP ester is decreased only 1.6-fold in D_2O. These results are strongly suggestive of a transition state in acylation which does not involve proton transfer (*i.e.*, transition state III), since the rates of nucleophilic attack of imidazole on *p*-NP esters are not reduced in D_2O (Johnson, 1967).

Is the Acylation of Chymotrypsin by Nitrophenyl Esters of Nonspecific Substrates Catalyzed by the Enzyme? Assuming that the mechanism of acylation is as described in Scheme II, it is important to consider whether the reaction involves the adsorption of the substrate onto the active site near the imidazole side chain of His-57 followed by simple nucleophilic attack, or if the displacement reaction is subject to additional catalysis by the enzyme. The rates of reaction of a number of nucleophiles, including chymotrypsin, with *p*-nitrophenyl benzoate are collected in Table VIII. The values given for H_2O and Ser-OH probably represent upper limits for these nucleophiles, since they are based on rate constants for the hydrolysis of an ester, a reaction which probably proceeds by a pathway involving general-base catalysis by other solvent molecules (Jencks and Carriuolo, 1961). The rate of reaction of chymotrypsin is greater than that of any of the other nucleophiles including the fully developed anion of the serine hydroxyl group, yet the acyl substituent effects indicate a transition state in which there has been little, if any, activation of the serine by general-base catalysis, *i.e.*, the nucleophile if it were the OH of serine would look more like Ser-OH than Ser-O^-, and other catalytic facets of chymotrypsin would have to explain the 10^{12}–10^{14} difference between the enzymatic and serine rates. One such factor, that due to the ΔF gained on forming the Michaelis complex with a dissociation constant = K_s, can be estimated with a reasonable degree of certainty. Many substrates and inhibitors containing one aromatic ring, which is presumably accommodated in the "tosyl hole" (Blow and Steitz, 1970), have dissociation constants between 0.5 and 10 mM (*e.g.*, McConn *et al.*, 1971; Zerner and Bender, 1964; Ingles and Knowles, 1968; Knowles, 1965; Hymes *et al.*, 1965), while the polycyclic inhibitor, proflavin, has a $K_i = 3.7 \times 10^{-5}$ M (Bernhard *et al.*, 1966b). These figures taken together with the probability that substituted phenyl esters bind to the enzyme with the *leaving* group in the hydrophobic pocket (Steitz *et al.*, 1969) suggest that $K_s \sim 10^{-3}$–10^{-2} M is the probable range of dissociation constants for the esters under consideration. These figures can be used to convert the second-order rate constant for the acylation of the enzyme by *p*-nitrophenyl benzoate to the first-order rate constant for acylation by the bound substrate, yielding a value between 0.1–1 sec^{-1}. This range of values would be equal to the observed rate constants in the presence of 60–600 M imidazole. This is not a particularly large rate acceleration for an enzymatic reaction; indeed the lower end of the range corresponds quite closely with the figure of 55 M which has been suggested as appropriate for comparing inter- to intramolecular reactions (Koshland, 1962). Thus the mechanistic pathway of acylation can be described as an operation in which the ester is absorbed at the hydrophobic site followed by

simple reaction with the imidazole side chain of histidine-57 at a rate that is not significantly enhanced over that expected from the known rates of nonenzymatic reactions. The acyl group is then transferred from the reactive acylimidazole to serine-195 to form the metastable isolable acyl-enzyme.

Frankfater and Kézdy (1971) have recently reported that the rates of acylation of chymotrypsin by *p*-nitrophenyl acetate and by *p*-nitrophenyl thiolacetate are identical, and have argued, from this result and from previous model studies which showed that nitrogen nucleophiles react with *certain* thiol esters at much greater rates than with their oxygen analogs, that imidazole could not be the primary nucleophile in the chymotrypsin reactions. The rate constants for the reactions of the *p*-NP ester and thiol ester under consideration here with imidazole, however, differ by only a factor of two (Table VI). Very minor differences in orientation at the enzyme active site could easily account for this small difference, and therefore, the identity in the enzymatic rate constants does not militate against imidazole as the primary nucleophile. The reason that the rate constants for the reactions of imidazole with *this* ester and its thiol ester analog are similar is that imidazole is a more powerful nucleophile than is either one of these two leaving groups. For this reason the transition state is reached at an early time along the reaction coordinate, and the effects of electron withdrawal in the leaving group are of less consequence than they are for reactions involving late transition states (Kirsch and Jencks, 1964; Jencks and Gilchrist, 1968). Finally it should be pointed out that the similarity in the rate constants does not provide evidence concerning the possible existence of a tetrahedral intermediate along the reaction pathway as argued by Frankfater and Kézdy (1971) (see, for example, the discussion by Jencks and Gilchrist, 1968).

Generality of the Mechanism. While the results and conclusions described above only relate to active esters of nonspecific substrates, it is important to assess the possible applicability of this mechanism to other substrates. The main argument *against* the occurrence of acylimidazoles as intermediates in reactions of unreactive esters or of amides is thermodynamic. The ΔF for the transfer of an acetyl group from *p*-nitrophenol to imidazole at pH 7 is -40 cal/mole whereas the formation of acylimidazoles from ethyl esters or peptides is unfavorable by $+8250$ and $+12{,}500$ cal per mole, respectively (Gerstein and Jencks, 1964). The lower ρ values for the leaving group in the *N*-methanesulfonyl-L-phenylalanine and hippurate series of esters (Williams, 1970) compared to the phenyl acetates also suggest that a different mechanism is operative for substrates other than active esters of nonspecific substrates. It is interesting that the substrates which gave the first experimental evidence for an acyl-enzyme intermediate in chymotrypsin-catalyzed reactions (Hartley and Kilby, 1954) actually form this derivative by what is probably an anomalous pathway allowed only by favorable thermodynamic factors and an atypical mode of binding.

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Mechanism of Action of Aspartase. A Kinetic Study and Isotope Rate Effects with ^2H†

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ABSTRACT: The mechanism of the aspartase-catalyzed reaction has been investigated at pH 7.0 in the presence of 1 mM Mg^{2+} by kinetic analysis and isotope rate studies with ^2H. Product inhibition and initial velocity patterns for the forward and reverse reactions, respectively, are consistent with a random mechanism in which all steps prior to the interconversion of the central complexes are in rapid equilibrium. The Michaelis, dissociation, and inhibition constants for fumarate and NH_4^+, as well as the Michaelis constant for 2*S*-aspartate, have been determined. No primary isotope effect was observed when the initial rates of deamination of 2*S*,3*R*-[3-^2H]aspartate and unlabeled 2*S*-aspartate were compared. However, a secondary isotope rate effect of 1.11 ± 0.02 was obtained with 2*S*-[2-^2H]aspartate. Provided that the release of products is not rate limiting, these findings suggest that the rate-determining step of the reaction could be C–N bond breakage and imply that, as the amino group leaves, the resulting intermediate could be accompanied by considerable carbonium-ion development at carbon 2. The possibility that the release of one of the products could be rate limiting is discussed.

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the conversion of 2*S*-aspartate (equivalent to L-aspartate) to fumarate and NH_4^+ in the following reversible reaction

$$^-OOCCH_2\underset{\displaystyle NH_3^+}{\underset{|}{C}}HCOO^- \rightleftharpoons {}^-OOCCH{=}CHCOO^- + NH_4^+$$

In the direction of fumarate amination, NH_4^+ can be replaced by hydroxylamine as an alternate substrate (Emery, 1963).

Aspartase has long been regarded as a catabolic enzyme in the amino acid metabolic schemes of various kinds of bacteria and plants. However, unlike most catabolic reactions, the aspartase reaction is readily reversible, and its equilibrium constant actually favors aspartate formation (Bada and Miller, 1968). This, together with the allosteric properties of the enzyme (Williams and Lartigue, 1967, 1969), suggests that aspartase may be a regulatory enzyme that could function synthetically, particularly under conditions in which aspartate was removed. In fact, Chibata *et al.* (1970) have patented a method for synthesizing 2*S*-aspartic acid with 83% yield by passing ammonium fumarate through a TEAE-cellulose column to which aspartase has been bound.

Englard (1958) and Krasna (1958) have shown independently that the addition of NH_4^+ to fumarate and its elimination from 2*S*-aspartate are stereospecific. Using the trans nature of the fumarate–hydratase reaction as a model, Gawron and Fondy (1959) have concluded from the data of Krasna

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