Neurath, H., Gladner, J. A., and Davie, E. W. (1954), *in* Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., editors, Baltimore, The Johns Hopkins Press.
Niewiarowski, S., Kowalski, E., and Stachurska, J. (1959), *Acta Biochim. Pol. 6*, 42.
Pechet, L., and Alexander, B. (1960), *Fed. Proc. 19*, 64.
Remmert, L. F., and Cohen, P. P. (1949), *J. Biol. Chem. 181*, 431.
Ronwin, E. (1956), *Canad. J. Biochem. Physiol. 34*, 1169.
Schwert, G., Neurath, H., Kaufman, S., and Snoke, J. (1948), *J. Biol. Chem. 172*, 221.
Seegers, W. H., and Smith, H. P. (1942), *Am. J. Physiol. 137*, 348.
Seegers, W. H., McClaughry, R. I., and Fahey, J. L. (1950), *Blood 5*, 421.
Seegers, W. H. (1962), *in* Proceedings VIIIth Congress European Society of Hematology, Vienna, 1961, vol. II, Basel, S. Karger, p. 399.
Scheraga, H. A. (1961), Protein Structure, New York, Academic Press, Inc., pp. 129–174.
Shainoff, J. R., and Page, I. H. (1960), *Circulation Res. 8*, 1013.
Sherry, S., and Troll, W. (1954), *J. Biol. Chem. 208*, 95.
Troll, W., Sherry, S., and Wachman, J. (1954), *J. Biol. Chem. 208*, 85.
Waley, S. G., and Watson, J. (1951), *Nature 167*, 360.
Wallén, P., and Bergstrom, K. (1958), *Acta Chem. Scand. 12*, 574.
Waugh, D. F., and Livingstone, B. J. (1951), *Science 113*, 121.
Wu, F. C., and Laskowski, M. (1955), *J. Biol. Chem. 213*, 609.

The Effect of Substituents on the Deacylation of Benzoyl-Chymotrypsins

MICHAEL CAPLOW AND WILLIAM P. JENCKS

*From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts**

Received June 4, 1962

The effect of substituents on a single step of a chymotrypsin-catalyzed reaction was studied with isolated *para* and *meta* substituted benzoyl-chymotrypsins. Deacylation is facilitated by electron-withdrawing substituents, and a *rho* value of 2.1 was observed for p-CH_3O-, p-CH_3-, H-, p-Cl-, m-F-, and p-CF_3-benzoyl-chymotrypsins. The negative deviations of *para*- and *meta*-nitrobenzoyl-chymotrypsins from the linear relationship are attributed to steric effects of the bulky nitro group. The rates of deacylation of p-nitrobenzoyl-, anisoyl-, and benzoyl-chymotrypsins were found to depend upon the ionization of a group with a pK of 7.25–7.40. The hydrolysis of benzoyl-chymotrypsin at pH 8.24 is 3.6-fold faster in water than in deuterium oxide. This suggests, but does not prove, that the mechanism involves general base catalysis. The nonenzymatic reactions of a series of substituted p-nitrophenyl benzoates, studied for comparison with the enzymatic reaction, display *rho* values of 2.04 for alkaline hydrolysis, 1.19 for nucleophilic catalysis of hydrolysis by imidazole, and *ca.* 1.57 for a general base-catalyzed reaction with imidazole.

Certain acyl transfer and hydrolysis reactions catalyzed by chymotrypsin proceed by transfer of the acyl group of the substrate to the enzyme to form an acyl-enzyme, which is subsequently catalytically hydrolyzed (Neurath and Hartley, 1959). This acyl-enzyme is isolable when the substrate is an effective acylating agent and the rate of enzyme acylation greatly exceeds that of deacylation. The isolation of this covalently bound enzyme-substrate complex makes it possible to study the deacylation independent of the other reactions in the catalytic process, and to investigate the mechanism of this single step in the enzymatic reaction.

The acyl group of acyl-chymotrypsin is believed to be bound to the protein in an ester linkage (Bender, 1960). Since deacylation involves ester hydrolysis, a comparison of acyl-chymotrypsin hydrolysis with nonenzymatic esterolytic creations may be instructive in determining the nature of the enzymatic process. For example, if the important step in the deacylation involves a nucleophilic attack on the ester bond, as in alkaline saponification or nucleophilic catalysis, a large rate enhancement will be produced by electron-withdrawing substituents. If, however, the important catalytic process resembles acid catalysis of ester hydrolysis, in which there is a cancellation of the opposing polar effects on protonation and on hydrolysis of the protonated substrate, the rate would be expected to be insensitive to polar substituents.

The effects of changes in the electronic properties of substrates hydrolyzed by chymotrypsin and a number of other hydrolytic enzymes have been studied previously. In several of these studies the effects of substituents on substrate binding and on the hydrolytic step have not been separated and it is, therefore, not possible to attribute substituent effects solely to the hydro-

* Publication No. 176.

lytic mechanism (Aldridge and Davison, 1952; Mounter and Whittaker, 1953; Bergmann *et al.*, 1958; Mounter, 1958). In studies in which kinetic analysis has separated the effects of substrate structure on V_{max} and K_m (Gawron *et al.*, 1953; Nath and Rydon, 1954; Lumry and Smith, 1955; Dodgson *et al.*, 1956; Nimmo-Smith, 1960), it has still not been possible to attribute the substituent effects to a known single step in the enzymatic process. Studies with chymotrypsin, for which the kinetic parameters are fairly well defined, have been made with substrates in which the electronic modification is accompanied by major changes in substrate structure, so that it is not possible to assign the rate differences to polar effects alone (Bender and Turnquest, 1955; Dixon and Neurath, 1957a; McDonald and Balls, 1957; Manning and Niemann, 1958). The effect of polar substituents in the *leaving* group of esters on the rate of acylation of chymotrypsin has recently been examined by Bender and Nakamura (1962), who have found that the rate is increased by electron-attracting substituents in the phenyl group of a series of substituted phenyl esters. However, it is not entirely clear whether this result can be attributed only to the acylation step, since the effects on the acylation rate and on the binding of substrate were not separated for most of the compounds studied.

The acyl enzymes utilized for this study were a series of *para*- and *meta*-substituted benzoyl-chymotrypsins. These were chosen because electronic modification by *meta* and *para* substituents on a benzoyl group already bound to the enzyme involves relatively minor change in substrate structure, so that steric effects at the reaction center are minimized. For comparison to the enzymatic process, a study was also made of the rates of the nonenzymatic alkaline and imidazole-catalyzed hydrolysis of a series of *p*-nitrophenyl benzoates. A preliminary report of this work has appeared (Caplow and Jencks, 1962).

Experimental Procedure

p-Nitrophenyl acetate (m.p. 78–79°) was prepared according to Chattaway (1931), recrystallized from hexane, and stored over $CaSO_4$. Deuterium oxide, 99.8%, was prepared by the Atomic Energy Commission and was obtained through the courtesy of the Department of Chemistry, Harvard University. The deuterium oxide was glass distilled before use. For the studies carried out in D_2O, all reaction components with exchangeable hydrogen atoms, with the exception of benzoyl-chymotrypsin, were pre-equilibrated in 99.8% D_2O by twice dissolving the materials in deuterium oxide and evaporating the solutions to dryness. Buffer solutions were neutralized with DCl.

Spectrophotometric rate measurements were carried out with a Zeiss PMQII spectrophotometer, equipped with a thermostated cell compartment maintained at 25°. Ultraviolet spectra were recorded with a Cary recording spectrophotometer, model 14, or with the Zeiss PMQII instrument.

Acylimidazoles were prepared by a slight modification of the method of Gerngross (1913), by adding 0.01 mole of the appropriate acyl chloride dropwise to 1.36 g of recrystallized imidazole in 1 liter of chilled benzene or ether which had been redistilled from lithium aluminum hydride or sodium. The reaction mixture was stirred overnight at room temperature, filtered, and evaporated to dryness under reduced pressure. The acylimidazoles were recrystallized before use, with the exception of benzoylimidazole, which was estimated to be 97% pure by conversion to the hydroxamate and comparison of the optical density of the ferric chloride complex at 540 mμ with that of pure benzohydroxamic acid. Benzoyl chloride, which would also react in a hydroxamic acid assay, could not be detected by infrared analysis. Physical constants of the acylimidazoles are listed in Table I.

The rate of the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was followed by measuring the rate of *p*-nitrophenolate ion formation at 400 mμ in a reaction mixture containing 8×10^{-4} M substrate, 4% acetonitrile, and 0.03 M tris(hydroxymethyl)aminomethane at *p*H 7.50.

Substituted benzoyl-chymotrypsins were prepared from crystalline, salt-free α-chymotrypsin, obtained from the Worthington Biochemical Corporation. Acylation was carried out at room temperature at *p*H 6.0 with 10–30 mg/ml of chymotrypsin and a slight molar excess of acylimidazole. After acylation the reaction mixture was adjusted to *p*H 3.4 with 0.1 M HCl, extracted three times with ether, and then freeze dried. The acylated enzyme is catalytically inactive, and the completeness of acylation was ascertained by observing the loss of enzymatic activity toward *p*-nitrophenyl acetate.

Hydrolysis of the acyl-chymotrypsins was carried out at 25° with 10–16 mg/ml of freshly prepared material, in 1-ml stoppered test tubes. The deacylated enzyme is able to hydrolyze *p*-nitrophenyl acetate and, since the rate of this reaction is proportional to the concentration of free enzyme, this assay was utilized to measure the concentration of deacylated enzyme. At substrate concentrations which are well above the apparent K_m, the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate occurs with a rapid acylation, which releases an amount of *p*-nitrophenolate equivalent to the free chymotrypsin concentration, followed by a slow steady state hydrolysis, which is also proportional to the concentration of free enzyme (Hartley and Kilby, 1954; Neurath and Hartley, 1959). The appearance of free chymotrypsin was therefore determined from measurements of the amount of *p*-nitrophenolate released in the initial acylation, as determined from either a 10-second reading or an extrapolated zero-time reading, and from the

TABLE I
PHYSICAL PROPERTIES OF ACYLIMIDAZOLES

Compound	m.p.	O[a] ‖ C / \ μ	λ_{max}[b] mμ	Recrystallization Solvent	k_{H^+}[c] min^{-1}
p-Nitrobenzoylimidazole, $C_{10}H_7N_3O_3$	120–122.5°	5.81	261	cyclohexane	24.
calc. C 55.27 H 3.25 N 19.25					
obs.[d] C 54.82 H 3.40 N 19.35					
m-Nitrobenzoylimidazole, $C_{10}H_7N_3O_3$	85.5–86.5°	5.80	259	cyclohexane	23.
calc. C 55.27 H 3.25 N 19.25					
obs. C 54.90 H 3.40 N 19.35					
p-Methoxybenzoylimidazole, $C_{11}H_{10}N_2O_2$	69–71°	5.84	285	cyclohexane	0.45
calc. C 65.30 H 4.99 N 13.86					
obs. C 65.32 H 4.93 N 13.83					
p-Chlorobenzoylimidazole, $C_{10}H_7N_2OCl$	85–86.5°	5.83	250	60–110° petroleum ether	4.4
calc. C 58.09 H 3.42 N 13.56					
obs. C 57.59 H 3.55 N 13.12					
p-Methylbenzoylimidazole, $C_{11}H_{10}N_2O$	69–71°	5.83	253	60–110° petroleum ether	0.94
calc. C 70.92 H 5.42 N 15.05					
obs. C 70.77 H 5.38 N 14.80					
m-Fluorobenzoylimidazole, $C_{10}H_7N_2OF$	49–49.5°	5.79	270	37–55° petroleum ether	6.5
calc. C 62.15 H 3.71 N 14.73			240 sh.		
obs. C 62.76 H 3.80 N 14.83					
p-Trifluoromethylbenzoylimidazole, $C_{11}H_7N_2OF_3$	78–79.5°	5.79	270 225	60–110° petroleum ether	
calc. C 55.00 H 2.94 N 11.66					
obs. C 54.98 H 3.13 N 11.76					
Benzoylimidazole, $C_{10}H_8N_2O$	[e]	5.83	243		2.2
Furoylimidazole, $C_8H_6N_2O_2$	48–49°	5.86	284	40–60° petroleum ether	1.2
calc. C 59.25 H 3.73 N 17.28		5.91			
obs. C 59.09 H 3.80 N 17.41					

[a] In CCl_4. [b] In water. [c] Rates measured for the fully protonated compound, in 0.1 M HCl, at 25°, ionic strength 0.1. Rates were followed spectrophotometrically, except for the *para* and *meta* nitro compounds, which were measured by the hydroxamic acid procedure. Rate constants for the *para* and *meta* nitro compounds are approximate values. [d] Analyses by Scandinavian Microanalytical Laboratory. [e] Gerngross (1913) reported a melting point of 18–19° for this compound.

slope of the steady-state hydrolytic rate. It may be noted that the determination of first order rate constants depends on the measurement of the percent change in activity, not the absolute level of activity. In addition, the rate of disappearance of anisoyl-chymotrypsin was measured directly by measuring the concentration of remaining ester with a slight modification of the Hestrin alkaline hydroxylamine procedure (Hestrin, 1949). Aliquots of 0.20 ml were removed from a 2×10^{-3} M solution and were incubated for 5 minutes with 0.5 ml of a solution which was freshly prepared by adding two volumes of 3.5 M NaOH to one volume of 4 M $NH_2OH \cdot HCl$. The ferric-hydroxamate color was developed by the addition of 1.3 ml of 10% $FeCl_3 \cdot 6H_2O$ in 0.7 M HCl, and 1 minute later 0.2 ml of 50% trichloracetic acid was added for deproteinization. The samples were filtered twice through glass wool, and the ferric-hydroxamic acid complex was measured spectrophotometrically at 540 mμ.

p-Nitrophenyl benzoates were a gift of Dr. Bruce M. Anderson. Rates of hydrolysis were measured spectrophotometrically by following the appearance of *p*-nitrophenolate at 400 mμ. The base-catalyzed hydrolysis was carried out in 0.25 M triethylamine HCl buffer at an apparent *p*H of 11.26, in 33% acetonitrile.

Measurements of *p*H were made with a Radiometer *p*H meter, type PHM 4b, with a G-200 B glass electrode. The *p*D values of deuterium oxide solutions were estimated from the formula *p*D = *p*H + 0.40 (Glasoe and Long, 1960). Hydroxide ion was estimated from the measured *p*H, taking $K_w = 10^{-14}$.

Rate constants were obtained by plotting the extent of the reaction, $x_\infty - x_t$, against time on semilogarithmic graph paper and by calculating the first order rate constants from the equation, $k = 0.693/t^1/_2$. Second order rate constants for reactions which are first order in respect to amine concentration were determined from the slope of a plot of observed pseudo first order rate constants against amine concentration. Third order rate constants were determined from the slope of plots of $k_{obs}/[RNH_2]$ *vs.* $[RNH_2]$. The intercepts of such plots gave the second order rate constants for these reactions.

RESULTS

The rates of deacylation of benzoyl-chymotrypsins were followed by measuring the amount of

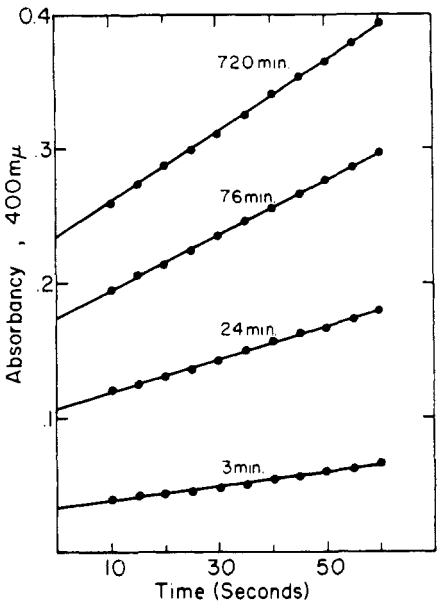


FIG. 1.—Hydrolysis of *p*-nitrophenyl acetate by aliquots of a deacylating anisoyl-chymotrypsin reaction mixture. See text for reaction conditions of the *p*-nitrophenyl acetate assay.

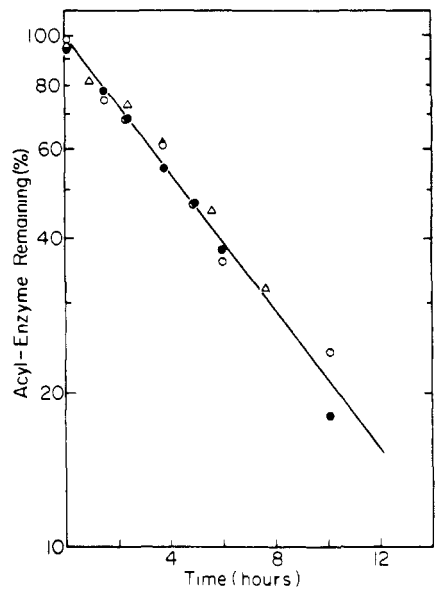


FIG. 2.—Hydrolysis of anisoyl-chymotrypsin at *p*H 7.07 in 0.1 M phosphate buffer at 25°, followed by the initial burst, ●, steady state rate, O, and hydroxamate, Δ, methods.

p-nitrophenolate released in the initial burst when aliquots of the deacylating acyl-enzyme reaction mixture were allowed to react with *p*-nitrophenyl acetate. An example, which shows the initial burst and the steady state reaction with *p*-nitrophenyl acetate of aliquots removed at different times from a solution of anisoyl-chymotrypsin, is shown in Figure 1. It may be seen that both the initial burst and the steady state hydrolytic rate increase as deacylation proceeds. The rate constants reported in this study were determined from measurements of the initial burst, since this method proved to be simplest and most accurate. The validity of the method was confirmed in several cases by comparison of the rates obtained by following the increase in the initial burst and in the steady state hydrolytic rates, and by directly following the disappearance of the acyl-enzyme as the hydroxamate. As shown in Figure 2, the deacylation of anisoyl-chymotrypsin follows the expected first-order kinetics and the three methods of analysis give results which are equivalent, within the error of the determinations. This is in contrast to the results reported by Viswanatha and Lawson (1961), who found that the rate of deacylation of C^{14}-acetyl-chymotrypsin did not appear to parallel the return of enzymatic activity toward acetyltyrosine ethyl ester, measured under somewhat different experimental conditions.

The effect of polar substituents on the rates of deacylation of substituted benzoyl-chymotrypsins is summarized in Table II. A good correlation

TABLE II

RATES OF DEACYLATION OF SUBSTITUTED BENZOYL-CHYMOTRYPSINS IN 0.1 M PHOSPHATE BUFFER, *p*H 7.07, AT 25°

Substituent	σ^a	$k \times 10^3$ (min.$^{-1}$)
p-CH_3O	−0.27	2.43
p-CH_3	− .17	3.24
H	.00	12.3
p-Cl	.23	19.7
m-F	.34	53.3
p-CF_3	.54	122.
m-NO_2	.71	85.3
p-NO_2	.78	21.7

[a] Brown *et al.* (1959).

is observed between the rate of hydrolysis and the electron-withdrawing ability, as indicated by Hammett *sigma* constants, of the *p*-CH_3O, *p*-CH_3, H, *p*-Cl, *m*-F, and *p*-CF_3 substituents (Fig. 3). The *para* and *meta* nitro groups show negative deviations from this relationship. This is attributed to a steric effect, because the smaller *m*-fluoro and *p*-trifluoromethyl groups, which are also strongly electron-withdrawing, show a satisfactory fit to the same *sigma-rho* relationship as the other substituted benzoyl-chymotrypsins. The nitro group is considerably larger than any of the other substituents examined, and might be expected to distort the acyl-enzyme or to provide steric inhibition to the planar form of this substituent which is required for resonance electron-withdrawal. The *p*-methyl and *p*-trifluoromethyl groups have similar orbital radii (Pauling, 1960), but differ by 39-fold in their effect on the rate of benzoyl-chymotrypsin hydrolysis; this difference is clearly a polar, and not a steric, effect.

The rates of deacylation of *p*-nitrobenzoyl- and anisoyl-chymotrypsin were found to depend upon the ionization of a group with a pK_a' of 7.25; the corresponding pK_a' value for benzoyl-chymotrypsin is 7.40 (Fig. 4). The small difference between these values makes it possible to attribute the differences in the rates of deacylation to polar effects rather than to an alteration of the *p*H dependence of the rate with the different substrates.

A specific buffer effect was observed with tris-(hydroxymethyl)aminomethane (Tris), a 0.1 M concentration of which, at *p*H 8.0, was found to increase the rate of deacylation of benzoyl-, *p*-nitrobenzoyl-, and anisoyl-chymotrypsin 2.7, 3.1, and 3.7-fold, respectively, over that observed at the same *p*H in 0.1 M borate or collidine buffer. This may reflect acylation of the Tris. Kerr and Niemann (1958) have shown that concentrated Tris buffers increase the rate of chymotrypsin-catalyzed disappearance of α - *N* - nicotinyl - L-tyrosinhydrazide. These authors attribute this result to an ionic strength effect.

The rate of deacylation of benzoyl-chymotrypsin at *p*H 8.24 was found to be decreased 3.6-fold in deuterium oxide (Fig. 4), confirming the observation made previously by Bender *et al.* (1961b), with cinnamoyl-chymotrypsin. The rate of deacylation is unaffected by small *p*H variations in this *p*H region, and the result, therefore, cannot be attributed to a small error in estimating *p*D from *p*H.

The ultraviolet difference spectra of benzoyl- and furoyl-chymotrypsins are compared to the spectra of the corresponding acylimidazoles and alkyl esters in Table III and Figure 5.

Nonenzymatic Reactions of p-Nitrophenyl Benzoates.—The rates of alkaline hydrolysis and imidazole-catalyzed hydrolysis of a series of acyl-substituted *p*-nitrophenyl benzoates were determined for comparison with the chymotrypsin esterolytic reaction and are summarized in Table IV. The rates of alkaline hydrolysis were measured in triethylamine buffer at an apparent *p*H of 11.26 in 33% acetonitrile. The rate of the reaction with *p*-nitrophenyl benzoate was shown to be directly proportional to hydroxide ion activity between *p*H 9.8 and 11.0, and it was shown that the rate of hydrolysis of *p*-nitrophenyl *p*-nitrobenzoate was not increased by doubling the concentration of triethylamine buffer at constant *p*H. The rates follow the *sigma-rho* correlation with a *rho* value of 2.04 (Fig. 6, curve B).

The rates of imidazole-catalyzed hydrolysis of *p*-nitrophenyl benzoates with electron-withdrawing substituents on the benzoyl group were found to increase more rapidly than the first power of the imidazole concentration at constant *p*H. The results obtained with *p*-nitrophenyl *p*-nitrobenzoate are shown in Figure 7. This behavior suggests that the imidazole reaction is subject to general base catalysis, involving a second molecule of base, as has been shown for several reactions of esters with nitrogen bases (Bunnett and

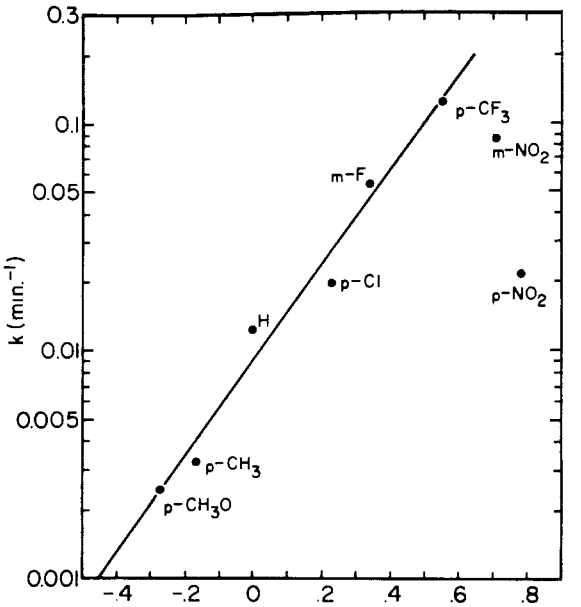


FIG. 3.—*Sigma-rho* plot of the deacylation of benzoyl-chymotrypsins at 25°, in 0.1 M phosphate buffer, *p*H 7.07; ρ = 2.1.

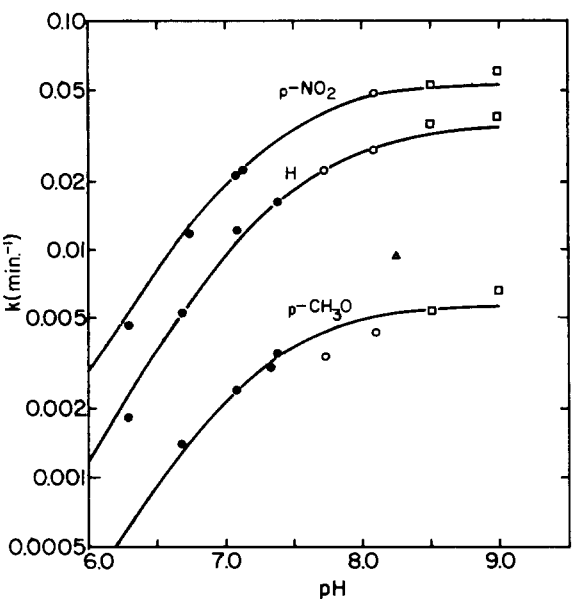


FIG. 4.—Dependence on *p*H of the rates of deacylation of *p*-nitrobenzoyl-, benzoyl-, and anisoyl-chymotrypsin at 25°. The solid lines are the theoretical lines calculated for *pK* values of 7.25, 7.25, and 7.40 for the reactions of *p*-nitrobenzoyl-, anisoyl-, and benzoyl-chymotrypsin, respectively. Phosphate buffer, 0.1 M, ●, collidine buffer, 0.1 M, ○, borate buffer, 0.1 M, □, collidine buffer, 0.1 M, in deuterium oxide, △.

Davis, 1960; Jencks and Carriuolo, 1960). The third-order catalytic constant for this catalyzed reaction was calculated by plotting the observed second-order constants against imidazole concentration (Jencks and Carriuolo, 1960) (Fig. 7, upper

TABLE III

SPECTRA OF ACYL-ENZYMES, ACYLIMIDAZOLES, AND METHYL ESTERS IN AQUEOUS SOLUTION

Acyl Group	Acylimidazole	λ_{max}, mμ Acyl-Enzyme[a]	Methyl Ester
p-NO_2-benzoyl	261	265	265
m-NO_2-benzoyl	259	260	261
p-CH_3O-benzoyl	285	268	257
m-F-benzoyl	270	275	277
	240 sh.	<240 (main peak)[b]	228 (main peak)
p-CF_3-benzoyl	270	275	275
	225	<240 (main peak)[b]	226 (main peak)
p-Cl-benzoyl	250	240	242
Benzoyl	243	272	272
		<240 (main peak)[b]	230 (main peak)
Furoyl	284	256	255

[a] Difference spectrum of acyl-chymotrypsin *vs.* chymotrypsin, approximately 0.75 mg/ml, recorded 10 minutes after preparation. The acyl-enzymes were prepared in 0.01 M acetate buffer, *p*H 4.67, with a substrate–enzyme ratio of 1.00–1.16. The extent of acylation, determined by the *p*-nitrophenyl acetate assay, varied from 70–96%. [b] Absorption by the protein prevented measurement of the difference spectra below this wave length.

curve). The values of the rate constants so obtained, as well as the second-order rate constants for the uncatalyzed reaction with imidazole, are given in Table IV. *Rho* for the uncatalyzed reaction is equal to 1.19 (Fig. 6, curve C). The *rho* value for the imidazole-catalyzed reaction of imidazole is difficult to determine precisely, because the contribution of the general base-catalyzed reaction becomes small in the absence of electron-donating substituents; the available data indicate that the *rho* value is approximately 1.57 (Fig. 6, curve D).

The third-order term in the imidazole reaction is not a salt or solvent effect, since the reaction

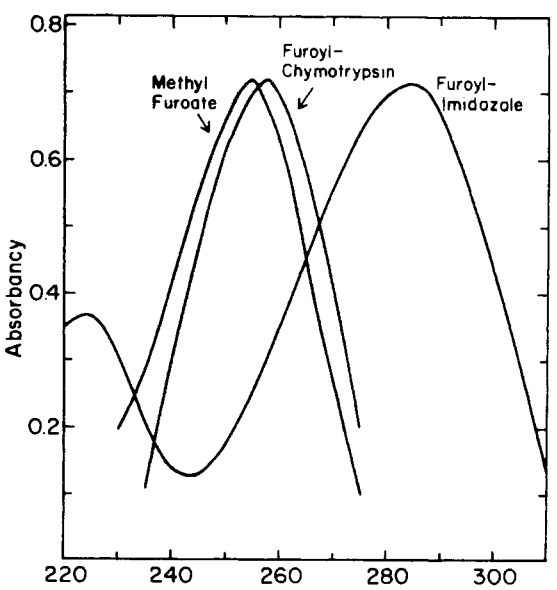


FIG. 5.—Spectra of furoylimidazole, methyl furoate, and difference spectrum of furoyl-chymotrypsin *vs.* chymotrypsin in aqueous solution. The spectra have been corrected to the same absorbancy at the λ_{max}. for purposes of comparison; the observed Δ absorbancy of the furoyl-chymotrypsin was 0.14.

was carried out at a constant ionic strength of 0.1 and the reaction with *p*-nitrophenyl *p*-nitrobenzoate was shown to be independent of ionic strength from 0.05 to 0.2. Furthermore, a change in acetonitrile concentration from 20% to 50% decreased the rate five-fold, from 2.6 to 0.51 M^{-1} $min.^{-1}$, which is in the opposite direction from the effect observed with increasing imidazole concentration. The rate of the uncatalyzed reaction with imidazole is decreased only slightly in D_2O, with k_{H_2O}/k_{D_2O} = 1.13, whereas the rate of the imidazole-catalyzed reaction with imidazole is considerably decreased in D_2O, with k_{H_2O}/k_{D_2O} = 1.81, as expected for a general base-catalyzed reaction (Table IV) (Jencks and Carriuolo, 1961; Bender *et al.*, 1962). With *N*-methylimidazole, which has no proton to remove by general base catalysis, no such third-order catalytic term was observed. A third-order term due to general base catalysis was also observed in the reaction of *p*-nitrophenyl *p*-nitrobenzoate with glycine ethyl ester (Table IV). No such term was found in the reaction with methylamine, but this reaction is too fast to study by ordinary techniques at concentrations of base similar to those at which general base catalysis contributes a significant part of the over-all rate in the reactions with other, less basic amines.

The immediate product of the reaction of imidazole with *p*-nitrophenyl *p*-nitrobenzoate was shown to be the acylimidazole, by conversion to the hydroxamic acid. After the reaction with imidazole had proceeded for four half-times, measured by *p*-nitrophenolate release, 74% of the hydroxylamine-reacting material which was originally present was detected upon addition of hydroxylamine, and 10% was found after 20 half-times. The imidazole reaction, therefore, involves the formation, followed by the hydrolysis, of *p*-nitrobenzoylimidazole. The rates of hydrolysis of the series of substituted benzoylimidazoles in acid solution, in which the compounds are completely converted to their conju-

TABLE IV
RATES OF REACTIONS OF ACYL-SUBSTITUTED *p*-NITROPHENYL BENZOATES AT 25°

Acyl Group	Alkaline Hydrolysis		Imidazole		N-methyl-imidazole	Methyl-amine	Glycine Ethyl Ester	
	$k_{obs.}$ at pH 11.26[a] (min.$^{-1}$)	k_{OH^-}[a,b] (M^{-1} min.$^{-1}$)	k_2[c,d] (M^{-1} min.$^{-1}$)	k_3[c,d] (M^{-2} min.$^{-1}$)	k_2[c,d] (M^{-1} min.$^{-1}$)	k_2[c,e] (M^{-1} min.$^{-1}$)	k_2[c,f,g] (M^{-1} min.$^{-1}$)	k_3[c,g] (M^{-2} min.$^{-1}$)
p-CH_3O-benzoyl	0.0042	2.32	0.036	[h]				
p-CH_3-benzoyl	.0076	4.23	.067	[h]				
Benzoyl	.020	11.1	.105[f]	0.04				
p-Cl-benzoyl	.051	28.5	.167[f]	.08				
m-NO_2-benzoyl	.46	251.	.59[f]	.52				
p-NO_2-benzoyl	.77	428.	.89[f]	.78	0.35	690.	0.53	0.41
p-NO_2-benzoyl in deuterium oxide			.79[f]	.43				
rho	2.04		1.19	1.57				

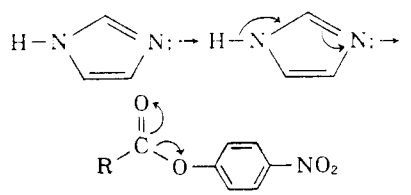
[a] In 33% acetonitrile in one-tenth neutralized triethylamine buffer, 0.25 M, apparent *p*H 11.26, ionic strength 1.0. [b] Calculated assuming $K_w = 10^{-14}$. [c] Based on the concentration of amine as the free base. [d] Measured in 27% acetonitrile, apparent *p*H 7.7, ionic strength 0.1, amine present as 90% free base. [e] Measured in 29% acetonitrile, apparent *p*H 8.5, 0.1 M tris(hydroxymethyl)aminomethane buffer, ionic strength 0.6. [f] Extrapolated value, from a plot of $k_{obs.}$/[free amine] *vs.* [free amine]; see Figure 7 for example. [g] Measured in 29% acetonitrile, apparent *p*H 7.97; glycine ethyl ester was present as 70% free base, ionic strength 1.0. [h] Not detectable.

gate acids (Jencks and Carriuolo, 1959a; Marburg and Jencks, 1962) are given in Table IV. The rates follow the Hammett relationship with a *rho* value of 1.7 (Fig. 6, curve A).

DISCUSSION

Nonenzymatic Reactions of Acyl-Substituted p-Nitrophenyl Benzoates.—The rate of alkaline hydrolysis of a series of *p*-nitrophenyl benzoates, substituted in the benzoyl group, is increased by electron - withdrawing substituents. The *rho* value of 2.04 is similar to that found for other substituted benzoate esters (Tommila and Hinshelwood, 1938; Jaffé, 1953). The rate of nucleophilic catalysis of the hydrolysis of these compounds by imidazole is somewhat less sensitive to substituents in the acyl group, with a *rho* value of 1.19. This is in contrast to the effect of substituents in the leaving group, which have a greater effect on the reaction with imidazole ($\rho = 1.90$) than on alkaline hydrolysis ($\rho = 1.0$) in a series of substituted phenyl acetates (Bruice and Schmir, 1957). Nucleophilic catalysis involving intramolecular participation of imidazole in the hydrolysis of acyl-substituted phenyl benzoates has a *rho* value of 1.67 (Pandit and Bruice, 1960).

It is of interest that the imidazole-catalyzed hydrolysis of *p*-nitrophenyl benzoates is itself subject to general base catalysis by imidazole, since no reaction of either imidazole or *p*-nitrophenyl esters has previously been found to be subject to general base catalysis. The evidence for such general base catalysis is (1) the third-order term in the rate expression for the reaction with imidazole, (2) the decrease in the rate of the general base-catalyzed reaction in deuterium oxide, (3) the absence of such catalysis with *N*-methylimidazole, and (4), by analogy, the finding of similar catalysis in the reaction with glycine ethyl ester. There are several possible mechanisms for such catalysis, similar to those observed for general base catalysis of other ester aminolyses (Bunnett and Davis, 1960; Jencks and Carriuolo, 1960); the simplest mechanism for the imidazole reaction involves removal of a proton by one imidazole molecule from a second molecule as it attacks the acyl group as shown in (1).



(1)

Since it is probable that the rate-determining step in the reaction of imidazole with *p*-nitrophenyl esters is the attack on the acyl group and since the imidazole anion is a rather unstable species, alternative mechanisms involving pre-equilibrium imidazole addition or general acid catalyzed reactions of the imidazole anion are less likely. Although, for technical reasons, the *rho* value for the imidazole-catalyzed reaction is not precise, it is certain that this reaction is more sensitive to the structure of the acyl group (ρ = *ca.* 1.57) than is the uncatalyzed reaction of imidazole ($\rho = 1.19$), because the general base-catalyzed reaction can only be detected with those substrates which contain electron - withdrawing substituents. Again, this is in contrast to the situation with phenyl-substituted phenyl acetates, in which general base-catalyzed aminolysis is observed only with relatively electron-donating substituents on the leaving group (Jencks and Carriuolo, 1960; Bruice and Mayahi, 1960).

Hydrolysis of Acyl-Chymotrypsins.—The effect of polar substituents on the rate of ester hydroly-

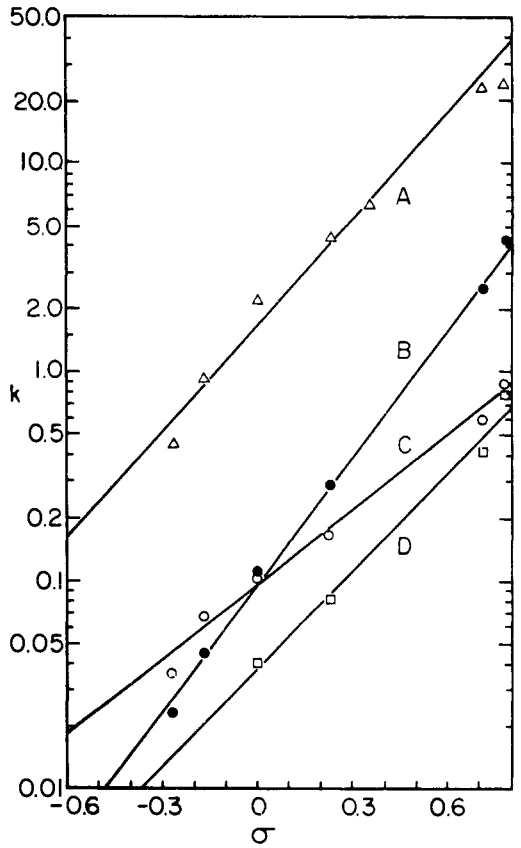


FIG. 6.—*Sigma-rho* plots for the hydrolysis of *p*-nitrophenyl benzoates and benzoylimidazolium compounds at 25°. Curve A: k (min.$^{-1}$) for hydrolysis of benzoylimidazoles in 0.1 M HCl; ρ = 1.7. Curve B: k (M^{-1} min.$^{-1}$) for alkaline hydrolysis of *p*-nitrophenyl benzoates; ρ = 2.04. Curve C: k (M^{-1} min.$^{-1}$) for nucleophilic catalysis by imidazole of the hydrolysis of *p*-nitrophenyl benzoates; ρ = 1.19. Curve D: k (M^{-2} min.$^{-1}$) for general base catalysis by imidazole of the reaction of imidazole with *p*-nitrophenyl benzoates; ρ = 1.57.

sis is dependent on the mechanism of catalysis of the reaction (Gould, 1959). If the important aspect of the catalytic process is a nucleophilic attack on the labile bond, a marked rate enhancement will be produced by electron-withdrawing substituents. This occurs because electron withdrawal increases the electrophilic character of the carbonyl carbon atom and facilitates nucleophilic attack on the ester linkage. If, however, the catalysis proceeds by protonation of the carbonyl group, accompanied by nucleophilic attack of a water molecule on the protonated substrate, the rate will be comparatively insensitive to substituents. This is because the electron withdrawal, which enhances nucleophilic attack, also hinders the protonation necessary for reaction. The reverse effect occurs with electron-donating substituents, which increase the extent of protonation of the ester bond but also decrease the electropositive nature of the carbonyl carbon atom, making nucleophilic attack more difficult. The approximate cancellation of these two effects accounts for the relative insensitivity of the rates of acid-catalyzed ester hydrolysis to substituent effects. For example, the alkaline hydrolyses of methyl benzoates (Tommila and Hinshelwood, 1938) and *p*-nitrophenyl benzoates are highly sensitive to polar substituents, with *rho* values of 2.3 and 2.04, respectively, while *rho* for the acid-catalyzed hydrolysis of methyl benzoates is only 0.11 (Timm and Hinshelwood, 1938).

The simplest and most direct conclusion that can be drawn from the effect of substituents on the rate of deacylation of benzoyl-chymotrypsins is that the participation of basic or nucleophilic groups is of greater importance than that of acidic groups in the enzymatic reaction. A similar conclusion has been drawn independently by Bender and Nakamura (1962) from their work on the acylation of chymotrypsin by phenyl-substituted phenyl acetates. This conclusion may be stated more precisely as follows: If the transition state for deacylation involves proton removal from an attacking water molecule and/or proton addition to the leaving alkoxide molecule, the proton removal is very nearly complete, with considerable stretching of the bond to hydrogen, while any proton donation by an acidic group has proceeded to only a small extent.

Two possible transition states for the deacylation involving general base catalysis are shown in Scheme 1. As discussed previously (Anderson *et al.*, 1961), these two mechanisms are kinetically indistinguishable, because they contain the same stoichiometric composition and

$$[B][H_2O] = K_a/K_{H_2O}[BH^+][OH^-]$$

from the equilibrium expressions for the dissociation of the enzymatic base and water. The difference between these two mechanisms is that in mechanism I the enzymatic base is acting to remove a proton from a water molecule or a water analogue, such as methanol or hydroxylamine, while in mechanism II the base removes a proton from the serine hydroxyl group.

The symmetry of the acylation and deacylation and considerations of microscopic reversibility require that the transition states for the two reactions be very similar (scheme 1) (Anderson *et al.*, 1961; Bender *et al.*, 1961b; Bruice, 1961). This is a consequence of the fact that many reactions catalyzed by chymotrypsin are reversible and, presumably, involve catalysis of the acylation and deacylation of serine, which are similar processes except for direction. The two reactions, therefore, exhibit a similar dependence on *p*H (Neurath and Hartley, 1959), deuterium oxide (Bender *et al.*, 1961a), and substituents (Bender and Nakamura, 1962). It also requires that any mechanism which includes general base catalysis in one direction must involve general acid catalysis in the opposite direction. Thus, it might be argued that both mechanisms I and

II in Scheme 1 should not show a large substituent effect, since both proceed by general acid catalysis in one direction. The general acid-catalyzed reactions should be independent of polar effects, because the electronic factors which favor anionic attack also retard proton transfer. The observed absence of a cancellation of these effects requires that proton transfer to the leaving alkoxide molecule must occur to only a slight extent in the transition states for acylation by mechanism I and deacylation by mechanism II. These modifications are shown in Scheme 1 by indicating that proton transfer to the leaving alkoxide ion in the transition states of the general acid catalyzed reactions has occurred to only a small extent, while proton transfer to the general base in the reverse reaction is almost complete. The incorporation of these considerations into Scheme 1 illustrates the conclusion that basic groups play a dominant role in the catalytic process.

Although the nature of the groups involved in the deacylation cannot be precisely determined, the *p*H dependence of the rate is consistent with the involvement of a histidine residue in the catalytic process. The imidazole group of histidine can function as either a nucleophilic or general base catalyst and has been implicated in the active site of chymotrypsin by convincing chemical evidence (Weil *et al.*, 1953; Whitaker and Jandorf, 1956; Schoellman and Shaw, 1962). Nucleophilic catalysis by an imidazole group with the formation of a highly reactive acylimidazole is thermodynamically unfavorable (Stadtman, 1954) but cannot be categorically ruled out, since this intermediate could be formed in low concentrations as a transient intermediate. General base catalysis has been suggested as a role for the imidazole group of histidine in chymotrypsin (Cunningham, 1957; Dixon and Neurath, 1957b; Bernhard and Gutfreund, 1957; Dixon *et al.*, 1958; Jencks and Carriuolo, 1959b; Spencer and Sturtevant, 1959) and is supported by the observation that imidazole is able to act as a general base catalyst in the deacylation of *N,O*-diacetylserinamide (Anderson *et al.*, 1961), a model for the active site of chymotrypsin and other esterolytic enzymes. It is, however, not possible to distinguish kinetically between general base and nucleophilic catalysis in the deacylation reaction, since the rates of both reactions are proportional to the concentration of the dissociated form of the enzymatic base. These two mechanisms can sometimes be differentiated by a study of the effect of deuterium oxide on the rates of catalysis. This is possible because a bond to hydrogen is stretched in general base catalysis of ester hydrolysis, which generally results in a two- to three-fold rate decrease in deuterium oxide (Anderson *et al.*, 1961; Jencks and Carriuolo, 1961). The observation of a 3.6 fold decrease in the rate of deacylation of benzoylchymotrypsin, as well as the observation of similar kinetic isotope effects in other chymotrypsin-catalyzed reactions (Bender *et al.*, 1961a,b) supports a general base catalysis mechanism. The possibility of a change in the protein structure produced by the deuterium oxide (Calvin *et al.*, 1959; Hermans and Scheraga, 1959) cannot be ruled out, however, and general base catalysis cannot be definitely implicated.

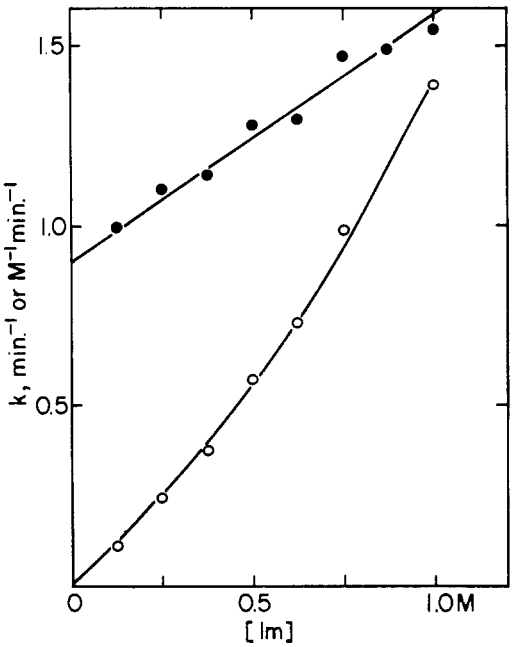
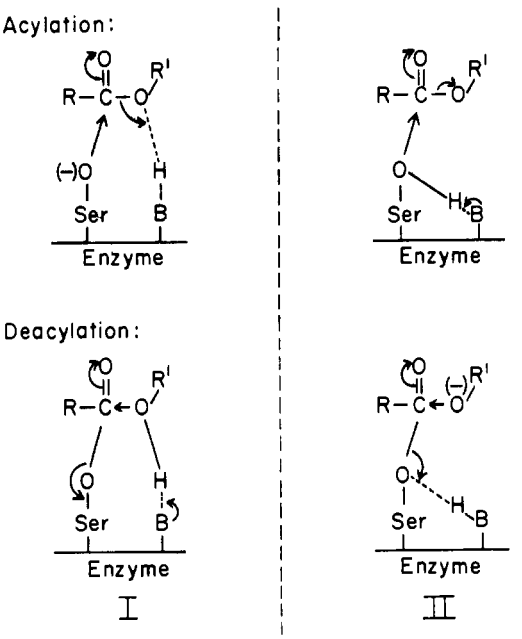


FIG. 7.—Hydrolysis of *p*-nitrophenyl *p*-nitrobenzoate as a function of imidazole concentration at 25° in 27% acetonitrile, at an apparent *p*H of 7.7, ionic strength 0.1. Rates were calculated for the concentration of amine present as the free base; k_{obs}, O; second order rate constant, ●, = k_{obs}/ [free amine].



Scheme 1

The high sensitivity to polar substituents of the general base-catalyzed reaction of *p*-nitrophenyl benzoates with imidazole indicates that the substituent effect observed in the deacylation of benzoyl-chymotrypsins is consistent with a general base-catalyzed mechanism. However, nucleophilic catalysis by imidazole with *p*-nitrophenyl benzoates as well as nucleophilic catalysis of several other reactions (Bruice and Schmir, 1957; Bruice and Sturtevant, 1959; Bruice and Mayahi, 1960; Gaetjens and Morawetz, 1960) show a similar substituent sensitivity, and this parameter cannot be used to differentiate between nucleophilic and general base catalysis.

A comparison of the ultraviolet difference spectra of the acyl-chymotrypsins provides further evidence that the site of acylation of the isolated acyl-enzyme is not the imidazole group of a histidine residue. Although the differences are relatively small, the spectra of the benzoyl-chymotrypsins are more similar to those of the benzoate methyl esters than to the corresponding benzoylimidazoles. Schonbaum *et al.* (1959) and Bender *et al.* (1961a) have previously shown that the absorption maximum of the difference spectrum of cinnamoyl-chymotrypsin (288 mμ) is somewhat closer to that of methyl cinnamate (279 mμ) than to that of cinnamoylimidazole (307 mμ). The more definitive results obtained with the furoyl derivatives of chymotrypsin ($\lambda_{max.}$ = 256 mμ), methanol ($\lambda_{max.}$ = 255 mμ), and imidazole ($\lambda_{max.}$ = 284 mμ, Fig. 5) provide further strong evidence that the acyl enzyme is, in fact, an ester. It may be noted that the difference spectra obtained with the benzoyl- and furoyl-enzymes are almost certainly not the result of changes in the conformation of the enzyme upon acylation, since the observed spectral differences are more than an order of magnitude larger than the changes in absorption at 290 mμ which have been attributed to changes in enzyme conformation (Havsteen and Hess, 1962).

Acknowledgments

We are grateful to Dr. Bruce Anderson for supplying the *p*-nitrophenyl benzoates and for carrying out some preliminary experiments on their reactions, to Mary Gilchrist for her excellent technical assistance, and to Dr. Myron Bender for communicating his results before publication. Supported by grants from the National Science Foundation and from the National Cancer Institute of the National Institutes of Health (C-3975). This work was carried out (by M.C.) during the tenure of a postdoctoral fellowship awarded by the National Institute of Dental Research, National Institutes of Health.

References

Aldridge, W. N., and Davison, A. N. (1952), *Biochem. J. 51*, 62.

Anderson, B. M., Cordes, E. H., and Jencks, W. P. (1961), *J. Biol. Chem. 236*, 455.

Bender, M. L. (1960), *Chem. Revs. 60*, 53.

Bender, M. L., and Nakamura, K. (1962), *J. Am. Chem. Soc. 84*, 2577.

Bender, M. L., Pollock, E. J., and Neveu, M. C. (1962), *J. Am. Chem. Soc. 84*, 595.

Bender, M. L., Schonbaum, G. R., and Hamilton, G. A. (1961a), *J. Polymer Sci. 49*, 75.

Bender, M. L., Schonbaum, G. R., Hamilton, G. A., and Zerner, B. (1961b), *J. Am. Chem. Soc. 83*, 1255.

Bender, M. L., and Turnquest, B. W. (1955), *J. Am. Chem. Soc. 77*, 4271.

Bergmann, F., Rimon, S., and Segal, R. (1958), *Biochem. J. 68*, 493.

Bernhard, S. A., and Gutfreund, H. (1957), Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, Maruzen, Tokyo, p. 124.

Brown, H. C., Okamoto, Y., and Inukai, T. (1958), *J. Am. Chem. Soc. 80*, 4964.

Bruice, T. C. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1924.

Bruice, T. C., and Mayahi, M. F. (1960), *J. Am. Chem. Soc. 82*, 3067.

Bruice, T. C., and Schmir, G. L. (1957), *J. Am. Chem. Soc. 79*, 1663.

Bruice, T. C., and Sturtevant, J. M. (1959), *J. Am. Chem. Soc. 81*, 2860.

Bunnett, J. F., and Davis, G. T. (1960), *J. Am. Chem. Soc. 82*, 665.

Calvin, M., Hermans, J., Jr., and Scheraga, H. A. (1959), *J. Am. Chem. Soc. 81*, 5048.

Caplow, M., and Jencks, W. P. (1962), *Fed. Proc. 21*, 248.

Chattaway, F. D. (1931), *J. Chem. Soc.* 2495.

Cunningham, L. W. (1957), *Science 125*, 1145.

Dixon, G. H., and Neurath, H. (1957a), *J. Biol. Chem. 225*, 1049.

Dixon, G. H., and Neurath, H. (1957b), *J. Am. Chem. Soc. 79*, 4558.

Dixon, G. H., Neurath, H., and Pechère, J. F. (1958), *Ann. Rev. Biochem. 27*, 489.

Dodgson, K. S., Spencer, B., and Williams, K. (1956), *Biochem. J. 64*, 216.

Gaetjens, E., and Morawetz, H. (1960), *J. Am. Chem. Soc. 82*, 5328.

Gawron, O., Grelecki, C. J., and Duggan, M. (1953), *Arch. Biochem. Biophys. 44*, 455.

Gerngross, O. (1913), *Berichte 46*, 1908.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.

Gould, E. S. (1959), Mechanism and Structure in Organic Chemistry, New York, Henry Holt and Co., pp. 318, 321.

Hartley, B. S., and Kilby, B. A. (1954), *Biochem. J. 56*, 288.

Havsteen, B. H., and Hess, G. P. (1962), *J. Am. Chem. Soc. 84*, 448.

Hermans, J., Jr., and Scheraga, H. A. (1959), *Biochim. Biophys. Acta 36*, 534.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Jaffé, H. H. (1953), *Chem. Revs. 53*, 191.

Jencks, W. P., and Carriuolo, J. (1959a), *J. Biol. Chem. 234*, 1272.

Jencks, W. P., and Carriuolo, J. (1959b), *J. Biol. Chem. 234*, 1280.

Jencks, W. P., and Carriuolo, J. (1960), *J. Am. Chem. Soc. 82*, 675.

Jencks, W. P., and Carriuolo, J. (1961), *J. Am. Chem. Soc. 83*, 1743.

Kerr, R. J., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1469.

Lumry, R., and Smith, E. L. (1955), *Disc. Far. Soc. 20*, 105.
Manning, D. T., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1478.
Marburg, S., and Jencks, W. P. (1962), *J. Am. Chem. Soc. 84*, 232.
McDonald, C. E., and Balls, A. K. (1957), *J. Biol. Chem. 227*, 727.
Mounter, L. A. (1958), *Biochim. Biophys. Acta 27*, 219.
Mounter, L. A., and Whittaker, V. P. (1953), *Biochem. J. 54*, 551.
Nath, R. L., and Rydon, H. N. (1954), *Biochem. J. 57*, 1.
Neurath, H., and Hartley, B. S. (1959), *J. Cellular and Comp. Physiol. 54*, suppl. 1, 179.
Nimmo-Smith, R. H. (1960), *Biochem. J. 75*, 284.
Pandit, U. K., and Bruice, T. C. (1960), *J. Am. Chem. Soc. 82*, 3386.
Pauling, L. (1960), The Nature of the Chemical Bond, ed. 3, Ithaca, New York, Cornell University Press, p. 260.
Schoellman, G., and Shaw, E. (1962), *Biochem. Biophys. Res. Commun. 7*, 36.
Schonbaum, G. R., Nakamura, K., and Bender, M. L. (1959), *J. Am. Chem. Soc. 81*, 4746.
Spencer, T., and Sturtevant, J. M. (1959), *J. Am. Chem. Soc. 81*, 1874.
Stadtman, E. R. (1954), *in* Symposium on the Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., editors, Baltimore, Johns Hopkins Press, p. 581.
Timm, E. W., and Hinshelwood, C. N. (1938), *J. Chem. Soc.* 862.
Tommila, E., and Hinshelwood, C. N. (1938), *J. Chem. Soc.* 1801.
Viswanatha, T., and Lawson, W. B. (1961), *Arch. Biochem. Biophys. 93*, 128.
Weil, L., James, S., and Buchert, A. R. (1953), *Arch. Biochem. Biophys. 46*, 266.
Whitaker, J. R., and Jandorf, B. J. (1956), *J. Biol. Chem. 223*, 751.

The Amino Terminal Sequence of Bovine Trypsinogen*

KENNETH A. WALSH, DOROTHY L. KAUFFMAN, AND HANS NEURATH

From the Department of Biochemistry, University of Washington, Seattle, Washington

Received July 2, 1962

As part of an investigation of the amino acid sequence of bovine trypsinogen, three peptides have been isolated which appear to be derived from the amino terminus of the molecule. These peptides have been obtained from tryptic and chymotryptic digests of *S*-sulfotrypsinogen and from chymotryptic digests of trypsinogen. Integration of their sequences establishes the N-terminal 20 amino acids of trypsinogen.

Studies of the structure and activation of bovine trypsinogen have established that the N-terminal residue is valine (Rovery *et al.*, 1953) and that the N-terminal sequence is Val.Asp.Asp.Asp.Asp.-Lys.Ileu.Val.Gly (Davie and Neurath, 1955; Desnuelle and Fabre, 1955). During the activation process, the peptide bond between lysine and isoleucine is opened, giving rise to the hexapeptide valyl-(aspartyl)$_4$-lysine (Davie and Neurath, 1955) and the enzymatically active protein, trypsin, with an N-terminal isoleucine (Rovery *et al.*, 1953). This single hydrolytic event is accompanied by an appreciable decrease in the levorotation of the molecule (Neurath *et al.*, 1956; Pechère and Neurath, 1957), which has been taken as an indication of changes in the secondary or tertiary structure of the molecule during its activation. The hypothesis has been put forward by Neurath and Dixon (1957) that such conformational changes might include a reorientation of the N-terminal region of the molecule which, by bringing histidine into juxtaposition with a particular DFP-sensitive[1] serine, might play a role in giving rise to the active center.

In the course of a program designed to elucidate the complete amino acid sequence of trypsinogen, peptides have been isolated from enzymatic digests of *S*-sulfotrypsinogen (Walsh *et al.*, 1961, and unpublished experiments) which permit the deduction of the amino acid sequence of the N-terminal twenty amino acids. The sequence is presented here prior to determination of the entire primary structure of the protein with the object of extending the detailed knowledge of the particular region of the molecule which is modified in the course of activation.

Materials and Methods

Crystalline bovine trypsinogen, purchased from Worthington Biochemicals (Lot TG711, contain-

* A preliminary report of this work has been published (Walsh *et al.*, 1961). This work was supported in part by the American Cancer Society (P-79, P-45), by the Public Health Service (RG-4617) and by the Office of Naval Research (NONR 477-04).

[1] The following abbreviations are used: DFP, diisopropylphosphofluoridate; Tris, tris(hydroxymethyl)aminomethane; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-, dinitrophenyl-.