Specificity and Stereospecificity of α-Chymotrypsin

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1. The optically pure *p*-nitrophenyl esters of the D and L enantiomers of *N*-acetyltryptophan, *N*-acetylphenylalanine and *N*-acetyl-leucine, and the *p*-nitrophenyl ester of *N*-acetylglycine, have been prepared. 2. These materials are all substrates of α-chymotrypsin, and the rates of deacylation of the corresponding acyl-α-chymotrypsins have been determined. 3. As the size of the amino acid side chain increases, the L series deacylate progressively faster than the *N*-acetylglycyl-enzyme, and the D series progressively more slowly. 4. The results are interpreted in terms of a three-locus model of the enzyme's active site, which accounts for the interrelationship between substrate specificity and stereospecificity observed. 5. The concepts of negative specificity and of specificity saturation are introduced.

One of the consequences of the multi-step nature of enzyme-catalysed reactions is that the specificity of the enzyme is not necessarily manifest in a single step. In the simplest case specificity arises only in the binding step (that is, at the stage of Michaelis complex-formation) and here the questions of rate and specificity are nicely separated. In common with many enzymes, however, the specificity of α-chymotrypsin is not very narrow, and selection by the enzyme between substrates is evident both in the binding step and in catalytic steps subsequent to it. This enzyme provides therefore a particularly useful system for an investigation of the forces responsible for an interaction between a protein and a small molecule, and of the origins and effects of both binding and kinetic specificity (Bender, Kézdy & Gunter, 1964).

There is good evidence (see e.g. Bender & Kézdy, 1964) that hydrolytic reactions catalysed by α-chymotrypsin proceed by a mechanism involving at least three steps:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} AE \xrightarrow{k_{+3}} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

where formation of the Michaelis complex (ES) is followed by acylation to the acyl-enzyme (AE), which then deacylates to yield the enzyme and the acid moiety of the substrate (P_2). From a Michaelis–Menten treatment of the reaction rates according to $v_0 = k_0[E][S_0]/(K_m + [S_0])$, the observed parameters k_0 and K_m are related to the constants of eqn. (1) as: $k_0 = k_{+2}k_{+3}/(k_{+2} + k_{+3})$ and $K_m = (k_{-1} + k_{+2})\, k_{+3}/k_{+1}(k_{+2} + k_{+3})$. In cases where deacylation is genuinely rate-determining, k_0 becomes k_{+3}, and (if $k_{-1} \gg k_{+2}$) K_m becomes $K_s k_{+3}/k_{+2}$. This is the condition which holds in the present work.

With few exceptions (Bender *et al.* 1964; Bender & Kézdy, 1965) previous discussions of the specificity of α-chymotrypsin have been based on the use of the kinetically complex parameters k_0 and K_m (Hein & Niemann, 1961, 1962*b*; Knowles, 1965*a*), which effectively prevents the definition of the type and degree of specificity exercised by the enzyme at a given stage of the reaction. Moreover, as has been argued by Bender & Kézdy (1965), the possibility of 'non-productive binding modes' (Huang & Niemann, 1952; Hein & Niemann, 1962*b*) invalidates the use of kinetic parameters other than k_0/K_m, or k_{+3}, for structure–specificity correlations.

In the experiments now reported, the hydrolysis of a series of *N*-acetylamino acid *p*-nitrophenyl esters (for which $k_0 = k_{+3}$) catalysed by α-chymotrypsin has been investigated to throw light on two aspects of the specificity problem: the question of side-chain specificity (that is, how the rate of deacylation of a given acyl-α-chymotrypsin is affected by the amino acid side chain), and the question of stereospecificity (that is, at what stage the enzyme distinguishes between enantiomeric amino acid derivatives). With one assumption, the data presented here allow a complete interpretation of the substrate specificity of α-chymotrypsin. A preliminary account of some of this work has appeared elsewhere (Ingles, Knowles & Tomlinson, 1966).

MATERIALS AND METHODS

All melting points are corrected. Measurements of optical rotation were performed on a Bendix–NPL Automatic

Polarimeter type 143. Microanalyses were done by Dr Weiler and Dr Strauss in this Department.

N-*Acetyl*-L-*tryptophan* p-*nitrophenyl ester*. *N*-Benzyloxycarbonyl-L-tryptophan was prepared according to the method of Smith (1948). This material was converted into *N*-benzyloxycarbonyl-L-tryptophan *p*-nitrophenyl ester by the general method of Bodanszky & du Vigneaud (1959*a*). Debenzyloxycarbonylation by the method of Greenstein & Winitz (1961) gave L-tryptophan *p*-nitrophenyl ester hydrobromide. The crude ester hydrobromide was dissolved in chloroform and the solution cooled to 0°. An exact equivalent of triethylamine dissolved in chloroform and cooled at 0° was added. Redistilled acetic anhydride was then added dropwise with shaking to the cooled solution until a spot test employing ninhydrin reagent showed the complete acetylation of all free amino groups. The reaction mixture was washed successively with very dilute solutions of $NaHCO_3$ and HCl and finally with water. The chloroform layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure, to leave a yellow oil. This oil was dissolved in ethyl acetate and chromatographed on a column of silica gel with ethyl acetate as the eluent. The resulting oil was then crystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give pale-yellow crystals. Two recrystallizations gave very pale-yellow needles, m.p. 130–131·5°, $[\alpha]_D^{20}-46{\cdot}3°$ (*c* 1 in chloroform) (Found: C, 62·2; H, 4·7; N, 11·6. $C_{19}H_{17}N_3O_5$ requires C, 62·1; H, 4·6; N, 11·4%).

N-*Acetyl*-D-*tryptophan* p-*nitrophenyl ester*. This was prepared from D-tryptophan (Sigma Chemical Co., St Louis, Mo., U.S.A.) as described for the L enantiomer. Recrystallization of the crude reaction product from ethyl acetate–light petroleum (b.p. 40–60°) gave pale-yellow needles, m.p. 130–130·5°, $[\alpha]_D^{20}+46{\cdot}8°$ (*c*1 in chloroform) (Found: C, 61·6; H, 4·4; N, 11·5. $C_{19}H_{17}N_3O_5$ requires C, 62·1; H, 4·6; N, 11·4%).

The optically pure *N*-acetyl *p*-nitrophenyl esters of the enantiomers of leucine and phenylalanine were prepared by the general method outlined above for tryptophan.

N-*Acetyl*-L-*leucine* p-*nitrophenyl ester*. This was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give white needles, m.p. 88–89°, $[\alpha]_D^{20}-55{\cdot}0°$ (*c*1 in chloroform) (Found: C, 57·0; H, 6·3; N, 9·4. $C_{14}H_{18}N_2O_5$ requires: C, 57·1; H, 6·1; N, 9·5%).

N-*Acetyl*-D-*leucine* p-*nitrophenyl ester*. This was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give white needles, m.p. 88–89°, $[\alpha]_D^{20}+54{\cdot}1°$ (*c*1 in chloroform) (Found: C, 56·9; H, 6·1; N, 9·3. $C_{14}H_{18}N_2O_5$ requires C, 57·1; H, 6·1; N, 9·5%).

N-*Acetyl*-L-*phenylalanine* p-*nitrophenyl ester*. This was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give white needles, m.p. 140–140·5, $[\alpha]_D^{20}-18{\cdot}6°$ (*c*2 in chloroform) (Found: C, 61·8; H, 4·7; N, 8·9. $C_{17}H_{16}N_2O_5$ requires: C, 62·2; H, 4·9; N, 8·5%).

N-*Acetyl*-D-*phenylalanine* p-*nitrophenyl ester*. This was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give white needles, m.p. 135–137°, $[\alpha]_D^{20}+17{\cdot}4°$ (*c*2 in chloroform) (Found: C, 61·6; H, 5·0; N, 8·5. $C_{17}H_{16}N_2O_5$ requires: C, 62·2; H, 4·9; N, 8·5%).

N-*Acetylglycine* p-*nitrophenyl ester*. *N*-Acetylglycine was esterified by a modification of the general method of Bodanszky & du Vigneaud (1959*a*). The crude reaction product was recrystallized three times from ethyl acetate–light petroleum (b.p. 40–60°) to give white fluffy needles (yield 62%), m.p. 126–128°. Zahn & Otten (1962) give m.p. 129–130°. Lorand, Brannen & Rule (1962) give m.p. 116–118° (Found: C, 50·0; H, 4·1; N, 11·6. Calc. for $C_{10}H_{10}N_2O_5$: C, 50·4; H, 4·2; N, 11·7%).

N-*Benzyloxycarbonyl*-L-*tryptophan* p-*nitrophenyl ester*. The sample of this material used for kinetic studies was Sigma Chemical Co. lot C-33B-206, used without further purification, m.p. 102–103·5°, $[\alpha]_D^{20}-4{\cdot}1°$ (*c*5 in dimethylformamide). Wilchek & Patchornik (1963) give m.p. 105°, $[\alpha]_D^{25}-4{\cdot}5°$ (*c*5 in dimethylformamide).

N-*Benzyloxycarbonyl*-L-*leucine* p-*nitrophenyl ester*. This was Sigma Chemical Co. lot 83B-1770, used without further purification, m.p. 94–95°, $[\alpha]_D^{20}-33{\cdot}6°$ (*c*2 in dimethylformamide). Bodanszky & du Vigneaud (1959*a*) give m.p. 95°, $[\alpha]_D^{20}-33{\cdot}5°$ (*c*2 in dimethylformamide).

N-*Benzyloxycarbonyl*-L-*phenylalanine* p-*nitrophenyl ester*. This was a gift from Mr J. H. Jones and had m.p. 126°, $[\alpha]_D^{20}-25{\cdot}0°$ (*c*2 in dimethylformamide). Bodanszky & du Vigneaud (1959*b*) give m.p. 126–126·5°, $[\alpha]_D^{20}-24{\cdot}7°$ (*c*2 in dimethylformamide).

N-*Benzyloxycarbonylglycine* p-*nitrophenyl ester*. This was Sigma Chemical Co. lot 15B-0670, used without further purification, m.p. 127–128·5°. Farrington, Hextall, Kenner & Turner (1957) give m.p. 128°.

N-*Acetyl*-D-*tryptophan amide*. A solution of *N*-acetyl-D-tryptophan methyl ester in dry methanol was saturated with ammonia and the saturated solution allowed to stand at 0° in a refrigerator for 2 days. After removal of the solvent under reduced pressure, the solid residue was recrystallized twice from ethanol–light petroleum (b.p. 40–60°) to give fine white needles of the amide (yield 92%), m.p. 191·5–192·5°, $[\alpha]_D^{20}-18{\cdot}8°$ (*c*1 in methanol). Huang & Niemann (1951) give m.p. 192–193°, $[\alpha]_D^{25}-19{\cdot}0°$ (*c*2 in methanol) (Found: C, 62·9; H, 5·8; N, 17·0. Calc. for $C_{13}H_{15}N_3O_2$: C, 63·6; H, 6·1; N, 17·1%).

N-*Acetyl*-D-*phenylalanine amide*. Ammonolysis of a solution of *N*-acetyl-D-phenylalanine methyl ester in dry methanol gave 89% of the amide as colourless needles from ethanol–light petroleum (b.p. 40–60°), m.p. 179–180°, $[\alpha]_D^{20}-27{\cdot}2°$ (*c*1 in methanol). Huang, Foster & Niemann (1952) give m.p. 176–177°, $[\alpha]_D^{25}-27{\cdot}0°$ (*c*1 in methanol) (Found: C, 63·3; H, 6·5; N, 13·5. Calc. for $C_{11}H_{14}N_2O_2$: C, 64·0; H, 6·8; N, 13·6%).

N-*Acetyl*-D-*leucine amide*. Ammonolysis of a solution of *N*-acetyl-D-leucine methyl ester in dry methanol gave crude *N*-acetyl-D-leucine amide. Recrystallization from chloroform–light petroleum (b.p. 40–60°) gave white needles, m.p. 131–132°, $[\alpha]_D^{20}+26{\cdot}0°$ (*c*1 in methanol) (Found: C, 55·6; H, 9·0. $C_8H_{16}N_2O_2$ requires C, 55·8; H, 9·3%).

N-*Acetylglycine amide*. Ammonolysis of a solution of *N*-acetylglycine ethyl ester in dry ethanol gave 64% of the amide as white needles from ethanol–light petroleum (b.p. 40–60°), m.p. 138·5–140°. Kurtz & Niemann (1961) gave m.p. 138–139·5° (Found: C, 41·1; H, 7·0; N, 24·2. Calc. for $C_4H_8N_2O_2$: C, 41·4; H, 6·9; N, 24·1%).

α-Chymotrypsin. This was a salt-free freeze-dried powder from three-times-crystallized α-chymotrypsin, purchased from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks. The titration of α-chymotrypsin by the method of Schonbaum, Zerner & Bender (1961) with *N*-cinnamoylimidazole was used to obtain the operational normality of the enzyme solutions. The enzyme preparation was found to be 85% active on a weight basis, assuming molecular weight 25000.

Acetonitrile. This was purified by the method of Cooper & Waters (1964).

Buffers. All buffers were made up with AnalaR-grade reagents and deionized water. pH measurements were made on a Radiometer instrument TTT1c with scale-expander pHA 630, standardized against standard buffer solutions (British Drug Houses Ltd.).

Kinetic measurements. All kinetic measurements were made with a Unicam SP.500 spectrophotometer fitted with a photomultiplier and coupled to a Smith's Servoscribe pen recorder. The temperature was controlled by using an Adkins constant-temperature block. Scale expansion of 75–100% transmission and 95–100% transmission was possible and chart speeds of up to 10mm./sec. were available.

(*a*) *N*-Acetyl L-amino acid *p*-nitropheny esters. The α-chymotrypsin-catalysed hydrolysis of the *N*-acetyl *p*-nitrophenyl esters of L-tryptophan, L-leucine and L-phenylalanine were followed by measuring the liberation of the *p*-nitrophenoxide anion at 400mμ. The extinction coefficient for the *p*-nitrophenoxide anion at a given pH value was calculated by using pK_a 7·04 for *p*-nitrophenol and ϵ_{400} 18380 for the *p*-nitrophenoxide anion (Kézdy & Bender, 1962). A typical run is described: 2·9ml. of 0·1*I* phosphate buffer and 100μl. of stock enzyme solution were pipetted into a 1cm. silica cell. After allowing 15min. for temperature equilibration, the recorder was adjusted to zero and 50μl. of a stock solution of substrate in acetonitrile was added. Recording was started as soon as possible (generally within 5sec.). The co-ordinates of 10points from the transmission versus time plot were fed into a best-fit polynomial computer programme to obtain the initial velocity (Knowles, 1965*b*). For both *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester and *N*-acetyl-L-tryptophan *p*-nitrophenyl ester the non-enzymic rate of hydrolysis was only 2·5% of the enzymic rate, even for the highest substrate concentration used. Substrate-blank corrections were therefore not applied for these two compounds. The non-enzymic blank rate of *N*-acetyl-L-leucine *p*-nitrophenyl ester amounted to 11% of the enzymic rate at the highest substrate concentration used and in this case the necessary corrections were made. The kinetic parameters K_m and k_0 and their probable errors were computed from reciprocal plots (Lineweaver & Burk, 1934) by a least-squares treatment (Knowles, 1965*b*).

The inhibition of the α-chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-leucine *p*-nitrophenyl ester by a series of *N*-acetyl amino acid amides was measured by a modification of the above procedure, the inhibitor being introduced in solution in the phosphate buffer.

(*b*) *N*-Acetylglycine *p*-nitrophenyl ester. The experimental technique was identical with that described in (*a*) except that the enzyme solution was made up in the phosphate buffer solution, since the enzyme concentration employed was approx. 1000 times greater (see details in Table 2).

(*c*) *N*-Acetyl D-amino acid *p*-nitrophenyl esters. Although the experimental procedure was essentially identical with that described above for the L enantiomers, the use of Lineweaver–Burk plots to obtain the kinetic parameters was ruled out by the narrow range of substrate concentrations which could be employed. The upper limit was determined by the solubility of the substrate and the lower limit by the necessity to maintain steady-state conditions (i.e. $[S_0] \gg [E]$). The use of high enzyme concentrations was necessitated by the relative magnitude of the non-enzymic blank reaction. Fortunately, the upper limit of solubility was sufficient to permit measurements under substrate saturation conditions (this was verified by the constancy of the observed enzymic rates over the range of substrate concentrations employed). The initial velocities (v), computed from the transmission versus time plots as described under (*a*), were corrected for the appreciable substrate blank reaction by means of the equation:

$$v_0 = v - ([S_0] - \pi) k_{obs.}$$

where $k_{obs.}$ is the pseudo-first-order rate constant for the non-enzymic reaction at the pH of the run and π is the observed burst of *p*-nitrophenol. Values of k_0 were then calculated from the limit equation:

$$v_0 = k_0 . [E]$$

(*d*) Non-enzymic hydrolysis rates. The hydroxide-ion-promoted hydrolysis of the various *p*-nitrophenyl esters was measured by following the liberation of *p*-nitrophenoxide anion at 400mμ. To 3·0ml. of buffer solution equilibrated in the cell compartment was added 50μl. of a stock solution of the ester in acetonitrile. Points from the transmission versus time trace were then used to obtain first-order plots from which a pseudo-first-order rate constant

Table 1. *Rate constants for the hydroxide-ion-promoted hydrolyses of* N-*acetyl amino acid* p-*nitrophenyl esters and* N-*benzyloxycarbonyl amino acid* p-*nitrophenyl esters at* 25°

p-Nitrophenyl ester	pH ranges	$10^5 \times$ Concn. of ester (M)	$10^{-3} . k_{OH^-}$ ($M^{-1}.sec.^{-1}$)
N-Acetyl-L-Phe	6·94–8·41*	4·23	7·4
N-Acetyl-L-Trp	6·94–8·41*	4·23	3·5
N-Acetyl-L-Leu	6·94–8·41*	5·70	5·3
N-Acetylglycine	7·00–8·67*	1·29	2·1
N-Benzyloxycarbonyl-L-Phe	7·94–8·94†	0·577	0·54
N-Benzyloxycarbonyl-L-Trp	8·59–9·09‡	0·456	0·16
N-Benzyloxycarbonyl-L-Leu	8·38–9·01‡	0·661	0·35
N-Benzyloxycarbonylglycine	8·38–8·94†	0·674	0·51

* 0·1 M-phosphate and 0·05 M-tris–HCl buffers.
† 0·05 M-tris–HCl buffer.
‡ 0·05 M-tris–HCl and 0·2 M-borate buffers.

($k_{obs.}$) for the particular pH was determined. Finally a graph of $k_{obs.}$ versus [OH^-] enabled the separate determination of k_{OH^-} and k_{H_2O} in the equation:

$$k_{obs.} = k_{H_2O} + k_{OH^-}.[OH^-]$$

RESULTS

The second-order rate constants for the hydroxide-ion-promoted hydrolysis of the series of *p*-nitrophenyl esters were calculated from the slopes of plots of $k_{obs.}$ (the pseudo-first-order rate constants) versus [OH^-] and are listed in Table 1. The plots for the *N*-acetyl amino acid *p*-nitrophenyl esters (e.g. *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester shown in Fig. 1) showed no deviation from linearity over a range of 1·5 pH units and overlapping buffer systems. Specific buffer catalysis must be negligible in this series. Similar plots for the *N*-benzyloxycarbonyl amino acid *p*-nitrophenyl esters were performed over narrower pH ranges (0·5–1·0 pH unit) and were reasonably linear. The sizeable intercepts on the $k_{obs.}$ axis (approx. 10^{-3} sec.$^{-1}$) together with the fact that the values determined in borate buffers generally fell a little above the line for values determined in tris buffers (see, for example, the plot for *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester shown in Fig. 2) suggest that specific buffer catalysis by both uncharged tris and borate anion is significant. The second-order rate constants quoted in Table 1 are, however, probably no more than 10% in error since all points were determined in tris–HCl buffers at pH values above the pK_a of tris (8·06). Under these conditions the slope of the $k_{obs.}$ versus [OH^-] plot will not deviate much from the pure hydroxide-ion slope (in the limit, as pH increases, the two slopes will become identical). Thus, the k_{OH^-} values for

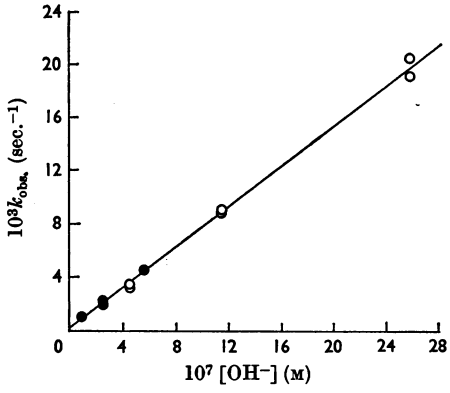

Fig. 1. Second-order plot for the hydroxide-ion-promoted hydrolysis of *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester at 25°: ●, 0·1 M-phosphate buffer; ○, 0·05 M-tris–HCl buffer. [Ester] $4·23 \times 10^{-5}$ M.

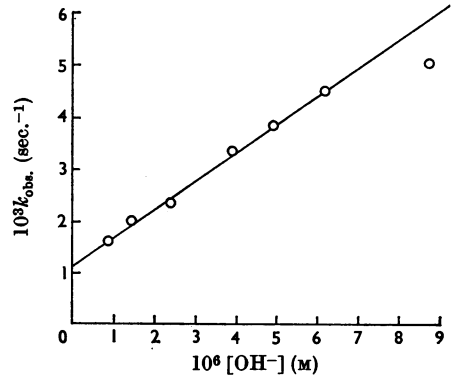

Fig. 2. Second-order plot for the hydroxide-ion-promoted hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester at 25°: 0·05 M-tris–HCl buffer. [Ester] $5·77 \times 10^{-6}$ M.

Table 2. *Deacylation rate constants of acyl-α-chymotrypsins*

Temp. 25°; 0·1 *I* phosphate buffer, pH 7·00 ± 0·02, 1·6% (v/v) acetonitrile.

Acyl group	[S_0] range (M)	[E_0] (M)	k_{+3} (sec.$^{-1}$)	K_m (M)
N-Acetyl-L-Trp	$6·2 \times 10^{-7}$–$1·10 \times 10^{-5}$	$7·8 \times 10^{-9}$*	52·0 ± 6·1	$4·90 (\pm 0·74) \times 10^{-6}$
N-Acetyl-L-Phe	$5·0 \times 10^{-7}$–$1·06 \times 10^{-5}$	$6·67 \times 10^{-9}$*	94·8 ± 10·0	$4·83 (\pm 0·68) \times 10^{-6}$
N-Acetyl-L-Leu	$2·65 \times 10^{-6}$–$3·34 \times 10^{-5}$	$4·74 \times 10^{-8}$*	5·04 ± 0·07	$6·08 (\pm 0·17) \times 10^{-6}$
N-Acetylglycyl	$2·95 \times 10^{-5}$–$5·19 \times 10^{-4}$	$6·15 \times 10^{-6}$	0·298	$5·1 \times 10^{-5}$
N-Acetyl-D-Leu	$1·27 \times 10^{-4}$–$5·82 \times 10^{-4}$	$8·00 \times 10^{-6}$	0·034 ± 0·0039†‡	—
N-Acetyl-D-Phe	$6·88 \times 10^{-5}$–$2·63 \times 10^{-4}$	$9·27 \times 10^{-6}$	0·0148 ± 0·0012†	—
N-Acetyl-D-Trp	$1·36 \times 10^{-5}$–$3·34 \times 10^{-5}$	$3·86 \times 10^{-6}$	0·00284 ± 0·00025†	—

* This value was checked for adsorption of enzyme on to the apparatus walls by performing experiments at double the enzyme concentration. The points for the two enzyme concentrations fell on the same straight line on an $[E_0]/v_0$ versus $1/[S_0]$ plot.

† Average of at least seven measurements. The errors quoted are the standard deviation from the mean.

‡ This value was quoted incorrectly in Ingles *et al.* (1966).

the *N*-benzyloxycarbonyl esters listed in Table 1, though of limited precision, are sufficiently accurate for use in the normalization procedure described below (see the Discussion section).

The kinetic parameters K_m and k_{+3} for the α-chymotrypsin-catalysed hydrolysis of the series of *N*-acetyl D and L-amino acid *p*-nitrophenyl esters are listed in Table 2. The values for the *N*-acetyl *p*-nitrophenyl esters of L-phenylalanine, L-tryptophan and L-leucine were computed from the Lineweaver–Burk plots as indicated in the Materials and Methods section. The plots for the L-phenylalanine and L-tryptophan compounds are shown in Figs. 3 and 4 respectively. Zerner & Bender (1963) have shown that, for this class of substrate, deacylation is rate-limiting, *i.e.* $k_{+2} \gg k_{+3}$, and the kinetic parameter k_0 obtained from the Lineweaver–Burk plot is thus a direct measure of k_{+3}. Zerner, Bond & Bender (1964) have previously reported deacylation rate constants for *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester [k_{+3} 73 sec.$^{-1}$; 25°, 0·1 M-calcium chloride, 15% (v/v) acetonitrile–water] and *N*-acetyl-L-tryptophan *p*-nitrophenyl ester [k_{+3} 30·5 sec.$^{-1}$; 25°, 0·1 M-calcium chloride, 3·17% (v/v) acetonitrile–water]. Their results were obtained by analysis of the progress curves for the hydrolysis of the racemic esters assuming and allowing for a slow irreversible acylation of the enzyme by the D esters. We have more confidence in our values, since they are derived from experiments with the optically pure material, uncomplicated by the presence of the partially competitive and (effectively) irreversible inhibitor, the D enantiomer.

In the α-chymotrypsin-catalysed hydrolysis of *N*-acetylglycine *p*-nitrophenyl ester, extrapolation of the extinction versus time plots to zero time gave positive intercepts on the extinction axis indicating a burst of *p*-nitrophenol. However, the observed burst (π) was found to vary with substrate concentration ([S_0]). This situation arises when k_{+2} is larger, but not much larger, than k_{+3}, and it can be shown that π varies with [S_0] according to eqn. 2:

$$\frac{1}{\sqrt{\pi}} = \frac{k_{+2}+k_{+3}}{k_{+2}.\sqrt{[E_0]}} + \frac{k_{+3}.K_m}{k_{+2}.\sqrt{[E_0]}} \cdot \frac{1}{[S_0]} \qquad (2)$$

A plot of $1/\sqrt{\pi}$ versus $1/[S_0]$ gave a good straight line (see Fig. 5). The value of $(k_{+2}+k_{+3})/k_{+2}$ obtained from the intercept was combined with the value of k_0 [$=k_{+2}.k_{+3}/(k_{+2}+k_{+3})$] obtained from the Lineweaver–Burk plot, to calculate individual values of k_{+2} and k_{+3}. Under the conditions employed (see Table 2) the analysis gave k_{+2} 5·85 sec.$^{-1}$, k_{+3} 0·298 sec.$^{-1}$.

The burst of *p*-nitrophenol observed in the α-chymotrypsin-catalysed hydrolysis of the D-esters was also found to vary with substrate concentration. The actual dependence of π on [S_0] was, however, much smaller than that observed for *N*-acetylglycine *p*-nitrophenyl ester ($k_{+2}/k_{+3} \sim 20$). Hence, for the D enantiomers the k_{+2}/k_{+3} ratio must be appreciably greater than 20 and little error attaches to the assumption that the observed enzymic rate reflects solely the deacylation step k_{+3}.

The inhibition constants (K_i) for a series of *N*-acetyl D-amino acid amides were determined against *N*-acetyl-L-leucine *p*-nitrophenyl ester as the substrate, and are listed in Table 3. In all cases inhibition was found to be fully competitive, as indicated by the constancy of k_0 in the final column

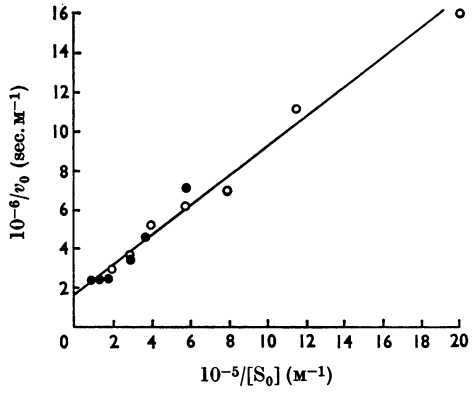

Fig. 3. Lineweaver–Burk plot for the α-chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanine *p*-nitrophenyl ester at pH 7·00, 25°, 0·1 *I* phosphate buffer, 1·6% (v/v) acetonitrile–water. ○, [E] $6{\cdot}67 \times 10^{-9}$ M; ●, [E] $1{\cdot}25 \times 10^{-8}$ M (these velocities are corrected to [E] $6{\cdot}67 \times 10^{-9}$ M; see the text).

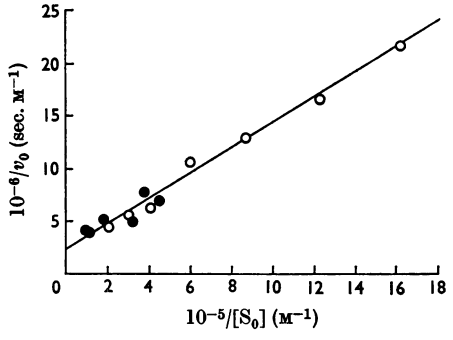

Fig. 4. Lineweaver–Burk plot for the α-chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester at pH 7·00, 25°, 0·1 *I* phosphate buffer, 1·6% (v/v) acetonitrile–water. ○, [E] $7{\cdot}8 \times 10^{-9}$ M; ●, [E] $1{\cdot}24 \times 10^{-8}$ M (these velocities are corrected to [E] $7{\cdot}8 \times 10^{-9}$ M; see the text).

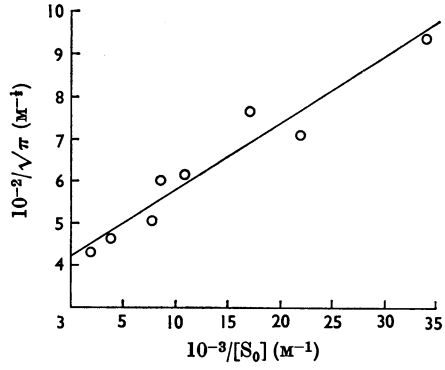

Fig. 5. $1/\sqrt{\pi}$ versus $1/[S_0]$ plot for *N*-acetylglycine *p*-nitrophenyl ester at pH 7·00, 25°, 0·1*I* phosphate buffer, 1·6% (v/v) acetonitrile–water. [E] $6{\cdot}15\times10^{-6}$ M.

Table 3. *Inhibition constants for a series of* N-*acetyl* D-*amino acid amides*

Temp. 25·0°, 0·1 *I* phosphate buffer, pH 7·00 ± 0·02, 1·6% (v/v) acetonitrile–water, determined against *N*-acetyl-L-leucine *p*-nitrophenyl ester as substrate.

Amino acid	$[I_0]$ (M)	K_i (M)	k_0 (sec.$^{-1}$)
Trp	$3{\cdot}37\times10^{-3}$	$1{\cdot}48\times10^{-3}$	5·12
Phe	$2{\cdot}15\times10^{-2}$	$1{\cdot}06\times10^{-2}$	4·92
Leu	$6{\cdot}48\times10^{-2}$	$1{\cdot}41\times10^{-1}$	4·89
Gly	$3{\cdot}72\times10^{-1}$	$6{\cdot}92\times10^{-1}$	5·12

of the Table. The K_i values for *N*-acetyl-D-tryptophan amide and *N*-acetyl-D-phenylalanine amide agree reasonably with the values of 2·3 mM and 12 mM respectively reported by Foster, Shine & Niemann (1955).

DISCUSSION

It has been pointed out earlier that pathways of enzyme-catalysed reactions involving several intermediates complicate the study of enzyme specificity. There is no *a priori* reason why the observed overall specificity of an enzyme reaction occurs in each or any one of the elementary steps, and an unambiguous account of specificity can only be made if each step is examined independently. Moreover, the substrate specificity (aside from the specificity of reaction) of an enzyme can often be formally subdivided, and, in the present case of α-chymotrypsin, the principal areas of interest are (*a*) the nature of the side chain and (*b*) the configuration of the asymmetric centre. [We shall not be concerned here with effects under either of these two heads relating to amino acid residues other than the one whose carbonyl group is being attacked, even though for related enzymes (e.g. carboxypeptidase, trypsin and papain) it is known that the configuration of neighbouring amino acid residues affects the rate of catalysis markedly (Schechter & Berger, 1966).] As will be seen, while the separation of these two types of specificity facilitates the discussion, a highly specific amino acid side chain affects the stereospecificity, producing a larger rate difference between D and L substrates in the catalytic steps.

The specificity of α-chymotrypsin for different amino acid side chains (*a* above) has been discussed by many groups (see e.g. Hein & Niemann, 1961; Knowles, 1965*a*; Bender & Kézdy, 1965), the common conclusion being that chymotrypsin shows a predilection for derivatives of the bulkier (often aromatic) amino acids. The question of stereospecificity (*b* above) has been less studied, the relevant conclusions being that K_i values for D inhibitors are close to and slightly lower than K_s values for the corresponding L substrates (see e.g. Knowles, 1965*a*) and that the degree of relative stereospecificity is determined (on the basis of k_0/K_m values) by the size of the groups attached to the asymmetric carbon atom (Hein & Niemann, 1962*a*). A report by Zerner *et al.* (1964) showed that *p*-nitrophenyl esters of *N*-acylated D-amino acids probably acylate the enzyme, and the present work exploits the possibility of preparing D- and L-acyl-enzymes, to provide detailed information about the kinetic specificity of α-chymotrypsin.

The 10^4-fold spread in the deacylation rate constants shown in Table 2 reflects both the inherent difference in reactivities of the series of acyl-chymotrypsins due to electronic and steric factors and the specificity contribution from the enzymic process. The k_{+3} values therefore require normalization for the inherent differences in reactivity before a strict relative measure of the contribution from enzymic catalysis is obtained. Since acyl-chymotrypsins are known to be serine esters (Oosterbaan, van Adrichem & Cohen, 1962; Shalitin & Brown, 1966), Bender *et al.* (1964) chose the saponification rates of the corresponding ethyl esters for the normalization process. We have used the saponification rates of the *p*-nitrophenyl esters since these (*a*) are more easily accessible (the rate constants for saponification of the ethyl esters are small and difficult to determine accurately) and (*b*) probably reflect more accurately the true reactivities of the acyl-chymotrypsins, which must themselves be 'active esters' (the Hammett ρ-values for enzyme deacylation and *p*-nitrophenyl ester saponification are 2·1 and 2·04 respectively; Caplow & Jencks, 1962). The hydrolysis of *N*-acetyl amino acid *p*-nitrophenyl esters promoted by hydroxide ion is

known, however, to proceed via the intramolecular formation of an oxazolone intermediate (Goodman & Stueben, 1962; Jersey, Kortt & Zerner, 1966), whereas the hydrolysis of the same compounds catalysed by α-chymotrypsin involves no such cyclic mechanism. [In support of this we have shown that while the rate constants for non-enzymic hydrolysis of *N*-acetyl-L-leucine *p*-nitrophenyl ester and *N*-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester differ by a factor of 15 (see Table 1), the deacylation rate constants for the α-chymotrypsin-catalysed hydrolysis differ only by a factor of 2·3 (D. W. Ingles, unpublished work). The hydrolysis of *N*-benzyloxycarbonyl-amino acid *p*-nitrophenyl esters promoted by hydroxide ion is known not to involve oxazolone intermediates, and the corresponding hydrolysis of *N*-acetyl derivatives is believed to go exclusively via the oxazolone. Since enzymically, *N*-benzyloxycarbonyl compounds are hydrolysed about half as fast as *N*-acetyl compounds, whereas the propensity for oxazolone formation differs by a factor of very much more than two, it is unlikely that enzymic deacylation involves a cyclic mechanism. Moreover, the compound D-3-carboxymethyldihydroisocarbostyril (which could not form an oxazolone intermediate) is hydrolysed by α-chymotrypsin at a rate almost identical with that of the analogue *N*-acetyl-L-phenylalanine methyl ester (which could form an oxazolone in the deacylation step) (Hein & Niemann, 1962*a*; Jones, Kunitake, Niemann & Hein, 1965)]. The deacylation rate constants of Table 2 have accordingly been normalized by using the hydroxide-ion-promoted hydrolysis rates of the *N*-benzyloxycarbonyl amino acid *p*-nitrophenyl esters. Setting the deacylation rate of *N*-acetylglycyl-α-chymotrypsin equal to unity, the $k_{+3}^{norm.}$ values of Table 4 are obtained by using $k_{+3}^{norm.i} = k_{+3}^{i}.k_{OH^-}^{Gly}/k_{+3}^{Gly}.k_{OH^-}^{i}$ (where i indicates Trp, Phe or Leu). It is evident from this Table that for acyl-enzymes of the L configuration, as the size of the amino acid side chain increases, the rate constant of the deacylation reaction increases. This is expected from the increase in the hydrophobic character of the substrate moiety as its bulk increases (Knowles, 1965*a*), and is in accord with the experiments and theory of kinetic specificity advanced by Bender *et al.* (1964). More significant, however, is the fact that the deacylation rate constants for the D series decrease in the same order. Indeed, a plot of log $k_{+3}^{norm.}$ for D- versus L- acyl-α-chymotrypsins (Fig. 6) is linear. This linearity implies that, as the size of the amino acid side chain increases, deacylation of an L-acyl-enzyme is favoured and deacylation of a D-acyl-enzyme is proportionately disfavoured. That is, the members of the D series exhibit what may be termed an inverse or 'negative' specificity. [The gradient of the line of Fig. 6 shows that the rate of deacylation is rather more sensitive to changes in side chain in the L than in the D series.]

The above argument indicates that the stereospecificity of α-chymotrypsin is intimately linked to the side-chain specificity, and leads to a simple interpretation of these phenomena in terms of a three-point attachment model, which is the simplest for a system displaying stereospecificity with respect to a single asymmetric centre. The model is shown in Fig. 7. For a specific substrate of the L configuration [e.g. an *N*-acyl-L-phenylalanyl derivative: Fig. 7(ii)] the substrate moiety of the acyl-enzyme is fixed at three loci: (*a*) by a covalent bond to the serine hydroxyl group [A] of the enzyme (Ser-195; Hartley, 1964); (*b*) by a hydrophobic interaction between the side chain (in Fig. 7, the benzyl group) and its corresponding locus at the active site [B] (Knowles, 1965*a*); (*c*) by the hydrogen bond(s) of the peptide link of the acyl amino group [C] (Bender & Kézdy, 1965; D. W. Ingles & J. R. Knowles, unpublished work). If each of these three interactions is present, the orientation of the susceptible

Table 4. *Normalized deacylation rate constants of a series of acyl-α-chymotrypsins*

For the normalization procedure see the text.

Acyl group	$k_{+3}^{norm.}$	log $k_{+3}^{norm.}$
N-Acetyl-L-Trp	560	2·748
N-Acetyl-L-Phe	296	2·471
N-Acetyl-L-Leu	24·1	1·382
N-Acetylglycyl	1·0	0·00
N-Acetyl-D-Leu	0·166	−0·780
N-Acetyl-D-Phe	0·047	−1·328
N-Acetyl-D-Trp	0·0302	−1·520
Acetyl	0·185	−0·733

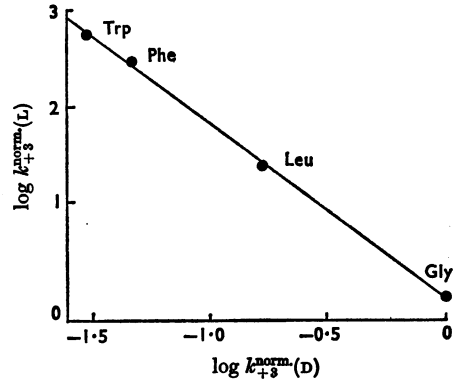

Fig. 6. Plot of log $k_{+3}^{norm.}$ for D- versus L-acyl-α-chymotrypsins.

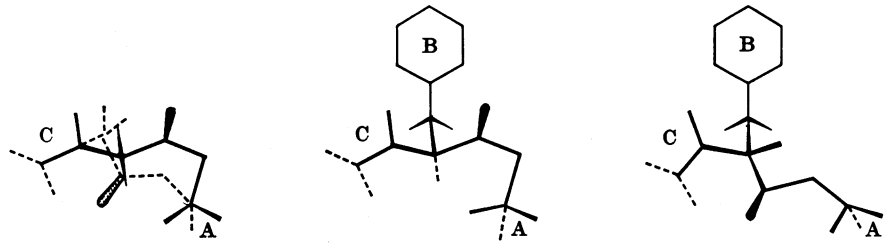

Fig. 7. Models for acyl-α-chymotrypsins. (i) *N*-Acetylglycyl-; (ii) *N*-acetyl-L-phenylalanyl-; (iii) *N*-acetyl-D-phenylalanyl-.

carbonyl group is fixed, presumably ideally with respect to the catalytic functionalities of the enzyme. If either of the interactions (*b*) or (*c*) is absent, then the substrate moiety has more degrees of freedom, and this is reflected in a lower deacylation rate constant. Thus if (*b*) is absent [the case of the *N*-acetylglycyl-enzyme: Fig. 7(i)], rotation of the $-CH_2-CO-O-$ group is possible between the fixed loci of [A] and [C] [one alternative orientation is shown dotted in Fig. 7(i)]. A similar argument can be advanced for when interaction (*c*) is absent (with the β-phenylpropionyl-enzyme). When both (*b*) and (*c*) are absent (acetyl-chymotrypsin), the acyl group has even more freedom, and the deacylation rate falls below that of the *N*-acetylglycyl-enzyme. If in the *N*-acetylglycyl group the hydrogen which results in a D-amino acid being produced is replaced by a benzyl group (giving the *N*-acetyl-D-phenylalanyl group), and interactions (*a*), (*b*) and (*c*) (above) are assumed to occur in the same regions of space as before, we obtain Fig. 7(iii), in which the only significant difference is that the susceptible carbonyl is pointing the opposite way from Fig. 7(ii) (presumably non-ideally with respect to the catalytic functionalities of the enzyme). This, we believe, may be responsible for the fact that the D-acyl-enzymes are disfavoured (over the *N*-acetylglycyl-enzyme) and that the extent of the disfavour is directly related to changes in the interaction at [B], in just the same way that the L series is favoured (over the *N*-acetylglycyl-enzyme) by the interaction at [B].

In summary, the model of Fig. 7 (in which, of course, the actual positions of groups are quite arbitrary) accounts for the following: (i) *N*-acetylglycyl-chymotrypsin (two-loci interaction) deacylates faster than acetyl-chymotrypsin (one locus only); (ii) all the D-acyl-enzymes deacylate more slowly than the glycyl-enzyme; (iii) the stronger the binding interaction of the amino acid side chain, the faster the L series deacylate and the more slowly

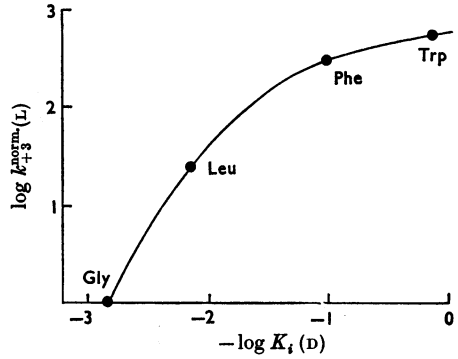

Fig. 8. Plot of log $k_{+3}^{norm.}$ (L) for acyl-α-chymotrypsins versus $-\log K_i$ (D) (K_i in mM) for the corresponding acyl amides.

the D series deacylate; (iv) the origin of the rate differences in deacylation of different acyl-chymotrypsins is largely entropic (Bender *et al.* 1964).

One requirement of the model presented in Fig. 7 is that while the free energy of the binding interactions at [B] and [C] increases on going to better substrates (here we are principally concerned with changes at the [B] locus), there is presumably a limit to how 'correctly' the susceptible carbonyl group can be oriented. We should expect therefore to see the value of k_{+3} rising increasingly less, as the strength of the interaction at [B] increases, giving a curve of hyperbolic form to a plot of k_{+3} versus a measure of binding interaction. To test this requirement, values of log $k_{+3}^{norm.}$ are plotted against the K_i values for the corresponding D-amides in Fig. 8. The K_i values provide a good measure of the relative free energies of binding. As can be seen from Fig. 8, the prediction of 'specificity saturation' is realized, the deacylation rate constant increases on going from Phe to Trp much less than would be expected on the basis of the difference in

binding free energy. It appears that the Trp derivative is close to the maximum possible deacylation rate for *N*-acetyl L-α-aminoacyl-α-chymotrypsins.

Finally, it should be pointed out that the proposed model takes no account of flexibility of enzyme (i.e. the possibility of induced-fit mechanisms; Koshland, 1958) or, apart from rotation about single bonds, of substrate (i.e. the possibility of rack mechanisms; Eyring, Lumry & Spikes, 1954; Jencks, 1967). Either or both of these processes may operate in chymotrypsin-catalysed reactions. [There is, indeed, evidence for a protein isomerization step in these reactions (see Oppenheimer, Labouesse & Hess, 1966), though this is believed to occur before the acylation step in the forward reaction.] A rigid enzyme–rigid substrate model is clearly adequate, however, to account for the data presented in this paper, and the postulates advanced here would require but little modification to accommodate induced-fit or rack processes.

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