Comparative Studies of the Specificities of α -Chymotrypsin and Subtilisin BPN'

STUDIES WITH FLEXIBLE SUBSTRATES

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A series of arylalkanoate esters and α -acetamidoarylalkanoate esters were tested as substrates for α -chymotrypsin and subtilisin BPN'. Chymotrypsin hydrolysed *N*-acetyl-L-phenylalanine methyl ester and methyl 4-phenylbutyrate faster than their respective higher and lower homologues, whereas methyl 2-acetamido-6-phenylhexanoate and methyl 6-phenylhexanoate were better substrates for subtilisin than their lower homologues. *N*-Acetyl-L-tryptophan methyl ester and its analogue, *N*-acetyl-3-(1-naphthyl)-alanine methyl ester, were hydrolysed 23 times faster by chymotrypsin than by subtilisin. These results indicate that the binding site of α -chymotrypsin is roughly 1.1 nm (11 Å) long and curved, whereas that of subtilisin is a longer system and less curved. The stereospecificity during the hydrolysis of typical substrates by both enzymes was found to vary over a wide range. The enhancing effect of the α -acetamido group in the L-series of substrates and the detrimental effect in the D-series of substrates also varies considerably.

Despite their wide differences in biological origin and in their primary structures, α -chymotrypsin and subtilisin BPN' exhibit remarkable similarity in their catalytic action (Wright *et al.*, 1969; Morihara & Tsuzuki, 1969; Alden *et al.*, 1970). Both these proteases display broad substrate specificity, the preferential point of attack being at the carboxyl end of an amino acid with an aromatic or hydrophobic side chain (Barel & Glazer, 1968; Morihara *et al.*, 1968, 1971; Blow, 1971). The X-ray crystallographic models of α -chymotrypsin (Birktoft *et al.*, 1970) and subtilisin BPN' (Wright *et al.*, 1969) show close similarities in the 'charge relay system' and other features in the active-site region although the hydrophobic site in the latter enzyme seems to be less well-defined. Alden *et al.* (1970) have detailed the similarities in the functional residues of these two proteolytic enzymes.

A major goal in studies with α -chymotrypsin has been to define the most productive mode of orientation of a typical, flexible substrate like *N*-acetyl-L-phenylalanine methyl ester (L-compound II) (Fig. 1). A wide variety of model substrates have been examined for this purpose during the past 10 years (Hein *et al.*, 1960; Hayashi & Lawson, 1969; Cohen & Lo, 1970; Rumsh *et al.*, 1970; Silver *et al.*, 1970). Almost all these locked substrates have been found to be inferior to L-compound II after making correction for their intrinsic reactivities. Whereas L-compound II appears to be hydrolysed with virtually absolute stereospecificity, the locked model substrates show a finite optical specificity that varies over a wide range.

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The high reactivity and near absolute stereospecificity in the hydrolysis of compound II appear to be caused by the interaction of the α -acetamido group with a suitable site at the active centre region. A hydrogen-bonding interaction between the NH group of the α -acetamido moiety of the substrate and the carbonyl group of serine-214 of α -chymotrypsin has been proposed by Steitz *et al.* (1969). Ingles & Knowles (1968) have summarized the chemical evidence for such hydrogen bonding. Freer *et al.* (1970) have suggested that such an interaction may not be operating in subtilisin BPN'. [Another carbonyl group may be postulated for this function in subtilisin BPN' (D. M. Blow and J. Kraut, personal communications).] Silver *et al.* (1970) suggested, however, that most of the effect of an α -acetamido group results from its electronic character and other factors. Barel & Glazer (1968) observed that *N*-acetyl amino acid esters are hydrolysed much faster than free amino acid esters by subtilisin, as is well known with chymotrypsin. In any case, an α -acylamido group appears to be responsible for the high reactivity and the stereospecificity during hydrolysis by both the enzymes. Replacement of an α -acetamido group by a formamido (Hein & Niemann, 1962a), hydroxyl or chlorine group (Snoke & Neurath, 1949; Cohen & Weinstein, 1964) decreases the stereospecificity and, at least in the last two cases, decreases the reactivity during chymotryptic hydrolysis.

In the present paper the effect of the α -acetamido group in a series of flexible substrates during hydrolysis by α -chymotrypsin and subtilisin BPN' is evaluated. The observations on the hydrolysis of

Fig. 1. Structures of substrates

these substrates indicate that stereoselectivity is not absolute. The differences in the binding sites in subtilisin BPN' and α -chymotrypsin are delineated.

Experimental

Materials

Salt-free α -chymotrypsin (EC 3.4.4.5), recrystallized three times, was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. The concentration of chymotrypsin solutions was determined spectrophotometrically at 280nm by the method of Wu & Laskowski (1955). Subtilisin BPN' (Nagarse) (EC 3.4.4.16) was supplied by Nagase Co., Osaka, Japan, through the Enzyme Development Corp., New York, N.Y., U.S.A. The enzyme was dialysed for 24h at 4°C, against 0.1M-KCl. The protein concentration after dialysis was determined from the E_{278} (Matsubara *et al.*, 1965). The operational normalities of α -chymotrypsin and dialysed subtilisin were determined by the method of Bender *et al.* (1966) by using *N-trans*-cinnamoylimidazole as titrant. The enzyme preparations were 69.2% (α -chymotrypsin) and 72.4% (subtilisin BPN') active, based on their concentrations determined from the extinction at 280nm (chymotrypsin) and at 278nm (subtilisin). Anhydrous dimethyl sulfoxide was purchased from Matheson, Coleman and Bell (East Rutherford, N.J., U.S.A.) and was distilled under vacuum before use. *N*-Acetyl-L-phenylalanine methyl ester was purchased from Mann Research Laboratories (New York, N.Y., U.S.A.). DL-2-Amino-4-phenylbutyric acid was supplied by the Cyclo Chemical

Co. (Los Angeles, Calif., U.S.A.). D-Phenylalanine and D-tryptophan were obtained from Schwarz-Mann (Orangeburg, N.Y., U.S.A.). L-Phenyl-lactic acid was obtained from the Aldrich Chemical Co. (Milwaukee, Wisc., U.S.A.). DL-2-Amino-2-phenylacetic acid and indol-3-ylpropionic acid were purchased from Eastman Kodak Co. (Rochester, N.Y., U.S.A.). Ethyl acetamidocyanoacetate was supplied by Winthrop Laboratories (New York, N.Y., U.S.A.). All other reagents were analytical-grade commercial chemicals.

Preparation of compounds

Melting points were uncorrected. I.r. and n.m.r. spectra were determined with Perkin-Elmer model 21 and Varian model A-60 spectrometers, respectively. Optical rotations were measured in a Rudolph model 80 polarimeter with a model 200 photoelectric attachment. A model TTT1c Radiometer autotitrator with a GK2302B combination electrode was used for kinetic studies. Elemental analyses (Table 1) were performed by Schwarzkopf Microanalytical Laboratory (Woodside, N.Y., U.S.A.) or by G. I. Robertson, Jr. (Florham Park, N.J., U.S.A.).

The general procedure for the isolation and purification of the esters involved the extraction of the material with the organic solvent indicated in individual cases. The extract was washed with water, aq. 5% (w/v) NaHCO₃, saturated NaCl and water, and was treated with charcoal and dried over anhydrous Na₂SO₄. The filtrate was evaporated under vacuum and the resulting product was crystallized

Table 1. *Elemental analysis of compounds*

For experimental details see the text.

Compound	Analysis (%)					
	Found			Calculated		
	C	H	N	C	H	N
$\text{C}_6\text{H}_5\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	63.41	6.48	6.89	63.75	6.32	6.76
$\text{L-C}_6\text{H}_5\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	63.85	6.42	6.87	63.75	6.32	6.76
$\text{D-C}_6\text{H}_5\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	63.59	6.15	6.88	63.75	6.32	6.76
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_2\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	66.38	7.13	6.04	66.36	7.28	5.95
$\text{L-C}_6\text{H}_5\text{--[CH}_2\text{]}_2\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	66.14	7.30	6.14	66.36	7.28	5.95
$\text{D-C}_6\text{H}_5\text{--[CH}_2\text{]}_2\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	66.20	7.38	6.08	66.36	7.28	5.95
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--C(CN)(NHCOCH}_3\text{)--CO}_2\text{C}_2\text{H}_5$	66.40	6.90	9.65	66.64	6.99	9.72
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_4\text{--C(CN)(NHCOCH}_3\text{)--CO}_2\text{C}_2\text{H}_5$	67.68	7.23	9.52	67.52	7.34	9.27
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--CH(NH}_2\text{)--CO}_2\text{H}$	68.43	7.85	7.49	68.37	7.82	7.25
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_4\text{--CH(NH}_2\text{)--CO}_2\text{H}$	69.36	8.27	6.85	69.53	8.27	6.76
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--CH(NHCOCH}_3\text{)--CO}_2\text{H}$	66.55	7.21	5.90	66.36	7.29	5.95
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_4\text{--CH(NHCOCH}_3\text{)--CO}_2\text{H}$	67.20	7.71	5.74	67.44	7.68	5.62
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	67.37	7.62	5.70	67.44	7.68	5.62
$\text{L-C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	67.70	7.66	5.88	67.44	7.68	5.62
$\text{D-C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	67.37	7.69	5.63	67.44	7.68	5.62
$\text{C}_6\text{H}_5\text{--[CH}_2\text{]}_4\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	68.64	7.89	5.32	68.41	8.04	5.32
$\text{L-C}_6\text{H}_5\text{--[CH}_2\text{]}_4\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	68.44	7.96	5.06	68.41	8.04	5.32
$\text{D-C}_6\text{H}_5\text{--[CH}_2\text{]}_3\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$	68.56	8.17	5.25	68.41	8.04	5.32

as indicated for the individual compounds. Similarly, for the isolation of the acids, the aqueous solution was acidified with HCl and extracted with the organic solvent. The extract was washed with water, saturated NaCl and water, and processed as described above.

The synthesis and optical resolution of *N*-acetyl-3-(1-naphthyl)-alanine methyl ester (compound XIV) were described by Hayashi & Lawson (1969). Methyl 3-(1-naphthyl)propionate (compound XV) was prepared as described by Fieser & Gates (1940).

N-Acetyl-L-tryptophan methyl ester (L-compound XII). This ester was prepared from L-tryptophan (3.4 g) by acetylation (du Vigneaud & Sealock, 1932) followed by esterification with methanol and thionyl chloride by the general procedure of Brenner & Huber (1953) to give 2.9 g (66% yield), m.p. 152.5–153.5°C and $[\alpha]_D^{25} +13.2^\circ$ (c 1.1 in methanol) [Huang & Niemann (1951) give m.p. 152.5°C and $[\alpha]_D^{25} +11.5^\circ$ in methanol].

N-Acetyl-D-tryptophan methyl ester (D-compound XII). This D-isomer (D-compound XII) was prepared similarly from D-tryptophan in 60% yield, m.p. 153.0–153.5°C and $[\alpha]_D^{25} -13.52^\circ$ (c 1.76 in methanol) [Huang & Niemann (1951) report m.p. 152.5°C and $[\alpha]_D^{25} -12^\circ$ in methanol].

Methyl L-phenyl-lactate (L-compound XI). This material was prepared from L-phenyl-lactic acid (3.1 g) by esterification with methanol and thionyl chloride. The product was crystallized from ether–

light petroleum (b.p. 30–60°C) to give 1.7 g (50.5% yield), m.p. 46.5–47.0°C and $[\alpha]_D^{25} -12.1^\circ$ (c 1.55 in benzene) [Snoke & Neurath (1949) give m.p. 45–46°C and $[\alpha]_D$ in benzene -11.9°].

Methyl D-phenyl-lactate (D-compound XI). This was synthesized in a similar way from the D-acid (Cohen & Weinstein, 1964) in 62% yield, m.p. 46–47°C and $[\alpha]_D^{25} +11.8^\circ$ (c 1.4 in benzene).

Methyl 3-(indol-1-yl)propionate (compound XIII). This ester was prepared from the corresponding acid (2.5 g) by the method of Brenner & Huber (1953) to yield 1.8 g (66% yield), m.p. 77.5–78.5°C [Manske & Robinson (1927) give m.p. 79–80°C].

5-Phenylvaleric acid. 3-(Phenylpropyl)malonic acid (11.5 g), which was prepared as described by Braun & Kruber (1911), was decarboxylated by heating in an oil bath at 150–160°C until CO₂ evolution ceased (20 min). The resulting oil was distilled under vacuum and the fraction boiling at 187–190°C and 1.1 kN·m⁻² (8 mmHg) was collected. The colourless oil (8.25 g, 91% yield) solidified into white flakes, m.p. 57–58°C [Braun (1910) gives m.p. 58–59°C].

6-Phenylhexanoic acid. This compound was prepared similarly by decarboxylating 13 g of 4-(phenylbutyl)malonic acid (Braun & Kruber, 1911), at 160–170°C. The fraction distilling at 194.5–195.0°C and 1.1 kN·m⁻² (8 mmHg) was collected, and the colourless oil (9.55 g, 90.5% yield) solidified into

white flakes on cooling in ice, m.p. 21–22°C [Staudinger & Müller (1923) report m.p. 22–24°C].

Methyl 2-phenylacetate (compound VI), methyl 3-phenylpropionate (compound VII), methyl 4-phenylbutyrate (compound VIII), methyl 5-phenylvalerate (compound IX) and methyl 6-phenylhexanoate (compound X) were prepared from the corresponding acids by the Fischer method (Fischer & Speier, 1895).

Methyl DL-2-acetamido-2-phenylacetate (compound I). Methyl DL-2-amino-2-phenylacetate hydrochloride was prepared by esterification of the amino acid by the Fischer method, m.p. 218–222°C (with decomposition) [Kossel (1891) gives m.p. 224°C]. This ester (6.05 g, 0.03 mol), in 14 ml of 1.5 M-NaOH, was treated with 8 ml (0.085 mol) of acetic anhydride and 6 M-NaOH in portions at pH 6–7. After 20 min, the solution was acidified to pH 1 with 6 M-HCl, then extracted with methylene chloride and processed. The product was crystallized from ether–light petroleum (b.p. 30–60°C) to give 4.5 g (72.5% yield) of compound I, m.p. 75.5–76.5°C.

N-Acetyl-D-phenylalanine methyl ester (D-compound II). This ester was prepared by acetylation, and subsequent esterification of D-phenylalanine in 50% yield, m.p. 88.5–89.5°C, $[\alpha]_D^{25} -16.1^\circ$ (c 1.8 in methanol) [Cohen & Schultz (1968) report m.p. 89.5–90°C and $[\alpha]_D^{26} -19.4^\circ$ in methanol].

Methyl DL-2-acetamido-4-phenylbutyrate (DL-compound III). DL-2-Amino-4-phenylbutyric acid (3.6 g, 20 mmol) was acetylated with 4.5 ml (47.7 mmol) of acetic anhydride in the presence of NaOH at pH 7.0 to give 3.4 g (76.5% yield) of methyl DL-2-acetamido-4-phenylbutyric acid, m.p. 148–149°C [Andrako *et al.* (1960) report m.p. 147–148°C]. This compound (3 g, 13.6 mmol) in 50 ml of methanol was treated with 1.2 ml of thionyl chloride to yield 2.6 g (81.5% yield) of DL-compound III as long white needles, m.p. 55.5–57.0°C.

Ethyl 2-acetamido-2-cyano-5-phenylvalerate. Sodium (2.53 g, 0.11 g-atom) was dissolved in 100 ml of dry ethanol in a 500 ml round-bottomed flask fitted with a condenser and protected from the atmosphere. To this solution maintained at 50°C, 18 g (0.106 mol) of ethyl acetamidocyanoacetate was added with stirring, followed by 20 g (0.1 mol) of 3-phenylpropyl bromide. This solution was refluxed for 2 h, and ethanol was removed by evaporation under vacuum. The residue was shaken with 200 ml of water, acidified to pH 5 with HCl and extracted with ethyl acetate. The extract was processed and the product was crystallized from ethyl acetate as colourless cubes (12.65 g, 44% yield), m.p. 94.5–95.5°C.

Ethyl 2-acetamido-2-cyano-6-phenylhexanoate. This compound was synthesized in a similar way by condensing 32 g (15.1 mmol) of 4-phenylbutylbromide with 27 g (15.9 mmol) of ethyl acetamidocyanoacetate. The product was recrystallized from ethanol–

water (1:1, v/v) to give 24 g (52.5% yield) of colourless needles, m.p. 122–123°C.

2-Amino-5-phenylvaleric acid. Ethyl 2-acetamido-2-cyano-5-phenylvalerate (20 g) was refluxed with 20 g of NaOH in 200 ml of water for 18 h. About 200 ml of water was added and the solution was neutralized with 6 M-HCl. The white precipitate was collected and dissolved in 500 ml of boiling water containing 5 ml of 2 M-HCl. After filtration and neutralization with aq. NH₃, white fluffy crystals (11.1 g, 86% yield) separated, m.p. 230–234°C (with decomposition, softens at 205°C) [Braun & Kruber (1912) give m.p. 203–206°C].

2-Amino-6-phenylhexanoic acid. This acid was prepared in a similar way from 27 g of ethyl 2-acetamido-2-cyano-6-phenylhexanoate. The amino acid was obtained as shiny white needles (15 g, 81% yield), m.p. 261–266°C (with decomposition, softens at 235°C) [Braun & Kruber (1912) give m.p. 237–242°C].

Methyl DL-2-acetamido-5-phenylvalerate (compound IV). 2-Acetamido-5-phenylvaleric acid (4.5 g) in 70 ml of methanol was esterified with 1.6 ml of thionyl chloride. The product was extracted with ethyl acetate, processed and crystallized from ether–light petroleum (b.p. 30–60°C) as stout colourless needles (3.5 g, 73.5% yield), m.p. 59.5–60.0°C.

Methyl DL-2-acetamido-6-phenylhexanoate (compound V). This compound was prepared in a similar way from 2-acetamido-6-phenylhexanoic acid (5 g) to yield 4.4 g (83.5% yield), m.p. 78.5–79.0°C.

Resolution of methyl DL-2-acetamido-2-phenylacetate (compound I) by α -chymotrypsin. A stirred solution of DL-compound I (4.5 g, 21.75 mmol) in 115 ml of water and 10 ml of 1.5 M-NaCl was treated with a solution of 250 mg of α -chymotrypsin in 25 ml of water at room temperature, at pH 7 (substrate concentration, 145 mM; enzyme concentration, 66.6 μ M). In 1 h 10.5 ml of 1 M-NaOH (10.5 mmol, 48.4% hydrolysis) was consumed and the reaction rate became very slow. NaHCO₃ (2 g) was added. The mixture was extracted with ether and the extract was processed to give the crude neutral fraction that was crystallized twice from ether–light petroleum (b.p. 30–60°C) to give 1.2 g (60% yield) of D-compound I, m.p. 112–113°C and $[\alpha]_D^{25} -175.8^\circ$ (c 0.95 in ethanol).

The aqueous solution was acidified to pH 1.5 with 6 M-HCl and freeze-dried. The residue was extracted with acetone, and the product, L-2-phenyl-2-acetamidacetic acid, was crystallized from ether–light petroleum (b.p. 30–60°C), yield 1.1 g (52.5% yield), m.p. 188–190°C and $[\alpha]_D^{26} +190.6^\circ$ (c 0.76 in water) [Cohen *et al.* (1966) give m.p. 194–195°C and $[\alpha]_D^{27} +191^\circ$ in 1 M-NaOH]. The (+)-acid (1 g) in 5 ml of methanol was treated with 0.4 ml of thionyl chloride, and the product was crystallized twice from ether–light petroleum (b.p. 30–60°C) to give

0.72 g (67.3% yield) of the pure L-ester, m.p. 110–112°C and $[\alpha]_D^{25} +180.5^\circ$ (*c* 1.05 in ethanol).

Resolution of methyl DL-2-acetamido-4-phenylbutyrate (compound III) by α -chymotrypsin. A solution of 2.6 g (11.05 mmol) of DL-compound III in 50 ml of dimethyl sulphoxide, 124 ml of water and 16 ml of 1.2M-NaCl was treated with a solution of 40 mg of α -chymotrypsin in 10 ml of water at pH 7 (substrate concentration, 55.25 mM; enzyme concentration, 8 μ M). Alkali consumption (5.5 ml of 1M-NaOH, 5.5 mmol, 49.75% hydrolysis) abated after about 50 min. NaHCO_3 (1 g) was added and the solution was extracted with ether. The ether extract was processed, and the product was crystallized from ether–light petroleum (b.p. 30–60°C) to give 0.75 g (58% yield) of D-compound III, m.p. 56–57°C and $[\alpha]_D^{25} -2.08^\circ$ (*c* 1.85 in ethanol).

The aqueous layer was acidified to pH 1.5 with HCl and extracted with ethyl acetate and the extract was processed. The crude acidic portion was recrystallized from ethyl acetate to yield 0.75 g (60.5% yield) of L-2-acetamido-4-phenylbutyric acid, m.p. 173–174°C and $[\alpha]_D^{25} +25.5^\circ$ (*c* 1.40 in ethanol) [du Vigneaud *et al.* (1939) give m.p. 179–180°C and $[\alpha]_D +25.8^\circ$ in ethanol]. This L-acid (700 mg) was esterified to yield 450 mg (60.5% yield) of L-compound III, m.p. 56–57°C and $[\alpha]_D^{25} +2.42^\circ$ in ethanol (*c* 2.72 in ethanol).

Resolution of methyl DL-2-acetamido-5-phenylvalerate (DL-compound IV) by α -chymotrypsin. The compound (1.74 g, 7 mmol) in 100 ml of dimethyl sulphoxide, 210 ml of water and 40 ml of 1.2M-NaCl was treated with a solution of 500 mg of α -chymotrypsin in 50 ml of water at pH 8.0, at room temperature (substrate concentration, 17.5 mM; enzyme concentration, 50 μ M). In 3 h, 3.52 ml of 1M-NaOH (3.52 mmol, 50.2% hydrolysis) was consumed and the reaction rate became very slow. The solution was extracted with ethyl acetate, the extract was processed, and the product, D-compound IV, was crystallized twice from ether–light petroleum (b.p. 30–60°C) as fine white needles to give 750 mg (86% yield), m.p. 64.5–66.0°C and $[\alpha]_D^{20} +6.92^\circ$ (*c* 2.03 in ethanol).

The aqueous layer was acidified to pH 1.5 with HCl and extracted with ethyl acetate. The oily residue obtained on evaporation of the extract was dissolved in dilute NaOH (pH 8.0). On acidification L-2-acetamido-5-phenylvalerate crystallized as long needles (550 mg, 66.5% yield), m.p. 136–138°C and $[\alpha]_D^{20} +17.72^\circ$ (*c* 1.05 in ethanol). This acid (450 mg) was esterified with methanol in the presence of thionyl chloride, and the product was crystallized twice from ether–light petroleum (b.p. 30–60°C) to give 360 mg (67.2% yield) of stout white needles, m.p. 65.5–66.5°C and $[\alpha]_D^{20} -7.56^\circ$ (*c* 1.1 in ethanol).

Resolution of methyl DL-2-acetamido-6-phenylhexanoate (DL-compound V) by α -chymotrypsin. The

ester (1.85 g, 7.0 mmol), in 300 ml of dimethyl sulphoxide, 410 ml of water and 90 ml of 1.2M-NaCl, was treated with a solution of 1.0 g of chymotrypsin in 100 ml of water at pH 8.0 at room temperature (substrate concentration, 7.8 mM; enzyme concentration, 44.4 μ M), and 3.15 ml of 1M-NaOH was consumed in 22 h (3.15 mmol, 45% hydrolysis) and the reaction rate became slow. The solution was extracted with ethyl acetate, and the extract was processed to yield crude D-compound V. This was crystallized twice from ether–light petroleum (b.p. 30–60°C) to yield 750 mg (81% yield) of the optically active ester, m.p. 69.5–70.5°C and $[\alpha]_D^{20} +8.1^\circ$ (*c* 1.08 in ethanol).

The aqueous layer was stirred with 25 g of NaCl, acidified and extracted with ethyl acetate, and the extract was processed. The product was crystallized twice from ethyl acetate–light petroleum (b.p. 30–60°C) to give 500 mg (64.5% yield) of L-2-acetamido-6-phenylhexanoic acid, m.p. 124–125.5°C and $[\alpha]_D^{20} +10.93^\circ$ (*c* 0.89 in ethanol). This acid (400 mg) was esterified with methanol and thionyl chloride and the product was crystallized twice from ether–light petroleum (b.p. 30–60°C) to give 310 mg (65% yield) of the L-ester, m.p. 70.5–71.0°C and $[\alpha]_D^{20} -8.52^\circ$ (*c* 1.0 in ethanol).

Hydrolysis of methyl D-2-acetamido-2-phenylacetate (D-compound I) by α -chymotrypsin and isolation of the product. The ester (840 mg, 4.06 mmol), in 125 ml of dimethyl sulphoxide, 50 ml of 1M-NaCl and 175 ml of water, was treated with a solution of 500 mg of α -chymotrypsin in 50 ml of water and titrated with 0.1M-NaOH at pH 8.0. The initial rate (240 μ mol/h) of enzymic hydrolysis was 108 times faster than the rate without the enzyme (spontaneous hydrolysis). In 3 h, 0.44 mmol of alkali was consumed and an additional 50 ml solution of α -chymotrypsin (500 mg) was added and the reaction continued for a further 4 h. At the end of this period a total of 1 mmol of alkali (24.6% hydrolysis) was consumed. NaHCO_3 (2 g) was added and the solution was extracted with 50 ml of ethyl acetate five times. The aqueous layer was acidified and extracted with ethyl acetate, and the extract was processed and evaporated. The resulting oil was dissolved in ether and the product was crystallized from ether–light petroleum (b.p. 30–60°C) to yield 110 mg (57% yield) of D-2-acetamido-2-phenylacetic acid, m.p. 188–189°C and $[\alpha]_D^{21} -183^\circ$ (*c* 0.52 in water) [Neubauer & Warburg (1910) give m.p. 191°C and $[\alpha]_D -197.4^\circ$ in water].

Hydrolysis of N-acetyl-D-phenylalanine methyl ester (D-compound II) by α -chymotrypsin and isolation of the product. The ester (884 mg, 4 mmol), in 50 ml of dimethyl sulphoxide, 62.5 ml of water and 12.5 ml of 1M-NaCl, was treated with a solution of 500 mg of chymotrypsin in 50 ml of water and titrated with 0.5M-NaOH at pH 8. The initial rate (42 μ mol/h) of enzymic hydrolysis was 56 times faster than the rate of alkali consumption without the

enzyme (spontaneous hydrolysis). In 24h, 1.44ml of 0.5M-NaOH (720 μ mol, 18% reaction) was consumed. NaHCO₃ (2g) was added and the solution was extracted with 300ml of ethyl acetate in six portions. The aqueous layer was acidified and extracted with 400ml of ethyl acetate in eight portions. This extract was processed and evaporated. The resulting oil was shaken with ether and light petroleum (b.p. 30–60°C) was added. The precipitate was collected and dissolved in dilute NaOH (pH 8), filtered and acidified to give 80mg (53.7% yield) of *N*-acetyl-D-phenylalanine, m.p. 168–169°C and $[\alpha]_D^{27.5}$ -40° (*c* 1.25 in methanol) [Birnbau *et al.* (1952) give m.p. 171°C and $[\alpha]_D$ in methanol -40.3°].

Assignment of absolute configurations

D-2-Amino-5-phenylvaleric acid. (+)-Methyl 2-acetamido-5-phenylvalerate (620mg), prepared as described under 'resolution of compound IV', was refluxed with 13ml of 3M-HCl for 20h, filtered while hot, and the solution was neutralized with pyridine to give the amino acid as a crystalline precipitate. This was recrystallized by dissolving it in dilute HCl and neutralizing the pyridine, to yield 320mg of white plates, m.p. 230–234°C (with decomposition). A negative shift in the molecular rotation was observed on acidification of the solution, indicating that the product had the D-configuration (Greenstein & Winitz, 1961). The compound had $[\alpha]_D^{21}$ -21.75° (*c* 0.9 in 1M-HCl), $[M]_D$ -41.98° ; $[\alpha]_D^{21}$ -1.305° (*c* 0.26 in water), $[M]_D$ -2.52° ; $[M]_D$ in 1M-HCl $-[M]_D$ in water $= -39.46^\circ$.

D-2-Amino-6-phenylhexanoic acid. This amino acid was prepared in a similar way from 640mg of (+)-methyl 2-acetamido-6-phenylhexanoate (D-compound V). The product was crystallized from dilute HCl on neutralization with pyridine to yield 350mg, m.p. 260–263°C (with decomposition). The compound had $[\alpha]_D^{20}$ -18.85° (*c* 0.35 in 1M-HCl), $[M]_D$ -36.76° ; $[\alpha]_D^{20}$ -12.56° (*c* 0.33 in 0.1M-HCl), $[M]_D$ -24.49° ; $[\alpha]_D^{20}$ -7.48° (*c* 0.10 in 5mm-HCl, $[M]_D$ -14.59° ; $[M]_D$ in 1M-HCl $-[M]_D$ in 5mm-HCl $= -22.17^\circ$. The insolubility of the compound precluded optical rotation measurements in water at neutral pH. However, the negative shift in molecular rotation observed on increasing the acidity is indicative of the D-configuration.

Kinetic procedures

Kinetic studies were performed at 25°C under N₂ at an apparent pH of 8.0 in aq. (41.7%) dimethyl sulphoxide. The assay system for studies with α -chymotrypsin consisted of 10ml of substrate solution in aq. 50% dimethyl sulphoxide, 1ml of 1.2M-NaCl and 1ml of enzyme solution in water. For studies with subtilisin BPN' the system consisted of 5ml of sub-

strate solution in aq. 50% dimethyl sulphoxide containing 0.1M-KCl and 1ml of enzyme solution in 0.1M-KCl. Titration was done with 0.1M-, 0.02M- or 0.01M-NaOH, depending on the reactivities of substrates, delivered from the 0.5ml automatic burette of the Radiometer titrator. Lineweaver-Burk plots of the initial hydrolysis rates at various substrate concentrations gave the kinetic parameters k_{cat} and $K_{m(app.)}$. The reactivities of all substrates were compared by means of k_{cat}/K_m ratios, after correction for their intrinsic reactivities. The intrinsic reactivity (rate of hydroxide ion-catalysed hydrolysis) of all substrates was determined by the titration method with 0.1M-NaOH described by Hayashi & Lawson (1969). The first-order kinetic constants were compared at pH 11.0 (apparent) for all compounds.

Results

Substrates

2-Amino-5-phenylvaleric acid and 2-amino-6-phenylhexanoic acid were synthesized by the ethyl acetamidocyanacetate method (Albertson & Tullar, 1945) and converted into the corresponding α -acetamido methyl esters. The other substrates used in these studies were synthesized as described in the Experimental section. The structures of hitherto unreported compounds were established by elemental analysis (Table 1), i.r. spectra and n.m.r. spectra. Compounds I, III, IV and V were resolved into their optical antipodes with chymotrypsin and the absolute configurations of the more reactive optical isomers of compounds I–V were all found to belong to the L-series.

The intrinsic reactivities of the substrates used in these studies, and the concentration of the enzymes and substrates employed for evaluation of the kinetic parameters, are listed in Table 2. The intrinsic reactivities are the rate of the first-order hydrolysis of ester by OH[−], relative to the rate of hydrolysis of *N*-acetyl-L-phenylalanine methyl ester (L-compound II), which is assigned an intrinsic reactivity of 1.0. These values were obtained in dimethyl sulphoxide–water (1:1, v/v) and 0.1M-NaCl.

Enzymic experiments

All kinetic experiments were performed in aq. 41.7% dimethyl sulphoxide. The rates of hydrolysis of substrates by α -chymotrypsin were observed to be 15–20 times slower in this solvent system and the decrease in rate was found to be primarily due to an increase in K_m (Hayashi & Lawson, 1969). The effect of various concentrations of dimethyl sulphoxide on the hydrolysis of L-compound II by α -chymotrypsin and subtilisin BPN' is presented in Table 3. Increasing the concentration of dimethyl

Table 2. *Intrinsic reactivity of esters and substrate and enzyme concentrations used in kinetic experiments*

Compound	Experimental details are given in the text.				
	Intrinsic reactivity	α -Chymotrypsin		Subtilisin BPN'	
		$10^7 \times [E]_0$ (M)	$10^3 \times [S]_0$ (M)	$10^7 \times [E]_0$ (M)	$10^3 \times [S]_0$ (M)
L-I	2.74	330	2.0–20.0	19.4	4.0–40.0
D-I		333	5.0–25.0	388	4.0–40.0
L-II	1.0	0.83	1.0–10.0	1.71	2.0–20.0
D-II		480	2.0–20.0	776	3.0–30.0
L-III	0.714	3.3	0.9– 9.0	7.76	1.6–16.0
D-III		432	0.5– 8.0	776	3.0–30.0
L-IV	0.625	330	1.0–10.0	7.76	3.0–30.0
D-IV		333	2.0–10.0	388	2.0–16.0
L-V	0.45	330	1.0–10.0	1.94	3.0–18.0
D-V		333	3.0–10.0	388	2.0–16.0
L-XI	3.09	8.80	1.0–10.0	16.2	2.0–20.0
D-XI		33.3	2.0–20.0	130	2.0–20.0
L-XII	0.574	0.67	2.0–20.0	13.6	2 –20.0
D-XII		417	8.0–20.0	342	10 –20.0
L-XIV	1.0	3.79	1.0–10.0	1.60	2.5–25.0
D-XIV		333	3.0–10.0	388	4.0–20.0
VI	0.548	333	2.0–40.0	388	4.0–40.0
VII	0.153	167	2.0–20.0	77.6	2.0–20.0
VIII	0.118	66.6	1.0–10.0	388	1.6–16.0
IX	0.091	333	0.7– 7.0	388	0.7– 7.0
X	0.062	333	0.9– 2.1	388	0.3– 2.7
XIII	0.224	133	2.0–20.0	136	2.0–20.0
XV	0.205	167	1.0–10.0	341	1.3–10.6

Table 3. *Effect of dimethyl sulphoxide on the hydrolysis of L-C₆H₅-CH₂-CH(NHCOCH₃)-CO₂CH₃ by α -chymotrypsin and subtilisin BPN'*

Hydrolysis was at pH8.0, 25°C. Further details are given in the text.

Dimethyl sulphoxide (%)	α -Chymotrypsin			Subtilisin BPN'		
	$k_{cat.}$ (s ⁻¹)	K_m (mM)	$k_{cat.}/K_m$ (s ⁻¹ ·M ⁻¹)	$k_{cat.}$ (s ⁻¹)	K_m (mM)	$k_{cat.}/K_m$ (s ⁻¹ ·M ⁻¹)
0	75.1	1.19	63 100	318	44.5	7140
10	81.9	3.19	25 700	185	32.9	5610
20	75.9	4.55	17 100	121	36.4	3330
30	57.6	6.66	8 650	113	38.4	2930
40	40.3	8.70	4 630	59.8	37.0	1620
50	27.8	12.2	2 280	35.0	38.5	909
60	11.7	90.0	130	12.1	43.5	278
70	0.0	—	0.0	5.64	57.1	98.8

sulphoxide decreased the reactivity of both the enzymes, the effect being more pronounced with chymotrypsin. α -Chymotrypsin is completely inactivated in aq. 70% dimethyl sulphoxide, whereas

subtilisin is still active at this concentration. Higher concentrations of dimethyl sulphoxide could not be tested because of the slow electrode response. The K_m value for this substrate shows a progressive

Table 4. Kinetic parameters for the hydrolysis of $L\text{-C}_6\text{H}_5\text{--}[\text{CH}_2]_n\text{--CH(NHCOCH}_3\text{)--CO}_2\text{CH}_3$, at pH 8.0, 25°C, in aq. 41.7% dimethyl sulphoxide

Experimental details are given in the text.

Compound	<i>n</i>	<i>k</i> _{cat.} (s ⁻¹)	<i>K_m</i> (mM)	<i>k</i> _{cat.} / <i>K_m</i>	Normalized
				(s ⁻¹ ·M ⁻¹)	(s ⁻¹ ·M ⁻¹)
α-Chymotrypsin					
L-I	0	0.714	69.5	10.3	3.75
L-II	1	40.0	9.52	4200	4200
L-III	2	11.1	7.87	1410	1980
L-IV	3	0.364	26.4	13.8	22.1
L-V	4	0.053	24.4	2.16	4.8
Subtilisin BPN'					
L-I	0	2.08	152	13.7	5.0
L-II	1	57.2	38.4	1490	1490
L-III	2	14.9	23.3	640	896
L-IV	3	18.2	83.3	219	350
L-V	4	96.2	66.6	1444	3210

Table 5. Kinetic parameters for the hydrolysis of $\text{C}_6\text{H}_5\text{--}[\text{CH}_2]_n\text{--CH}_2\text{--CO}_2\text{CH}_3$, at pH 8.0, 25°C, in aq. 41.7% dimethyl sulphoxide

Experimental details are given in the text.

Compounds	<i>n</i>	$10^3 \times k_{\text{cat.}}$	<i>K_m</i> (mM)	<i>k_{cat.}</i> / <i>K_m</i>	Normalized
		(s ⁻¹)		(s ⁻¹ ·M ⁻¹)	(s ⁻¹ ·M ⁻¹)
α-Chymotrypsin					
VI	0	4.4	50.0	0.088	0.161
VII	1	71.9	14.7	4.89	31.9
VIII	2	66.0	6.89	9.58	81.2
IX	3	2.97	7.69	0.386	4.24
X	4	—	—	0.038*	0.617
Subtilisin BPN'					
VI	0	5.57	86.2	0.065	0.118
VII	1	83.3	77.0	1.08	7.07
VIII	2	14.3	47.6	0.300	2.54
IX	3	8.48	27.3	0.311	3.42
X	4	50.0	50.5	0.990	16.1

* (*v*)/[*E*][*S*], average of four values.

increase with dimethyl sulphoxide concentration in the case of chymotrypsin whereas with subtilisin, no such alteration was noticeable.

The kinetic parameters for the hydrolysis of compounds L-I–L-V by chymotrypsin and subtilisin are shown in Table 4. *N*-Acetyl-L-phenylalanine methyl ester (L-compound II) is hydrolysed three times faster

by chymotrypsin than by subtilisin. The differences in reactivity are due to a poorer binding (*K*_m) in the case of subtilisin. Methyl L-2-acetamido-2-phenylacetate (L-compound I), the lower homologue of L-compound II, is hydrolysed much more slowly by both enzymes. A high *K*_m and a low *k*_{cat.} contribute to the low reactivity of L-compound I. Methyl L-2-

Table 6. *Hydrolysis of substrates at pH8.0, 25°C, in aq. 41.7% dimethyl sulphoxide*

Experimental details are given in the text.				Normalized
Compound	$k_{\text{cat.}}$ (s^{-1})	K_m (mM)	$k_{\text{cat.}}/K_m$ ($\text{s}^{-1} \cdot \text{M}^{-1}$)	$k_{\text{cat.}}/K_m$ ($\text{s}^{-1} \cdot \text{M}^{-1}$)
α -Chymotrypsin				
L-XI	2.33	13.3	174	56.4
D-XI	0.381	32.0	11.9	3.85
L-XII	49.5	7.43	6670	11 600
D-XII	—	—	0.006*	0.011
L-XIV	49.2	25.1	1960†	1960
D-XIV	0.003	23.3	0.126	0.126
XIII	0.159	13.2	12.1	53.8
XV	0.056	20.0	2.78	13.6
Subtilisin BPN'				
L-XI	2.33	55.5	42	13.6
D-XI	0.026	27.4	0.932	0.302
L-XII	12.7	42.4	300	523
D-XII	—	—	Not reactive	—
L-XIV	5.0	58.8	85	85
D-XIV	—	—	Not reactive	—
XIII	0.011	23.6	0.48	2.14
XV	0.014	27.8	0.514	2.51

* $(v)/[E][S]$, average of four values.

† Hayashi & Lawson (1969).

acetamido-4-phenylbutyrate (L-compound III) is hydrolysed by both chymotrypsin and subtilisin approximately half as fast as L-compound II. The K_m values for compounds L-II and L-III for the same enzyme are comparable. L-Compound IV, which has a side chain longer by one more methylene group than L-compound III, is two orders of magnitude less reactive with chymotrypsin. It appears that the length of the hydrophobic site in chymotrypsin is not capable of effectively accommodating any moiety larger than phenethyl in α -acetamido esters. The next higher homologue L-compound V is 4.6 times less reactive than L-compound IV, showing that maximal hindrance has been achieved with L-compound IV, and further elongation of the side chain causes only a minor effect. However, subtilisin hydrolyses L-compound IV at approximately half the rate of that of its lower homologue. L-Compound V is hydrolysed 10 times faster than L-compound IV, and it is therefore the most reactive substrate in the series for subtilisin. It is likely that the binding-site region in subtilisin can productively accommodate a group at least as large as a phenylbutyl system in this type of substrate.

The results for the hydrolysis of phenylalkanoate esters (Table 5) support the above conclusions. Methyl 4-phenylbutyrate (compound VIII) is the most reactive substrate for chymotrypsin compared

with its higher and lower homologues, whereas subtilisin hydrolysed methyl 6-phenylhexanoate (compound X) faster than any of its lower homologues. Because of the decrease in the solubility of substrates with increasing chain length, higher homologues of compounds V and X were not tested.

N-Acetyl-L-tryptophan methyl ester (L-compound XII) is a better substrate than L-compound II for chymotrypsin, whereas subtilisin hydrolyses L-compound XII 23 times slower (Table 6). The naphthalene analogue (L-compound XIV) is half as reactive as L-compound II with chymotrypsin, whereas subtilisin also hydrolyses this ester 23 times more slowly. The side chain of L-compound XII, and to a lesser extent that of L-compound XIV, can be accommodated productively at the curved binding-site region of chymotrypsin (Hayashi & Lawson, 1969). However, the corresponding region in subtilisin does not seem to be curved and the side chains of compounds L-XII and L-XIV are not accommodated as effectively as with chymotrypsin.

Table 7 gives the results on the hydrolysis of the D-enantiomers of α -acetamidophenylalkanoate esters. These substrates are hydrolysed by both enzymes at very slow rates. The initial rates were determined from the rate of alkali consumption over an extended period of time (1–3h) for each experiment. The

Table 7. Kinetic parameters for the hydrolysis of $D-C_6H_5-[CH_2]_n-CH(NHCOCH_3)-CO_2CH_3$, at pH 8.0, 25°C, in aq. 41.7% dimethyl sulphoxide

Experimental details are given in the text.

Compound	<i>n</i>	$10^3 \times k_{\text{cat.}}$	<i>K_m</i> (mM)	<i>k_{cat.}/K_m</i>	Normalized
		(s ⁻¹)		(s ⁻¹ · M ⁻¹)	(s ⁻¹ · M ⁻¹)
α-Chymotrypsin					
D-I	0	11.8	80.0	0.147	0.054
D-II	1	0.333	8.33	0.04	0.040
D-III	2	2.95	6.90	0.427	0.60
D-IV	3	0.595	6.25	0.095	0.152
D-V	4	0.840	5.13	0.164	0.364
Subtilisin BPN'					
D-I	0	28.6	246	0.116	0.042
D-II	1	0.862	62.5	0.014	0.014
D-III	2	1.18	31.2	0.038	0.053
D-IV	3	3.57	50.4	0.071	0.113
D-V	4	22.2	69.0	0.322	0.716

Table 8. Stereospecificity in the hydrolysis of substrates

The results are expressed as the L/D ratio of $k_{cat.}/K_m$.

Compound	<i>α</i> -Chymotrypsin	Subtilisin BPN'
I	70	118
II	105 000	108 000
III	3 300	1 700
IV	145	3 090
V	13.2	4 490
XI	14.3	45
XII	106 000	—
XIV	15 500	—

spontaneous hydrolyses of the substrates under identical conditions were negligible. The slow rates observed, however, do not appear to be caused by contamination of the more active optical antipodes, for the following reasons. With the high enzyme concentrations used in these experiments, any trace amounts of the L-isomers should be hydrolysed within a short span of time. At least in one case (D-compound III), the chymotryptic hydrolysis was allowed to proceed for 36 h (substrate concentration, 12 μmol; enzyme concentration, 0.52 μmol) and 97% of the substrate was hydrolysed during this time, based on the amount of alkali consumed. To eliminate the possibility of the presence of a hitherto unknown isomerase in the enzyme preparations that could convert the D-isomers into their antipodes, the products of chymotryptic hydrolysis of compounds D-I and D-II (see the Experimental section) were isolated

Table 9. Effect of *α*-NHCOCH₃, in the hydrolysis of substrates

The enhancing effect of *α*-NHCOCH₃ in the L-series of substrates represents the increase in the normalized $k_{cat.}/K_m$ ratios over their corresponding analogues lacking this group. The detrimental effect in the D-series of substrates represents the decrease in the normalized $k_{cat.}/K_m$ ratios compared with the corresponding arylalkanoate esters.

Substrate	<i>α</i> -Chymotrypsin		Subtilisin BPN'	
	L	D	L	D
I	23.3	3.0	42.3	2.79
II	132	798	210	512
III	24.3	135	353	48.0
IV	5.21	27.9	102	30.2
V	7.77	1.69	200	22.5
XII	216	4890	244	—
XIV	145	108	33.9	—

and were shown to be D-*α*-acetamido acids by their optical rotation values.

Table 8 shows the stereospecific ratios in the hydrolysis of optically active substrates. The ratios for the two enzymes are not always comparable. Although for compounds I and II they are in the same range, compounds III, IV, V and XI are hydrolysed more stereospecifically by subtilisin. The ratios for the stereospecific hydrolysis by subtilisin of compounds XII and XIV could not be determined be-

cause of the inertness of the D-enantiomers. It is reasonable to conclude that the ratios will be of the same order of magnitude for both enzymes, if not higher with subtilisin, from the results obtained for the other esters.

Table 9 shows the contribution of α -acetamido groups in substrates. The enhancing effect of this group in the case of the L-series of substrates represents the increase in the normalized $k_{\text{cat.}}/K_m$ ratios over their corresponding analogues that lack this group. Similarly, the detrimental effect in the case of the D-series is evaluated. The results indicate that the positive contribution of α -acetamido groups in the L-series varies over a wide range. In no case is the enhancement much larger than two orders of magnitude. In compounds L-IV and L-V, with respect to chymotrypsin, the effect is almost negligible. The enhancement is generally greater in the case of subtilisin. Similarly the detrimental effect of α -acetamido groups in the D-series of substrates varies over a wide range. Whereas for compounds D-II and D-XII (the most stereospecifically hydrolysed substrates) the effect is large, for D-compound I with respect to both the enzymes and for D-compound V with respect to chymotrypsin, the detrimental effect is negligible.

Discussion

The effect of dimethyl sulphoxide on the hydrolysis of *N*-acetyl-L-phenylalanine methyl ester (L-compound II) by α -chymotrypsin and subtilisin BPN' provides some information on the rigidity of the conformation of the active-site regions of these enzymes. Chymotrypsin is more sensitive to the effect of the solvent than is subtilisin. In the case of chymotrypsin, in addition to the lowering of the $k_{\text{cat.}}$ values with increase in dimethyl sulphoxide concentration in the medium, the K_m value also increases. Clement & Bender (1963) observed that the slower rate of α -chymotrypsin-catalysed hydrolyses in mixtures of organic solvents and water is primarily caused by an increase in K_m . With subtilisin, no alteration in the K_m was noticeable. It is likely that the conformation of the active site of subtilisin is more stable to solvent effects, including denaturation. This is consistent with the observation that α -chymotrypsin is rapidly denatured by urea and guanidine hydrochloride (Rossi & Bernhard, 1971), whereas subtilisin BPN' is extremely stable to these denaturing agents (Ottesen & Svendsen, 1970).

A comparison of the relative rates of hydrolysis of arylalkanoate esters and α -acetamidoaryalkanoate esters by α -chymotrypsin and subtilisin BPN' shows the differences in the geometry of the binding sites of these two enzymes. The dimensions of the binding site of α -chymotrypsin are such that it cannot, without interfering with productive binding, accommodate

site chains of typical substrates longer than a phenethyl group. Jones *et al.* (1965) have studied the chymotryptic hydrolysis of a series of *N*-acetyl-L-amino acid esters of the general formula $\text{H}-[\text{CH}_2]_n-\text{CH}(\text{NH}-\text{CO}-\text{CH}_3)-\text{CO}_2\text{CH}_3$, where $n = 1$ to 6. The rate of reaction was found to be greatest when the number of carbon atoms in the acyl group was five. Hofstee (1957) noted that chymotryptic hydrolysis rates in a series of alkanoate esters of *m*-hydroxybenzoic acid increase with increasing chain length, reaching a maximum with the octanoate ester. From these and other studies (Hofstee, 1967; Enriquez & Gerig, 1969; Milestein & Fife, 1969; Dupaix *et al.*, 1970), it was concluded that the binding site of α -chymotrypsin can accommodate 5–6 methylene groups [1.0–1.1 nm (10–11 Å)]. The present studies are in agreement with this conclusion.

With subtilisin BPN', the pattern looks somewhat different. Methyl L-2-acetamido-6-phenylhexanoate (L-compound V) and methyl 6-phenylhexanoate (compound X) are hydrolysed faster than their lower homologues, suggesting that the binding site of subtilisin is long enough to accommodate productively a group as large as a phenylpentyl moiety. The binding site of α -chymotrypsin has been shown to be elongated and curved (Wallace *et al.*, 1963; Hayashi & Lawson, 1969; Pattabiraman & Lawson, 1972a). The faster rate of hydrolysis of *N*-acetyl-L-tryptophan methyl ester compared with that of L-compound II by chymotrypsin is explicable on this basis. L-Compound XII and its 1-naphthalene analogue (L-compound XIV) are hydrolysed 23 times more slowly by subtilisin, suggesting that the hydrophobic region of subtilisin is not curved and that the side chains of these substrates can be accommodated only with some spatial constraint.

It is well established that α -chymotrypsin hydrolyses locked substrates with a stereoselectivity that varies over a wide range (Pattabiraman & Lawson, 1972a, and references therein). The present studies indicate that the stereospecificity during the hydrolysis of typical substrates (α -acetamido esters) by chymotrypsin and subtilisin is not absolute and also exhibits a broad spectrum of variation. Applewhite *et al.* (1958) noted that *N*-acetyl-D-tryptophan methyl ester (D-compound XII) undergoes slow hydrolysis in the presence of large amounts of α -chymotrypsin. Ingles & Knowles (1968) showed that the stereospecific ratios for the hydrolysis (deacylation rates) of the *p*-nitrophenyl esters of *N*-acetyl phenylalanine and *N*-acetyl tryptophan are 18300 and 6400, respectively, in favour of the L-isomers. Silver *et al.* (1970) confirmed these observations. Because *p*-nitrophenyl esters show decreased stereospecificity, these authors predicted that the ratios for the corresponding methyl esters would be two orders of magnitude greater. The stereospecific ratios reported here are not far from those predicted.

A good correlation between reactivity and stereospecificity is obtained for chymotryptic hydrolysis. The highest stereospecificity (10^6) was observed with compound XII, the most reactive substrate. Compound II, which is half as reactive as compound XII, displays a stereospecificity that is decreased tenfold. With compounds III and XIV, which are half as reactive as compound II, the stereoselectivity decreases by approximately an order of magnitude. With the least reactive ester, compound V, the L-isomer is hydrolysed only 13 times faster than its enantiomer. In no case was a complete inversion of the antipodal specificity observed. The variation in stereospecificity is due mainly to changes in reactivity of the L-enantiomers. Cohen *et al.* (1966) have demonstrated that ethyl 2-acetamido-2-phenyl acetate is hydrolysed by chymotrypsin at a much slower rate than that of compound II and with L-specificity. Whereas our results are in agreement with their observation concerning the decreased reactivity of the phenylglycine derivative, we observe, in contrast, that the stereospecificity is limited rather than being absolute. A high K_m and low k_{cat} contribute to the low reactivity of L-compound I. The reason for this seems to be, that with the ester group oriented towards the catalytic region and the acetamido group directed towards a polar site on the surface of the enzyme, the phenyl ring projects towards the hydrophobic binding site, but makes poor interaction with it. This effect is slightly more pronounced with chymotrypsin, since the ratio of the reactivity of compounds L-II to L-I is more with chymotrypsin (1120) than with subtilisin (296). In the case of subtilisin no correlation between reactivity and stereospecificity could be made. The ratios observed are of the same magnitude or greater than those observed with chymotrypsin. This result appears to be a reflexion of the less stringent requirement on the size of the substrates' side chains, which are accommodated at a more spacious binding site in the case of subtilisin. The high stereospecific ratios observed during hydrolysis of compounds IV and V by subtilisin are solely caused by the enhanced reactivities of the L-antipodes with subtilisin compared with the rates of hydrolysis observed with chymotrypsin.

The arylalkanoate esters are less reactive than the corresponding L- α -acetamido analogues as substrates both for chymotrypsin and subtilisin. The enhanced reactivities can be considered to be caused by a positive contribution of the interaction of the α -acetamido groups, as the binding constants (K_m values) for the α -acetamido esters and the corresponding substrates without this group are in the same range. Similarly the α -acetamido groups in compounds D-I-D-V, D-XII and D-XIV cause a detrimental effect. In the case of the L- α -acetamido substrates, this group should be accommodated at a polar site and the interaction at this site produces an

enhancement of the reactivity. In the D-series, the acetamido group may be directed towards a region of restricted volume (Cohen & Lo, 1970), or might be interfering with nucleophilic or electrophilic attack on the carbonyl group of the ester moiety of the substrate, and with subsequent hydrolysis as envisaged by Hein & Niemann (1962*b*). Alternatively, the acetamido and alkaryl groups might bind properly and thus hinder proper interaction of the ester group. Compounds VI-X, XIII and XV can be considered as typical bifunctional substrates that use only the catalytic and hydrophobic regions. The results in Table 9 indicate that the positive contribution of an α -acetamido group in the L-series of substrates and the detrimental effect in the D-series both vary over a wide range. The maximum positive contribution (216 in L-compound XII for chymotrypsin, 352 in L-compound III for subtilisin) is less than the maximum detrimental effect observed (4890 in D-compound XII for chymotrypsin and possibly an equal order of magnitude for subtilisin). Comparison of the reactivities of the optical isomers of compound XI, which has an α -hydroxyl group, with that of compound II indicates that this group does not enhance the reactivity of L-compound XI, but has a detrimental effect of an order of magnitude in D-compound XI for both the enzymes. A hydrogen-bonding interaction between the NH part of the α -acetamido group and a suitable site on the surface of the enzyme has been proposed by many workers as the factor responsible for the high reactivity of typical substrates in the case of α -chymotrypsin (Ingles & Knowles, 1968; Steitz *et al.*, 1969), and another carbonyl group may be postulated for this function in subtilisin BPN' (D. M. Blow and J. Kraut, personal communications). Silver *et al.* (1970), however, suggested that the effect could be related to other factors, such as the electronic character of the α -acetamido group. The present results do not provide any direct evidence for or against a hydrogen-bonding interaction with a specific group on the enzymes. We believe that there is a definite site for the acylamido group, but this site may not necessarily involve a particular residue in these enzymes. The site could consist merely of the hydrated acylamido group itself, in contact with solvent. The relationship between the acylamido groups and the side chains of the various substrates, in conjunction with the nature of the different 'aromatic' binding sites of chymotrypsin and subtilisin, is likely to result in varying probabilities of productive interaction of the ester groups. The widely different reactivities and stereospecificities observed result from the totality of all these factors.

In conclusion, these studies help in understanding some differences in the geometry of the binding sites of α -chymotrypsin and subtilisin BPN'. The stereospecificity during the hydrolysis of typical substrates

is not absolute and displays a broad spectrum for both the enzymes, as is also observed with locked substrates (see Pattabiraman & Lawson, 1972b).

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