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The pH-Dependence of the Binding of Competitive Inhibitors to Pepsin

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1. The pH-dependence of the binding to pepsin of four dipeptide competitive inhibitors is reported. Values of K_i obtained from equilibrium-dialysis experiments agree closely with those from kinetic measurements. 2. The binding of uncharged *N*-acyl-dipeptide amides to pepsin is essentially independent of pH from 0·2 to 5·8. Values of K_i for the corresponding *N*-acyl-dipeptide acids rise rapidly above pH 3·5, and depend on the ionization of a group of apparent pK_a 3·6. 3. The data indicate that pepsin does not undergo any gross conformation change (at least none that affects binding) over the whole pH range of its catalytic activity. The pH-dependence of the dipeptide acid inhibitors indicates that the acid anions do not bind to pepsin, presumably because of electrostatic repulsion between the inhibitor anion and a negative centre at or near the active site of the enzyme. 4. The binding of all four stereoisomers of *N*-acetylphenylalanylphenylalanine, of the depside analogues of the L–L- and D–L-compounds and of *N*-acetylglycyl-L-phenylalanine and *N*-acetyl-L-phenylalanylglycine was studied at pH 2·2. 5. These results throw further light on the binding specificity of pepsin and on the charge nature of the active site of this enzyme.

The mechanistic proposals based on the findings reported in this and the following papers are presented in Knowles (1969).

One of the important first steps in the delineation of an enzyme mechanism is the determination of pH-dependence of the catalysed process. For many reasons, particularly in pH regions where K_m increases, the kinetic breakdown of initial-velocity data into k_0 and K_m is fraught with possible error, and it is very desirable to check the variation of analogous parameters by other methods. One approach that can provide very useful information is to study the binding of substrate analogues that are competitive inhibitors by a direct method, such as gel filtration (e.g. Fairclough & Fruton, 1966), spectral perturbation (e.g. Foster & Coahran, 1963) or equilibrium dialysis (e.g. Johnson & Knowles, 1966). If the competitive inhibitors used are closely analogous to substrates, then it is normally presumed that the dependence of the inhibition constant K_i on pH parallels the dependence of K_s, the corresponding dissociation constant for the enzyme–substrate complex, ES. Moreover if the kinetic step immediately following the formation of the ES complex is rate-determining in the overall catalysis, then K_m for the substrate catalysis will normally reduce to K_s. In the case of pepsin there are many indications that K_m equals K_s (see, e.g., Denburg, Nelson & Silver, 1968), and so the information gained from inhibitor-binding studies is very probably applicable to the binding behaviour of substrates to the enzyme. We report here the pH-dependence of the binding of a number of competitive inhibitors to pepsin, determined both by equilibrium dialysis and by kinetic experiments. These data are readily interpretable alongside the pH-dependence of the kinetic parameters for pepsin-catalysed reactions of substrates reported in the next paper (Cornish-Bowden & Knowles, 1969), and throw some light on the question of the charge nature of the active site of this enzyme.

MATERIALS

Pepsin. Pepsin (ex hog stomach mucosa), three-times recrystallized, activity 1:60000, was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks. The concentration of enzyme solutions was determined by measuring E_{280}, assuming $E_{280}^{0.1\%}$ 1·47 for 10mm. path length (Perlmann, 1959).

Acetic anhydride. [*Me*-^{3}H]Acetic anhydride (batch 21, specific radioactivity 100mc/m-mole; batch 22, specific radioactivity 327mc/m-mole) was purchased from The Radiochemical Centre, Amersham, Bucks. A.R. acetic anhydride was used as a carrier.

Scintillation solvent. This was prepared by dissolving 1·75g. of 2,5-diphenyloxazole and 0·060g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene (both obtained from Thorn Electronics Ltd., Tolworth, Surrey) in 350ml. of A.R. toluene and 160ml. of dry ethanol. The solution was stored in a dark bottle.

Buffer solutions. These were made up with A.R.-grade

reagents and deionized water. Below pH4·5 Sørensen buffer was used (Sørensen, 1909), and from pH4·5 to 5·8 0·1M-citrate buffer was used.

N[*Me*-^{3}H]-*Acetyl*-D-*phenylalanyl*-L-*phenylalanine*. *N*-Benzyloxycarbonyl-D-phenylalanine was prepared by the method of Greenstein & Winitz (1961*a*). The crude product was extracted into ethyl acetate and washed with 0·05M-HCl and then with water. After being dried over $MgSO_4$ the product was precipitated with light petroleum (b.p. 40–60°). The yield was 58%, m.p. 85–86°, $[\alpha]_D^{20}$ −5·2° (*c* 1 in ethanol) (Found: C, 68·5; H, 5·5; N, 4·7. Calc. for $C_{17}H_{17}NO_4$: C, 68·2; H, 5·7; N, 4·7%). L-Phenylalanine ethyl ester hydrochloride was prepared by the method of Curtius & Goebel (1888). After recrystallization the yield was 88%, m.p. 156–157°, $[\alpha]_D^{20}$ −7·6° (*c* 3 in water) (Found: C, 57·5; H, 7·0; Cl, 15·3; N, 6·0. Calc. for $C_{11}H_{16}ClNO_2$: C, 57·5; H, 7·0; Cl, 15·5; N, 6·1%).

N-Benzyloxycarbonyl-D-phenylalanine (3g., 10m-moles) and L-phenylalanine ethyl ester hydrochloride (2·3g., 10m-moles) were coupled by the method of Greenstein & Winitz (1961*b*) with dicyclohexylcarbodi-imide (2·1g., 10m-moles). The dipeptide was recrystallized from ether. The yield was 69%, m.p. 111–112° (Found: C, 70·8; H, 6·4; N, 6·1. Calc. for $C_{28}H_{30}N_2O_5$: C, 70·9; H, 6·3; N, 5·9%).

The benzyloxycarbonyl group was removed by treatment (at room temperature) with a saturated solution of HBr in acetic acid. The product was precipitated with dry ether and recrystallized from ethanol by addition of ether. The yield was 79%, m.p. 118–120°.

D-Phenylalanyl-L-phenylalanine ethyl ester hydrobromide (2·24g., 5·4m-moles) was suspended in dry chloroform (50ml.) cooled to 0°. Triethylamine (0·55g., 5·3m-moles) in chloroform (50ml.) was added and a slight excess of triethylamine was added dropwise until all the ester had dissolved.

[*Me*-^{3}H]Acetic anhydride diluted with unlabelled material (0·543g., 5·3m-moles, 25mc) in chloroform (20ml.) was then added dropwise over a period of about 1hr. until the reaction mixture gave no significant reaction with ninhydrin. The reaction mixture was maintained at 0° throughout the addition of acetic anhydride. The chloroform solution was then washed successively with water (50ml.), 0·2M-HCl (50ml.), 0·2M-$NaHCO_3$ (50ml.) and water (50ml.), and then dried over $MgSO_4$. After filtration the solution was divided into two portions and each evaporated to dryness.

One portion of the acetylated ester was saponified by dissolving the ester (1·08g.) in acetone (25ml.) and treating it with 0·5M-NaOH (6ml.). After standing at room temperature for 45min. the solution was acidified (to Congo Red), and on cooling to 5° the precipitated acid was filtered off and recrystallized from ethyl acetate. The yield was 52%, m.p. 193–194°, $[\alpha]_D^{20}$ +5·0° (*c* 1 in methanol) (Found: C, 67·4; H, 6·3; N, 8·2. Calc. for $C_{20}H_{22}N_2O_4$: C, 67·8; H, 6·2; N, 7·9%).

N[*Me*-^{3}H]-*Acetyl*-D-*phenylalanyl*-L-*phenylalanine amide*. This was prepared from *N*[*Me*-^{3}H]-acetyl-D-phenylalanyl-L-phenylalanine ethyl ester by the action of NH_3 gas dissolved in dry methanol. The ester (0·4g.) was dissolved in methanol (50ml.) and NH_3 gas was bubbled through for about 1hr. at 0°. The flask was stoppered and kept at room temperature for 24hr. The solution was then evaporated to dryness, redissolved in a small amount of methanol and the amide precipitated by the addition of acetone. This material was recrystallized from methanol–acetone. The yield was 60%, m.p. 237–239°, $[\alpha]_D^{20}$ −19·6° (*c* 1 in dimethylformamide) (Found: C, 68·0; H, 6·3; N, 11·8. Calc. for $C_{20}H_{23}N_3O_3$: C, 68·0; H, 6·5; N, 11·9%).

N[*Me*-^{3}H]-*Acetyl*-L-*phenylalanyl*-D-*phenylalanine*. This was prepared in the same way as its enantiomer. It had m.p. 193–194°, $[\alpha]_D^{20}$ −4·6° (*c* 1 in methanol) (Found: C, 67·7; H, 6·1; N, 8·1. Calc. for $C_{20}H_{22}N_2O_4$: C, 67·8; H, 6·2; N, 7·9%).

N[*Me*-^{3}H]-*Acetyl*-L-*phenylalanyl*-D-*phenylalanine amide*. This was prepared in the same way as its enantiomer. It had m.p. 239–240°, $[\alpha]_D^{20}$ +19·6° (*c* 1 in dimethylformamide) (Found: C, 68·1; H, 6·9; N, 10·6. Calc. for $C_{20}H_{23}N_3O_3$: C, 68·0; H, 6·5; N, 11·9%).

3,5-*Dinitro*-L-*tyrosine*. This compound was prepared by the nitration of L-tyrosine as described by Chalmers, Dickson, Elks & Hems (1949), and had m.p. 228–229° (decomp.). Chalmers *et al.* (1949) give m.p. 230–232° (decomp.).

N-*Acetyl*-3,5-*dinitro*-L-*tyrosyl*-L-*phenylalanine*. This material was prepared by the same route as that used for *N*-acetyl-D-phenylalanyl-L-phenylalanine (see above). Relevant data on the intermediates in this preparation are as follows. *N*-Benzyloxycarbonyl-3,5-dinitro-L-tyrosine had m.p. 86–87·5°, $[\alpha]_D^{20}$ −11·9° (*c* 1 in methanol) (Found: C, 49·1; H, 3·9; N, 10·7. Calc. for $C_{17}H_{15}N_3O_9$: C, 50·4; H, 3·7; N, 10·4%). *N*-Benzyloxycarbonyl-3,5-dinitro-L-tyrosyl-L-phenylalanine ethyl ester had m.p. 160–161° (Found: C, 58·1; H, 5·0; N, 9·3. Calc. for $C_{28}H_{28}N_4O_{10}$: C, 57·8; H, 4·9; N, 9·6%). 3,5-Dinitro-L-tyrosyl-L-phenylalanine ethyl ester hydrobromide had m.p. 165–170° (Found: C, 45·7; H, 4·6; Br, 13·2; N, 9·4. Calc. for $C_{20}H_{23}BrN_4O_8$, C, 45·6; H, 4·4; Br, 15·1; N, 10·0%). *N*-Acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine ethyl ester had m.p. 151–155° (Found: C, 54·3; H, 5·1; N, 10·7. Calc. for $C_{22}H_{24}N_4O_9$: C, 54·3; H, 4·9; N, 11·5%). *N*-Acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine had m.p. 208–209°, $[\alpha]_D^{20}$ −4·6°, $[\alpha]_{546}^{20}$ −8·0° (*c* 1 in methanol) (Found: C, 51·8; H, 4·6; N, 12·1. Calc. for $C_{20}H_{20}N_4O_9$: C, 52·2; H, 4·4; N, 12·1%).

N-*Acetyl*-L-*phenylalanyl*-L-*phenylalanine and* N-*acetyl*-L-*phenylalanyl*-L-*phenylalanylglycine*. These were prepared as described by Cornish-Bowden & Knowles (1969).

N-*Acetyl*-L-*phenylalanine*. This was prepared from L-phenylalanine and acetic anhydride under Schotten–Baumann conditions. The crude product was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) as colourless plates, m.p. 167–170°, $[\alpha]_D^{20}$ +46·1° (*c* 2 in ethanol). Coffy, Green & Kenner (1959) give m.p. 169–170°, $[\alpha]_D^{22}$ +47·1° (*c* 2 in ethanol).

N-*Acetyl*-D-*phenylalanine*. This was prepared in exactly the same way as the L-enantiomer, but from D-phenylalanine The material had m.p. 166–170°, $[\alpha]_D^{20}$ −46·1° (*c* 2 in ethanol).

N-*Acetyl*-D-*phenylalanyl*-D-*phenylalanine*. A very marked difference in solubility in various solvents between acetyl-L-phenylalanyl-L-phenylalanine and its D-L-analogue was noticed. For example, the L-L-compound can be conveniently recrystallized from pure methanol, whereas the D-L-compound is readily soluble in cold methanol and is not precipitated on the addition of considerable amounts of ether. It therefore seemed probable that any of the four stereoisomers could be conveniently and rapidly prepared by separating the two products from a synthesis that allowed complete racemization at the asymmetric carbon

atom of the carboxyl moiety. The following preparation of the D-D-compound uses this approach.

N-Acetyl-L-phenylalanine (1·24g., 6m-moles) and triethylamine (0·61g., 6m-moles) were dissolved in 15ml. of toluene and cooled to −5°, whereupon an oily precipitate was formed. Ethyl chloroformate (0·65g., 6m-moles) was added and the mixture was stirred for 25min. D-Phenylalanine (0·99g., 6m-moles) in 1M-NaOH (5ml.) was added and the mixture was stirred vigorously for 12hr., during which time it was allowed to reach room temperature. The aqueous layer was separated from the mixture, and was washed once with ether, and then acidified (to Congo Red) with conc. HCl. A copious precipitate of N-acetyl-DL-phenylalanyl-D-phenylalanine was formed, which was collected and dried. Since the L-D-component is easily soluble in cold methanol, it was expected that recrystallization from methanol would yield almost pure D-D-compound. The crystals from methanol had m.p. 230–238° (decomp.), which is in agreement with this expectation [m.p. of L-L-compound 245–249° (decomp.), m.p. of L-D-compound 193–195°]. The compound was recrystallized from methanol (Found: C, 66·6; H, 6·2; N, 7·2. Calc. for $C_{22}H_{22}N_2O_4$: C, 67·8; H, 6·3; N, 7·9%). The material had $[\alpha]_D^{20}$ −13·3° (*c* 1 in pyridine). The L-L-compound had $[\alpha]_D^{20}$ +14·3° (*c* 1 in pyridine).

N-*Acetyl*-L-*phenylalanyl*-D-*phenylalanine*. This was readily obtained from the mother liquor of the first crystallization from methanol (above), by removing the methanol under reduced pressure and crystallizing the L-D-material from ethyl acetate (Found: C, 67·6; H, 6·3; N, 7·7. Calc. for $C_{22}H_{22}N_2O_4$: C, 67·8; H, 6·3; N, 7·9%). The material had $[\alpha]_D^{20}$ −5·2° (*c* 1 in methanol). The enantiomer had $[\alpha]_D^{20}$ +5·0° (*c* 1 in methanol).

N-*Acetylglycyl*-L-*phenylalanine ethyl ester*. *N*-Acetylglycine (2·34g., 20m-moles) was dissolved in 60ml. of tetrahydrofuran and 8ml. of dimethylformamide, and triethylamine (2·02g., 20m-moles) was added. The solution was cooled in an ice–salt bath, and ethyl chloroformate (1·92ml., 20m-moles) was added. After 5min. L-phenylalanine ethyl ester hydrochloride (4·59g., 20m-moles) and triethylamine (2·02g., 20m-moles) were added to the solution of mixed anhydride. The solution was stirred overnight, during which time it was allowed to reach room temperature. The precipitated triethylamine hydrochloride was removed by filtration and washed with tetrahydrofuran and dimethylformamide, the washings being added to the filtrate. The filtrate was then evaporated to dryness under high vacuum to remove the last traces of dimethylformamide, and a brown oil was obtained. This was taken up in ethanol and decolorized with charcoal. On evaporating the solvent a pale-yellow oil was obtained that was recrystallized from ethanol–light petroleum (b.p. 30–40°). The material had m.p. 85·5–87°, $[\alpha]_{365}^{20}$ +23·6° (*c* 2 in dimethylformamide), $[\alpha]_D^{20}$ 0°, approx. (Found: C, 61·5; H, 6·7; N, 9·1. Calc. for $C_{15}H_{20}N_2O_4$: C, 61·7; H, 6·8; N, 9·6%).

N-*Acetylglycyl*-L-*phenylalanine*. *N*-Acetylglycyl-L-phenylalanine ethyl ester was saponified by the method used for *N*-acetyl-D-phenylalanyl-L-phenylalanine ethyl ester (see above). The product was twice recrystallized from water to give colourless needles, m.p. 163–167° with resolidification at 167–170° and final m.p. 177–179° (decomp.), $[\alpha]_D^{20}$ +39·3° (*c* 2 in ethanol) (Found: C, 59·0; H, 6·1; N, 9·3. Calc. for $C_{13}H_{16}N_2O_4$: C, 59·1; H, 6·0; N, 10·6%).

N-*Benzyloxycarbonyl*-L-*phenylalanylglycine methyl ester*. *N*-Benzyloxycarbonyl-L-phenylalanine and glycine methyl ester hydrochloride were coupled by the method described for the preparation of *N*-benzyloxycarbonyl-D-phenylalanyl-L-phenylalanine ethyl ester. On account of the great ease with which free glycine ethyl ester forms piperazine-2,5-dione, the reaction was carried out at −5°. The product was recrystallized from ether and had m.p. 120–121°. Vogler, Lanz & Lergier (1962) give m.p. 122–123° (Found: C, 64·9; H, 6·0; N, 8·7. Calc. for $C_{20}H_{22}N_2O_5$: C, 64·9; H, 6·0; N, 7·6%).

Benzyloxycarbonyl-L-phenylalanylglycine methyl ester was debenzyloxycarbonylated by the method described above. The product was an amorphous white powder, m.p. 135° (Found: C, 45·2; H, 5·4; Br, 25·0; N, 9·0. Calc. for $C_{12}H_{17}BrN_2O_3$: C, 45·5; H, 5·4; Br, 25·2; N, 8·8%).

N-*Acetyl*-L-*phenylalanylglycine methyl ester*. L-Phenylalanylglycine methyl ester hydrobromide was acetylated by the method described for L-phenylalanyl-L-phenylalanine ethyl ester. After being washed with hot ether, the compound had m.p. 144·5–146° (Found: C, 61·0; H, 6·4; N, 10·1. Calc. for $C_{14}H_{18}N_2O_4$: C, 60·4; H, 6·5; N, 10·1%).

N-*Acetyl*-L-*phenylalanylglycine*. *N*-Acetyl-L-phenylalanylglycine methyl ester was saponified by the method described above. No significant precipitation of product occurred on acidification of the solution. The product was therefore extracted into ethyl acetate, dried over $MgSO_4$, filtered and evaporated to dryness. The resulting white crystalline solid was washed with ether and collected. After recrystallization from ethyl acetate it had m.p. 164·5–165·5°. Behrens, Doherty & Bergmann (1940) give m.p. 159–161° for the enantiomer of this compound. The material had $[\alpha]_{365}^{20}$ +10·7° (*c* 1 in ethanol) (Found: C, 59·0; H, 6·4; N, 10·8. Calc. for $C_{13}H_{16}N_2O_4$: C, 59·1; H, 6·5; N, 10·6%).

L-*β-Phenyl-lactic acid*. The procedure was based on the reaction of Dakin & Dudley (1914) for the deamination of L-phenylalanine. L-Phenylalanine (25g., 0·15mole) was dissolved in 1M-H_2SO_4 (465ml.) and the solution cooled to −5°. Then $NaNO_2$ (15·1g., 0·22mole), dissolved in water (80ml.) and cooled on ice, was added over 2hr. by using a narrow Teflon tube as a siphon, the mixture being vigorously stirred and maintained below 0°. The mixture was stirred overnight, then extracted into ether. The combined extracts were dried over $MgSO_4$ and evaporated under reduced pressure to yield a pale solid. This was recrystallized, after charcoal decolorization, from chloroform–light petroleum (b.p. 60–80°) to give colourless needles (8·6g., 34%). These had m.p. 124–125°, $[\alpha]_D^{20}$ −21·5° (*c* 2 in water). Shchukina, Gromova & Ravdel (1966), who prepared the compound by resolution of DL-β-phenyl-lactic acid, give m.p. 124–125°, $[\alpha]_D^{20}$ −22°.

N-*Acetyl*-DL-*phenylalanyl*-L-*β*-*phenyl*-*lactic acid*. Lokshina, Orekhovich & Sklyankina (1964) reported the synthesis of *N*-acetyl-L-phenylalanyl-L-β-phenyl-lactic acid by the direct coupling of *N*-acetyl-L-phenylalanine and L-β-phenyl-lactic acid by using a 'mixed-anhydride' method, and obtained a product of m.p. 168–170°. The most commonly used mixed-anhydride reagents are benzenesulphonyl chloride and ethyl chloroformate. These reagents were tried several times under the conditions suggested by Schröder & Lubke (1965) to minimize the racemization of the acyl moiety that generally occurs.

Thus *N*-acetyl-L-phenylalanine (1g., 4·8m-moles) was

dissolved in sodium-dried tetrahydrofuran (20ml.) at −15°, together with triethylamine (0·49g., 4·8m-moles) and ethyl chloroformate (0·52g., 4·8m-moles). A precipitate of the amine salt formed over 30min., indicating the formation of the mixed anhydride, to which was then added L-β-phenyllactic acid (0·8g., 4·8m-moles) in tetrahydrofuran (15ml.) at −15°. The mixture was stirred overnight, the amine salt removed by filtration and the filtrate evaporated down to leave a colourless oil, which crystallized from water–ethanol as colourless needles (0·85g., 50%). A similar procedure with benzenesulphonyl chloride in dioxan at 0° gave a 37% yield.

All the specimens prepared in this way melted over large parts of the temperature range 125–175°, but were homogeneous in the t.l.c. system (silica-gel plates, eluted with methanol–ethyl acetate, 1:4, v/v) used by Lokshina *et al.* (1964). On the basis of the following evidence these materials obtained by the mixed-anhydride synthesis were shown to be mixtures of the two diastereoisomers *N*-acetyl-L-phenylalanyl-L-β-phenyl-lactic acid and *N*-acetyl-D-phenylalanyl-L-β-phenyl-lactic acid. (i) The samples were analytically pure (Found: C, 67·3; H, 6·2; N, 3·4. Calc. for $C_{20}H_{21}NO_5$: C, 67·6; H, 5·9; N, 3·9%). (ii) The mass spectrum of a sample gave the expected molecular ion (*m/e* 355), and all the major peaks were explicable in terms of the breakdown of these molecules. (iii) Eight recrystallizations of a sample from ethanol–water, acetic acid–water and chloroform–light petroleum (b.p. 60–80°) changed the m.p. from 125–165° to 155–173° and $[\alpha]_D^{20}$ (*c* 2 in methanol) from −16·7° to −18·2°. (iv) A preparation starting with *N*-acetyl-D-phenylalanine rather than the L-enantiomer gave material of m.p. 146–174° and $[\alpha]_D^{20}$ −20·8° (*c* 2 in methanol). (v) The tests of optical purity described below indicated that 50–60% of the phenylalanyl residue in these samples was in the D-configuration.

Extensive trials were carried out with various solvent combinations with 5cm. × 20cm. silica-gel t.l.c. plates to find a method of separating the two depsides. A modification of the system used by Ting & Dugger (1965) was found to be effective. This was diethyl ether saturated with water and mixed with 1–5% of formic acid. At 1% formic acid the R_F values for the diastereoisomers were: D–L-isomer 0·30; L–L-isomer, 0·38. At 5% formic acid these became: D–L-isomer, 0·62; L–L-isomer, 0·69. Higher percentages of acid decreased the separation. The volatility of the solvent made the use of chromatographic columns difficult. Accordingly, preparative t.l.c. plates (100cm. × 20cm.) were used, made up with a 1mm. layer of Kieselgel HF254. In u.v. light of wavelength 254nm. the phosphor in this material was quenched by the compounds, which showed up as dark-purple strips against a green background. Only about 300mg. of the mixture could be separated on each of the plates, since larger amounts caused the silica layer to disintegrate in the solvent. The 1%-formic acid mixture was used so that multiple elution would be effective. The plates were developed twice. The two bands were marked out in u.v. light and the compounds were washed off the collected silica overnight by using acetone in a Soxhlet extractor.

N-*Acetyl*-L-*phenylalanyl*-L-β-*phenyl-lactic acid.* The extract of the faster-running band from the above t.l.c. separation was crystallized from chloroform–light petroleum (b.p. 40–60°) and then from ethanol–water to give colourless needles, m.p. 133–134°. The recovery was 70%, assuming an initial 1:1 mixture. The material had $[\alpha]_D^{20}$ −8·47° (*c* 2 in methanol). The tests described below showed the compound to be 99±2% L–L-diastereoisomer (Found: C, 66·9; H, 5·8; N, 4·0. Calc. for $C_{20}H_{21}NO_5$: C, 67·6; H, 5·9; N, 3·9%).

N-*Acetyl*-D-*phenylalanyl*-L-β-*phenyl-lactic acid.* The extract of the slower-running band was recrystallized as described above to give colourless needles, m.p. 174–182°, $[\alpha]_D^{20}$ −23·1° (*c* 2 in methanol), which were shown to be 91±2% D–L-diastereoisomer. Therefore it was put through the separation process again to give colourless needles, m.p. 182–184°, 50% overall recovery, $[\alpha]_D^{20}$ −30·3° (*c* 2 in methanol), 97±2% D–L-diastereoisomer (Found: C, 67·2; H, 5·8; N, 4·2. Calc. for $C_{20}H_{21}NO_5$: C, 67·6; H, 5·9; N, 3·9%).

Tests of optical purity. The following methods were used to assess the optical purity of preparations of *N*-acetyl-DL-phenylalanyl-L-β-phenyl-lactic acid.

Hydrolysis with carboxypeptidase A was carried out under the conditions used by Bethune, Ulmer & Vallee (1964) for the hydrolysis of hippuryl-DL-β-phenyl-lactic acid. A solution about 0·3mM with respect to the substrate was made up in 0·2M-NaCl solution. Then 20μl. of the enzyme preparation was dissolved in 1ml. of 2M-NaCl solution to give a stock solution containing 0·4mg. of protein/ml. (11·7μM). The progress curve of the hydrolysis reaction was followed to completion on a pH-stat at pH7·5. This took 10–30min., depending on the sample. The asymptotes to which the curves tended were reproducible to ±5%, and for each diastereoisomeric compound corresponded to close to 100% hydrolysis. Lokshina *et al.* (1964) supposed that their preparation of the depside was fairly pure on the grounds that it was 90% hydrolysed by carboxypeptidase A. This seemed a fair test because the literature indicates that this enzyme is completely stereospecific for *C*-terminal residues of the L-configuration, and almost completely so with regard to the configuration of the penultimate residue. An apparently close parallel to the present case arises from the work of Yanari & Mitz (1954), who found that, whereas L-leucyl-L-tyrosine and D-leucyl-L-tyrosine are hydrolysed by carboxypeptidase at equal rates, the introduction of an *N*-acetyl group makes the D–L-diastereoisomer practically resistant to carboxypeptidase A, in contrast with the L–L-diastereoisomer, which becomes more susceptible by the factor 10^4.

Hence it is surprising that during the present work samples of what were believed to be *N*-acetyl-DL-phenylalanyl-L-β-phenyl-lactic acid were 100% hydrolysed in the presence of carboxypeptidase A. However, the progress curves of the hydrolysis were detectably biphasic, and it was assumed that the second slower part of the titration curve represents hydrolysis of the D–L-diastereoisomer. This was confirmed when the pure materials were isolated, and a more sensitive check of optical purity was found. This simply involved the determination of the optical rotation of the alkaline hydrolysate (pH10, room temperature). With this method the following percentages of L–L-diastereoisomer were found: (i) in the reaction product from *N*-acetyl-L-phenylalanine prepared by the method of Lokshina *et al.* (1964), 42±2%; (ii) in the reaction product from *N*-acetyl-D-phenylalanine prepared similarly, 32±2%; (iii) the more mobile component from t.l.c. separation of the diastereoisomers, 99±2%; (iv) the less mobile component from the t.l.c. separation, 3±2%.

METHODS

Microanalyses were performed by Dr Weiler and Dr Strauss in this Department.

Melting points were taken on a Kofler block and are uncorrected.

Optical rotations were measured with a Perkin–Elmer PE 141 automatic polarimeter.

pH measurements were made with a Radiometer TTT1c titrator, with a pHA630 scale-expander attachment, standardized against standard buffer solutions supplied by British Drug Houses Ltd., Poole, Dorset.

Measurements of E_{280} were made with a Unicam SP.500 spectrophotometer.

Scintillation counting was performed with automatic Beckman instruments (models DPM200 and DPM100).

Equilibrium-dialysis experiments. These were carried out as described by Johnson & Knowles (1966).

Kinetic experiments. All kinetic runs were performed at 37° on an apparatus designed for the continuous analysis of the products of peptide hydrolysis by using the analytical system of a Technicon automatic amino acid analyser (Cornish-Bowden & Knowles, 1965, 1969). The K_i values were determined against either *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine or *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine as substrate. Values of the kinetic parameters were obtained from plots of $[S_0]/v_0$ versus $[S_0]$ (Dowd & Riggs, 1965) by using the computational procedure described by Knowles (1965).

Activity checks. To estimate the loss of enzymic activity over 24hr. at 5°, the catalytic activity of 100μM-pepsin solution was assayed by a modification of the haemoglobin method of Anson (1948). Portions (100μl.) of the pepsin solution at the appropriate pH, having been incubated at 5° for 24hr., were added to 3ml. of a 2% solution of haemoglobin in 0·06M-HCl at 35°. After the mixture had stood at 35° for 10·0min., 5ml. of aq. 5% (w/v) trichloroacetic acid was added rapidly, and the mixture was left for a further 5min. and then centrifuged for 10min. The E_{280} of the supernatant was then measured and compared with a blank (prepared freshly by using 100μl. of buffer in place of enzyme solution). These experiments indicated that there was only a small decrease in enzyme activity (less than 5%) over the 24hr. period over the whole pH range investigated (0·2–5·85).

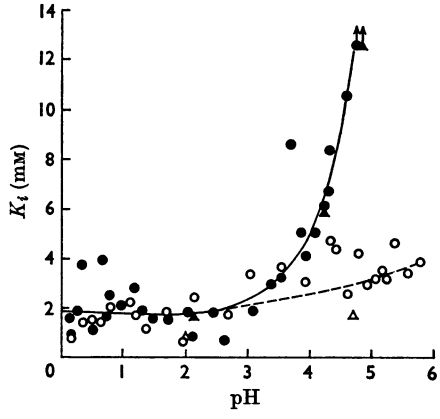

Fig. 1. pH-dependence of K_i for the binding to pepsin of *N*-acetyl-D-phenylalanyl-L-phenylalanine (● and ▲) and the corresponding amide (○ and △). ● and ○, Equilibrium-dialysis experiments; ▲ and △, kinetic experiments.

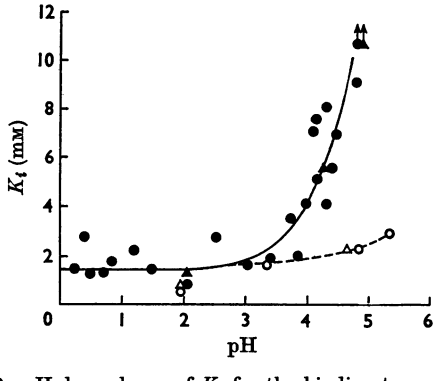

Fig. 2. pH-dependence of K_i for the binding to pepsin of *N*-acetyl-L-phenylalanyl-D-phenylalanine (● and ▲) and the corresponding amide (○ and △). ● and ○, Equilibrium-dialysis experiments; ▲ and △, kinetic experiments.

RESULTS AND DISCUSSION

The results of the inhibitor-binding experiments reported here can be summarized as follows. (1) The binding of the neutral inhibitor *N*-acetyl-D-phenylalanyl-L-phenylalanine amide is essentially independent of pH over the range pH 0·22–5·84 (Fig. 1). Measurements over a wider range than this were not attempted, since this spans the pH of detectable catalytic activity of pepsin, and since the enzyme denatures above about pH 6. Under the conditions of the equilibrium-dialysis experiments (22hr. at 0–5°) loss of pepsin activity even at the pH extrema was less than 5%. The corresponding L–D-dipeptide amide behaves similarly (Fig. 2). (2) The binding of the *N*-acetyl-dipeptides themselves (*N*-acetyl-D-phenylalanyl-L-phenylalanine and its L–D-enantiomer) is independent of pH from pH 0·2 to approx. pH 3·5, but above this latter value the K_i values increase rapidly (Figs. 1 and 2). A Dixon (1953) plot (Fig. 3) of pK_i versus pH indicates that the binding of each of the charged inhibitors depends on the ionization of a single group of pK_a 3·6. The change in slope of the Dixon plot requires that this ionizing group is either on the free enzyme or on the free inhibitor. (3) The values of K_i for the above inhibitors determined kinetically (against *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate) agree well in magnitude and in pH-dependence (Table 1 and Figs. 1 and 2) with

those determined by equilibrium dialysis. Each of the four inhibitors, at all pH values at which kinetic K_i determinations were made, is a competitive inhibitor. The agreement between the values determined by the different methods indicates that the only significant binding of the inhibitors under scrutiny is at the active site of the enzyme.

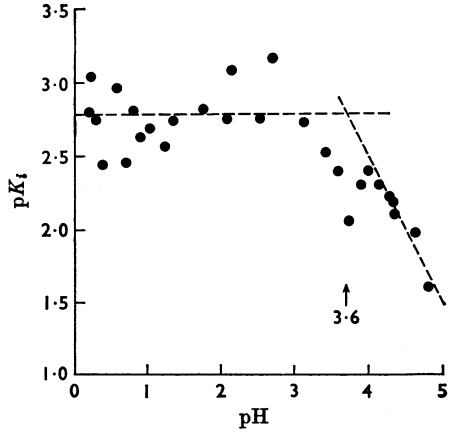

Fig. 3. Plot of pK_i versus pH for the binding to pepsin of *N*-acetyl-D-phenylalanyl-L-phenylalanine. The lines have the theoretical slopes of 0 and −1.

The constancy of K_i for the enantiomeric neutral inhibitors shows that pepsin does not undergo any gross conformational change (at least, not one that measurably affects inhibitor binding at the active site) over the pH range of its catalytic activity. This contrasts with the situation observed for α-chymotrypsin, for which the integrity of the active site is lost [in terms of binding of substrates as measured by K_s (Himoe, Parks & Hess, 1967) and of inhibitors as measured by K_i (Johnson & Knowles, 1967)] at pH 8–9, owing to the ionization of the *N*-terminal isoleucine-16 (Oppenheimer, Labouesse & Hess, 1966). Although pH-dependent conformational changes have been reported for pepsinogen (Frattali, Steiner & Edelhoch, 1965; Perlmann, Oplatka & Katchalsky, 1967), none has been observed with pepsin itself (the changes in pepsinogen in any case occur at high pH values at which pepsin is irreversibly denatured). The pH-independence of K_i for neutral inhibitors makes it probable that K_m (or, in this case, K_s) for neutral substrates is also invariant with pH. This has been shown directly by two groups of workers, by Clement & Snyder (1966) with *N*-acetyl-L-phenylalanyl-L-tyrosine methyl ester and by Denburg *et al.* (1968) with *N*-acetyl-L-phenylalanyl-L-tyrosine amide. These data support the simple though not axiomatic view that the pH-dependent behaviour of K_s for a substrate parallels the behaviour of K_i for analogous inhibitors.

Table 1. *K_i values determined kinetically and by equilibrium dialysis*

K_i values were determined in kinetic experiments (*a*) versus *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine at 37° at pH 2·2 and (*b*) versus *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine at 37° at the pH stated. K_i values obtained in equilibrium-dialysis experiments were taken from Figs. 1 and 2.

	K_i (mM)		
	Kinetic experiments		
Inhibitor	(*a*)	(*b*)	Equilibrium-dialysis experiments
N-Acetyl-D-phenylalanyl-L-phenylalanine	1·1	1·8 (pH 2·1)	1·5 (pH 2·1)
		6·0 (pH 4·3)	6·2 (pH 4·3)
		∞ (pH 4·8)	∞ (pH 4·8)
N-Acetyl-L-phenylalanyl-D-phenylalanine	1·1	1·3 (pH 2·1)	1·5 (pH 2·1)
		5·7 (pH 4·3)	5·5 (pH 4·3)
		∞ (pH 4·8)	∞ (pH 4·8)
N-Acetyl-D-phenylalanyl-D-phenylalanine	0·76	—	—
N-Acetyl-L-phenylalanyl-L-phenylalanine	1·5* (pH 2·2)		—
N-Acetyl-D-phenylalanyl-L-phenylalanine amide	—	0·61 (pH 2·0)	~1·5 (pH 2·0)
		1·7 (pH 4·7)	~3·0 (pH 4·7)
N-Acetyl-L-phenylalanyl-D-phenylalanine amide	—	0·55 (pH 2·0)	0·5 (pH 2·0)
		2·2 (pH 4·7)	2·2 (pH 4·7)
N-Acetyl-L-phenylalanylglycine	~50	56 (pH 2·0)	—
N-Acetylglycyl-L-phenylalanine	13	17 (pH 2·0)	—
N-Acetyl-L-phenylalanyl-L-β-phenyl-lactic acid	1·4	—	—
N-Acetyl-D-phenylalanyl-L-β-phenyl-lactic acid	1·3	—	—

* K_m value.

Each of the enantiomeric dipeptide acid inhibitors shows a pK_a in the pK_i–pH profile of about 3·6 (Fig. 3). Aside from the arguments about the absence of any major conformational change in pepsin between pH 0·2 and 5·8 put forward above, the fact that the free dipeptide acid inhibitors have a pK_a at 3·6 due to the ionization of the carboxyl group makes it virtually certain that it is this ionization that governs the pH dependence of K_i for these materials. The increase in K_i above pH 3·5 presumably arises from the repulsion of the dipeptide acid anion by a negative charge (or charges) at the active site of the enzyme, in the neighbourhood of the carboxyl group of the *C*-terminal amino acid of the bound inhibitor.

The mechanistic significance of this negative charge at (or near) the active site of pepsin may be negligible. Pepsin is a very acidic protein indeed, having an isoelectric point near to 1, and its surface is presumably peppered with carboxylate anions above this pH. It is of course possible, as has been shown for papain (Schechter & Berger, 1967) and carboxypeptidase (Schechter & Berger, 1966), that pepsin has a number of amino acid receptor sites, and that the anions of dipeptide acid inhibitors are repelled from the active site by a carboxylate group that is functional catalytically. The data do not bear on this point. However, Hollands & Fruton (1968) have obtained results consistent with the existence of a negative charge on the enzyme close to the imidazolium group of bound *N*-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester. The enzymic group concerned has an apparent pK_a of about 3·8. In this case, too, the data do not distinguish between the negative charge being one of, or simply near to, the catalytic functionalities of the enzyme. Finally, the experiments of Erlanger, Vratsanos, Wassermann & Cooper (1967) have shown that *p*-bromophenacyl bromide reacts with a single aspartyl carboxyl group in pepsin with a concomitant loss of most of the enzymic activity. By contrast, the corresponding diazo ketone reacts with a different single carboxyl group, with concomitant loss of all the enzymic activity. The latter is very probably the same as the aspartyl residue that is labelled by the substrate analogues *N*-diazoacetyl-L-phenylalanine ethyl ester (Bayliss & Knowles, 1968) and 1-diazo-4-phenylbutanone (Fry, Kim, Spona & Hamilton, 1968), and that is believed to be one of the catalytic functionalities of the enzyme. The fact that a single carboxyl group of pepsin that is believed not to be directly involved in the catalysis reacts with *p*-bromophenacyl bromide with substantial (although incomplete) loss of activity supports the view that the area around the active site of pepsin contains at least one non-essential carboxylate group.

Binding specificity. To probe the specificity

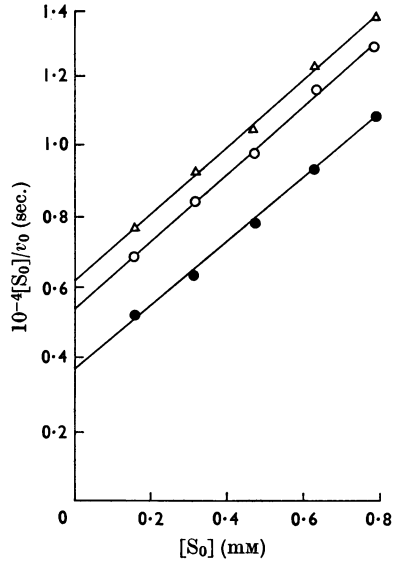

Fig. 4. Plot of $[S_0]/v_0$ versus $[S_0]$, showing competitive inhibition of the hydrolysis of *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine (●) by *N*-acetyl-L-phenylalanyl-D-phenylalanine (0·53 mM) (○) and by *N*-acetyl-D-phenylalanyl-D-phenylalanine (0·53 mM) (△) at pH 2·2 at 37°. $[E_0]$ was 2·05 μM.

requirements for pepsin substrates and inhibitors further, the inhibition of pepsin by various analogues of *N*-acetylphenylalanylphenylalanine was studied. The results of this work are shown in Table 1 and Fig. 4. The following deductions can be made.

The D–L-, L–D- and D–D-stereoisomers of *N*-acetylphenylalanylphenylalanine all bind to pepsin with similar energies and are competitive inhibitors. Moreover the K_m value for the L–L-substrate stereoisomer is close to these values. The similarity of values of K_i for the three inhibitors makes it appear that the free energy of binding of dipeptides is governed largely by hydrophobic forces (as has been proposed by, among others, Tang, 1963). That is, hydrophobic interactions between inhibitor and enzyme seem to be essentially unaffected by changing the configuration of the inhibitor peptide backbone. If K_m equals K_s for dipeptide substrates of pepsin (Denburg *et al.* 1968), then it is apparent that, as with other proteolytic enzymes (see, e.g., Ingles & Knowles, 1967), the stereospecificity of pepsin is a kinetic specificity, arising from the unfavourable arrangement of the susceptible peptide linkage in the enzyme–inhibitor complexes. In fact, the data above do not by themselves demand that both aromatic side chains are involved in hydrophobic interactions with the enzyme, but the

results discussed in the following paragraph do indicate that this is required. It should be noted here that, although a complete set of the four stereoisomeric dipeptides has not previously been studied, three groups have recently demonstrated the close similarities in binding between different stereoisomers. Inouye & Fruton (1967) studied the L–L–L-, L–L–D- and L–D–L-isomers of *N*-benzyloxycarbonylhistidylphenylalanylphenylalanine ethyl ester, and found the values of K_m (or K_i) to be 0·18mM, 0·28mM and 0·19mM respectively; Clement & Snyder (1966) determined the binding constants for the L–L- and D–D-isomers of *N*-acetylphenylalanyltyrosine methyl ester as 2·3mM and 1·9mM respectively; and Jackson, Schlamowitz & Shaw (1965) showed that the binding constants at pH2·0 of the L–L- and D–L-isomers of *N*-acetylphenylalanyldi-iodotyrosine are 0·075mM and 0·08mM respectively.

As a test of the importance of each of the two postulated hydrophobic binding loci of *N*-acetylphenylalanylphenylalanine isomers, each of the phenylalanine residues was replaced by glycine. Neither of these materials had any detectable substrate activity. It is notable that the K_i value for *N*-acetyl-L-phenylalanylglycine is much higher than that of *N*-acetylglycyl-L-phenylalanine, and that the latter is about an order of magnitude higher than the K_m of *N*-acetyl-L-phenylalanyl-L-phenylalanine. These data, coupled with the finding by Jackson, Schlamowitz & Shaw (1966) that the K_i for *N*-acetylglycylglycine is about 500mM, confirm the conclusion that for effective binding to the active site of pepsin a substrate or inhibitor requires at least two hydrophobic side chains. The importance of kinetic specificity can also be stressed, since, although *N*-acetylglycyl-L-phenylalanine binds only about tenfold less effectively than *N*-acetyl-L-phenylalanyl-L-phenylalanine to the enzyme, substrate activity is not detectable for the former.

Finally, the binding of the depside analogues of L–L- and D–L-*N*-acetylphenylalanylphenylalanine to pepsin was studied. It is now known from the work of Inouye & Fruton (1967) that pepsin is an esterase, and catalyses the hydrolysis of the depside analogues of peptide substrates with roughly comparable values of K_m and k_0. [The experiments of Lokshina *et al.* (1964) with *N*-acetyl-L-phenylalanyl-L-β-phenyl-lactic acid are probably unreliable, since their synthetic route to this compound results in a product containing at least 50% of the D–L-material, which is not separated from the L–L-diastereoisomer by their t.l.c. system (see the Materials section).] However, since our assay method only responds to the hydrolysis products of peptides, the apparent value of K_i equals the value of K_m (not the K_s) of a depside acting as a competitive substrate. The values of K_m and K_i for the L–L- and D–L-stereoisomers of *N*-acetylphenylalanyl-β-phenyl-lactic acid are 1·43mM and 1·25mM respectively. These numbers are very close to the corresponding values for the peptide analogues, and one can conclude that the –NH– group of a dipeptide substrate does not contribute measurably to the binding free energy by acting as a hydrogen-bond donor in the enzyme–substrate complex.

In summary, the studies of inhibitor binding to pepsin lead to the following conclusions: (*a*) that no gross conformational change that affects inhibitor binding occurs in pepsin between pH0·2 and 5·8; (*b*) that *N*-acylated dipeptide acids bind to pepsin only as the conjugate acids, i.e. below about pH3·6; (*c*) that, in the absence of charged groups on the substrate or inhibitor, the binding free energy is largely determined by hydrophobic interactions involving the amino acid side chains, and is unaffected by changing the peptide to a depside; (*d*) that at least two hydrophobic amino acid side chains are required for effective binding to the enzyme, and *a fortiori* for a measurable rate of breakdown of the enzyme–substrate complex.

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