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The pH-Dependence of Pepsin-Catalysed Reactions

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1. The pH-dependence of the pepsin-catalysed hydrolysis of three peptide substrates was studied by using a method for the continuous monitoring of the formation of ninhydrin-positive products. 2. Two peptide acid substrates, *N*-acetyl-L-phenylalanyl-L-phenylalanine and *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine, show apparent pK_a values of 1·1 and 3·5 in the plots of k_0/K_m versus pH. By contrast a neutral substrate, *N*-acetyl-L-phenylalanyl-L-phenylalanine amide, shows apparent pK_a values of 1·0 and 4·7. 3. Together with the data of the preceding paper (Knowles, Sharp & Greenwell, 1969), these results are taken to indicate that the rate of pepsin-catalysed hydrolysis is controlled by the ionization of two groups, which on the free enzyme have apparent pK_a values of 1·0 and 4·7. It is apparent that the anions of peptide acid substrates are not perceptibly bound to the enzyme, resulting in apparent pK_a values of 3·5 for the dependence of k_0/K_m for these materials.

A considerable amount of work has been published on the pH-dependence of pepsin-catalysed reactions, both for protein and for synthetic substrates, but the origins of the dependences observed, and the cause of the variations with the substrate, are far from clear. For protein substrates the pH-dependence is markedly affected by the state of denaturation of the substrate: for such substrates as casein, haemoglobin etc. the pH optimum is about pH 1·0 if the substrate is 'native', but is higher and less well defined when the substrate is denatured before the start of the experiment (Christensen, 1955; Schlamowitz & Peterson, 1959). This is consistent with the opinion expressed by Linderstrøm-Lang, Hotchkiss & Johansen (1938), that proteins are susceptible to enzyme-catalysed proteolysis only when they are in a denatured form. Thus with native proteins the pH-dependent, often reversible, denaturation of substrate in acid is a major contributor to the observed pH-dependence. In addition to this complication, a more serious objection to the use of protein substrates for the study of pepsin catalysis is that for any protein the number of sites where hydrolysis can occur is large and varied, so that interpretation of the observations is rarely clear-cut, and meaningful kinetic analysis is impossible. For these reasons, most recent workers have preferred to study synthetic substrates that are hydrolysed at only one site. However, recent data on the pH-dependence of the pepsin-catalysed hydrolysis of synthetic substrates have not provided a completely consistent picture. A considerable body of data is available on the pH-dependence of the rate of pepsin-catalysed reactions based on measurements of the extent of reaction after a definite time (see, e.g., Inouye, Voynick, Delpierre & Fruton, 1966). Under certain conditions such measurements can yield meaningful results: if the total extent of reaction is small, then the measurement of extent of reaction will give an approximate value for the initial velocity. If K_m is negligible in comparison with [S] at all times, these velocities provide a measure of k_0, and conversely, if [S] is negligible compared with K_m at all times, the velocities provide a measure of k_0/K_m. For model synthetic substrates of pepsin neither of these conditions is normally fulfilled, and the results of the present work are discussed below in relation to experiments in which meaningful kinetic parameters have been derived from the rate data.

An attempt was made here to obtain meaningful information about the pH-dependence of the pepsin-catalysed hydrolysis of three simple substrates: *N*-acetyl-L-phenylalanyl-L-phenylalanine, *N*-acetyl-L-phenylalanyl-L-phenylalanine amide and *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine. These three substrates, in which the susceptible link is the same, were selected in order to determine the approximate pK_a values of any enzymic groups involved in the catalytic action, and to investigate the effect of various derivatives of the carboxyl function on the catalysis, since *N*-acyl-dipeptide acids have so frequently been used as model pepsin substrates in the past. It was hoped that *N*-acetyl-L-phenylalanyl-L-phenylala-

nine amide would show whether the pH behaviour of *N*-acetyl-L-phenylalanyl-L-phenylalanine was dependent on the ionization of the carboxyl group in the latter, as was suggested by Inouye *et al.* (1966). *N*-Acetyl-L-phenylalanyl-L-phenylalanylglycine was used to ascertain whether the presence of an ionizable group more remote from the hydrolysed bond affects the catalysis. Coupled with the results reported in the preceding paper (Knowles, Sharp & Greenwell, 1969), the pH-dependence of pepsin-catalysed reactions is interpreted in terms of the ionization of two groups on the enzyme, with pK_a values of 1·0 and approx. 4·7, which affect the catalytic activity as expressed by k_0 [in $v_0 = k_0[E_0][S_0]/(K_m + [S_0])$]. In agreement with the findings of Denburg, Nelson & Silver (1968) it is proposed that the binding of peptide substrates to the enzyme is affected by the ionization of the carboxyl terminus of acid substrates.

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 $E^{0.1\%}_{280}$ 1·47 for a 10mm. path length.

Ninhydrin. Ninhydrin (puriss grade) was obtained from Koch–Light Laboratories Ltd.

Hydrindantin. Hydrindantin (pure grade) was generally obtained from Koch–Light Laboratories Ltd. A sample was also prepared by reduction of ninhydrin with ascorbic acid (West & Rinehart, 1942). After thorough drying *in vacuo* over P_2O_5 it had m.p. 227–234° (decomp.) (Found: C, 66·2; H, 3·2. Calc. for $C_{18}H_{10}O_6$: C, 67·1; H, 3·1%).

MethylCellosolve. MethylCellosolve (2-methoxyethanol) was obtained from Union Carbide Ltd., London W.1. It was purified by the method recommended by Technicon Instruments Co. Ltd. (1966). The distilled material had b.p. 120–124° and gave no colour with KI solution. It was stored under N_2 in the dark.

Haemo-Sol. The detergent Haemo-Sol was produced by Mainecke and Co. Inc., New York, N.Y., U.S.A., and was obtained from Alfred Cox (Surgical) Ltd., Coulsdon, Surrey. It was dissolved in water to give an approx. 0·4% (w/w) solution, which was used for cleaning the analytical system for kinetic experiments.

Ninhydrin reagent. Ninhydrin reagent was made up by the method recommended by Technicon Instruments Co. Ltd. (1966). This method is as follows: (i) ninhydrin (10g.) and hydrindantin (0·75g.) are dissolved in methylCellosolve (325ml.) and the solution is poured into a dark bottle; N_2 (O_2-free) is bubbled through the solution for 15min.; (ii) 4M-acetate buffer (see below) (175ml.) is mixed with this solution and N_2 is bubbled through for 30min.; (iii) aq. 50% (v/v) methylCellosolve (1500ml.) is added and N_2 is bubbled through the solution for 20min. The reagent is stored in the dark under N_2. It is allowed to stand for 1 day before use and any remaining after 14 days is thrown away. Since commercial N_2 generally contains traces of O_2 that may, over a period of days, oxidize hydrindantin, the N_2 is passed over, but not through, the solution while it is being used.

Acetonitrile. Acetonitrile for use in kinetic experiments was purified by fractional distillation from P_2O_5 after refluxing with $KMnO_4$, by the method of Cooper & Waters (1964).

NN-*Dimethylformamide.* This was dried over CaH_2 and redistilled twice before use.

L-*Phenylalanylglycine.* L-Phenylalanylglycine (for use as a ninhydrin colour standard) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. This compound was found to have a ninhydrin colour value of 107% of that of phenylalanine under the conditions of the kinetic experiments.

Glycine methyl ester hydrochloride. This compound was prepared by the method of Curtius & Goebel (1888). It had m.p. 176·5–177°. Curtius & Goebel (1888) give m.p. 175°.

L-*Phenylalanine ethyl ester hydrochloride.* This compound was prepared by the method of Curtius & Goebel (1888), and had m.p. 156–157°. Vejdělek (1950) gives m.p. 149° for this compound, but 155° for its enantiomer. The material had $[\alpha]_D^{20}$ −7·6° (*c* 3 in water). Fischer & Schoeller (1907) give $[\alpha]_D^{20}$ −7·6° (*c* 3 in water), and Vejdělek (1950) gives $[\alpha]_D^{20}$ −7·5° (*c* 2 in water).

N-*Benzyloxycarbonyl*-L-*phenylalanine.* The benzyloxycarbonylation of L-phenylalanine was carried out by the general method given by Greenstein & Winitz (1961). After precipitation of the product by acidification of the aqueous solution, the product was extracted as described by Grassman & Wünsch (1958) and was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give a product of m.p. 87°. Grassman & Wünsch (1958) give m.p. 88–89° and discuss the reasons for the higher values reported by earlier authors (e.g. 126–128° given by Smith & Brown, 1941) (Found: C, 69·7; H, 5·8; N, 4·8. Calc. for $C_{17}H_{17}NO_4$: C, 68·3; H, 5·7; N, 4·7%). The material had $[\alpha]_D^{20}$ +5·7° (*c* 1 in ethanol).

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-L-*phenylalanine ethyl ester.* *N*-Benzyloxycarbonyl-L-phenylalanine (2·49g., 8m-moles) was dissolved in chloroform and the solution was cooled to 0°. L-Phenylalanine ethyl ester hydrochloride (1·83g., 8m-moles) was added, together with triethylamine (0·81g., 8m-moles). Dicyclohexylcarbodi-imide (1·65g., 8m-moles) was added in portions during 20min. After standing in the cold for about 1hr. the reaction mixture did not give a positive reaction to ninhydrin. The reaction mixture was filtered and the filtrate was washed successively with water, 0·5M-HCl, water, 0·5M-Na_2CO_3 and water, and was dried over $MgSO_4$. The solution was evaporated to dryness and the product was recrystallized from ethyl acetate–light petroleum (b.p. 60–80°). It had m.p. 134–136°. Fruton & Bergmann (1942) give m.p. 140° (Found: C, 71·5; H, 6·7; N, 6·5. Calc. for $C_{28}H_{30}N_2O_5$: C,70·7; H, 6·8; N, 5·9%).

L-*Phenylalanyl*-L-*phenylalanine ethyl ester hydrobromide.* L-Phenylalanyl-L-phenylalanine ethyl ester hydrobromide was prepared by treatment of benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine ethyl ester (2·24g., 4·73m-moles) with a saturated solution of HBr in acetic acid (3·5ml.) in a flask protected from the atmosphere by a $CaCl_2$ tube. After 1 hr. no further evolution of CO_2 could be observed, and about 100ml. of dry ether was added to the reaction mixture, whereupon copious precipitation occurred. This preparation was carried out several times, and in most cases it was necessary to wash the precipitate

several times with ether in order to obtain a pure white solid. The product had m.p. 174–176° (Found: C, 56·6; H, 6·0; Br, 19·8; N, 6·7. Calc. for $C_{20}H_{25}BrN_2O_3$: C, 57·1; H, 5·9; Br, 19·0; N, 6·4%).

N-Acetyl-L-phenylalanyl-L-phenylalanine ethyl ester. L-Phenylalanyl-L-phenylalanine ethyl ester hydrobromide (2·44g., 5·8m-moles) was suspended in 40ml. of dry chloroform and the mixture was cooled to 0°. Triethylamine (0·59g., 5·8m-moles) dissolved in 40ml. of chloroform and cooled to 0° was added to the suspension. An excess of 2 drops of triethylamine was added to dissolve the small amount of undissolved material. During a period of 45min. acetic anhydride (0·60g., 5·8m-moles) was added to the reaction mixture, which was kept at 0°. After the last addition the reaction mixture gave no significant reaction with ninhydrin. It was washed successively with water, 0·2M-HCl, 0·2M-K_2CO_3 and water, dried over $MgSO_4$, filtered and evaporated to dryness. The product was recrystallized from methanol–water. It had m.p. 146–149° (Found: C, 68·5; H, 6·7; N, 7·3. Calc. for $C_{22}H_{26}N_2O_3$: C, 69·1; H, 6·8; N, 7·3%). Smith & Spackman (1955) give m.p. 148–149°.

N-Acetyl-L-phenylalanyl-L-phenylalanine. The saponification conditions given by Smith & Spackman (1955) for the preparation of this compound were found to be unsatisfactory, on account of the incomplete miscibility of acetone and M-NaOH. The following method is very similar, except for the addition of water to the mixture. *N*-Acetyl-L-phenylalanyl-L-phenylalanine ethyl ester (1·00g., 2·6m-moles) was dissolved in acetone (about 15ml.) and 0·5M-NaOH (5·2ml., 2·6m-moles) was added during 30min. Sufficient water was added to the mixture to maintain a single phase. The reaction mixture was kept in the refrigerator overnight and was then acidified (to Congo Red) with 5M-HCl. The product was precipitated and was collected. A second crop was precipitated from the filtrate, and the two crops were combined and dried under vacuum for 24hr. The product was recrystallized twice from methanol, m.p. 245–249° (decomp.). Smith & Spackman (1955) give m.p. 264–265° (decomp.), but the m.p. was very dependent on the rate of heating, and values from 230° to 260° could be obtained for material from the same source (Found: C, 67·9; H, 6·2; N, 7·9. Calc. for $C_{20}H_{22}N_2O_4$: C, 67·8; H, 6·3; N, 7·9%). The material had $[\alpha]_D^{20}$ +14·9° (*c* 1 in pyridine). Smith & Spackman (1955) give $[\alpha]_D^{20}$ +16·2° (*c* 1 in pyridine). The value of +14·9° was not altered by recrystallization.

N-Acetyl-L-phenylalanyl-L-phenylalanine amide. A 100ml. portion of dry ethanol was saturated with NH_3 at 0° and the solution was poured on to *N*-acetyl-L-phenylalanyl-L-phenylalanine ethyl ester (1·6g.). The reaction vessel was tightly stoppered and placed in the refrigerator. The progress of the reaction was examined daily by t.l.c. (Kieselgel G plates, eluted with ethyl acetate). After 8 days there was no longer a significant amount of starting material present, and precipitation of product had begun. The solution was evaporated to dryness, the residue dissolved in ethanol and the solution again evaporated to dryness. The product was recrystallized from ethanol and had m.p. 256–260° (decomp.) (Found: C, 67·7; H, 6·6; N, 12·2. Calc. for $C_{20}H_{23}N_3O_3$: C, 68·0; H, 6·6; N, 11·9%). The material had $[\alpha]_D^{20}$ −32·5° (*c* 1 in dimethylformamide), and after a second recrystallization, $[\alpha]_D^{20}$ −33·0° (*c* 1 in dimethylformamide).

L-Phenylalanine amide hydrochloride. About 40ml. of dry methanol was saturated with NH_3 at 0° and immediately poured on to L-phenylalanine ethyl ester hydrochloride (1g., 4·4m-moles) in a pressure bottle that had been cooled to 0°. The bottle was sealed at 0°, and the mixture was allowed to reach room temperature and then left for 25 days. After this time the solution was filtered and evaporated to dryness to yield a white solid. This material was taken up in methanol and again evaporated to dryness. The product was recrystallized twice from methanol–ethyl acetate. The melting behaviour was complex: phase change (needles to plates) 180–190°, melted and resolidified (as brown needles) 223–230°, melted finally at about 270° (Found: C, 54·3; H, 6·5; Cl, 17·3; N, 13·7. Calc. for $C_9H_{13}ClN_2O$: C, 53·8; H, 6·5; Cl, 17·2; N, 13·9%). This compound was found to have a ninhydrin colour value of 75% of that of phenylalanine under the conditions of the kinetic experiments.

N-Benzyloxycarbonyl-L-phenylalanylglycine methyl ester. *N*-Benzyloxycarbonyl-L-phenylalanine and glycine methyl ester hydrochloride were coupled by the method described for the coupling of *N*-benzyloxycarbonyl-L-phenylalanine and 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. It 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%).

L-Phenylalanylglycine methyl ester hydrobromide. *N*-Benzyloxycarbonyl-L-phenylalanylglycine methyl ester was debenzyloxycarbonylated by the method described above. The product was an amorphous white powder (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-Benzyloxycarbonyl-L-phenylalanyl-L-phenylalanylglycine methyl ester. *N*-Benzyloxycarbonyl-L-phenylalanine and L-phenylalanylglycine methyl ester hydrobromide were coupled by the method described above. The product was recrystallized from methanol–ether. It had m.p. 184·5–188° (Found: C, 65·3; H, 5·7; N, 8·8. Calc. for $C_{29}H_{31}N_3O_6$: C, 67·3; H, 6·0; N, 8·1%).

L-Phenylalanyl-L-phenylalanylglycine methyl ester hydrobromide. *N*-Benzyloxycarbonyl-L-phenylalanyl-L-phenylalanylglycine methyl ester was debenzyloxycarbonylated by the method described above. The resulting oil was unusually resistant to crystallization on treatment with ether, although eventually a white solid was obtained by addition of ether to a warm solution of the oil in ethyl acetate. It had m.p. 194–198° (Found: C, 53·6; H, 5·7; Br, 16·3; N, 8·4. Calc. for $C_{21}H_{26}BrN_3O_4$: C, 54·3; H, 5·6; Br, 17·0; N, 9·0%).

N-Acetyl-L-phenylalanyl-L-phenylalanylglycine methyl ester. L-Phenylalanyl-L-phenylalanylglycine methyl ester hydrobromide was acetylated by the method described above. The white powder was washed thoroughly with ether but was not recrystallized. It had m.p. 199–201° (Found: C, 64·4; H, 6·4; N, 9·6. Calc. for $C_{23}H_{27}N_3O_5$: C, 64·9; H, 6·4; N, 9·9%).

N-Acetyl-L-phenylalanyl-L-phenylalanylglycine. *N*-Acetyl-L-phenylalanyl-L-phenylalanylglycine methyl ester was saponified by the method described above, except that the reaction was carried out in aqueous methanol instead of acetone, the methanol being evaporated off before acidification. The product was recrystallized from water. It had m.p. 207–210° (Found: C, 63·7; H, 6·0; N, 10·1. Calc. for

$C_{21}H_{25}N_3O_5$: C, 64·3; H, 6·1; N, 10·2%). The material had $[\alpha]_D^{20}$ −25·8° (*c* 1 in methanol), in reasonable agreement with a value of −25·2° for a sample from a separate synthesis that had been recrystallized from methanol–ethyl acetate.

METHODS

Microanalyses were carried out in this Department by Dr Weiler and Dr Strauss.

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

Measurements of optical rotation were carried out on a Perkin–Elmer PE 141 polarimeter.

General method of following peptide hydrolysis. The rate of hydrolysis of peptide substrates was followed by the continuous monitoring of ninhydrin-positive material by using an apparatus based on the analytical system of a Technicon automatic amino acid analyser. Since the method of operation has been outlined by Cornish-Bowden & Knowles (1965) and essentially the same method has been described by Lenard, Johnson, Hyman & Hess (1965), only a brief account need be given here. The reaction mixture is contained in a 10 ml. stopped bottle in a thermostatically controlled jacket at 37°. The reaction is started by the addition of either enzyme or substrate, as appropriate. From the time of mixing, the first 'sample' emerges from the proportioning pump, where the reaction is stopped by mixing with ninhydrin reagent at pH 5·5, in about 1 min. The stream is segmented with N_2 and passes through a mixing coil, whence it enters the heating bath at 95°. On emerging from the heating bath the stream passes through a cooling coil and a debubbler, before entering the 15 mm.-path-length colorimeter flow cell. The E_{570} value due to the ninhydrin-positive material is recorded continuously on a pen recorder. The course of each hydrolysis is recorded for between 10 and 25 min., and the percentage transmission readings replotted in E_{570} units. The gradients of the linear plots so obtained were estimated by eye, and these values of initial velocity used for the computation of the kinetic parameters as described below. The parts supplied by Technicon were the proportioning pump, heating bath, colorimeter, voltage stabilizer, mixing coil and cooling coil. The proportioning pump tubes were Tygon for the aqueous solutions and Solvaflex for the solutions containing methyl-Cellosolve. The remainder of the apparatus consisted of a Circotherm thermostat, supplied by Shandon Scientific Co. Ltd., London N.W.10, to which three thermostatically controlled jackets were connected, and a Leeds–Northrup $2\frac{1}{2}$ mv recorder. The ninhydrin reservoir was a darkened 3 l. flask, and provision was made for N_2 to be passed over the ninhydrin solution during operation. Narrow-bore tubing was used for the waste outflow of the debubbler, because it was found that a resistance of this kind produced a considerable stabilization of the flow rate.

It was possible to alter the proportioning-pump rate in each channel, by using different-bore tubing, in the range 0·015–3·9 ml./min. In practice it was generally found convenient to use the following arrangement: ninhydrin line: 0·045 in. Solvaflex (0·80 ml./min.); nitrogen line: 0·035 in. Tygon (0·42 ml./min.); sample line: 0·025 in. Tygon (0·235 ml./min.); effluent: 0·040 in. Solvaflex (0·60 ml./min.).

Enzyme solutions were made up initially in pH 3·4 citrate buffer and purified as described below. The concentration of enzyme in the stock solution was determined by measuring E_{280} in a Unicam SP. 500 spectrophotometer. The stock solution was then diluted with buffer components appropriate for the desired pH and made up into equal portions. These were stored in the refrigerator until required. Substrate solutions were made up in 0·1 M-NaOH, apart from *N*-acetyl-L-phenylalanyl-L-phenylalanine amide, which was made up in dimethylformamide. In two runs the substrate concentrations were high enough to have a measurable effect on the pH of the reaction mixtures; in these cases the substrate solutions were made up in alkali sufficiently concentrated for the resultant solution to be 0·1 M with respect to NaOH. In all other cases no allowance for the buffering due to the substrate was necessary. Before each reaction mixture was made up the pepsin solution, the substrate solution and the pipette were warmed to 37°.

Pepsin solutions were made up freshly for each day's work and were never kept for more than a few hours. In order that any progressive deterioration of the pepsin solutions should have been detectable, reaction mixtures were run sequentially from the most concentrated (in substrate) to the most dilute and then in reverse order. Substrate solutions were also made up freshly for each day's work, apart from solutions of *N*-acetyl-L-phenylalanyl-L-phenylalanine amide in dimethylformamide, which were kept in sealed bottles for several days, each stock solution being used for four runs.

Measurement of pH. pH measurements were made on a Radiometer TTT 1c instrument with scale-expander PHA 630 Ta, standardized against standard buffer solutions (British Drug Houses Ltd., Poole, Dorset).

Measurement of pK_a *values.* For measuring the pK_a values of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine and *N*-acetyl-L-phenylalanyl-L-phenylalanine, a Radiometer TTT 1c instrument connected to a Radiometer Titrigraph type SBR 2c was used. The substrate was dissolved in dilute NaOH (6 ml.) and titrated against 0·2 M-HCl. Since in both cases the pK_a is below pH 4, i.e. in the region where the strong acid–strong base titration plot is highly curved, it is difficult to obtain accurate values of the pK_a from a single titration curve. Accordingly a second titration was carried out under identical conditions but with the substrate omitted. Subtraction of the first plot from the second gives a curve with a well-defined point of inflexion at the pK_a value.

With *N*-acetyl-L-phenylalanyl-L-phenylalanine considerable difficulty arises because of the insolubility of this compound in acid solutions. The highest concentration that could be used without rapid precipitation was about 0·55 mM. This difficulty was partially circumvented by using a mixture in equal proportions of *N*-acetyl-L-phenylalanyl-L-phenylalanine and the corresponding D–D-enantiomer, so that an effective concentration of 1·1 mM could be achieved. Since these two compounds are optical isomers they must have the same pK_a value.

With *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine the solubility in acid is much greater, and it was found possible to use a concentration of 12 mM without precipitation during the titration.

The concentrations of alkali were 33 mM (for the *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine determination) and 5 mM (for the *N*-acetyl-L-phenylalanyl-L-phenylalanine determination), and the concentrations of acid were 2 M and 0·2 M respectively.

The pK_a values found by these methods were $3\cdot7\pm0\cdot05$ for *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine and $3\cdot6\pm0\cdot05$ for *N*-acetyl-L-phenylalanyl-L-phenylalanine.

Buffers. The 4M-acetate buffer used for making up the ninhydrin reagent was made as follows: sodium acetate trihydrate (544·4g.) and acetic acid (100ml.) were dissolved in water and made up to 1l. The solution was filtered and stored at room temperature in the dark. The pH was checked, and if found to be below the range $5\cdot51\pm0\cdot03$ it was corrected by the addition of a little solid NaOH.

Buffers used in making up reaction mixtures were 0·1M-sodium citrate–HCl buffers made up to the specification of Sørensen (1909). The pH of each buffer was measured at 25°. According to Walbum (1920) the temperature correction for citrate buffers is very small in the range 10–70°, and no correction was made in this work. For convenience the buffers were prepared from three stock solutions: (i) 0·1M-HCl, (ii) 0·1M-NaOH and (iii) a solution containing 42·016g. of citric acid and 300ml. of M-NaOH/l. Equal quantities of solutions (ii) and (iii) were used to obtain the specifications of Sørensen (1909). Since the use of this buffer system is limited to the pH range 1–5 (and in practice a somewhat smaller range on account of the necessity of dissolving the substrate in alkaline solution), some deviations from the correct specifications were necessary at the extremes of the range: pH values less than 1·5 were achieved by using acid more concentrated than 0·1M and pH values greater than 4·8 were achieved by using more than the prescribed amount of 0·1M-alkali. This latter practice may be expected to result in loss of buffer capacity, but it was found that the pH values of all reaction mixtures were constant during a run.

All buffers were prepared with deionized water and A.R.-grade reagents. Since commercial standard solutions of HCl were found to contain appreciable amounts of ninhydrin-positive material (probably NH_3), solutions were made up by diluting redistilled constant-boiling HCl with water.

Purification of pepsin solutions. Solutions of the commercial pepsin used were found to have an inconveniently high ninhydrin colour value. It was found that this colour value could be decreased by about 65% by the removal of low-molecular-weight impurities by chromatographing the enzyme solutions on Sephadex G-25 (fine grade). The dimensions of the Sephadex column used were 25cm.× 2cm.^{2}. The pepsin was added in 5ml. of a pH3·4 citrate buffer, and was eluted with the same buffer.

The purification was carried out at pH3·4 because it is known (Blumenfeld, Léonis & Perlmann, 1960) that pepsin is most stable to denaturation and autolysis in the pH range 3·0–3·5. Moreover, pepsin is not easily dissolved in high concentrations at pH values much lower than this.

Product analysis. Although it was thought very unlikely that pepsin would catalyse the hydrolysis of *N*-acetyl-L phenylalanyl-L-phenylalanylglycine at the phenylalanylglycine bond, experiments were carried out to show that no hydrolysis occurred at this bond. It was found that the system butan-1-ol–acetic acid–water (3:3:1, by vol.) gave a good separation between glycine and L-phenylalanylglycine on Whatman no. 1 paper. Accordingly this system was used to chromatograph a reaction mixture containing pepsin and *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine at pH2·6 that had been allowed to stand at room temperature for 24hr. The reaction mixture gave a strong reaction with ninhydrin for L-phenylalanylglycine (R_F 0·64) and no detectable spot for glycine (R_F 0·24). Since in practice reaction times were very much less than 24hr. (typically about 25min.), it may be presumed that hydrolysis only occurs at the phenylalanyl-phenylalanine bond.

Computation of kinetic parameters from rate data. The kinetics of the reactions studied have been interpreted in terms of the Michaelis–Menten equation:

$$v_0 = k_0[E_0][S_0]/(K_m+[S_0])$$

This equation may be rewritten in linear form in various ways, and Dowd & Riggs (1965) have discussed the merits of three different linear transformations from a theoretical point of view. They conclude that the plot of $[S_0]/v_0$ against $[S_0]$ is considerably more satisfactory than the very widely used plot of $1/v$ against $1/[S_0]$. Both of these plots were introduced by Lineweaver & Burk (1934). For this reason the plot of $[S_0]/v_0$ against $[S_0]$ has been used throughout this work. Computational procedures for obtaining values of k_0/K_m from the raw data were similar to those described by Knowles (1965).

Computation of pK_a values. To derive values of pK_1 and pK_2 from the bell-shaped pH profiles of k_0/K_m, a programme was written that computes the values of K_1 and the optimum pH that give the best fit of the equation:

$$1/k = (H+H_{max.}^2/H)/K_1\tilde{k}+1/\tilde{k}$$

to the experimental values. k is the experimental value of the parameter (in this case, k_0/K_m), $\tilde{k}$ is the 'pH-independent parameter', H is the H^+ ion concentration, $H_{max.}$ is the H^+ ion concentration at the pH optimum and K_1 is one of the acid dissociation constants for the pH-dependent equilibria. The above equation is a linearized form of the Michaelis function:

$$k = \tilde{k}/(1+H/K_1+K_2/H)$$

where K_2 is replaced by $H_{max.}^2/K_1$ (see Bender, Clement, Kezdy & Heck, 1964). The programme input includes an approximate value of $H_{max.}$, and the value of $H_{max.}$ is then computed that gives the best least-squares fit to a plot of $1/k$ against $(H+H_{max.}^2/H)$. The values of $1/k$ are weighted so that each value has a weight proportional to the magnitude of k for that point. A weighting procedure of this nature is necessary to combat the tendency of the equation to give greatest weight to the smallest (and least accurately known) values. With the neutral substrate (Fig. 5), where there are necessarily fewer points at the pH extremes, the pK_a values were estimated by the method described by Denburg *et al.* (1968).

RESULTS

Under ideal conditions, when the enzyme concentration is sufficiently low for the enzyme blank to be negligible, and when the substrate is sufficiently soluble for concentrations greater than K_m to be used, the continuous assay method described here yielded highly satisfactory results. The self-consistency of the results may be assessed from the plot of $[S_0]/v_0$ versus $[S_0]$ for a typical run with *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate, as shown in Fig. 1. Rather less satisfactory plots are obtained with the poorer

substrates, *N*-acetyl-L-phenylalanyl-L-phenylalanine and *N*-acetyl-L-phenylalanyl-L-phenylalanine amide, largely because it was necessary to use higher enzyme concentrations, which, even for purified enzyme, result in a significant background ninhydrin colour.

Solvent effects. For experiments carried out above pH 2, 0·1 M-citrate buffers were used, but below pH 2 it was necessary to use buffers of higher ionic strength to achieve the desired pH values. Jackson, Schlamowitz & Shaw (1965) report that the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-3,5-di-iodo-L-tyrosine is not dependent on ionic strength in the range 0·01–0·1, from pH 2 to pH 5. Moreover, Zeffren & Kaiser (1967) have shown that at pH 2 the rate of pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-3,5-dibromo-L-tyrosine is the same at *I* 0·02 and *I* 0·12; and Inouye *et al.* (1966) have found that there are no significant specific effects of acetate, formate, chloroacetate and citrate on the peptic hydrolysis of *N*-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester at pH 3·75. We can conclude that there are no large salt effects affecting the measurement of pepsin-catalysed reactions of model peptide substrates.

A more important problem is the effect of organic solvents on the catalysis. With *N*-acetyl-L-phenylalanyl-L-phenylalanine and *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine it was possible to carry out experiments without any organic solvent in the system, but with *N*-acetyl-L-phenylalanyl-L-phenylalanine amide it was found to be impossible to dissolve adequate amounts of the substrate in purely aqueous solution. The choice of an organic solvent was limited by the necessity that it must not boil in the analytical system (i.e. its boiling point must be greater than 95°). For this reason the most obviously suitable solvent, methanol, which is known to be only a feeble inhibitor of pepsin (Tang, 1965), could not be used. It was therefore considered that dimethylformamide might be suitable, since Lokshina, Orekhovich & Sklobovskaya (1961) had reported that concentrations of dimethylformamide as high as 30% have little effect on the pepsin-catalysed hydrolysis of haemoglobin. No information was available on the effect of dimethylformamide on the pepsin-catalysed hydrolysis of small substrates.

To determine the effect of dimethylformamide on the pepsin-catalysed hydrolysis of dipeptides, experiments were carried out at pH 2·2 and 3·7 with *N*-acetyl-L-phenylalanyl-L-phenylalanine and 1·2% (v/v) dimethylformamide. The results for the experiment at pH 2·2 are illustrated in Fig. 2. The results at pH 3·7 were very similar. The effect of 1·2% dimethylformamide is to produce a decrease in velocity of about 40%. It is not of course possible to determine whether the effect on *N*-acetyl-L-phenylalanyl-L-phenylalanine amide is the same as this, but experiments with this substrate with 1·2% and 5·8% dimethylformamide suggested that this is the case; it was found that no advantage is to be gained by using concentrations of dimethylformamide greater than about 1%, because the increased solubility of the substrate is almost exactly offset by the decrease in activity of the enzyme.

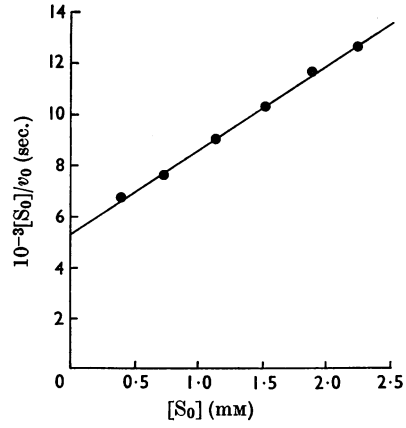

Fig. 1. Plot of $[S_0]/v_0$ versus $[S_0]$ for the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine at pH 2·57 at 37°. $[E_0]$ was 0·809 μM.

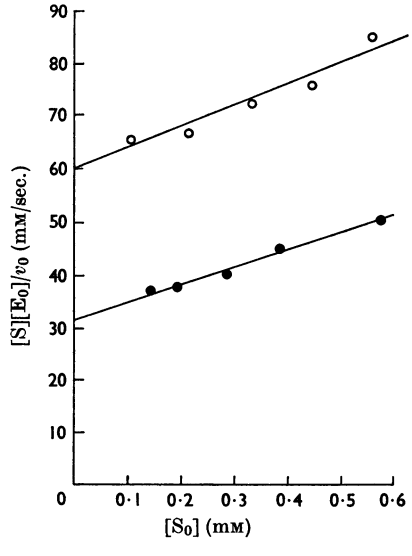

Fig. 2. Plot of $[S_0][E_0]/v_0$ versus $[S_0]$ for the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanine at pH 2·2 at 37° in the presence (○) and absence (●) of 1·2% (v/v) dimethylformamide.

The apparent discrepancy between these results and those of Lokshina *et al.* (1961) mentioned above is comprehensible in view of the statement of Anson & Mirsky (1932) that, at the concentration of haemoglobin used in the standard haemoglobin assay for pepsin, the rate of hydrolysis is independent of the concentration of haemoglobin. This implies that in this assay the enzyme is saturated with substrate, and thus the rate is independent of K_m. Consequently, even quite large increases in K_m, such as 30% dimethylformamide would be expected to produce, would have little or no effect on the observed velocity.

Enzyme-blank correction. The principal non-random error that arose in the kinetic runs described here was the enzyme-blank rate, presumed to be caused by autolysis. The value of this rate could be determined either by measuring the rate of production of ninhydrin-positive materials in the absence of substrate, or by extrapolating a plot of v_0 against $[S_0]$ to zero $[S_0]$. With α-chymotrypsin-catalysed reactions a satisfactory method of correcting for enzyme blank has been used by Ingles & Knowles (1966), which depends on the assumption that only the free enzyme (and not the Michaelis complex) can autolyse. In the present work a simpler procedure, that of subtracting the enzyme blank from each rate, was used, which does not greatly differ from the procedure of Ingles & Knowles (1966) unless $[S_0]$ is larger than K_m. The actual blank rate used was the measured rate, except in a few experiments where the measured rate was very ill-defined, and instead a value was estimated by extrapolating a plot of v_0 versus $[S_0]$. In most cases there was no significant difference between the values obtained by the two methods.

Rate data. The values of k_0/K_m for *N*-acetyl-L-phenylalanyl-L-phenylalanine at pH values between 0·94 and 4·44 are listed in Table 1 and shown in Fig. 3. The values of k_0/K_m for *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine at pH values between 0·47 and 5·67 are listed in Table 2 and shown in Fig. 4. The values of k_0/K_m for *N*-acetyl-L-phenylalanyl-L-phenylalanine amide at pH values between 0·71 and 5·21 are shown in Table 3 and plotted in Fig. 5.

DISCUSSION

The first continuous method of following pepsin reactions was reported by Freimuth, Seidel & Krüger (1962), utilizing the change in electrical conductivity during the hydrolysis of protein substrates. One limitation of this method is clearly the need to keep the ionic strength low, and it is not clear that this method could be a general one of adequate sensitivity for kinetic work. Silver, Denburg & Steffens (1965) have successfully used a

Table 1. *Values of k_0/K_m for the pepsin-catalysed hydrolysis of* N-*acetyl*-L-*phenylalanyl*-L-*phenylalanine*

The temperature was 37°; citrate buffers (for composition see the text) were used. The errors quoted are probable errors from the plots of $[S_0]/v_0$ against $[S_0]$ and are not accuracy estimates. $[S_0]$ was commonly 0·11–1·3 mM; $[E_0]$ was 5–8 μM.

pH	k_0/K_m (M^{-1} sec.$^{-1}$)	pH	k_0/K_m (M^{-1} sec.$^{-1}$)
0·94	12·7±0·4	2·53	25·3±0·3
1·05	16·5±0·3	2·79	24·6±0·3
1·37	23·0±0·6	3·09	22·0±0·3
1·67	22·2±0·4	3·52	15·0±0·2
1·82	24·6±0·4	3·69	15·1±0·4
2·03	29·2±0·4	3·85	8·6±0·2
2·19	29·0±0·7	4·29	4·3±0·2
2·31	27·3±0·4	4·44	3·8±0·3

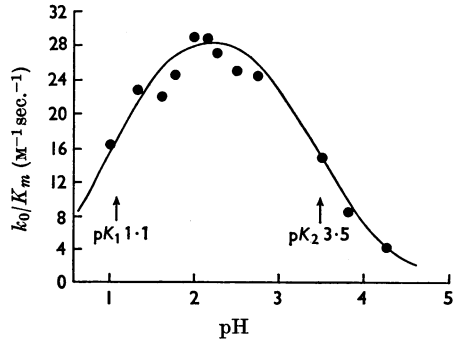

Fig. 3. pH-dependence of k_0/K_m for the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanine at 37°. The solid line is the theoretical line for pK_a of 1·1 and 3·5.

Table 2. *Values of k_0/K_m for the pepsin-catalysed hydrolysis of* N-*acetyl*-L-*phenylalanyl*-L-*phenylalanylglycine*

The temperature was 37°; citrate buffers (for composition see the text) were used. The errors quoted are probable errors from the plots of $[S_0]/v_0$ against $[S_0]$ and are not accuracy estimates. $[S_0]$ was commonly 0·25–4·8 mM; $[E_0]$ was 0·1–5·0 μM.

pH	k_0/K_m (M^{-1} sec.$^{-1}$)	pH	k_0/K_m (M^{-1} sec.$^{-1}$)
0·47	37±1	2·75	215 ±3
0·72	74±1	3·28	164 ±1
1·12	176±1	3·98	70·6 ±0·5
1·67	237±2	4·59	23·0 ±0·5
1·80	192±3	4·79	13·2 ±0·1
2·08	292±4	5·20	3·5 ±0·1
2·30	198±1	5·67	0·98±0·03
2·57	233±4		

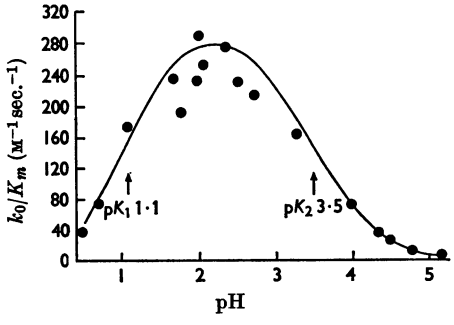

Fig. 4. pH-dependence of k_0/K_m for the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine at 37°. The solid line is the theoretical line for pK_a values of 1·1 and 3·5.

Table 3. *Values of k_0/K_m for the pepsin-catalysed hydrolysis of* N-*acetyl-*L-*phenylalanyl-*L-*phenylalanine amide*

The temperature was 37°; citrate buffers (for composition see the text) containing 1·2% (v/v) of dimethylformamide were used. The errors quoted are probable errors from the plots of $[S_0]/v_0$ against $[S_0]$, and are not accuracy estimates. $[S_0]$ was commonly 0·10–0·62 mM; $[E_0]$ was 5–9 μM.

pH	k_0/K_m (M^{-1} sec.$^{-1}$)	pH	k_0/K_m (M^{-1} sec.$^{-1}$)
0·71	7·1±0·3	2·44	19·8±0·7
0·95	9·3±0·2	2·91	22·4±0·2
1·12	10·7±0·2	3·32	20·5±0·5
1·26	14·8±0·3	3·81	20·9±0·3
1·45	16·9±0·5	4·31	16·0±0·2
1·59	15·7±0·3	4·34	15·4±0·5
1·94	17·8±0·3	4·77	11·6±0·3
1·98	20·7±0·5	5·21	7·5±0·3

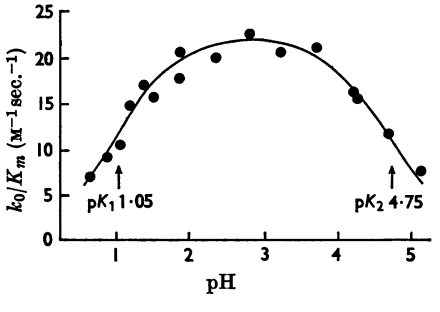

Fig. 5. pH-dependence of k_0/K_m for the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanine amide at 37° in 1·2% (v/v) dimethylformamide. The solid line is the theoretical line for pK_a values of 1·05 and 4·75.

difference-spectra method, although the wavelength used by these workers (237nm.) is on the edge of the protein absorption, and, as has been pointed out by Inouye & Fruton (1968), this limits the sensitivity of the method at relatively high enzyme concentrations. This difficulty does not arise with the method developed by Fruton and his co-workers (see, e.g., Inouye & Fruton, 1967), who utilize the u.v. difference spectrum at 310nm. arising on the cleavage of peptide links involving *p*-nitrophenylalanine on the *N*-terminal side. This method is sensitive, although it is limited to chromophoric substrates, and is less sensitive at low pH, where the change from ·CO·NH· to ·CO·OH does not perturb the aromatic chromophore as much as the change from ·CO·NH· to ·CO·O$^-$. However, it makes a usefully complementary pair with the continuous ninhydrin method used here.

The continuous automatic ninhydrin method described in this paper (also see Cornish-Bowden & Knowles, 1965; Lenard *et al.* 1965) can be used at any pH value, and does not limit the choice of amino acid side chain in model peptide substrates. There is, of course, a practical limit on the enzyme concentration, since even after passage down Sephadex G-25 pepsin gives a significant ninhydrin colour, and autolytic processes give rise to a significant 'blank' rate in unfavourable cases. However, the inherent sensitivity of the method enables one to use relatively low concentrations of enzyme, and provides good initial-rate data at very low conversions of substrate. In most of the kinetic runs reported here the extent of hydrolysis during the observed part of the reaction was less than 3%.

Because of the limited water-solubility of most good substrates of pepsin, it is not usually possible to obtain initial-rate data at substrate concentrations much greater than K_m, and this makes an analysis of the pH-dependence of the individual parameters k_0 and K_m a dangerous procedure. In the present work no attempt has been made to obtain directly the pH-dependence of the individual kinetic parameters, and we have relied on the determination of the most accurately determined parameter, k_0/K_m. As is well-known, the value of this pseudo-second-order rate constant is the most precisely defined from linear transformations of the Michaelis–Menten equation, and, as has been shown by Peller & Alberty (1959), the pH-dependence of this constant reflects simply the ionizations in the free enzyme (and of the free substrate, if any) that affect the catalytic activity.

Since all three substrates show a pK_a value in the k_0/K_m–pH profile of about 1·0, it is reasonable to assume that this pK_a value relates to the ionization of a single enzymic group. The value of 1·0 is remarkably low for any amino acid side chain commonly found in proteins, but since the isoelectric point of the enzyme is about 1 (Perlmann, 1955) there must be at least some acidic groups on the enzyme with abnormally low pK_a values. This

value of the isoelectric point is at least 2 units lower than that expected on the basis of the amino acid content of the enzyme, and is due in part to the presence of the single serine phosphate residue in the molecule. Removal of the phosphate group raises the isoelectric point only to 1·7, and it is therefore clear that some of the groups in this enzyme are abnormally acidic. The most likely candidates responsible for the low isoelectric point are carboxyl groups involved in interactions or fixed in environments that stabilize the ionized rather than the un-ionized form. The possibility that the observed ionizing group of pK_a about 1·0 is due to the phosphate group is ruled out both by the fact that removal of this group does not affect the catalytic activity of the enzyme (Perlmann, 1952), and by the isolation of the naturally occurring pepsin D (Lee & Ryle, 1967), which contains no phosphate group and yet has the same catalytic activity as pepsin A towards haemoglobin and towards *N*-acetyl-L-phenylalanyl-3,5-di-iodo-L-tyrosine.

At the high-pH end of the scale the neutral and the ionizable substrates behave differently: *N*-acetyl-L-phenylalanyl-L-phenylalanine shows an upper pK_a value of 3·5 in k_0/K_m, but the upper pK_a value for the corresponding amide is about 4·7. Since the pK_a value of *N*-acetyl-L-phenylalanyl-L-phenylalanine itself is about 3·6 it is tempting to ascribe the upper pK_a value for the hydrolysis of this substrate to the carboxyl group of the substrate. Attempts to identify it definitely as a pK_a affecting K_m, rather than k_0, were unsuccessful because the low solubility of the substrate precluded the accurate determination of k_0 and K_m individually. However, the possibility that this pK_a related to an effect on K_m is supported by the observation that the K_i–pH profiles of both *N*-acetyl-L-phenylalanyl-D-phenylalanine and the corresponding D–L stereoisomer exhibit pK_a values of about 3·6. It has been argued that K_m (or K_s) for pepsin substrates will behave similarly (Knowles *et al.* 1969). Indeed, Denburg *et al.* (1968) have obtained direct experimental evidence for this supposition, using *N*-acetyl-L-phenylalanyl-L-tryptophan and *N*-acetyl-L-phenylalanyl-L-tyrosine as substrates. Thus we envisage that the observed fall in k_0/K_m for dipeptide acid substrates that occurs at about pH 3·5 arises from the repulsion of the ionized form of the substrate from the active site, presumably by a negative charge or charges at or near the *C*-terminus of the dipeptide substrate when the latter is bound.

In the hydrolysis of the neutral substrate *N*-acetyl-L-phenylalanyl-L-phenylalanine amide, pK_a values of approx. 1·0 and 4·7 are observed in k_0/K_m. In this case, with a neutral substrate, the pK_a values must relate to ionizing groups in the free enzyme (Peller & Alberty, 1959). The question remains, however, as to whether these groups are functional directly (i.e. participating in the covalency changes that constitute the catalytic process) or indirectly (e.g. by controlling an active conformation of the enzyme, such as is found for the higher pK_a observed in k_0/K_m for α-chymotrypsin-catalysed reactions; Oppenheimer, Labouesse & Hess, 1966). An indication that these ionizing groups relate directly to the catalytic reaction comes from the equilibrium-dialysis data reported in the preceding paper (Knowles *et al.* 1969). Thus the binding of *N*-acetyl-D-phenylalanyl-L-phenylalanine amide (and the corresponding L–D-isomer) to pepsin is independent of pH over the whole of the range of peptic activity from pH 0·2 to pH 5·8. This implies that there is no gross conformational change in the enzyme (at least, none that is reflected in a large change in the K_i of competitive inhibitors) over the whole pH region of pepsin stability. These data suggest, although they cannot prove, that the binding of L–L-substrate is also pH-independent, in which case the ionizing groups on the free enzyme with pK_a values of about 1·0 and 4·7 affect the catalysis (as expressed by k_0) directly.

The findings reported in this paper are in good agreement with those of Denburg *et al.* (1968), who obtain pK_a values of 1·17 and 3·4 in k_0/K_m with *N*-acetyl-L-phenylalanyl-L-tyrosine, and of 1·17 and 4·35 with the corresponding amide (in 3% methanol). Lutsenko, Ginodman & Orekhovich (1967) report upper pK_a values in k_0/K_m of 3·8 and of 4·2 for *N*-acetyl-L-phenylalanyl-L-tyrosine and its ethyl ester respectively (in 10% ethanol). Zeffren & Kaiser (1967) obtained values of 0·75 and 2·67 with *N*-acetyl-L-phenylalanyl-3,5-dibromo-L-tyrosine (in 5·0–5·8% methanol) and also suggested that only the protonated peptide acid substrate can bind to the enzyme.

Finally, the behaviour of the tripeptide acid substrate, *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine, is closely similar to the dipeptide acid discussed above. We observe two pK_a values in the dependence of k_0/K_m, of 1·1 and 3·5. As before, we suggest that the group of pK_a 1·1 is an enzyme group, required in the unprotonated form for the catalytic reaction (see Knowles, 1969); and the ionization of pK_a 3·5 relates to the terminal carboxyl group of the substrate. Mechanistic studies on pepsin have not yet advanced to the stage when it is profitable to speculate on the reasons for the almost identical behaviour of a dipeptide acid and a tripeptide acid substrate. As has been said, the surface of pepsin is presumably peppered with carboxyl and carboxylate groups, and one or more of these is probably responsible for the fact that di- and tri-peptide acid anions do not bind to the enzyme.

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