The Rate-Determining Step in Pepsin-Catalysed Reactions, and Evidence against an Acyl-Enzyme Intermediate

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To delineate further the pathway of pepsin-catalysed reactions, three types of experiments were performed: (a) the enzyme-catalysed hydrolysis of a number of di- and tri-peptide substrates was studied with a view to observing the rate-determining breakdown of a common intermediate; (b) the interaction of pepsin with several possible substrates for which 'burst' kinetics might be expected was investigated; (c) attempts were made to trap a possible acyl-enzyme intermediate with [^{14}C]methanol in both a hydrolytic reaction (with *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine) and in a 'virtual' reaction (with *N*-acetyl-L-phenylalanine) under conditions where extensive hydrolysis or ^{18}O exchange is known to occur. It is concluded that (i) intermediates in pepsin-catalysed reactions (aside from the Michaelis complex) occur subsequently to the rate-determining transition state, and (ii) an acyl-enzyme intermediate, if such is formed, cannot be trapped with [^{14}C]methanol in these systems.

Two sorts of reaction intermediate have been proposed for pepsin-catalysed reactions. On the one hand, there is good evidence for the intermediacy of an 'amino-enzyme' intermediate, enzyme-NH $\cdot$ Y, which is formed from a peptide X $\cdot$ CO $\cdot$ NH $\cdot$ Y and the enzyme. The evidence comes principally from studies of the transpeptidation reaction carried out by Neumann, Levin, Berger & Katchalski (1959) and by Fruton, Fujii & Knappenberger (1961). This postulate is supported by evidence concerning the ordered release of the products (X $\cdot$ CO $_2$ H and NH $_2$ Y, in that order) obtained from studies on the product inhibition of the hydrolysis of peptide substrates by Greenwell, Knowles & Sharp (1969) and by Inouye & Fruton (1968). On the other hand, the finding that pepsin catalyses the exchange of ^{18}O between water and acyl-amino acids has led, by analogy with the similar behaviour of 'neutral' proteases such as α -chymotrypsin (Sprinson & Rittenberg, 1951) and papain (Grisaro & Sharon, 1964), to the postulate that an acyl-enzyme (which can be hydrolysed by H $_2$ ^{18}O) is also a possible intermediate in pepsin reactions. That this ^{18}O -exchange process is a true enzyme-catalysed reaction is strongly suggested by the amino acid side-chain specificity (Kozlov, Ginodman & Orekhovich, 1967) and the amino acid stereospecificity (Sharon, Grisaro & Neumann, 1962) that the ^{18}O -exchange reaction displays.

In the present work three different types of experiment are described, each of which aims at the detection of reaction intermediates and a delineation

of the rate-determining step of pepsin-catalysed hydrolysis of peptide substrates.

MATERIALS

Pepsin. This was obtained as described by Knowles, Sharp & Greenwell (1969).

N-Acetyl-3,5-dinitro-L-tyrosine and L-phenylalanylglycine. These were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

N-Acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine and N-acetyl-L-phenylalanine. These were prepared as described by Knowles *et al.* (1969).

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 methyl ester. This was prepared by the esterification of *N*-acetyl-L-phenylalanine, and had m.p. 89–90°. Huang, Foster & Niemann (1952) give m.p. 89–90°. This compound was also prepared from *N*-acetyl-L-[G- ^3H]phenylalanine (a gift from Dr M. J. Hawkins), and had m.p. 89–90°. The radioactive ester was used as a radioactive marker for t.l.c. and had a measured radioactivity of 22500 c.p.m./mg.

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

3,5-Dinitro-L-tyrosine methyl ester hydrochloride. This was prepared by esterification of the amino acid, and was crystallized from methanol–water as pale-yellow needles. It had $[\alpha]_D^{20}$ –13.5° (c 1 in dimethylformamide) (Found: C, 35.8; H, 4.2; Cl, 10.6; N, 12.4. Calc. for C $_{10}$ H $_{12}$ ClN $_3$ O $_7$ ·H $_2$ O: C, 35.4; H, 4.2; Cl, 10.5; N, 12.4%).

N-Benzoyloxycarbonyl-L-phenylalanyl-3,5-dinitro-L-tyrosine methyl ester. This was prepared as follows. *N-Benzoyloxycarbonyl-L-phenylalanine* (see Cornish-Bowden & Knowles, 1969) (7 m-moles), 3,5-dinitro-L-tyrosine methyl ester hydrochloride (7 m-moles) and redistilled triethylamine (7 m-moles) were dissolved in chloroform at 0°. Dicyclohexylcarbodi-imide (7 m-moles) was added in portions over $\frac{1}{2}$ hr. and the mixture was stirred at 0° for a further 3 hr. At the end of this time the precipitated dicyclohexylurea was filtered off, and the filtrate was washed with water, dried over MgSO_4 and evaporated to dryness. The material was triturated with ether, and the combined ether extracts were recrystallized from ether to yield the desired product, m.p. 176–178° (Found: C, 57.1; H, 4.8; N, 9.7. Calc. for $\text{C}_{27}\text{H}_{26}\text{N}_4\text{O}_{10}$: C, 57.3; H, 4.6; N, 9.9%). The ether-insoluble residue (which formed more than half of the isolated material) was shown to be *N,O*-di(benzoyloxycarbonyl-L-phenylalanyl)-3,5-dinitro-L-tyrosine methyl ester (Found: C, 62.0; H, 4.8; N, 7.8. Calc. for $\text{C}_{44}\text{H}_{41}\text{N}_5\text{O}_{13}$: C, 62.0; H, 4.7; N, 8.1%).

L-Phenylalanyl-3,5-dinitro-L-tyrosine methyl ester hydrobromide. This was prepared by debenzoyloxycarbonylation of the above compound with HBr-acetic acid. This material was subjected to acetylation and saponification without characterization (see below).

N-Acetyl-L-phenylalanyl-3,5-dinitro-L-tyrosine. L-Phenylalanyl-3,5-dinitro-L-tyrosine methyl ester hydrobromide was suspended in dry chloroform and cooled to 0°. An equimolar amount of triethylamine in chloroform at 0° was added. To the cooled mixture, acetic anhydride was added slowly until the reaction mixture was ninhydrin-negative. A slight precipitate was removed by filtration, and the filtrate was washed successively with 0.5M-HCl and water. The solution was dried over MgSO_4 and evaporated to dryness. Since the most likely impurity at this stage was *N*-acetyl-L-phenylalanyl-3,5-dinitro-*O*-acetyl-L-tyrosine methyl ester, which would yield the desired product on saponification, the yellow powdery product was not purified. The material was suspended in water at room temperature and the pH adjusted to approx. 12 with 0.1M-NaOH. Further additions of base were made to maintain the pH at this level for several hours. On acidification a copious yellow precipitate was obtained. This material on crystallization from water and from ethyl acetate-light petroleum (b.p. 40–60°) had m.p. 185–187° and $[\alpha]_D^{20} + 10.5$ (c 1 in methanol) (Found: C, 52.3; H, 4.6; N, 11.7. Calc. for $\text{C}_{20}\text{H}_{20}\text{N}_4\text{O}_9$: C, 52.2; H, 4.4; N, 12.2%).

N-Benzoyloxycarbonyl-L-phenylalanine p-nitroanilide. *N-Benzoyloxycarbonyl-L-phenylalanine* (1 m-mole), *p*-nitroaniline (1 m-mole) and dicyclohexylcarbodi-imide (1 m-mole) were dissolved in 10 ml. of tetrahydrofuran, and the solution was kept overnight at room temperature. Ethyl acetate (20 ml.) was then added and the dicyclohexylurea removed by filtration. The filtrate was evaporated to an oil, which was washed with ether (2 × 5 ml.) to remove any excess of *p*-nitroaniline. On standing, the product crystallized, and had m.p. 155–156°, $[\alpha]_D^{20} + 16.4$, $[\alpha]_{436}^{20} + 65.7$ (c 1 in chloroform) (Found: C, 65.6; H, 4.9; N, 10.2. Calc. for $\text{C}_{23}\text{H}_{21}\text{N}_3\text{O}_5$: C, 66.0; H, 5.1; N, 10.0%).

N-Benzoyloxycarbonyl-L-phenylalanine p-methoxyanilide. This was prepared as above, by using freshly recrystallized *p*-anisidine instead of *p*-nitroaniline. Recrystallization of the material from ethanol gave a product of m.p. 170–171°, $[\alpha]_D^{20} + 5.8$, $[\alpha]_{436}^{20} + 14.8$ (c 1 in chloroform) (Found:

C, 71.7; H, 6.1; N, 7.1. Calc. for $\text{C}_{24}\text{H}_{24}\text{N}_2\text{O}_4$: C, 71.4; H, 6.0; N, 7.0%).

N-Acetyl-DL-phenylalanine phenethylamide. *N*-Acetyl-L-phenylalanine (5 m-moles) was dissolved in ethyl acetate and the solution cooled to 0°. Phenethylamine (5 m-moles) was added. To the resulting suspension of *N*-acetyl-L-phenylalanine phenethylammonium salt was added a solution of dicyclohexylcarbodi-imide (5 m-moles) in ethyl acetate. The mixture was warmed at approx. 50° for 1 hr. The mixture was cooled and the precipitate of dicyclohexylurea removed immediately by filtration. On standing at room temperature overnight, the product crystallized from the filtrate. A further crop of product was obtained on adding light petroleum (b.p. 30–40°). The material was recrystallized from ethyl acetate-light petroleum (b.p. 30–40°), and had m.p. 156.5–158.5°, $[\alpha]_D^{20} + 0.4$ (c 1 in ethanol) (Found: C, 73.5; H, 7.4; N, 9.1. Calc. for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}_2$: C, 73.6; H, 7.1; N, 9.0%).

p-Nitrobenzoyl-L-phenylalanine. L-Phenylalanine (18 m-moles) and *p*-nitrobenzoyl chloride (35 m-moles) were refluxed in dry ethyl acetate (100 ml.) for $2\frac{1}{2}$ hr. After removal of insoluble material by filtration, the solution was evaporated to dryness, and the residue crystallized from acetone-carbon tetrachloride. The material thus obtained was found to be *p*-nitrobenzoic acid. The mother liquor yielded a further crop of crystals on standing at room temperature. Recrystallization of this latter material from acetone-carbon tetrachloride yielded pale-yellow crystals, m.p. 148°, $[\alpha]_D^{20} - 63.3$ (c 2 in acetone) (Found: C, 61.0; H, 4.5; N, 9.0. Calc. for $\text{C}_{16}\text{H}_{14}\text{N}_2\text{O}_3$: C, 61.1; H, 4.5; N, 8.9%).

N-trans-3-(Indol-3-lactoyl)-L-phenylalanine. This was prepared as the dicyclohexylammonium salt by the method of McClure & Neurath (1966). It had m.p. 193–198°, $[\alpha]_D^{20} + 28.6$ (c 0.5 in methanol). McClure & Neurath (1966) give m.p. 197–198° (Found: C, 74.6; H, 8.1; N, 8.0. $\text{C}_{32}\text{H}_{41}\text{N}_3\text{O}_3$ requires: C, 74.3; H, 8.0; N, 8.1%).

Acetonitrile and NN-dimethylformamide. These were obtained as described by Cornish-Bowden & Knowles (1969).

Scintillation solvent. This was prepared as described by Knowles *et al.* (1969).

^{14}C Methanol. ^{14}C Methanol of specific radioactivity 9.2 mc/m-mole was purchased from The Radiochemical Centre, Amersham, Bucks. A.R. methanol was used as a carrier.

METHODS

The progress of hydrolytic reactions was followed as described by Cornish-Bowden & Knowles (1969).

Liquid-scintillation counting was carried out as described by Knowles *et al.* (1969). For the counting of material from t.l.c., the relevant portion of silica gel was scraped from the plate into a scintillation bottle containing 3 ml. of toluene-based scintillation solvent. The material to be detected (*N*-acetyl-L-phenylalanine ^{14}C methyl ester) is freely soluble in this medium. No significant quenching by the silica gel was detected.

All spectrophotometric measurements were carried out on a Unicam SP. 800 spectrophotometer, fitted with a scale-expansion attachment, constant-wavelength device and a slave-pen recorder.

Experiments involving the use of ^{14}C methanol were carried out in tightly stoppered test tubes, incubated for 3 hr. at 37.0°.

Hydrolytic experiment. A mixture of pepsin ($6.7\ \mu\text{M}$) and *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine ($0.366\ \text{mM}$) was incubated in citrate buffer ($10\ \text{ml.}$) (Sørensen, 1909) at pH 3.1 to which [^{14}C]methanol (1% , v/v; specific radioactivity $0.02\ \text{mc/m-mole}$) had been added. A duplicate solution was prepared, containing [^{14}C]methanol, but no pepsin. This mixture was used as a blank, in case of the appearance of any spurious peaks of non-enzymic origin on the t.l.c. plates (e.g. of acid-catalysed esterification products). This solution contained substrate ($0.182\ \text{mM}$), and the products *N*-acetyl-L-phenylalanine ($0.191\ \text{mM}$) and L-phenylalanylglycine ($0.208\ \text{mM}$), in approximate simulation of the 'half-time' concentrations of reactant and products in the test solution.

After incubation for 3 hr. the test solution and the control solution were shaken with chloroform ($5\ \text{ml.}$). The pepsin in the test solution was precipitated, and was removed by filtration through a Millipore filter by using a syringe and a Swinney adaptor. The aqueous layers were further extracted with chloroform ($4 \times 5\ \text{ml.}$). The combined chloroform extracts were dried over MgSO_4 and evaporated to dryness under reduced pressure. This material was then subjected to t.l.c. as described below.

'Virtual-substrate' experiment. The conditions of Kozlov, Ginodman & Orekhovich (1965) were followed. *N*-Acetyl-L-phenylalanine ($46.7\ \text{mM}$) and pepsin ($0.156\ \text{mM}$) were incubated in acetate buffer, pH 4.71 ($2.18\ \text{ml.}$), containing [^{14}C]methanol ($0.18\ \text{ml.}$; $2\ \text{M}$; specific radioactivity $0.4\ \mu\text{C/m-mole}$) at 37° . A control solution was made up as above, but contained no pepsin. After 3 hr. each solution was poured into acetone ($8\ \text{ml.}$). The precipitated enzyme in the first solution was centrifuged, and $9.0\ \text{ml.}$ of the supernatant was evaporated to dryness under reduced pressure. Each residue was dissolved in water ($1\ \text{ml.}$) and extracted with chloroform ($5 \times 0.5\ \text{ml.}$); the combined extracts from

each solution were concentrated to about $0.5\ \text{ml.}$ and each was subjected to t.l.c.

Thin-layer chromatography. T.l.c. plates of Kieselgel HF254 were used. The extracts from reaction mixtures in chloroform (approx. $0.5\ \text{ml.}$) were applied to t.l.c. plates, alongside the extracts of the relevant control experiments. These applications were flanked by spots of *N*-acetyl-L-phenylalanine methyl ester to define the expected location of trace amounts of any ^{14}C -labelled compound. The plates were eluted with ethyl acetate, the solvent front moving between 15 and $20\ \text{cm.}$ After drying, the plates were divided into bands $0.5\ \text{cm.}$ wide. These bands were scraped off into scintillation bottles and their radioactivity was measured as described above.

RESULTS AND DISCUSSION

As pointed out in the introduction, there is good evidence that pepsin-catalysed hydrolysis reactions involve an amino-enzyme intermediate. Moreover, there is some presumptive support for the view, at least for the ^{18}O exchange between acyl-L-amino acids and H_2^{18}O catalysed by pepsin, that acyl-enzymes are also possible reaction intermediates. It is therefore important to obtain additional information that bears on the existence of acyl- and amino-enzymes in pepsin-catalysed processes, and to discover whether the rate-determining step of the catalysed reaction can be related to the hydrolysis of either of these intermediates.

Reaction intermediates that occur before the rate-determining transition state of a reaction can in principle be observed either directly or indirectly by a study of the pre-steady-state kinetics or by the observation of an identical reaction rate (implying the breakdown of a common intermediate) for a

Table 1. *Catalytic constants for pepsin-catalysed reactions at pH 2.2 and 37°*

Substrate	K_m (mM)	k_0 (sec.^{-1})
<i>N</i> -Acetyl-L-phenylalanyl-3,5-dinitro-L-tyrosine	0.52	0.011
<i>N</i> -Acetyl-L-phenylalanyl-L-phenylalanylglycine	1.7	0.39
<i>N</i> -Acetyl-L-phenylalanyl-L-phenylalanine	1.4	0.038
<i>N</i> -Acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine	0.41	0.055

series of related reactants. In the present case, if the hydrolytic breakdown of either an acyl-enzyme or an amino-enzyme were completely rate-determining, then we should observe a common k_0 {in $v = k_0[E_0][S_0]/(K_m + [S_0])$ } for substrates in which either acyl or amino moiety is the same. The lack of any identity in k_0 values for the two series of peptide substrates ($X \cdot CO \cdot NH \cdot Y$) in which X or Y is kept constant but the other residue varied has been noted previously (e.g. Inouye & Fruton, 1967a) and is confirmed in the present work (see Table 1). By way of further confirmation that neither an amino-enzyme nor an acyl-enzyme occurs on the catalytic pathway before the rate-determining step, we have investigated the possible existence of a 'burst' of either acyl- or amino-component of *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine. For the detection of a burst of this type the following kinetic conditions must be satisfied: $[S_0] > K_m$ and $k_{+2}/k_{+3} \gg 1$ (where k_{+2} is the rate constant for the step leading to the intermediate, and k_{+3} that for the step leading from it; see Bender *et al.* 1966). In addition, $[E_0]$ must be high enough for one to be able to detect the breakdown of an equimolar amount of substrate, yet the condition $[S_0] > [E_0]$ must be satisfied for one to observe a significant amount of the steady-state reaction subsequent to the burst. For pepsin, whose substrates have low solubilities and relatively high K_m values, the conditions are very hard to satisfy. For the detection of a possible burst of L-phenylalanine the ninhydrin method (Cornish-Bowden & Knowles,

1969) is sensitive, and the condition $[S_0] > [E_0]$ can be met. At maximum substrate concentration, however, $[S_0]$ (0.67 mM in our experiments) is not much larger than K_m (0.41; see Table 1). Despite this, we believe that the rate of appearance of ninhydrin-positive product for this substrate (Fig. 1) confirms the deduction quoted above, that an acyl-enzyme, if such exists, occurs after the rate-determining transition state in hydrolytic reactions. Decision as to whether a burst of *N*-acetyl-3,5-dinitro-L-tyrosine occurs in the hydrolysis of the dipeptide *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine is thwarted by the lack of sensitivity of the method for detecting the rate of liberation of the acetyldinitrotyrosine. The method depends on the effect of hydrolysis of the peptide link on the u.v. absorption of the aromatic chromophore (compare Schwert & Takenaka, 1955; Inouye & Fruton, 1967a), but even at pH 4.1 (when $X \cdot CO \cdot NH \cdots$

goes to $X \cdot CO_2^- + NH_3^+$) the differential extinction coefficient for the above compound is only approx. 300. This demands an unacceptably high $[E_0]$ (since $[E_0]$ must be high enough for the hydrolysis of an equimolar quantity of substrate to be estimated), so that $[S_0]$ cannot be much larger than $[E_0]$. Inouye & Fruton (1967a) have reported that no burst of the acyl moiety of a dipeptide substrate is observable with a substrate with a higher differential extinction coefficient than that used here.

Since it appears that for dipeptide substrates of pepsin $k_{+2}/k_{+3} < 1$ (where k_{+2} and k_{+3} have only the general significance defined above), a number of non-peptide analogues of dipeptide substrates were prepared, in the hope of increasing k_{+2} while k_{+3} remained constant. This approach is exactly analogous to the use of esters as substrates for the neutral proteases. Thus for α -chymotrypsin it is generally true that, although acylation of enzyme is rate-determining for amide substrates, for ester substrates the acylation step is so much faster that deacylation becomes rate-determining. The reaction intermediate now becomes amenable to study by all the techniques for observing intermediates that occur before the rate-determining step (see, e.g., Bender & Kezdy, 1964). The observation by Inouye & Fruton (1967b) that the esterase activity of pepsin is only marginally greater than its peptidase activity is discussed more fully elsewhere (Knowles, 1969), but this fact effectively eliminates depsides from consideration in the present context. Two types of substrates were synthesized: those possessing an amino moiety (Y in $X \cdot CO \cdot NH \cdot Y$), which might result in rapid acylation of the enzyme, and those with a modified acyl moiety (X), which might form an amino-enzyme more rapidly than a dipeptide. For the first

Fig. 1. Rate of production of ninhydrin-positive product from the pepsin-catalysed hydrolysis of *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine at pH 4.1 at 20°. The concentration of enzyme is indicated, and the broken line represents the expected time-course of product formation for the rate-determining breakdown of an acyl-enzyme (see the text).

approach two possibilities were considered in selecting a new leaving group: on the one hand, if the reaction were essentially nucleophilic, as with α -chymotrypsin, then it would be expected that *p*-nitroaniline would be a better leaving group than phenylalanine. On the other hand, if the reaction were essentially electrophilic, as is perhaps more likely in view of the fact that pepsin is most active at low pH values (see also Knowles, 1969), it would be expected that *p*-methoxyaniline would be a better leaving group than phenylalanine. To test these two possibilities the two compounds *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitroanilide and *N*-benzyloxycarbonyl-L-phenylalanine *p*-methoxyanilide were prepared. Each of these compounds gave a significant difference spectrum when compared with the free amine, so that it is feasible to follow the hydrolysis spectrophotometrically. Both compounds are only sparingly soluble, and it was necessary to carry out experiments in 20% (v/v) acetonitrile. In each case no hydrolysis was detected in the presence of pepsin. It is probable that any activity that might have been observed would have been decreased by the inhibitory effect of the acetonitrile, but in view of the fact that no hydrolysis at all was observed it is more likely that the compounds are not capable of reaction with pepsin. A possible reason for this is that in these compounds the two phenyl groups are two carbon atoms closer together than in phenylalanylphenylalanine dipeptide substrates, and this may result in so much distortion of the positions of the atoms forming the anilide bond that catalysis is impossible. If this were the case then it would be expected that a compound containing phenethylamine as a leaving group would be a substrate. Accordingly the compound *N*-acetyl-DL-phenylalanine phenethylamide was prepared and its reaction with pepsin investigated. No reaction was detected under conditions (pH 2.5, 1.2% dimethylformamide, 37°) where another neutral substrate, *N*-acetyl-L-phenylalanyl-L-phenylalanine amide, shows high activity. It is noteworthy that Inouye & Fruton (1967a) have found that another compound (*N*-benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalaninol) lacking a carbonyl group adjacent to the susceptible peptide link is inactive as a pepsin substrate.

For the second approach (that of rapid formation of an amino-enzyme) *p*-nitrobenzoyl-L-phenylalanine was prepared, since it was thought that *p*-nitrobenzoic acid would be a better leaving group than acetylphenylalanine. No pepsin-catalysed hydrolysis of this compound could be detected, either spectrophotometrically or by the continuous ninhydrin method. *p*-Nitrobenzoyl-L-phenylalanine suffers from one of the same drawbacks as the anilides described above, in that the two aromatic

binding groups are not the correct distance apart. This drawback was overcome by preparing a compound that is known to be a substrate for another protease (carboxypeptidase A; McClure & Neurath, 1966), and in which the binding groups are correctly situated, namely *N-trans*-3-indol-3-ylacryloyl-L-phenylalanine. No hydrolysis of this compound in the presence of pepsin could be detected.

It appears from the above work that pepsin is much more specific than the neutral proteases, and that only very limited alterations in the structure of a peptide (such as the minimal change to an L-L-depside studied by Inouye & Fruton, 1967b) are possible if one is to retain substrate activity. [A notable exception to this statement is, however, the activity of sulphite esters as pepsin substrates, studied by Reid & Fahrney (1967).]

A third line of evidence that points to the fact that reaction intermediates in pepsin-catalysed hydrolyses (other than the Michaelis complex) occur after the transition state of the rate-determining step is the apparent equivalence of K_m and K_s for dipeptide substrates of pepsin. This point has been fully discussed by Denburg, Nelson & Silver (1968) and the supposition rests primarily on the near identity of K_m values for L-L-dipeptide substrates, and the K_i values of their enantiomeric and diastereoisomeric analogues (Knowles *et al.* 1969). Although this identity does not necessarily demand the identity of K_m and K_s , the evidence taken together supports the view that the rate-determining step of pepsin-catalysed reactions is that which follows the formation of the Michaelis complex. This means that a different approach has to be made to the problem of defining the obligatory intermediates in pepsin-catalysed reactions. Essentially the only way of detecting intermediates that occur after the rate-determining step of a reaction is by trapping experiments. Here the normal breakdown pathway of an intermediate is diverted by the addition of a reagent that reacts very rapidly with it. In fact, the reaction of *N*-acyl-L-amino acids with the amino-enzyme during pepsin-catalysed hydrolyses (i.e. transpeptidation) represents the trapping of the amino-enzyme, diverting this intermediate from its normal hydrolytic course into the synthesis of a new peptide. As is described by Greenwell *et al.* (1969), attempts have been made with an ethyl ester and a methyl thiol ester to trap the amino-enzyme more effectively, although these experiments were not designed to test the intermediacy of the amino-enzyme, since the evidence for this is firm. It is the possibility that an acyl-enzyme is an intermediate in pepsin-catalysed reactions that needs to be tested, since this proposal rests at present on the established fact that pepsin catalyses ^{18}O exchange between

H_2^{18}O and *N*-acyl-L-amino acids coupled with the possibly weak analogy that similar isotope-exchange reactions catalysed by neutral proteases do involve acyl-enzymes.

The intrinsic nucleophilicity of methanol is much greater than that of water (see Bender, Clement, Gunter & Kezdy, 1964), and this fact together with its hydroxylic nature makes it an obvious choice as a water analogue and as a trap for possible acyl-enzymes. Indeed, methanol has been used extensively for this purpose (see, e.g., Bender *et al.* 1964; Lowe & Williams, 1965) and has the advantage over hydroxylamine (which has also been much used; Caplow & Jencks, 1963; Bender *et al.* 1964) that species trapped by ^{14}C -labelled material can be detected in very low concentration. Two attempts to trap the putative acyl-enzyme have been made, one in a system undergoing the catalysed hydrolysis of a tripeptide substrate, the other in a system containing a virtual substrate under conditions in which ^{18}O exchange with H_2^{18}O is known to occur. In the first experiment a mixture of pepsin and substrate (*N*-acetyl-L-phenylalanyl-L-phenylalanylglycine) was incubated at pH 3.1 (close to the pH optimum for this substrate), 1% (v/v) with respect to ^{14}C methanol, for 3 hr. The extent of the hydrolysis reaction was monitored by the ninhydrin method (Cornish-Bowden & Knowles, 1969) and was approx. 70%. A chloroform extract of the reaction mixture was subjected to t.l.c. A control experiment in the absence of pepsin was performed, and the difference in the distribution of radioactivity along the t.l.c. plate in the two experiments is plotted in Fig. 2. It is apparent that no *N*-acetyl-L-phenylalanine ^{14}C methyl ester is detectable. A control experiment with a ^3H -labelled sample of *N*-acetyl-L-phenylalanine methyl ester demonstrated that the recovery of this material, after incubation with pepsin and subjection to the extraction and chromatographic procedures, was greater than 80%. Only an estimate can be made of the expected amount of *N*-acetyl-L-phenylalanine methyl ester on the basis of the known reactivities of methanol and water. The ratio of methanolysis to hydrolysis rate constants for carboxylic acid derivatives has been estimated as 100 (Bender *et al.* 1964). The molarity of methanol in aq. 1% methanol solution is 0.25 M. This, for the reaction under consideration, is probably an underestimate, since the local (enzyme-bound) concentration of methanol is very probably relatively higher than that in free solution (and probably accounts, for instance, for the larger ratios of apparent rate constants for methanolysis and hydrolysis of acyl- α -chymotrypsins, which range between 40 and 600; Bender *et al.* 1964). On the basis of this modest assumption, the ratio of methanolysis and hydrolysis products should be

Fig. 2. Distribution of radioactivity along t.l.c. plates from the reaction of pepsin with *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine in the presence of 1% (v/v) ^{14}C methanol. The radioactivity from corresponding fractions in the control experiment (without pepsin) was subtracted from that of the test solution, after a small correction for the difference in total radioactivity along the t.l.c. plate for the two tracks. The expected radioactivity for 10% and 1% trapping of putative acyl-enzyme is indicated in the position expected (for *N*-acetyl-L-phenylalanine ^{14}C methyl ester).

about 0.45. From the extent of substrate hydrolysis as determined by the appearance of phenylalanylglycine, and since we know that the *N*-acetyl-L-phenylalanine methyl ester is not hydrolysed by pepsin, we can calculate how much methyl ester would have been found had *N*-acetyl-L-phenylalanyl-pepsin been a reaction intermediate and been susceptible to methanolysis as well as hydrolysis. This estimate is plotted in Fig. 2, which shows that the method would have detected less than 1% of the 'expected' amount of methanolysis product.

In a second experiment pepsin was incubated with *N*-acetyl-L-phenylalanine at pH 4.7 and 37° for 3 hr. Under these conditions it has been shown by Kozlov *et al.* (1965) that approx. 40% of the carboxyl group oxygen atoms of the acyl-amino acid exchange with ^{18}O -enriched water. In our experiments the solution was 2.0 M with respect to ^{14}C methanol. Methanol is known (Tang, 1965) to be a competitive inhibitor of pepsin-catalysed hydrolyses, and very possibly inhibits the ^{18}O -

exchange reaction also. This would presumably decrease the extent of any acyl-enzyme formation, and consequently cut down the amount of methanolysis product observable. However, the K_i of methanol in the hydrolytic reaction is about 0.6M (Tang, 1965) and it is very unlikely that this effect could decrease the amount of *N*-acetyl-L-phenylalanine methyl ester by more than fivefold. Such a restriction is a trivial one, if one considers the detection capability of the method. After incubation the reaction was stopped by the addition of acetone and the precipitated pepsin was separated by centrifugation. A portion of the supernatant was subjected to t.l.c. As before, a control experiment was performed in the absence of enzyme, and the distributions of radioactivity along the two t.l.c. plates were compared. Once again it was found that no product from the methanolysis of an acyl-enzyme could be detected.

Negative experiments such as those described above cannot, of course, be conclusive. It is possible that an acyl-enzyme does form, but that the acyl link is inaccessible to the more bulky (albeit more reactive) nucleophile, methanol. However, argument by analogy with other hydrolytic enzymes such as α -chymotrypsin (Bender *et al.* 1964), papain (Lowe & Williams, 1965) and lysozyme (Rupley, Gates & Bilbrey, 1968) makes one less willing to accept a mechanism that allows nucleophilic attack by water but not by methanol. The sensitivity of the method is so high that a very small proportion (less than 1%) of trapped acyl-enzyme is detectable, and we conclude that neither pepsin-catalysed hydrolysis reactions nor pepsin-catalysed ^{18}O -exchange processes involve acyl-pepsin intermediates. The mechanistic implications of this statement are discussed more fully elsewhere (Knowles, 1969).

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