Towards a Mechanism for Pepsin

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The fact that proteins in their folded state are resistant to attack by proteolytic enzymes essentially creates a physiological requirement for a proteolytic enzyme which is both stable and active under conditions where most other proteins are unfolded. This problem might perhaps have been solved by the evolution of an enzyme that could rapidly degrade proteins in their native state, but such an enzyme would presumably be very susceptible to self-deeating autolytic digestion, and the first solution seems to be the preferred one. So we have the unusual enzyme, pepsin, which catalyses the hydrolysis of proteins at pH values at which most ingested protein material is either reversibly or irreversibly unfolded. It does not matter, of course, if the unfolding is reversible, since a few cuts in the protein chain would be sufficient to prevent refolding when the pH is raised again, resulting in long random-coil peptides in an ideal state for further digestion by the neutral proteases. Pepsin is of interest physiologically therefore, since it mediates the first, crucial, cuts in the breakdown of ingested protein.

The remarkably low pH range of stability ("pH" circa 0.2 to 6) and of activity (pH circa 1 to 5) of pepsin makes this enzyme of interest in protein chemical terms, and it is relevant to ask what features of pepsin contribute to this unusual behaviour. Pepsin has, in common only with other acid proteases like rennin and gastricsin (and also with the hormone gastrin), a very high ratio of acidic to basic amino acids. There are about 40 free carboxyl groups and only four basic groups (Rajagopalan et al. 1966a). This accounts in part for the low isoelectric point (of less than 1: Perlmann 1955), as does the existence in the molecule of a single phosphate group which is probably a diester and is attached on one side to a serine residue. Excision of this phosphate raises the isoelectric point to 1.7, and has no effect on the catalytic activity (Perlmann 1952). An isoelectric point of 1.7 is still too low to be accounted for even by the imbalance of acidic and basic groups, and we must conclude that there are in pepsin one or more ionizing groups of abnormally low pKₐ. We shall return to this point later. Pepsin is also very resistant to common protein denaturants.
such as urea, which further attests to its unusual stability.

The third reason which makes pepsin an interesting and perhaps useful system for investigation is that - at least superficially - it seems mechanistically more to resemble an acid-catalysed than a base-catalysed process. It has been recognized for some time that catalysis mediated by the neutral proteases $\alpha$-chymotrypsin and trypsin is dominated by nucleophilic/general base characteristics, this view having originally sprung from the rough parallelism between the rates of attack of hydroxide ion and of $\alpha$-chymotrypsin and trypsin on $p$-nitrophenyl esters, alkyl esters, and amides. The ratio of $k_{OH}$ for alkyl esters and analogous amides is commonly between $10^5$ and $10^4$, and this is in the range estimated for the rates of enzyme acylation by analogous ester and amide substrates of the above enzymes (Zernner et al. 1964). It can be argued that this comparison is naive - which it undoubtedly is - since the Hammett $\rho$-value for substituted anilide substrates is known to be negative (Inagami et al. 1965). This implies that the transition state of the rate-determining step here is electron deficient, and indicates a dominant electrophilic component for the rate-determining step for these substrates. On the other hand, the $\rho$-value for substituted aryl esters is positive (Caplow & Jencks 1962). These data have been discussed by Wang (1968) and it is possible that the rate-determining step of the acylation process (sic) or the actual mechanism for ester and anilide hydrolysis is different. However, these details will have to await experimental clarification. In contrast to the behaviour of $\alpha$-chymotrypsin and trypsin, the ester:amide (actually depside:peptide) rate ratio for pepsin is about 2 (Inouye & Fruton 1967). This is in the range of ratios observed for the acid-catalysed hydrolysis of esters and amides, which are normally less than 10. These comparisons tempt one to suggest that pepsin is complementary in chemical catalysis terms, to chymotrypsin and trypsin. It is very possible that pepsin catalysis is dominated by an electrophilic (acid) component whereas chymotrypsin and trypsin owe more to a nucleophilic (and/or general base) contribution. Carboxypeptidase A also has a low $k_{\text{peptide}}/k_{\text{peptide}}$ ratio (of about 4) (Davies et al. 1968) and for this enzyme there are good reasons for the belief that electrophilic catalysis dominates the rate-determining covalency changes (see Lipscomb et al. 1968).

**pH Dependence**

The dependence of pepsin-catalyzed reactions on pH is not straightforward: the low water solubility of many simple substrates precludes kinetic measurements over very wide ranges of substrate concentration, and the low pH optimum of the enzyme demands the use of assay procedures of limited sensitivity. However, the dependence of $k_{\text{cat}}/K_m$ for a neutral substrate is bell-shaped, with apparent pK_a-values of about 1 and 4.7 (Cornish-Bowden & Knowles 1969, see also Denburg et al. 1968, Lutsenko et al. 1967, Zeffren & Kaiser 1967). These results relate to the uncharged dipeptide substrate N-acetyl-l-phenylalanyl-l-phenylalanine amide, and the above pK_a-values therefore reflect ionizations of groups on the free enzyme. When more soluble charged substrates are used, the pH dependence of $k_{\text{cat}}/K_m$ changes, for instance that for N-acetyl- l-phenylalanyl- l-phenylalanine shows apparent pK_a-values at 1 and 3.5, the latter being very close to that of the free di-
peptide substrate. Two questions are posed by these data: does the apparent pK_a of 3.5 for N-acetyl-L-phenylalanyl-L-phenylalanine relate to the ionization of the carboxyl group of the substrate itself, and do the two pK_a-values on the free enzyme of 1 and 4.7 (observed with the neutral substrate N-acetyl-L-phenylalanyl-L-phenylalanine amide) reflect the ionizations of functional groups at the active site of pepsin which participate directly in the covalency changes that constitute catalysis, or of groups which control the stability or native conformation of the protein? Probable answers to each of these questions are provided by the results of inhibitor binding studies (Knowles et al. 1969). The binding of the neutral diastereoisomeric analogues of N-acetyl-L-phenylalanyl-L-phenylalanine amide (the corresponding L-D and D-L materials) to pepsin is essentially independent of pH over the whole range of pepsin activity, from pH 0.2 to 5.8. These materials are each cleanly competitive inhibitors of the catalysed reaction, and the pH-independence of K_i indicates that pepsin undergoes no major conformational change (at least, none that affects inhibitor binding at the active site) over this range. By contrast, the L-D and D-L analogues of the substrate N-acetyl-L-phenylalanyl-L-phenylalanine (which are each competitive inhibitors of the catalysis) do not discernably bind to pepsin above about pH 3.5. This indicates that these dipeptides only bind to the protein in their uncharged form, and that their anions are repelled from the active site. Thus the fall in k_cat/K_m for the L-L substrate can be ascribed to a rise in K_m with an apparent pK_a of 3.6. This interpretation agrees with that presented by Denburg et al. (1968).

The following conclusions can be made from the pH-dependence work outlined above. (A) The active site of pepsin contains a net negative charge which repels the anions of dipeptide acid substrates and inhibitors. (B) Pepsin suffers no gross conformational change over the whole range of its catalytic activity. (C) The catalytic activity of the enzyme depends upon the ionization of two groups with apparent pK_a-values of about 1 and 4.7 on the free enzyme.

Reaction Intermediates

For the peptidolytic reactions catalyzed by pepsin, it has been argued earlier (Knowles 1969) that the rate-determining step is that which immediately follows enzyme-substrate complex formation, and that no reaction intermediate is detectable by a direct method. This view is based on the following: (A) the apparent equivalence of K_m and K_i (which is a kinetic consequence of the rate-determining step immediately following binding; see Denburg et al. 1968), (B) the form of the pre-steady-state release of the two products from a dipeptide substrate (i.e., no “burst” of either product can be detected; see Inouye & Fruton 1967, Cornish-Bowden et al. 1969), and (C) the non-equivalence of k_cat in either of two sets of substrates X-CO-NH-Y in which either X or Y is invariant (i.e., the breakdown of neither enzyme-CO-X nor of enzyme-NH-Y is rate-determining; Inouye & Fruton 1968, Cornish-Bowden & Knowles 1969).

In these circumstances, detection of reaction intermediates has to rely on kinetic measurements relating to the ordered release of products, on the diversion of the intermediate from its normal course of breakdown, or on direct trapping experiments of an enzyme-substrate system during turnover. Each of these approaches has been taken.
The scheme is:

\[
\text{enzyme.X-CO-NH-Y} \xrightleftharpoons{} \text{NH}_3^+ + \text{enzyme} + X^-\text{COOH} \quad (1)
\]

From the nature of the products in transpeptidation reactions, Neumann et al. (1959) and Fruton et al. (1961) showed unequivocally that transpeptidation occurs by imino-transfer (i.e. transfer of enzyme-bound-NH-Y to acceptor X'-COOH) and not by acyl transfer (of enzyme-bound X-CO- to acceptor NH3+-Y'). These studies, in fact, first pointed to the intermediciy of an amino-enzyme in peptidase-catalyzed reactions. The non-competitive inhibition of product (X-COOH) mentioned above is in complete agreement with the transpeptidation product analysis data, the non-competitive inhibition simply arising from the diversion of amino-enzyme away from hydrolysis into the synthesis of new peptide.

On the basis that the amino-enzyme acts with X-COOH as an acceptor, we investigated the possibility that X-COOCMe would be a better acceptor, since an ester might be attacked by the nitrogen atom of the amino-enzyme more rapidly than the free acid. Moreover, since it is known that thiol esters are some 10⁴ times more susceptible to attack by nitrogen nucleophiles than are oxygen esters (Bruce & Benkovic 1966a), the inhibition of hydrolysis by X-COSMe was also studied (Greenwell et al. 1969). All of the tested materials (N-acetyl-L-phenylalanine ethyl ester, and N-methanesulphonyl-L-phenylalanine methyl and methyl thiol esters) act as non-competitive inhibitors. However, from a system containing pepsin, substrate, and N-acetyl-L-phenylalanine ethyl ester, no ethanol was detectable. The assay for ethanol (with alcohol dehydrogenase) would have detected less than 2 per cent of that expected if the whole of the decrease in hydrolysis rate of the substrate had been due to transpeptidation. Moreover, peptic-catalyzed hydrolysis in the presence of the thiol ester did not give rise to any detectable amount of methane thiol, showing that this ester too cannot act as an imine acceptor in transpeptidation. The observed non-competitive inhibition of these esters is therefore of the "dead-end" type, and their inability to act as transpeptidation acceptors must be due either to the fact that the spatial requirements for transpeptidation are so strict that enucleophilic esters may be dominated by an enucleophilic rather than a nucleophilic component.) Nevertheless, the data are all consistent with the intermediciy of an amino-enzyme in peptidase-catalyzed reactions.

The third type of trapping experiment involved the use of methanol as a water analogue, and was designed primarily to test whether in addition to the amino-enzyme, an acyl-enzyme is formed at any stage. By analogy with the neutral proteases α-chymotrypsin (Sprinson & Rittenberg 1951) and papain (Grisaro & Sharon 1964), it has been suggested that, since peptic catalyses the exchange
of $^{18}$O between $H_2^{18}O$ and the carboxyl group of $N$-acyl-$L$-amino acids, the reaction involves an acyl-enzyme. Certainly the acyl-enzyme postulate is the simplest interpretation of these results, as:

$$H_2^{18}O + \text{enzyme} \rightarrow \text{enzyme-CO-X} \rightarrow \text{X-CO}^{18}OH + \text{enzyme}$$

(2)

In order to reconcile the $^{18}$O exchange data with the unambiguous conclusions from the transpeptidation experiments, mechanisms involving the simultaneous attachment to the enzyme of each half of the substrate, were suggested (e.g. Sharon et al. 1962, Bender & Kerdy 1965, Zeffren & Kaiser 1967, Clement et al. 1968):

$$\text{X-CO-NH-Y} \rightarrow \text{enzyme-CO-X} \rightarrow \text{X-COOH}$$

As a test of acyl-enzyme intermediates, both partial reactions (equation 2) and hydrolysis reactions (equation 1) were run in the presence of a small concentration of very radioactive methanol (Cornish-Bowden et al. 1969). Methanol has been found to be very effective as a water analogue in reactions of other proteases (Bender et al. 1964, Lowe & Williams 1965) since the intrinsic nucleophilicity is much greater than that of water, and significant competition with water can be effected at low methanol concentrations. The results of all attempts to trap an acyl-pepsin intermediate, by searching for $N$-acyl-$L$-phenylalanine [14C] methyl ester in hydrolytic reactions (with $N$-acyl-$L$-phenylalanine-$L$-phenylalanylglycine) or in partial reactions (with $N$-acyl-$L$-phenylalanine), were negative (Cornish-Bowden et al. 1969). While such negative experiments cannot be conclusive, the success of this approach with other hydrolytic enzymes (chymotrypsin: Bender et al. 1964, papain: Lowe & Williams 1965, lysozyme: Rupley et al. 1968) makes it less easy to accept mechanisms for pepsin catalysis which involve acyl-enzyme intermediates. However, if no acyl-enzyme intermediate is involved in the mechanism, how does pepsin catalyse the exchange of $^{18}$O? Stating the question another way, if it is accepted that neither methanol nor water is explicitly involved in the $^{18}$O-exchange, how does $^{18}$O get into virtual substrates at all? A probable answer has recently been provided by the experiments of Shkarenkova et al. (1968), who showed that

* It should perhaps be emphasized that we cannot neglect the $^{18}$O-exchange reaction, nor does it make sense to propose a mechanism for $^{18}$O-exchange which differs from that for hydrolysis and transpeptidation. These three catalytic activities have broadly the same pH dependences, specificities for different amino acids (Ginodman et al. 1964), and stereospecificities (Sharon et al. 1962). There are no data which do not support the view that a single overall mechanism describes all of these reactions.
one or more carboxyl groups in pepsin, postulated to be at the active site, rapidly exchange oxygen directly with $H_2^{18}O$. Thus a possible alternative route for catalysed $^{18}O$-exchange involves transfer of $^{18}O$ from $H_2^{18}O$, via the protein itself, to the virtual substrate. As will be seen later, the rate data pertaining to the exchange reaction obtained by Shkarenkova et al. (1968) (which are more reliable than the absolute extent-of-labelling estimates) are in complete agreement with such a scheme.

Aside from the use of methanol as a possible acyl-enzyme trap, one might expect that this nucleophile would compete with water in amino-enzyme hydrolysis, if this is a covalent intermediate. We shall see below that an attractive formulation of the amino-enzyme is as a covalent intermediate involving an enzyme carboxyl group, as: enzyme-CO-NH-Y. Methanolysis of such an intermediate would lead to enzyme-CO.OMe, presumably an enzymically inactive protein. However, the hydrolysis of the amino-enzyme is postulated (vide infra) to involve a neighboring carboxyl group, and this group in the same way could rapidly catalyse the decomposition of the ester link (compare the carboxyl-group catalysed hydrolysis of half esters of phthalic acid: Bender et al. 1958a). Thus the fact that peptide hydrolysis in the presence of methanol does not lead to progressive enzyme inactivation (Tang 1965, Cornish-Bowden et al. 1969) cannot reasonably be used as evidence against the amino-enzyme intermediate.

**Direct trapping of the amino-enzyme**

The clearest evidence for the existence and nature of the amino-enzyme must come from direct trapping experiments. This approach is based upon the premise that the amino-enzyme (assuming that there is a covalent link between enzyme and "amino" moiety of the substrate) is hydrolysed rapidly solely by virtue of the juxtaposition of appropriate functional groups at the active site of the enzyme, and that if these groups are removed by unfolding the protein, rapid hydrolysis of the amino-enzyme will not occur. The experiment consists, therefore, of quenching a pepsin-catalysed hydrolysis while the enzyme is turning over into a solution which will unfold the protein more rapidly than the amino-moiety of the substrate is lost from it hydrolytically (P. Greenwell & T. M. Kitson, unpublished experiments). The major problem is that since, as has been argued above, the amino-enzyme follows the rate-determining step, the steady-state concentration of this intermediate will be low, and the proportion of pepsin which is amino-enzyme will be correspondingly difficult to detect. However, we argued that the ready observation of transpeptidation by imino-transfer must mean that the amino-enzyme has a finite lifetime, since it must exist long enough for $X$-COOH of the original peptide to depart and $X'$-COOH (the new acceptor) to bind. We therefore assumed that the rate constant for hydrolysis of amino-enzyme would not be more than $10^4$ times the rate constant for the reaction back to dipeptide. Accordingly, an appropriate substrate, very highly radioactive in the $Y$ moiety, was prepared. This was $N$-acetyl-$l$-phenylalanyl-$d$-[131I] -iodo-$l$-tyrosine. This substrate in the highest possible concentration (to maximize the occupancy of enzyme by substrate) was incubated with pepsin at pH 2 for about 1 minute, which is quite long enough for the steady-state to be reached. Portions of the reaction mixture were then transferred to filter discs and the discs dropped into acetone, which is known rapidly and efficiently to de-
nature pepsin (Bayliss et al. 1969). The filter discs were washed exhaustively alternately with acetone and water and, after drying, were counted in a scintillation counter. As a control in case of adventitious binding of radioactive material to the protein an analogous reaction was run with substrate and pepsin, specifically inactivated with N-diazoacetyl-L-phenylalanine methyl ester (Bayliss et al. 1969). It appears that radioactive material is bound to the native protein at the steady state, since our preliminary results show that the native pepsin has associated with it consistently higher radioactivity than the inactive control. This experiment is being validated by studying the effect of varying the enzyme and substrate concentrations and the quenching method, and by a double-labeling experiment in which N-[14C]-acetyl-L-phenylalanine di [125I]iodo-L-tyrosine is the substrate. The latter experiment is designed to show unequivocally that the enzyme is labelled with 125I but not with 14C, and that the trapped intermediate is indeed the amino-enzyme.

Catalytic Groups
From modification studies involving active-site-directed irreversible inhibitors, it has been shown that a single carboxyl group in pepsin is uniquely reactive and is essential for the proteolytic and peptidolytic activities (Delapierre & Fruton 1965, 1966, Rajagopalan et al. 1966b, Stepanov et al. 1967, Hamilton et al. 1967, Erlanger et al. 1967, Ong & Perlmann 1967, Kozlov et al. 1967, Bayliss & Knowles 1968). This group is the β-carboxyl group of an aspartic acid residue in a sequence Ile-Val-Asp-Thr-Gly-Thr-Ser (Knowles & Wybrant 1968). The above study utilized the substrate analogue N-diazoacetyl-L-phenylalanine methyl ester, and it seems probable that most if not all the specific diazo inhibitors used to inactivate pepsin modify this residue. The carboxyl group reagent β-bromphenacyl bromide also reacts stoichiometrically with pepsin, though it results in incomplete loss of proteolytic and peptidolytic activity (Erlanger et al. 1967, Dr. B. F. Erlanger, private communication). Since pepsin which has reacted with this reagent (with modification of an aspartic acid residue in a peptide of composition Gly2, Asp, Ser, Glu) will further react stoichiometrically with the analogous diazo compound (α-diazo-β-bromacetophenone; Erlanger et al. 1967), it is unlikely that this group is directly involved in the covalency changes that constitute the catalytic process. There is little doubt, however, that the first-mentioned aspartyl carboxyl group is essential for peptic activity.

On the basis of the pH-dependence of catalysis and of binding discussed earlier, it was stated that the catalytic activity of pepsin depends on two ionizing groups which, on the free enzyme, have apparent pK_a-values of about 1.0 and 4.7. Moreover, these groups appear to control the catalytic process itself, rather than (say) a conformational state of the protein. Despite the well-known dangers of assigning particular functional groups to apparent pK_a-values, there are rather few options in the case of pepsin. The enzyme has no sulphhydril groups, acylation of amino groups has no effect on the catalytic activity, photo-oxidation of the single histidine residue does not inactivate the enzyme, and the single serine phosphate group is known to be inessential. In the light of these facts, it is difficult not to deduce that each of the two ionizations observed kinetically is due to an enzyme carboxyl group. (The case against a protonated peptide link has been argued elsewhere: Knowles 1969.) We should then have a pair
of carboxyl groups, one protonated and one not, which mediate the covalency changes of the catalytic reaction. This postulate is consistent with all the data pertaining to pepsin including the modification studies of carboxyl and other groups, the pH-dependences of catalysis and of binding (which imply a net negative charge at the active site around pH 3.5), and the results of the trapping experiments described above. If this is so, then the question arises as to why the first $pK_a$ of the pepsin system is so low (at about 1.0). Actually this is not a real problem, and may be a further indication that there is a pair of carboxyl groups. The argument runs as follows:

If two ionizing groups of closely similar $pK_a$ form a hydrogen-bonded pair, then we may expect that the first $pK_a$ of the system will be much lower than the $pK_a$ of either group in isolation. This can be thought of as arising from the equal contribution of each of two hydrogen-bonded states to the singly ionized system. On the other hand, if the two groups have very different $pK_a$-values in isolation, then the two (macroscopic) $pK_a$-values of the system will be very little perturbed from the values of the two groups separately, there being no additional stabilization of the singly ionized state.

This proposal nicely accommodates the observed apparent $pK_a$-values for $\alpha$-chymotrypsin, papain, and pepsin. $\alpha$-Chymotrypsin activity shows a $pK_a$ of about 7 ($pK_a$ relates to a conformational change controlled by the ionization of the $\alpha$-amino group of isoleucine, and is not relevant here). This enzyme has a hydrogen-bonded trio of residues: Asp106, His57 and Ser195 (Blow, Birktorf & Hartley 1969) the intrinsic $pK_a$ values of which are all well separated, and the observed $pK_a$ is close to that of isolated histidine. Papain, on the other hand, has a duo of His158 and Cys25 (Drenth et al. 1968) which are probably hydrogen-bonded in the free enzyme, and the intrinsic $pK_a$-values are much closer to each other, allowing the doubly-charged thiolate:imidazolium system to contribute to the singly ionized state. It may be relevant, therefore, that the pH-dependence of $k_{\text{cat}}/K_m$ for papain-catalysed reactions shows apparent $pK_a$-values at about 4 and 8, the $pK_a$ of this system being significantly lowered. We postulate that pepsin contains two carboxyl groups, possibly hydrogen-bonded in the free enzyme, and that $pK_a$ of this system is much lower than that of an isolated carboxyl group. Although model systems are complicated by direct effects, the two $pK_a$-values of maleic acid are 1.83 and 6.07, and of fumaric acid, for which the proposed interaction is impossible, 3.08 and 3.15.

MECHANISTIC PROPOSALS

From what has been said above, we are in a position to suggest a tentative mechanism consistent with all results bearing on the pepsin mechanism. Any mechanism must accommodate the following, which are either established fact or are postulates which have been argued here or elsewhere. (1) The three catalytic activities of pepsin: hydrolytic, transpeptidation, and $^4$O-exchange, occur at a single active site, show similar specificities, and follow similar overall mechanisms. (2) The mechanism of hydrolysis and transpeptidation involves an amino-enzyme which is probably a covalent intermediate. (3) The catalytic step immediately following the Michaelis complex is rate-determining and is possibly dominated by an electrophilic catalytic component. (4) The binding of neutral substrates and inhibitors to the enzyme is in-
dependent of pH, though the active site appears to bear a net negative change above pH 3.5. (5) The catalytic steps are meditated by two groups of apparent pKₐ of about 1.0 and 4.7. (6) Certainly one, and possibly both, of these groups is a carboxyl group. (7) ^18O-exchange occurs rapidly between H₂^18O and one or more carboxyl groups on the enzyme, and the rate of loss of ^18O from labelled pepsin is, in the presence of an N-acylamino acid, close to the rate of ^18O uptake by the acylamino acid.

In recent years a number of groups have proposed mechanistic outlines for pepsin-catalysed reactions. Based primarily on the absence of a solvent isotope effect (Clement & Snyder 1966), some suggestions have been made which involve the reversible formation of an anhydride between two enzyme carboxyl groups (Bender & Kezdy 1965, Clement et al. 1968), and which do not involve proton transfers in, or prior to, the rate-determining transition state. The recent observation of Hollands & Fruton (1969) that pepsin-catalysed peptidolytic reactions can show a significant isotope effect has removed the necessity of avoiding proton transfers before the rate-determining step. The presence or absence of a solvent isotope effect appears to be substrate dependent (Clement & Snyder 1966, Reid & Fahney 1967, Hollands & Fruton 1969) and probably reflects a balance between nucleophilic-general base catalysis (which will show k₃[H₂O]/k₃[D₂O] > 1) and acid catalysis (which can have k₃[H₂O]/k₃[D₂O] < 1) for different substrates. It is interesting in this connection that carboxypeptidase A (for which it has been argued that, like pepsin, electrophilic catalytic components may dominate the mechanism, Knowles 1969) also shows kinetic solvent isotope effects which are substrate-dependent. Thus for peptide substrates small effects are seen (of 1.1–1.2, for references, see Lipscomb et al. 1968), while for decapeptide substrates for which one may expect a greater contribution from nucleophilic-general base catalysis, a value for k₃[H₂O]/k₃[D₂O] of about 2 has been observed (B. Kaiser & E. T. Kaiser, personal communication). These data strengthen the view that the size, and even the apparent absence, of a kinetic solvent isotope effect for an enzyme reaction must not be used as a criterion for mechanism. Some of the dangers of using the solvent isotope effect of an enzyme-catalysed reaction in this way have been further discussed elsewhere (Knowles 1969).

A concerted scheme for the transpeptidation reaction has been put forward (Bruce & Benkovic 1966b). This suggestion, while accounting for transpeptidation by imino-transfer, cannot (in an analogous hydrolytic reaction) allow the observed ordered release of products. However, the essence of this proposal has been incorporated into mechanistic suggestions involving the simultaneous attachment of both acyl and amino moieties of the substrate to the enzyme (Zeffren & Kaiser 1967, Clement et al. 1968). Aside from the details of these proposals (e.g. some unexpected covalency changes), they involve acyl-enzymes. In particular, ^18O-exchange is envisaged by these mechanisms to arise from attack by H₂[^18O] on an acyl-enzyme. We have preferred to accept a causal rather than a fortuitous relationship between the exchange of ^18O between H₂[^18O] and the enzyme itself, and the enzyme-catalysed exchange of the oxygen atoms of a virtual substrate (see below).

The above notwithstanding, it cannot be said that mechanisms involving an anhydride enzyme, or involving the transient formation of an acyl-aminio-enzyme, can be definitely ruled out at this time. What follows is a proposal which, in our view, is the simplest which
can accommodate all the relevant data. It contains the features of an outline suggestion for pepsin-catalysed hydrolysis and transpeptidation reactions put forward by Delpierre & Fruton (1965).

The proposed mechanism is outlined in Scheme 1. The attack of enzyme carboxylate ion at the peptide carboxyl (A) is an obvious first step to yield a tetrahedral intermediate (B). Peptide links are rather unreactive particularly to such a weak nucleophile as a carboxylate group, and we suggest that this process is made more facile by general-acid catalysis by the second carboxyl group. The protonation site is depicted as carbonyl oxygen rather than peptide nitrogen. While there is no a priori reason why this should be (protonation at either site would facilitate the formation of the tetrahedral intermediate), protonation on oxygen is aesthetically more attractive since it minimizes charge separation in the transition state. This conversion of A to B is likely to be the slowest step of the whole reaction (a step prior to amino-enzyme formation must be rate-determining, vide infra), and this view also fits with the suggestion that the rate-determining step of the reaction is dominated by an electrophilic component. Protonation of substrate must clearly be significant for attack by carboxylate to occur at all, and could even be complete before the nucleophile attacks (Wang 1968). From
the tetrahedral intermediate, B, we are faced with the question of how to satisfy the requirement for prior release of •- COOH and formation of amino-enzyme. Various possible routes and organic chemical models for this have been discussed elsewhere (Knowles 1969). A pathway via the diacylamine (cf. the acid-catalysed rearrangement of aspartyl peptides from α-linked to β-linked) would require dehydration of the tetrahedral intermediate, as would a route analogous to the N → O acyl shift seen in the rearrangement of carboxyl adducts of carbodiimides, and there is no obvious path for such a dehydration without the arbitrary introduction of more catalytic functionalities. More attractive is the formulation in Scheme 1, in which the tetrahedral intermediate breaks down directly in a four-centre reaction, liberating •- COOH and forming the amino-enzyme (C), as first suggested by Delpierre & Fruton (1965).

A similar problem in the formulation of a chemically reasonable mechanism exists for other "leaving group transfer" enzymes, e.g. coenzyme-A transferase (Hersh & Jencks 1967a). Here, a covalent coenzyme-A enzyme intermediate has been isolated which very possibly uses an enzyme carboxyl group, and the transfer of coenzyme A is believed to occur "either by a concerted four-centre reaction or through the intermediate formation of an anhydride" (Hersh & Jencks 1967b). In the case of pepsin there are arguments against the latter route, and the four-centre rearrangement (B → C) is, at the present time, the simplest working hypothesis.

The amino-enzyme, D, is formulated as a peptide in Scheme 1, though the identity of any covalent linkage must naturally await characterization of a trapped amino-enzyme. The amino-enzyme may now undergo a reversal of the steps A → B → C, in a transpeptidation reaction (D → E → F). Alternatively, hydrolysis of the amino-enzyme may occur, the reaction being catalysed by the neighbouring undissociated carboxyl group. The hydrolysis of the amide of phthalamic acid (Bender et al. 1958b) is catalysed by the neighbouring undissociated carboxyl group in this molecule, and may represent a close chemical model of the enzymic hydrolysis. For phthalamic acid, hydrolysis of the amide is about 10⁴-fold faster at pH 3 than that of the unsubstituted material, benzamide. Catalysis of amino-enzyme breakdown by a neighbouring carboxyl group answers the question of what is the kinetic advantage of a catalyst breaking one peptide link (of the substrate) and simply making another (of the amino-enzyme). What is proposed is that the "leaving group transfer" process catalyses the formation of an intermediate which is itself much more susceptible to hydrolysis than is the substrate.

Finally, we are left with the inclusion of the ¹⁸O-exchange activity of pepsin. As has been pointed out, if the failure of the methanol-trapping experiments is accepted at face value, then water should not be explicitly involved in the ¹⁸O-exchange reaction. Since the enzyme undoubtedly catalyses this reaction, the ¹⁸O must enter the virtual substrate (N-acylamino acid) via the enzyme itself. The formulation of ¹⁸O-exchange then falls easily into the framework of Scheme 1. The rapid exchange of an enzyme carboxyl group (or groups) directly with H₂¹⁸O (G → H) is succeeded by the steps I → J → K, which are exactly analogous to the hydrolytic (A → D) and transpeptidation (D → F) covalency changes. Scheme 1 demands that the rate of ¹⁸O loss from ¹⁸O-labelled pepsin is the same as the rate of ¹⁸O uptake by •- COOH. For the virtual substrate N-acetyl-l-phenylalanine,
this is indeed the case (Shkarenkova et al. 1968).

In summary, it appears that at this time Scheme 1 is the simplest formulation that satisfactorily accommodates all the data which bear on the mechanism of pepsin catalysis.

REFERENCES