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On the Mechanism of Action of Pepsin

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On the mechanism of action of pepsin

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The present status of the mechanism of action of pepsin is summarized, and a mechanism compatible with the hydrolytic, transpeptidation, and isotopic exchange activities of this enzyme is presented.

Despite a number of formulations (see, for example, Delpierre & Fruton 1965; Bender & Kezdy 1965; Bruice & Benkovic 1966), no completely acceptable proposal for the mechanism of action of pepsin has been put forward. In this paper, an attempt is made to collect and assess the data which bear directly on the mechanism of pepsin, and to present the evidence and organic chemical analogy for a scheme which rationally accommodates all the facts relating to the mechanism of this enzyme. The scheme proposed is similar to one put forward in outline by Delpierre & Fruton (1965), who suggested 'that a single carboxylate group ($ECOO^-$) of pepsin attacks the carbonyl carbon of the protonated amide ($RCONHR'$) to form reversibly a tetrahedral intermediate...(which)...is thought to undergo a reversible four-centre exchange reaction leading to the expulsion of RCOOH and the formation of $ECONHR'$. The reaction of this product with water (or a carboxylic acid) would liberate NH_2R' (or lead to transpeptidation by 'imino transfer') with the regeneration of $ECOO^-$.'

The pathway

(*a*) *The amino-enzyme*

The work of Neumann, Levin, Berger & Katchalski (1959) and of Fruton, Fujii & Knappenberger (1961) demonstrated that an intermediate in pepsin catalysed transpeptidation reactions is the amino-enzyme: pepsin-NH-Y, which arises during the cleavage of a peptide X–CO–NH–Y, by pepsin. The nature of the transpeptidation products rules out transpeptidation via an acyl-enzyme, and demands that the amino-enzyme lives long enough for the X–COOH moiety to leave, and X′–COOH to act as the amine acceptor resulting in the synthesis of the new peptide X′–CO–NH–Y. The concurrency of hydrolysis and transpeptidation processes, the enzyme specificity (for different amino acids) towards amino group acceptors (Mal'tsev, Ginodman, Orekhovich, Valueva & Akimova 1966), and analogy with the neutral proteinases, argue for a common mechanism for hydrolysis and transpeptidation. The amino-enzyme has a choice of two routes, one hydrolytic, releasing the amino component of the peptide: N^+H_3–Y, and the other essentially a reversal of the half hydrolysis, releasing the new peptide: X′–CO–NH–Y.

The kinetics of inhibition by products and product analogues (Greenwell, Knowles & Sharp 1969; Inouye & Fruton 1968) confirm the assumption that an amino-enzyme is an intermediate in the *hydrolytic* reaction. It has been shown that the non-competitive inhibition of the hydrolysis of X–CO–NH–Y by X–COOH coupled with the competitive nature of the inhibition by

N^+H_3–Y, point to a mechanism involving the ordered release of the two products in the hydrolysis, with X–COOH being released first. The evidence for the existence of an amino-enzyme intermediate in both types of pepsin catalysed reactions of peptide substrates is thus firm.

(*b*) *The acyl-enzyme*

A further important characteristic of pepsin which has been believed to bear on the question of the reaction intermediates is the fact that the enzyme catalyses the exchange of ^{18}O between the free carboxyl group of acylamino acids, and H_2O. That this process is truly enzyme catalysed, is indicated by the following: (i) the rate of exchange is proportional to the pepsin concentration, (ii) the reaction shows enzyme specificity (different amino acids exchange at different rates: Kozlov, Ginodman & Orekhovich 1967) and stereospecificity (*N*-benzyloxycarbonyl-L-phenylalanine exchanges, but its D enantiomer does not: Sharon, Grisaro & Neumann 1962), and (iii) there is a correlation between the ^{18}O exchange rate for different acylamino acids and the activity as imine acceptors in transpeptidation (Ginodman, Kolzlov, Mal'tsev & Orekhovich 1964). By analogy with the neutral proteases, e.g. α-chymotrypsin (Sprinson & Rittenberg 1951) and papain (Grisaro & Sharon 1964), these data were taken to indicate the intermediacy of an acyl-enzyme in the ^{18}O exchange reaction. Moreover, the above data were reasonably discussed on the basis that the mechanism for ^{18}O exchange follows a similar path, *mutatis mutandis*, to hydrolysis and transpeptidation. As a result of such studies, it was postulated that the enzyme combines with both acyl- and amino-moieties of the susceptible peptide bond (e.g. Sharon *et al.* 1962; Bender & Kezdy 1965). However, attempts to trap the putative acyl-enzyme by reaction with highly radioactive methanol both in systems allowing 'partial' reaction (e.g. pepsin + *N*-acetyl-L-phenylalanine) or in systems allowing hydrolysis (e.g. pepsin + *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine), have failed (Cornish-Bowden, Greenwell & Knowles 1969). Such failure does not, of course, disprove the intermediacy of an acyl-enzyme, but analogy with the neutral proteases, for which methanol is a very effective trap of acyl-enzymes (e.g. Inward & Jencks 1965; Lowe & Williams 1965), demands a reappraisal of the acyl-enzyme postulate in the case of pepsin. That is, can specific, enzyme catalysed ^{18}O exchange be accounted for without the intermediacy of an acyl-enzyme? Recent work of Shkarenkova, Ginodman, Kozlov & Orekhovich (1968) supports what is on other grounds an attractive alternative route for ^{18}O exchange (see below).

The rate determining step

Since we can say that pepsin catalysed reactions do not occur via a single transition state, but are mediated by an amino-enzyme, it is logical to ask whether the intermediate(s) occur before or after the rate determining step of the overall hydrolysis reaction. In principle, intermediates which occur before the rate determining step of a reaction are detectable either by the direct observation of the steady state concentration or by following the pre-steady state reaction during which time the rates of formation and destruction of the intermediate govern the rate of attainment of the steady state. Intermediates occurring after the rate determining step cannot, of course, normally be observed directly, but their existence may often be inferred from trapping experiments, in which one expects to see product diversion with no change in the rate of loss of substrate. For enzyme catalysed reactions, other approaches are possible. For

instance, the identity of catalytic rate constants for a series of substrates XY, XY′, XY″ can implicate a common intermediate, enzyme–X, which does not involve the varied group Y (see, for example, Zerner, Bond & Bender 1964).

In the case of pepsin we can cite the following, as evidence that the amino-enzyme occurs *after* the rate determining step of the hydrolytic reaction. (i) No 'burst' of the carboxyl product of a dipeptide substrate is observable. (This is true at least for N-benzyloxycarbonyl-L-histidyl-*p*-nitro-L-phenylalanyl-L-phenylalanine methyl ester: Inouye & Fruton 1967.) Since release of the amino moiety of the substrate occurs after liberation of the carboxyl moiety, this indicates that the release of both products occurs after the rate determining step. Attempts have been made to find substrates for which the first slow step would be speeded up, in the hope of observing a change in the rate determining step of the hydrolysis, such as is seen for some of the neutral proteases on going from amide substrates to ester substrates (see, for example, Zerner *et al.* 1964). These attempts were unsuccessful (Cornish-Bowden *et al.* 1969), for reasons which will be apparent from the subsequent discussion. (ii) Within sets of dipeptide substrates of the general type: X–CO–NH–Y, if X is kept the same and Y is varied, or if Y is kept the same and X is varied, values of the catalytic rate constant differ markedly (Inouye & Fruton 1968; Cornish-Bowden & Knowles 1969). This indicates that there is no reaction intermediate common to either set, the breakdown of which is rate determining. (iii) As has been argued before (for a recent summary, see Denburg, Nelson & Silver 1968), it appears that for pepsin catalysed hydrolysis of peptide substrates, K_m represents the thermodynamic dissociation constant of the enzyme–substrate complex, K_s. This condition can be satisfied if the step immediately following enzyme–substrate complex formation in the mechanistic pathway, is rate determining.

Since we can say that the amino-enzyme is formed subsequently to the rate determining step, it is pertinent to ask whether this intermediate can be trapped in the classical manner, with the observation of product diversion with no change in rate of loss of substrate. The requirement is for a reagent which will react faster with the amino-enzyme than water. For an enzyme catalysed reaction, it may not be possible to divert an intermediate since the naïve application of organic chemical principles may fail for a reaction which presumably involves the tight and precise approximation and orientation of catalytic groups and of water, in the enzymic intermediate. We have made two attempts to trap the amino-enzyme: one using radioactive methanol as a water analogue, and the other using a material designed to be peculiarly susceptible to –NH– group attack in transpeptidation. If methanol acts as a water analogue, then the expectation is that the methanolysis of the amino-enzyme (pepsin—CO–NH–Y) would lead to enzymically inactive, radioactive enzyme (pepsin–CO–$O^{14}CH_3$), plus the usual product N^+H_3–Y. However, from our experiments (Cornish-Bowden *et al.* 1969) and those of Tang (1965), it is clear that methanol does not inactivate pepsin in the presence of substrate. (It is possible, of course, that the product: pepsin–CO–$O^{14}CH_3$ *is* formed, and that this is rapidly hydrolysed, possibly by a mechanism analogous to that seen for half esters of phthalic acid (Bender, Chloupek & Neveu 1958; see also the discussion of amino-enzyme hydrolysis, below). In this case, any trapping action of methanol would have been unobservable.) The second attempt to trap the amino-enzyme was based on the fact that diversion of the amino-enzyme from hydrolysis into transpeptidation is an established phenomenon. Transpeptidation must involve attack by the –NH– group of the amino-enzyme on the carboxyl group of the acceptor amino acid, and it was hoped to increase the rate of this process by using a thiol ester, since such esters are known to be very much more suceptible to attack by nitrogen nucleophiles than

are their oxygen analogues. While the thiol ester behaved—as expected—as a non-competitive inhibitor of the hydrolytic reaction (Greenwell *et al.* 1969), its effectiveness was not dramatically different from that of its oxygen analogue. Moreover, no methane thiol was liberated, demonstrating that the thiol ester does not act as the hoped for amino-enzyme trap. The reason for this may simply be that the spatial requirements for transpeptidation are strict enough to prevent a thiomethyl ester acting as an analogue for a carboxylic acid, or that the mechanism of the covalency changes in pepsin catalysed reactions is such that the treatment of the –NH– group of the amino-enzyme as a nucleophilic centre is erroneous.

The nature of the rate determining step

It has been pointed out by many workers that the neutral proteinases appear mechanistically to resemble base or nucleophilic catalysed reactions (see, for example, Zerner *et al.* 1964). Although such statements can be misleading, and are often based on rather ill-defined premises, it is instructive to draw reasonable parallels with non-enzymic data. Two points merit discussion here: the apparent resemblance of pepsin catalysed processes to acid rather than base catalysed reactions, and the absence of a solvent kinetic isotope effect.

Table 1. Relative rates of catalysed hydrolysis of esters and amides

catalyst	ester	amide	k_{ester}/k_{amide}
OH^-	methyl acetate	acetamide	3400†
H_3O^+	methyl acetate	acetamide	9†
α-chymotrypsin	*N*-acetyl-L-tryptophan methyl ester	*N*-acetyl-L-tryptophan amide	1000‡
pepsin	*N*-benzyloxycarbonyl-L-histidyl-*p*-nitro-L-phenylalanyl-L-β-phenyllactic acid methyl ester	*N*-benzyloxycarbonyl-L-histidyl-*p*-nitro-L-phenylalanyl-L-phenylalanine methyl ester	2§
carboxypeptidase A	hippuryl-DL-β-phenyllactic acid	*N*-benzoylglycyl-L-phenylalanine	4‖

References: † Tables of Chemical Kinetics (1951). ‡ Zerner *et al.* (1964). § Inouye & Fruton (1967). ‖ Davies, Riordan, Auld & Vallee (1968).

For a neutral proteinase such as α-chymotrypsin, the rate of acylation of the enzyme is very much faster for esters than it is for amides or peptides, which parallels the much greater reactivity of esters than amides to nucleophilic attack by, for instance, hydroxide ion (see table 1). Indeed, the difference between the base catalysed rates of hydrolysis of alkyl esters and corresponding amides is commonly between 10^3 and 10^4. The ratio of rates of acylation of α-chymotrypsin by alkyl esters and analogous amides is certainly greater than 10^3 (Zerner *et al.* 1964). By contrast, the ester:amide hydrolysis rate ratios for non-enzymic acid catalysed reactions are commonly less than 10. It is significant, therefore, that the only analogous ester:amide pair the pepsin catalysed hydrolysis of which has been studied (Inouye & Fruton 1967), has a rate ratio of 2. (Peptidase activity of pepsin cannot be detected for simple acylamino acid amides, nor is esterase activity detectable for simple acylamino acid esters (H. C. Sharp, unpublished experiments).) This fact, that esters are not hydrolysed by pepsin very much faster than peptides, leads to the tentative conclusion that the rate determining step of pepsin catalysed

hydrolysis reactions owes more to acid-type catalysis than to mechanisms of the base or nucleophilic type. For an enzyme the pH optimum of which is as low as 3, this is hardly a surprising conclusion, though it underscores one of the fundamental differences between pepsin and the neutral mammalian proteinases. Carboxypeptidase A is also included in table 1, and like pepsin appears to be dominated by electrophilic catalysis rather than nucleophilic. Again, this is not surprising, since it is known from crystallographic studies (Reeke, Hartsuck, Ludwig, Quiocho, Steitz & Lipscomb 1967) that the oxygen atom of the susceptible carbonyl link is a ligand for the 'super-acid', zinc.

The effect of D_2O on the catalytic rate of enzyme catalysed reactions has been used to throw light on mechanism: in the case of α-chymotrypsin in an attempt to distinguish between general base and nucleophilic mechanisms (Bender, Clement, Kezdy & Heck 1964). However, even when the more obvious pitfalls are avoided, and checks are made on the effect of D_2O on protein conformation and experiments performed at pH (pD) values well clear of the pK_a values of functional ionizing groups on the enzyme, deductions from the size of the $k_0(H_2O)/k_0(D_2O)$ ratio are infirm. This is because—particularly for a hydrolytic enzyme for which water is one of the substrates—the following effects are either not defined, or are undefinable: the effect on pK_a values of catalytically important groups, the effect of changing the solvating medium on the 'nucleophilicity' of functional groups and on the stability of transition states and intermediates, the effect on the activity of bound substrate water, and the effect on the local conformation of the enzyme functionalities. These, and other, uncertainties make the interesting observation that the maximum rate of pepsin catalysed hydrolysis is unaffected by D_2O (Clement & Snyder 1966), hard to interpret. That is, if the apparent *size* of the isotope effect is believed to be a dangerous criterion for mechanism, then the apparent *absence* of a solvent isotope effect should not form the basis for mechanism either. Moreover, the apparent resemblance of pepsin catalysed reactions to acid catalysed processes makes a mechanism involving no rate determining proton transfers (which is what has been proposed on the basis of the absence of solvent isotope effect: Clement & Snyder 1966), less attractive.

The nature of the catalytic groups

The identification of enzymic groups directly involved in the covalency changes accompanying catalysis is a notoriously dangerous activity. Yet the combination of pH dependence data (which bear directly on the enzymic activity), and the use of active site directed irreversible inhibitors (which select uniquely reactive groupings at the active site), has produced results, e.g. for α-chymotrypsin and papain, which look encouraging when faced with the protein crystallographic information. The activity of pepsin depends on the ionization of two groups on the enzyme, of apparent pK_a 1.0 and 4.7. Possible candidates for these ionizations are the peptide link itself, carboxyl groups, and histidine. Pepsin contains no cysteine, the necessary perturbation for amino or phenolic groups would be enormous, and the single serine phosphate in pepsin is irrelevant to the catalytic activity (Perlmann 1952). Modification of pepsin by various reagents (e.g. ketene, acetylimidazole, *N*-bromosuccinimide, iodoacetate, etc.) has indicated that while modification of tryptophan and tyrosine residues affects the catalytic activity, the acylation of amino groups, the alkylation of methionine, and the photo-oxidation of histidine, do not affect the activity of the enzyme. We are left, therefore, with carboxyl groups, and very strong acids such as the protonated peptide link.

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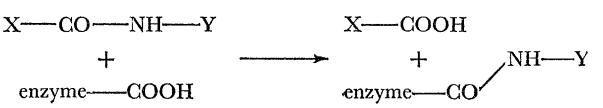
Single carboxyl groups in pepsin have recently been modified by a number of workers (see Bayliss, Knowles & Wybrandt 1969), and it appears that one aspartyl residue (in a sequence Ile-Val-Asp-Thr-Gly-Thr-Ser: Bayliss *et al.* 1969) is essential for the catalytic activity, and that modification of a second (in a portion of the chain of composition: Gly_2,Asp,Ser,Glu: Erlanger, Vratsanos, Wassermann & Cooper 1965) causes very significant loss of catalytic activity. It can definitely be stated that at least one carboxyl group is required for the catalytic action of pepsin and it is likely that this is one or other of the groups of apparent pK_a 1.0 and 4.7. The group of pK_a 1.0 is hard to assign, for this pK_a lies somewhere between the 'normal' pK_a values of peptide links and carboxyl groups, and either of these would require significant perturbation. If this group is a peptide link, then the lower pK_a would have to relate to the *acid* catalyst (see below), and the upper, to a carboxyl group required in the ionized form. This is very unsatisfying in efficiency terms, for it means that a very small proportion indeed of the enzyme would be in the catalytically active ionization state, even at pH 2.8. On the other hand, if this strong acid is a very strong carboxyl group, why is it so strong? Two possible reasons suggest themselves: either the carboxylate group can form a stronger bond with a hydrogen bond donor than can the carboxyl group, or the lower pK_a arises from the proximity of the carboxylate group to a positive charge (or the positive end of a dipole). The fact that acylation of amino groups and oxidation of the single histidine does not affect the catalytic activity, militates against the latter possibility. However, it is probable that a carboxylate group hydrogen bonds more strongly to a hydrogen bond donor than does a carboxyl group and this possibility can—at least in principle—account for the low pK_a of 1.0. [The above phenomenon presumably accounts in part for the low pK_1 values for such acids as malonic and phthalic acids.] In the subsequent discussion, we shall assume that both the observed ionizations refer to carboxyl groups.

Mechanistic proposals

On the basis of the foregoing, we are in a position to set up a tentative mechanism for the catalytic reactions mediated by pepsin. As a working hypothesis, we suggest that the active site of pepsin contains two carboxyl groups which are primarily responsible for the covalency changes occurring during catalysis. The most reasonable form of the amino-enzyme (which is the first formed intermediate subsequently to the enzyme–substrate complex) is then an amide, and the overall course of the first step of the breakdown of the enzyme–substrate complex is therefore a transacylation of an amine:



This path is inescapable, since we have to accommodate the phenomenon of transpeptidation by amino transfer which requires that the carboxyl moiety of the substrate be released first, *and* that the amino moiety remain bound to the enzyme. This reaction is not as unattractive as it appears at first sight, and possibly the best model for it is the rearrangement of aspartyl peptides from α-linked to β-linked, which is a facile reaction in acid solution (Hill 1965). Mechanistically this reaction is believed to proceed via an α, β-aspartyl cyclic diacylamine:

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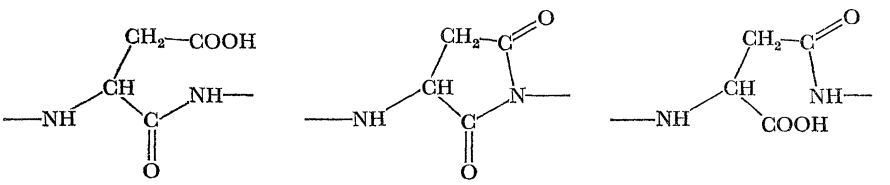
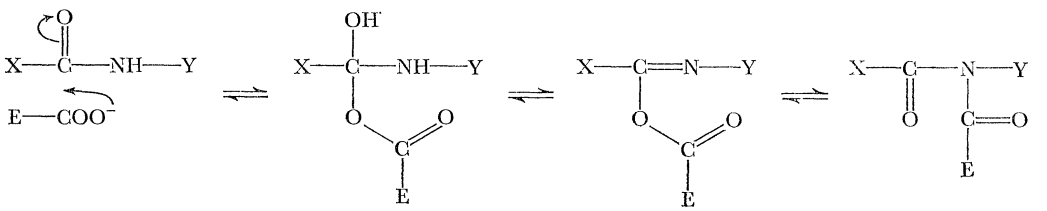


α-form α, β-form β-form

The formation of the diacylamine could in principle occur by attack of the (very poorly nucleophilic) amido nitrogen on the carboxyl group, in the usual manner of nucleophilic attack at the carbonyl group of acids and their derivatives (Bender 1960). However, a more attractive formulation is attack by the carboxylate anion on the amide group, resulting in a tetrahedral intermediate (I). (This step may be rendered more facile enzymically, by pretransition state protonation of the type suggested by Wang (Wang & Parker 1967). In this case, the carboxylate group would attack a protonated amide link.) If the α, β-peptide rearrangement of aspartyl peptides is taken as a model, then the tetrahedral intermediate (I) must break down to the diacylamine (III). The most logical route for this is via a dehydration by β-elimination to II, which now undergoes an O to N acyl shift exactly in the manner observed in reactions of diimides with carboxyl groups (Khorana 1953).

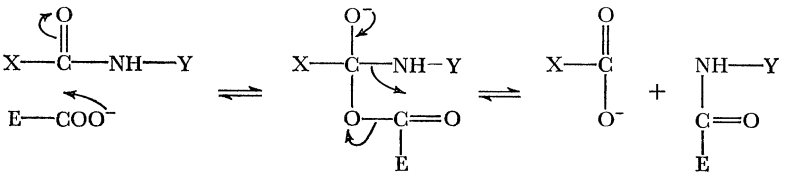


(I) (II) (III)

Two pieces of evidence militate against this formulation. First, the mechanism analogous to the above which is required to explain the catalysis of ^{18}O exchange with acylamino acids, would involve an acid anhydride intermediate which it is hard to imagine would not be trapped by $^{14}CH_3OH$. Secondly, if a diacylamine is a reaction intermediate which is then hydrolysed, we should expect to observe some competitive methanolysis in the presence of $^{14}CH_3OH$. As has been discussed above, none is found (Cornish-Bowden *et al.* 1969). (In addition it is very possible that the nucleophilic carboxylate group is that with the very low pK_a (about 1.0), and it is known that for unsymmetrical diacylamines (such as III) hydrolysis preferentially liberates the free carboxyl group of lower pK_a (Lamberton & Standage 1960). Hydrolysis of the diacylamine III is therefore expected preferentially to liberate E–COOH (regenerating the enzyme) and X–CO–NH–Y (regenerating the substrate).) The diacylamine hypothesis, therefore, does not appear to be very attractive, although it cannot be ruled out on the basis of the above. A further possibility, which should be considered here, is that the tetrahedral intermediate (I) *does* break down to give an acyl-enzyme (X–CO–O–CO–E), in which X is attached to the protein by an anhydride linkage. This would be very rapidly attacked by the nitrogen nucleophile NH_2–Y which has just been released by the breakdown of I. While it is known that amines attack anhydrides very much faster than does water (or, for that matter, alcohols like [^{14}C]methanol),

the more susceptible carbonyl link for amine attack would be that adjacent to X, rather than that adjacent to E. This would simply result in a reversal of the steps back to the enzyme–substrate complex. As with the diacylamine hypothesis presented above, this route is unattractive, but cannot be ruled out at this time.

As an alternative mode of breakdown of the tetrahedral intermediate, one can envisage a four centre reaction which forms the amino-enzyme without the intermediacy of an acyl-enzyme.



(IV) (V)

Such a four centre reaction is analogous to that involved in the II → III conversion above. Essentially this route has been proposed by Delpierre & Fruton (1965), as an alternative to a rather similar suggestion by Bender & Kezdy (1965) which involved an anhydride linkage between two enzymic carboxyl groups. This latter theory is not compatible with the analysis of the pH dependence data of Denburg *et al.* (1968), nor is it supported by experiments performed in the presence of radioactive methanol (Cornish-Bowden *et al.* 1969) or work on ^{18}O exchange of the free enzyme (Shkarenkova *et al.* 1968). However, it is of interest that Erlanger, Vratsanos, Wasserman & Cooper (1966) have reported that native pepsin contains two ester linkages (though these apparently involve glutamic acid residues), and the anhydro-enzyme postulate cannot definitely by ruled out. On the other hand, Gross & Morell (1966) apparently do not find any ester linkages in the native enzyme. This discrepancy is presently under investigation.

At this point, one may justifiably ask what is the kinetic advantage of breaking the substrate peptide link, only to form another peptide link with the enzyme? Here, the second postulated carboxyl group is of obvious importance, since the experiments of Bender, Chow & Chloupek (1958) have shown that the hydrolysis of the amide link of phthalamic acid is catalysed by the undissociated adjacent carboxyl group. The hydrolysis of phthalamic acid depends on the undissociated carboxyl group between 1 and 5, and at pH 3 is about 10^5-fold faster than the unsubstituted material, benzamide. This appears to be a splendid model for the hydrolysis of the amino-enzyme, and completes the rationale for the two ionizing groups in the free enzyme upon which the catalytic activity depends. Thus there is required a nucleophile for attack at the susceptible peptide link possibly provided by the postulated carboxylate group with the rather low pK_a of 1.0, and an undissociated carboxyl group for catalysis of the hydrolysis of the amino-enzyme, possibly identifiable with the ionizing group on the enzyme of pK_a 4.7. The postulate of an undissociated carboxyl group also enables us to reconcile this mechanism with the fact that pepsin catalysis apparently resembles an acid catalysed process. The scheme above involves a simple nucleophilic attack (leading to IV), and would surely not parallel acid rather than nucleophilic catalysis as it stands. However, the inclusion of acid catalysis by an undissociated carboxyl group, where the extent of substrate protonation at the transition state is significant (even if not complete: Wang & Parker 1967), embraces the acidic characteristic of pepsin catalysis.

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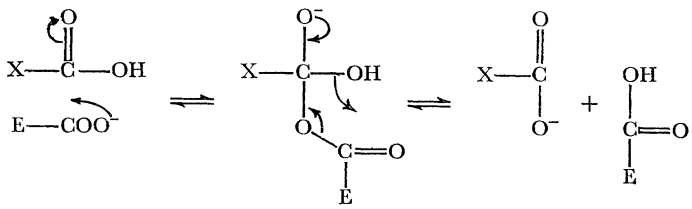
One loose end remains, which has provided most of the trouble in earlier formulations of pepsin mechanisms. That is, how is the ^{18}O exchange between H_2O and acylamino acids accommodated by this mechanism, especially since we have agreed above on a common reaction scheme for the three activities of pepsin: hydrolysis, transpeptidation, and ^{18}O exchange? We have already noted the dilemma that ^{18}O exchange occurs apparently *without* the intermediacy of an acyl-enzyme. The scheme proposed, with an acylamino acid instead of a peptide, as substrate, becomes:



Since H_2O is not explicitly involved in these steps, ^{18}O exchange with $H_2{}^{18}O$ cannot occur *unless the enzyme carboxyl group(s) become labelled first.* This interesting experiment has recently been carried out, and Shkarenkova *et al.* (1968) have found that ^{18}O is rapidly incorporated from $H_2{}^{18}O$ into active site carboxyl group(s) of pepsin. Most importantly, the *rate* of ^{18}O loss from previously ^{18}O labelled pepsin is very close to the pepsin catalysed rate of ^{18}O incorporation into *N*-acetyl-L-phenylalanine. It is thus tempting to suggest that the way that ^{18}O gets into *N*-acetyl-L-phenylalanine is via the active site carboxyl group(s) of pepsin itself. It is not profitable yet to speculate how the active site carboxyl groups incorporate ^{18}O so much more rapidly than others in the protein, although carboxyl group catalysis of hydrolytic processes of acid derivatives is well known (see, for example, Bender, Chow & Chloupek 1958; Bender, Chloupek & Neveu 1968; Capon 1964).

We may summarize the suggestions made by referring to figure 1. The enzyme's active site is postulated to contain two functional groups, one of which has a normal pK_a of about 4.7 and the other of which has a perturbed pK_a of 1.0. This is in accord with the pH dependence of the catalytic reaction (Denburg *et al.* 1968; Cornish-Bowden & Knowles 1969) and of the binding of competitive inhibitors (Knowles & Sharp 1969). Formation of the tetrahedral intermediate (VII) is an acid-nucleophilic catalysis, with acid catalysis dominating the bond forming processes at the transition state. Although the step VI → VII assumes protonation on amide oxygen, there are presently no data to indicate whether this is the case, or whether nitrogen is the proton acceptor. Thermodynamic arguments to the effect that oxygen is the more basic site than nitrogen in amides (Gillespie & Birchall 1963) are not of course relevant to the kinetic situation at issue here. A hydrogen bond (presumably the precursor of proton transfer in the reaction) in either position is consistent with the observed inhibition of hydrolysis of amino-enzyme (overall non-competitive inhibition) observed both for *N*-acetyl-L-phenylalanine ethyl ester (Greenwell *et al.* 1969) and *N*-acetyl-L-phenylalaninamide (H. C. Sharp, unpublished experiment.)

The four centre reaction (VII → VIII) results in the formation of the amino-enzyme and the carboxyl moiety of the substrate is now lost (VIII → IX). This fits with the experiments on the nature of transpeptidation (Neumann *et al.* 1959; Fruton *et al.* 1961) and on the kinetic requirement for the hydrolysis reaction that the carboxyl moiety be released first (Greenwell *et al.* 1969). The amino enzyme (IX) can bind an acylamino acid acceptor specifically (Mal'tsev *et al.* 1966)

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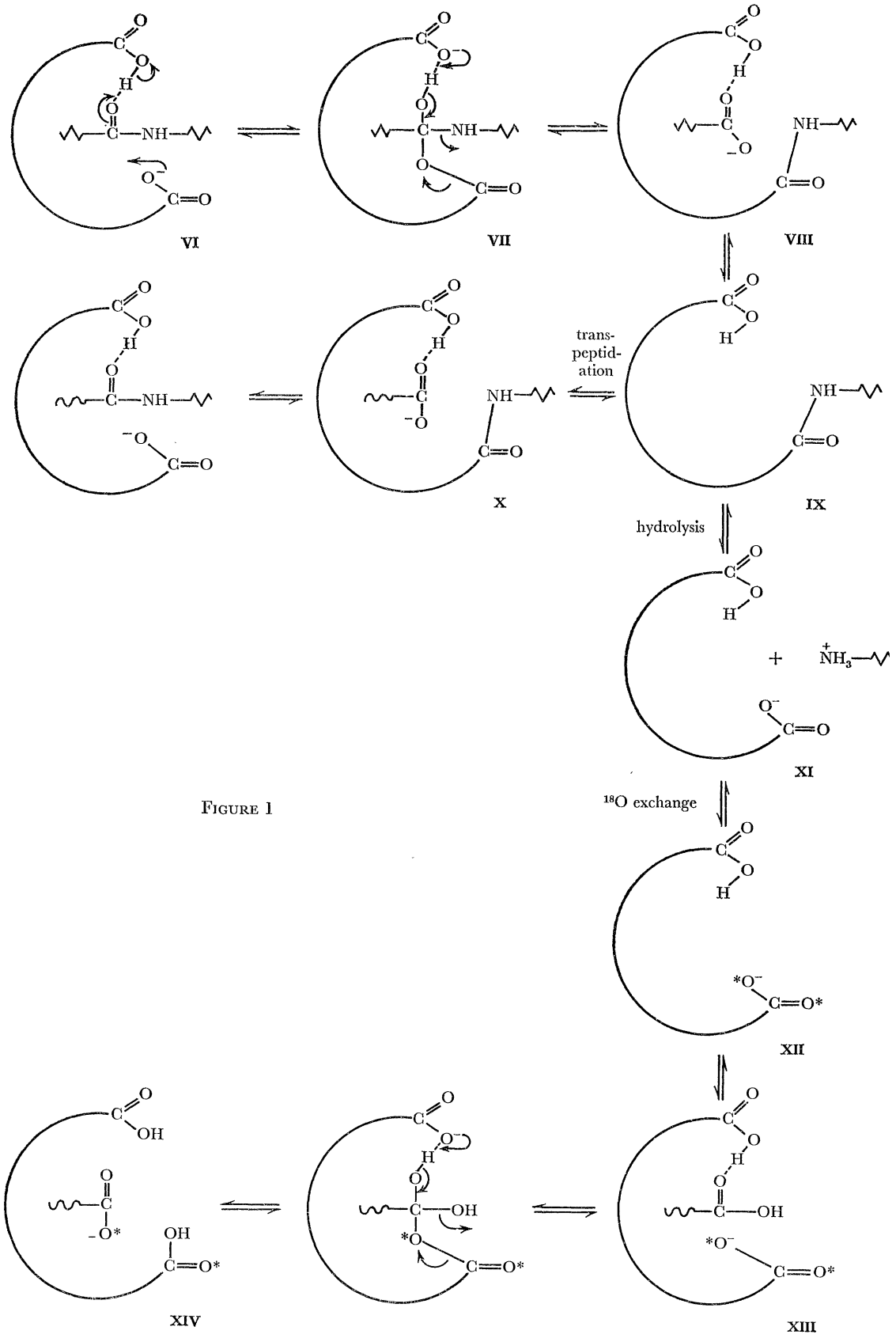

FIGURE 1

(as in X), and a reversal of VI → VII → VIII results in transpeptidation and the synthesis of a new peptide. The amino-enzyme can also hydrolyse (IX → XI), the organic model for this process being that of phthalamic acid hydrolysis at low pH (Bender, Chow & Chloupek 1958). The neighbouring carboxyl group in the free enzyme (XI) can catalyse the exchange of ^{18}O between the active site carboxyl groups and $H_2{}^{18}O$, resulting in a labelled enzyme (XII) (Shkarenkova *et al.* 1968). This enzyme can bind acylamino acids specifically (Sharon *et al.* 1962; Kozlov *et al.* 1967), resulting in the incorporation of ^{18}O into the acylamino acid, by a route (XIII → XIV) exactly analogous to the catalytic steps VI → VIII, and *not* involving an acyl-enzyme at any stage (Cornish-Bowden *et al.* 1969).

Finally, it must be pointed out that the present proposals have not dealt with the question of enzyme specificity. The primarily hydrophobic nature of the binding sites in pepsin has been well demonstrated, most recently and in most quantitative form by Inouye & Fruton (1967). Although for enzymic reactions, questions of rate and specificity cannot be separated, the time is clearly not yet ripe in the case of pepsin, for any detailed analysis of the contribution of kinetic specificity to the overall catalytic rate (see, for example, Ingles & Knowles 1967, 1968).

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