The Pathway of Pepsin-Catalysed Transpeptidation

EVIDENCE FOR THE REACTIVE SPECIES BEING THE ANION OF THE ACCEPTOR MOLECULE

By T. M. KITSON and J. R. KNOWLES

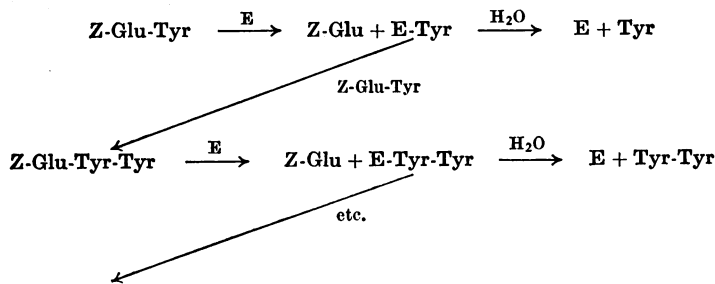*Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, U.K.*

1. The inhibition of pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by the acyl product and product analogues was studied at pH 4.3. 2. The acyl product, *N*-acetyl-L-phenylalanine, gives rise to linear competitive inhibition at pH 4.3, whereas at pH 2.1 it shows linear non-competitive behaviour. 3. The extent of transpeptidation to *N*-acetyl-L-[^{3}H]phenylalanine during the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine is significant at pH 4.7, but is undetectable at pH 1.3. 4. Both the inhibition and transpeptidation experiments are consistent with the anion of the acceptor molecule being the substrate in pepsin-catalysed transpeptidation. This conclusion supports the formulation of pepsin-catalysed reactions put forward by Knowles *et al.* (1970).

The first detailed study of the transpeptidation reaction catalysed by pepsin was made by Neumann, Levin, Berger & Katchalski (1959). These workers demonstrated that during the pepsin-catalysed hydrolysis of substrates such as *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine unexpected products such as *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine and L-tyrosyl-L-tyrosine appear in the reaction mixture. No such products could be detected when the substrate had no free α-carboxyl group and it appeared that a free α-carboxyl acceptor group is essential for the transpeptidation reaction to occur.

On the basis of these and other results Neumann *et al.* (1959) suggested that pepsin-catalysed transpeptidation occurs via amino (as distinct from

acyl) group transfer, and that this is most economically formulated as occurring via an 'amino-enzyme', as shown in eqn. (1) (Scheme 1).

The isotopic-tracer studies carried out by Fruton, Fujii & Knappenberger (1961) reinforced the above conclusion. They incubated with pepsin (*a*) a solution of benzyloxycarbonyl-L-tyrosyl-L-tyrosine, benzyloxycarbonyl-L-[^{14}C]tyrosine and L-tyrosine, and (*b*) a solution of benzyloxycarbonyl-L-tyrosyl-L-tyrosine, benzyloxycarbonyl-L-tyrosine and L-[^{14}C]tyrosine; after termination of the reaction, separation of the remaining substrate and debenzyloxycarbonylation, the L-tyrosyl-L-tyrosine obtained from (*a*) showed significant incorporation of radioactivity into its *N*-terminal residue, whereas that from (*b*) did not.

$$\text{Z-Glu-Tyr} \xrightarrow{\text{E}} \text{Z-Glu} + \text{E-Tyr} \xrightarrow{\text{H}_2\text{O}} \text{E} + \text{Tyr}$$

Scheme 1. Possible reactions in pepsin-catalysed transpeptidation involving an 'amino-enzyme' (Neumann *et al.* 1959). Abbreviation: Z, benzyloxycarbonyl.

Thus it appears that in the pathway of pepsin-catalysed transpeptidation there occurs an intermediate amino-enzyme, conveniently though by no means axiomatically formulated as a covalently linked species E–NH–Y (arising from the catalysed cleavage of X–CO–NH–Y). The intermediacy of such a species in the hydrolytic reaction can be investigated by studying the nature of inhibition of the hydrolytic reaction by a product or product analogue. This approach was taken in the preceding paper (Kitson & Knowles, 1971) and the results indicate that in the hydrolytic reaction there is non-random release of products, the order of which is consistent with the amino-enzyme postulate. Moreover, the simplest explanation for the observed linear non-competitive inhibition of the hydrolysis of N-acetyl-L-phenylalanyl-L-phenylalanylglycine by N-acetyl-L-phenylalanine (the acyl product) was if this molecule were binding non-productively both to the free enzyme and to the amino-enzyme intermediate. One reason for the lack of productive binding to the amino-enzyme would be if, at pH 2.1, N-acetyl-L-phenylalanine were in an inappropriate ionization state, i.e. if the first released product of the hydrolysis were the anion of N-acetyl-L-phenylalanine. This superficially surprising conclusion is actually required by the outline mechanism for pepsin-catalysed reactions put forward by Knowles et al. (1970), and the point is clearly an important one.

It was therefore decided to reinvestigate the inhibition of pepsin-catalysed hydrolysis at pH values above the pK_a of the inhibitor, at which the inhibition is likely to be primarily due to the presence of the inhibitor anion.

The problem can be restated in terms of the transpeptidation process: is the acceptor in such a process $R–CO_2H$ or $R–CO_2^-$? As a check on the results obtained from the study of inhibition, direct measurements of the extent of transpeptidation at pH values well above and well below the pK_a of the carboxyl group of the acceptor have been made. This was done by using radioactively labelled acyl product as acceptor and investigating the incorporation of radioactivity into remaining substrate.

MATERIALS

Pepsin, N-*acetyl*-L-*phenylalanyl*-L-*phenylalanylglycine,* N-*acetyl*-L-(*and* D-)*phenylalanine,* N-*acetyl*-L-(*and* D-)-*phenylalanine ethyl ester and* N-*acetyl*-L-(*and* D-)*phenylalanine amide.* These were obtained as described in the preceding paper (Kitson & Knowles, 1971).

N-*Acetyl*-L-[³H]*phenylalanine.* This compound was prepared from L-[³H]phenylalanine in the way described (for the unlabelled material) by Greenwell, Knowles & Sharp (1969); it had m.p. 169–171°C and $[\alpha]_D^{20}$ +46.1° (c 2 in ethanol). Coffy, Green & Kenner (1959) give m.p. 169–170°C and $[\alpha]_D^{20}$ +47.1° (c 2 in ethanol).

N-*Acetyl*-L-[³H]*phenylalanine amide.* N-Acetyl-L-[³H]-phenylalanine (0.97 g) was dissolved in dry ethanol (10 ml), and the solution was cooled in ice. Dry HCl was passed into the solution until saturation. The reaction mixture was left overnight at 0°C and then evaporated to dryness. The resulting oil was recrystallized from ethanol–water as fine white needles. The yield of N-acetyl-L-[³H]-phenylalanine ethyl ester was 0.86 g (65% yield); it had m.p. 89.5–90.5°C. Caplow & Jencks (1964) give m.p. 89.5–91°C. N-Acetyl-L-[³H]phenylalanine ethyl ester was converted into the amide by dissolving it in dry methanol that had previously been saturated with NH_3 and cooled to 0°C. After standing in the cold for 3 days the solution was evaporated to dryness and the resulting solid recrystallized from ethanol–light petroleum (b.p. 40–60°C) as white needles (0.53 g; 70% yield); it had m.p. 180–182°C and $[\alpha]_D^{20}$ +27.1° (c 1 in methanol). Huang, Foster & Niemann (1952) give m.p. 176–177°C and $[\alpha]_D^{20}$ −27.0° (c 1 in methanol) for the D-enantiomer.

2,5-*Diphenyloxazole and* 1,4-*bis*-(5-*phenyloxazol*-2-*yl*)-*benzene.* These were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

METHODS

The nature of the inhibition produced by the compounds studied, and the corresponding inhibition constants, were determined as described in the preceding paper (Kitson & Knowles, 1971), by using the continuous automatic ninhydrin method described by Cornish-Bowden & Knowles (1969). The extent of hydrolysis in the experiments to determine the amount of transpeptidation was also estimated by this method. The intensity of absorption produced by a sample of the reaction mixture with ninhydrin was compared with that produced by a standard solution of L-phenylalanylglycine.

Thin-layer chromatography. T.l.c. was used to separate substrate and acceptor in the experiments to determine the extent of transpeptidation. To terminate an incubation the reaction mixture was cooled in acetone–solid CO_2. The solid obtained was freeze-dried, the resulting material then being triturated with acetone. (When the reaction mixture had been at pH 4.7, the acetone was acidified sufficiently to ensure that the substrate and acceptor were in their un-ionized forms.) The insoluble material was filtered off, and the acetone solution of substrate and acceptor applied to a plate (20 cm × 20 cm) covered with a layer (0.1 cm) of Kieselgel HF 254 (E. Merck A.-G., Darmstadt, Germany). In the separation of N-acetyl-L-phenylalanine from N-acetyl-L-phenylalanyl-L-phenylalanylglycine the plates were eluted with diethyl ether–formic acid–water (94 : 3 : 3, by vol.); in the separation of N-acetyl-L-phenylalanine amide from N-acetyl-L-phenylalanyl-L-phenylalanylglycine the eluent was ethyl acetate–pyridine–acetic acid–water (120 : 20 : 6 : 11, by vol.). After elution and drying, the plates were divided into bands 0.5 cm wide. These bands were scraped off into scintillation bottles and their radioactivities measured as described below.

Liquid-scintillation counting. This was done on an automatic Beckman instrument (model DPM 100) with a scintillation solvent made up with 1.75 g of 2,5-diphenyloxazole and 0.060 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 350 ml of A.R. toluene and 160 ml of ethanol. For

measurement of the radioactivity of material from t.l.c., the relevant portion of the silica-gel layer was scraped from the plate into a scintillation bottle containing 6 ml of the solvent. No significant quenching by the silica gel was detected.

RESULTS

The nature of the inhibition produced by each inhibitor studied, together with the corresponding K_i value determined against N-acetyl-L-phenyl-alanyl-L-phenylalanylglycine, is recorded in Table 1. In each case the incubations were performed at pH 4.3 and at 37°C. The inhibition constants were calculated from the slopes of the Lineweaver–Burk plots by using the expression:

$$K_i = \frac{[I]}{(\text{slope})_i/(\text{slope})_{i=0} - 1}$$

Table 2 records the conditions and results of the transpeptidation experiments. In the first three cases the substrate was N-acetyl-L-phenylalanyl-L-phenylalanylglycine. The fourth entry in Table 2 is a result obtained by Mal'tsev, Ginodman, Orekhovich, Valueva & Akimova (1966), where the substrate used was N-acetyl-L-phenylalanyl-L-

phenylalanine, included for purposes of comparison. In all cases the incubations were performed at 37°C.

DISCUSSION

The results of the preceding paper (Kitson & Knowles, 1971) were consistent with a reaction pathway for pepsin-catalysed hydrolysis in which there occurs an intermediate amino-enzyme. The results also suggested that the anion of the acyl product is released first in the hydrolytic reaction, and the experiments reported here were designed to test this postulate.

Comparison of the present results (Table 1) with the data in Table 1 of the preceding paper (Kitson & Knowles, 1971) shows that, with the exception of N-acetyl-L-phenylalanine, the inhibitors behave very similarly at the two pH values. Thus N-acetyl-D-phenylalanine and the corresponding ester and amide are linear competitive inhibitors at both pH 4.3 and 2.1; N-acetyl-L-phenylalanine ethyl ester and the corresponding amide are linear non-competitive inhibitors at both pH values. At the higher pH value, also, it appears that the three compounds of the D-series inhibit

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Table 1. *Inhibition of pepsin-catalysed hydrolysis by a product and product analogues*

Inhibition was tested against N-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate, the concentration of which was 0.7–4.2 mM. The temperature was 37°C and the pH was 4.3. The terms for the type of inhibition are as defined by Hsu, Cleland & Anderson (1966).

Inhibitor	Type of inhibition	K_i (mM)	[I] (mM)
N-Acetyl-L-phenylalanine	Linear competitive	63	0, 24.4, 48.8
N-Acetyl-L-phenylalanine ethyl ester	Linear non-competitive	15	0, 6.0, 12.0
N-Acetyl-L-phenylalanine amide	Linear non-competitive	40	0, 9.1, 18.2
N-Acetyl-D-phenylalanine	Linear competitive	68	0, 25.1, 50.2
N-Acetyl-D-phenylalanine ethyl ester	Linear competitive	12	0, 6.0, 12.0
N-Acetyl-D-phenylalanine amide	Linear competitive	39	0, 9.1, 18.2

Table 2. *Results of the transpeptidation experiments*

The substrate was N-acetyl-L-phenylalanyl-L-phenylalanylglycine, except in the last case where it was N-acetyl-L-phenylalanyl-L-phenylalanine. The temperature was 37°C. Degree of hydrolysis of substrate and amount of transpeptidation product are expressed as percentages of initial amount of donor, taken as 100%.

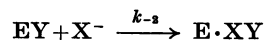| Acceptor	pH	[Pepsin] (mM)	[Acceptor] (mM)	[S_0] (mM)	Time of incubation (min)	Hydrolysis (%)	Transpeptidation (%)
N-Acetyl[^{3}H]phenylalanine	4.7	0.429	60	10	60	85	7.5
N-Acetyl[^{3}H]phenylalanine	1.3	0.029	25	2.5	30	85	<0.2
N-Acetyl[^{3}H]phenylalanine amide	4.7	0.143	20	10	50	80	<0.2
N-Benzyloxycarbonyl-phenylalanine*	4.7	0.429	10	10	360	79	8.5

* Data of Mal'tsev *et al.* (1966).

the forward reaction only by binding to the free enzyme, whereas the L-ester and L-amide inhibit the reaction by binding both to the free enzyme and to the amino-enzyme.

In terms of the actual K_i values at the two pH values, the two esters show very similar K_i values at pH 4.3 and 2.1, and the two amides each show only a small diminution in K_i on going from pH 2.1 to 4.3. N-Acetyl-D-phenylalanine shows a considerably larger K_i value at pH 4.3 than it does at pH 2.1 (68 and 26 mM respectively). This is not unexpected, for the binding between pepsin and its substrates and inhibitors is dominated by hydrophobic interactions (Hollands, Voynick & Fruton, 1969) and the raised K_i is an expected consequence of the ionization of the amino acid. The K_i of the acid anion may be further raised by electrostatic repulsion from such a notably anionic protein. Again, as at pH 2.1, there is close agreement between the K_i value of the L-ester or L-amide and the corresponding value for the D-analogue. The reason for this correspondence has been discussed in the preceding paper (Kitson & Knowles, 1971).

In summary, it appears that eqn. (2) (Scheme 2) is followed at both pH values for the three inhibitors of the D-series, whereas eqn. (3) operates at both pH values for the two product analogues of the L-series.
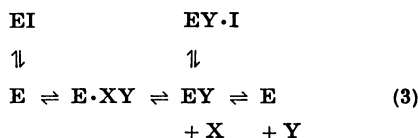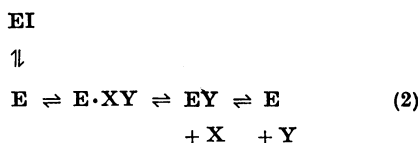
The situation for the product of the reaction itself, N-acetyl-L-phenylalanine, shows some important differences at the two pH values, which are discussed below.

Inhibition by N-*acetyl*-L-*phenylalanine.* For the acyl product of the reaction (here, X), eqns. (4) and (5) in Scheme 3 are relevant to the situations at low and high pH values respectively. The pathway of eqn. (4) will give rise to linear non-competitive inhibition by added X, as is observed at pH 2.1. The form of eqn. (5) requires that the anion of X binds to the free enzyme and also inhibits the forward reaction by binding productively to the amino-enzyme and participating in the back-reaction:
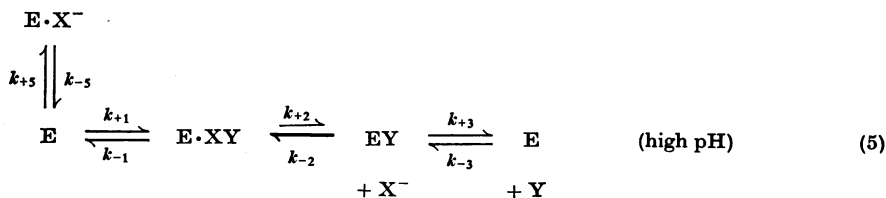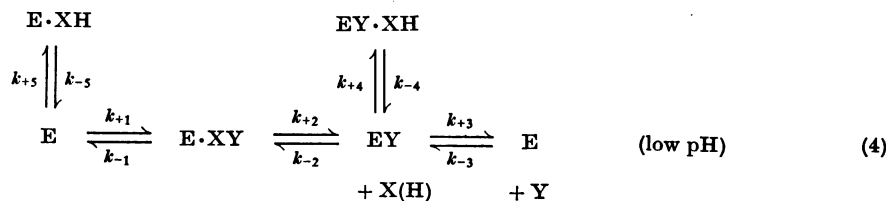
$$EY + X^- \xrightarrow{k_{-2}} E \cdot XY$$

Eqn. (5) predicts that inhibition by X^- will be non-linear non-competitive. (That is, in the Lineweaver–Burk plot we should see a vertical intercept that varies linearly with [X] and a horizontal intercept that varies in a non-linear manner with [X].)

How do the results fit this prediction? Inspection of Figs. 1 and 3 shows that, within experimental error, the vertical intercepts do not vary with [X] (i.e. the inhibition is competitive). Fig. 2 indicates, moreover, that the inhibition is linear. This is expected if the acyl product anion binds only to the free enzyme, without any interaction with the amino-enzyme. That this is not the case is seen from the ready observation of transpeptidation in this pH range. However, as pointed out in the preceding paper (Kitson & Knowles, 1971), the

EI

⇅

$$E \rightleftharpoons E \cdot XY \rightleftharpoons EY \rightleftharpoons E \qquad (2)$$
$$\qquad\qquad + X \quad + Y$$

EI   EY·I

⇅   ⇅

$$E \rightleftharpoons E \cdot XY \rightleftharpoons EY \rightleftharpoons E \qquad (3)$$
$$\qquad\qquad + X \quad + Y$$

Scheme 2.

E·XH   EY·XH

k_{+5} ⇵ k_{-5} k_{+4} ⇵ k_{-4}

$$E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot XY \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EY \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E \qquad \text{(low pH)} \qquad (4)$$
$$\qquad\qquad\qquad + X(H) \qquad + Y$$

E·X⁻

k_{+5} ⇵ k_{-5}

$$E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot XY \underset{k_{-2}}{\overset{k_{+2}}{\leftarrow}} EY \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E \qquad \text{(high pH)} \qquad (5)$$
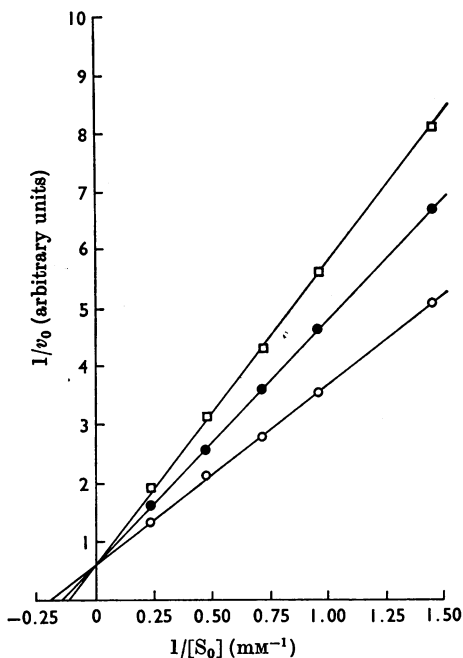$$\qquad\qquad\qquad + X^- \qquad + Y$$

Scheme 3.

Fig. 1. Lineweaver–Burk plot for the inhibition of the pepsin-catalysed hydrolysis of N-acetyl-L-phenylalanyl-L-phenylalanylglycine by N-acetyl-L-phenylalanine at pH 4.3 and at 37°C. O, Uninhibited; ●, [I] 24.4 mM; □, [I] 48.8 mM.

Fig. 2. Plot of the gradients of the lines of the Lineweaver–Burk plot for inhibition by N-acetyl-L-phenylalanine (Fig. 1) against inhibitor concentration.

Fig. 3. Plot of the vertical intercepts of the lines of the Lineweaver–Burk plot for inhibition by N-acetyl-L-phenylalanine (Fig. 1) against inhibitor concentration.

pathway of eqn. (5) results in inhibition that approximates to linear competitive when the rate constant k_{-2} is very small. The same condition explains the close similarity between the K_i values of N-acetyl-L-phenylalanine and its D-isomer (see Table 1 and Kitson & Knowles, 1971). It must be remembered that k_{-2} is a composite constant, made up of the binding constant of the acyl product to the amino-enzyme and the first-order rate constant for the synthetic reaction. Since at pH 4.3 both acceptor and amino-enzyme carry negative charges it is probable that the binding between the two species will be weak, and the small value of k_{-2} indicated by the kinetic results is not surprising.

Transpeptidation. Most investigations of pepsin-catalysed transpeptidation (e.g. Neumann *et al.* 1959; Fruton *et al.* 1961; Mal'tsev *et al.* 1966) have been carried out at relatively high pH values, typically between pH 4.0 and 4.7. This does not mean, of course, that it is necessarily the anion of the acceptor that is the reactant species, but rather because under these conditions pepsin, substrate and acceptor have more conveniently high solubilities than, say, at pH 2. The only specific claim in the literature that the anion of the acceptor is in-
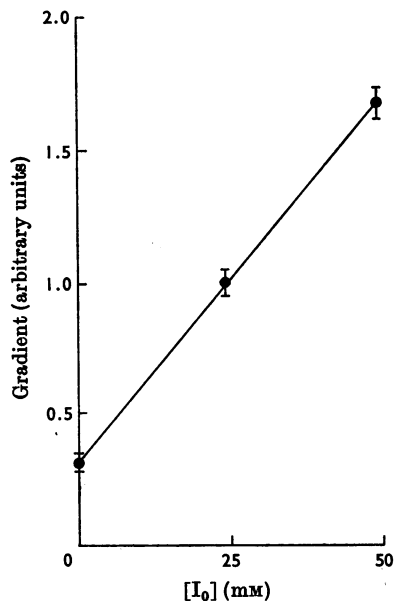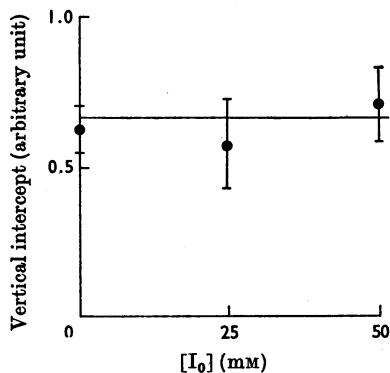
volved is that by Kozlov, Ginodman, Orekhovich & Valueva (1966), who calculate that the free energy of formation of an acyl-dipeptide ester is a minimum in the pH range 4–7 because of the state of ionization of the reactants.

The first entry in Table 2 records the extent of transpeptidation occurring at pH 4.7 in the reaction of pepsin with N-acetyl-L-phenylalanyl-L-phenylalanylglycine in the presence of N-acetyl-L-[³H]-phenylalanine. The small radioactive peak in Fig. 4

shows that under these conditions radioactivity is incorporated into the substrate (the larger peak is due to excess of acceptor), and the extent of transpeptidation is about 7.5% when the extent of hydrolysis is about 85% (expressed as a percentage of the initial amount of donor). This result parallels very closely the observations made by Mal'tsev et al. (1966); a typical result of the Russian workers is included for purposes of comparison in Table 2.

A second experiment was performed, similar to the one described above, but in which the pH of incubation was 1.3. The two experiments cannot be exactly comparable since several other variables have to be altered when the pH is changed. At the lower pH the solubilities of enzyme, substrate and acceptor are all lower, whereas the activity of pepsin is increased. However, reactant concentrations were adjusted such that the same extent of hydrolysis was observed as at the higher pH. These results are shown in Table 2, and depicted graphically in Fig. 5. No transpeptidation can be detected at the lower pH. This result, taken in conjunction with the positive result at the higher pH, further suggests that it is the anion of the acceptor that is the reactant species in pepsin-catalysed transpeptidation.

The third experiment was designed as a further test of this postulate. N-Acetyl-L-[^{3}H]phenyl-

alanine amide is sterically and in terms of chemical reactivity a reasonably close analogue of N-acetyl-L-[^{3}H]phenylalanine. The amide is not, however, analogous to the anion of N-acetyl-L-[^{3}H]phenylalanine. Under conditions very similar to those resulting in significant transpeptidation when N-acetyl-L-[^{3}H]phenylalanine is the acceptor, no transpeptidation could be detected with N-acetyl-L-[^{3}H]phenylalanine amide (see Table 2).

Mechanistic implications. In earlier papers (Knowles, 1970; Knowles et al. 1970) a scheme was proposed in outline that was the simplest formulation that satisfactorily accommodated all the data bearing on the problem of the pepsin mechanism. In this scheme (the relevant parts of which are shown in Scheme 4) two enzyme carboxyl groups react with peptide substrate (A) to form a tetrahedral intermediate (B). In order to satisfy, among other things, the requirement for prior release of the carboxyl moiety of substrate, it was suggested that this intermediate collapses via a four-centre reaction to an 'amino-enzyme' and the carboxyl part of the substrate (C). (The 'amino-enzyme' was con-
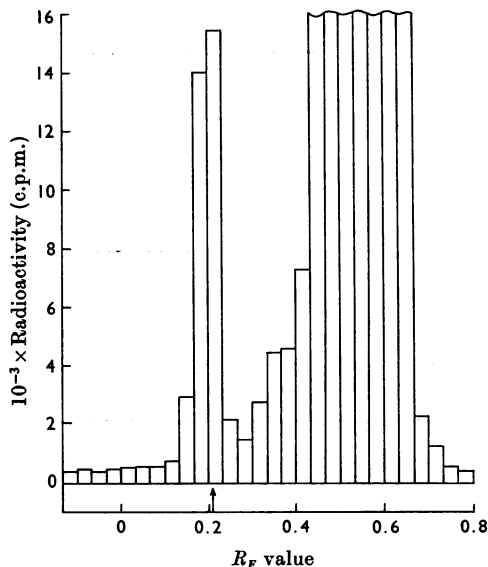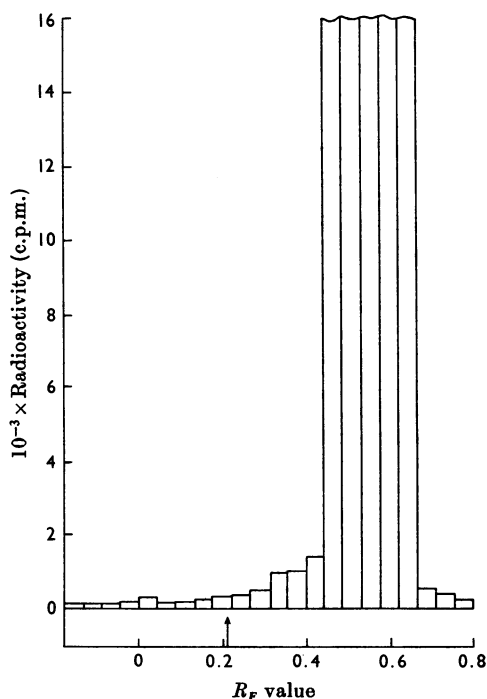

Fig. 4. Histogram of the distribution of radioactivity along a t.l.c. plate from the reaction of pepsin with N-acetyl-L-phenylalanyl-L-phenylalanylglycine in the presence of N-acetyl-L-[^{3}H]phenylalanine at pH 4.7. The vertical arrow indicates the R_F of the substrate.
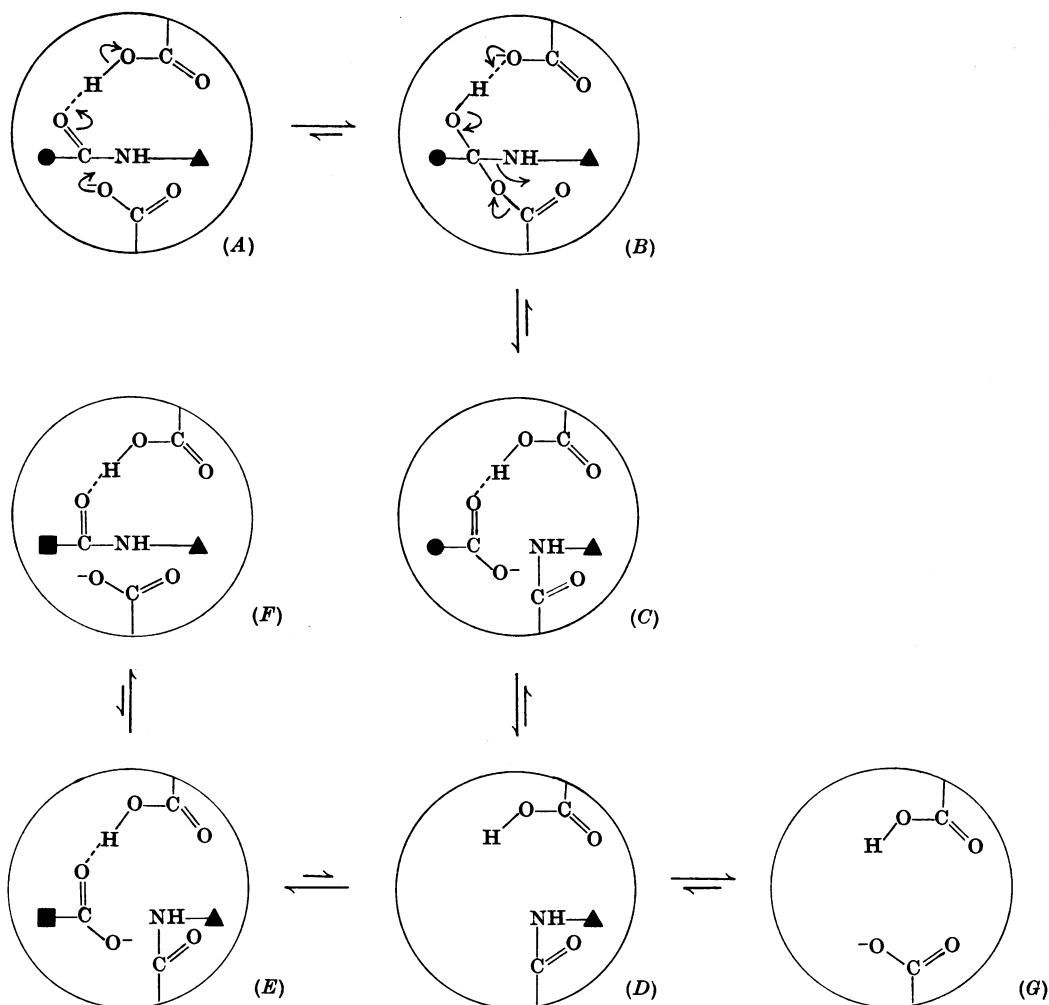

Fig. 5. Histogram of the distribution of radioactivity along a t.l.c. plate from the reaction of pepsin with N-acetyl-L-phenylalanyl-L-phenylalanylglycine in the presence of N-acetyl-L-[^{3}H]phenylalanine at pH 1.3. The vertical arrow indicates the R_F of the substrate.

Scheme 4. Possible mechanism for pepsin-catalysed hydrolysis and transpeptidation.

veniently represented as a covalent intermediate, though until definitive experiments have been performed the covalent nature of this species must remain hypothetical.) In that a neutral substrate interacts with an enzyme active site containing one un-ionized and one ionized carboxyl group, the state (C) necessarily contained the anion of the carboxyl moiety of the substrate. Moreover, since the transpeptidation reaction catalysed by pepsin (D→E→F) must follow the microscopically reverse pathway (i.e. D→C→B→A), our mechanism also required that it is the anion of the acceptor amino acid that reacts with the amino-enzyme (D) in transpeptidation.

It is significant therefore that, from the inhibition studies reported in this and the preceding paper (Kitson & Knowles, 1971) we are led to suggest that it is indeed the acyl product anion that is the product released first. Further, the fact that transpeptidation is only detectable (under our conditions, at least) at pH 4.7 and not at pH 1.3 is entirely consistent with the anion of the acceptor amino acid (of pK_a about 3.6) being the kinetically important species.

The results reported in this and the preceding paper (Kitson & Knowles, 1971) therefore support the formulation of the pepsin-catalysed peptidolytic and transpeptidation reactions as shown in Scheme 4.

Finally, reference must be made to the suggestion of Silver, Stoddard & Stein (1970) that the substrate for pepsin-catalysed ^{18}O exchange between

an acyl-L-amino acid and $H_2^{18}O$ is the anion of the acyl-amino acid. On the basis (*a*) that the ^{18}O-exchange reaction shows specificity and stereospecificity for the acyl-amino acid, (*b*) that direct transfer of ^{18}O from enzyme carboxyl groups to acyl-amino acid had been demonstrated (Ginodman & Shkarenkova, 1968) and (*c*) of mechanistic economy, we suggested that un-ionized acyl-amino acid might behave analogously to peptide substrate and account for the ^{18}O-exchange reaction (Knowles, 1970). However, since there is a correlation between the ^{18}O-exchange rate for different acyl-L-amino acids and the activity of these materials as acceptors in transpeptidation (Ginodman, Kozlov, Mal'tsev & Orekhovich, 1964), and since results of Silver *et al.* (1970) suggest that the anion of the acyl-amino acid is the substrate for the ^{18}O-exchange process, our original formulation of this exchange activity of pepsin may require modification. Until, however, more results bearing on this point become available, the question of which part of the peptidolytic and transpeptidation pathway most closely parallels the ^{18}O-exchange reaction must remain open.

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