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The Inhibition of Pepsin-Catalysed Reactions by Products and Product Analogues

KINETIC EVIDENCE FOR ORDERED RELEASE OF PRODUCTS

By P. GREENWELL, J. R. KNOWLES and HILARY SHARP
The Dyson Perrins Laboratory, University of Oxford

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1. The inhibition of pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by products and product analogues was studied. 2. The non-competitive nature of the inhibition by the product *N*-acetyl-L-phenylalanine confirms an ordered release of products, and points to a common mechanism (involving an amino-enzyme) for pepsin-catalysed transpeptidation and hydrolysis reactions. 3. *N*-Acetyl-L-phenylalanine ethyl ester is also a non-competitive inhibitor, but here the inhibition is of the 'dead-end' type. No ethanol is detectable in reaction mixtures, indicating that this ester cannot act as an amino group acceptor in a transpeptidation process. 4. The same is true for *N*-methanesulphonyl-L-phenylalanine methyl and methyl thiol esters. No methanethiol is liberated when the methyl thiol ester is present as an inhibitor of the hydrolytic reaction, and the hope that such a thiol ester would effectively trap the amino-enzyme was not fulfilled.

From the work of Neumann, Levin, Berger & Katchalski (1959) and of Fruton, Fujii & Knappenberger (1961) it is apparent that pepsin will catalyse transpeptidation reactions of the amino-transfer type. That is, an intermediate in the pepsin-catalysed hydrolysis of a dipeptide substrate X·CO·NH·Y is pepsin·NH·Y, and that this 'amino-enzyme' lives long enough for X·CO_2H to be released, and (when transpeptidation occurs) X'·CO_2H to bind, with the catalysed formation of a new peptide X'·CO·NH·Y.

A large body of evidence is now available to indicate that in the pepsin-catalysed hydrolysis of small peptide substrates the rate-determining step of the catalytic reaction immediately follows the formation of the Michaelis complex, and this fact precludes the direct observation of any subsequent intermediates (see Cornish-Bowden, Greenwell & Knowles, 1969). Classically, intermediates that follow the rate-determining step of a reaction can be detected by trapping experiments, where a reagent is added that reacts fast enough with the intermediate for one to observe product diversion with no change in overall reaction rate. For an enzyme-catalysed process there is the additional possibility of studying the nature of inhibition of the reaction by a product or product analogue. This approach is nicely exemplified by the work of Hsu, Cleland & Anderson (1966) on the inhibition of acid phosphomonoesterase. From the inhibition pattern these workers were able to deduce that this enzyme operates by a mechanism involving the ordered release of products, and moreover that two enzyme–phosphate complexes are involved in the catalytic reaction pathway.

In the present work studies are reported on both fronts: an attempt has been made to trap the amino-enzyme implicated in the pepsin mechanism by the transpeptidation experiments mentioned above, and a preliminary study of the pattern of inhibition by one type of product is discussed. The latter studies, taken with work reported by Inouye & Fruton (1968) on the inhibition of pepsin-catalysed reactions by the second type of product, allow a simple and self-consistent picture of the pathway of pepsin catalysis to be drawn.

MATERIALS

Pepsin. This was obtained as described by Cornish-Bowden & Knowles (1969).

N-*Acetyl-L-phenylalanyl-L-phenylalanylglycine.* This was obtained as described by Cornish-Bowden & Knowles (1969).

N-*Acetyl-L-phenylalanine.* This was prepared from L-phenylalanine and acetic anhydride under Schotten–Baumann conditions. The crude material was recrystallized from ethyl acetate–light petroleum (b.p. 40–60°) to give colourless plates, m.p. 167–170°, $[\alpha]_D^{20}$ +46·1° (*c* 2 in ethanol). Coffy, Green & Kenner (1959) give m.p. 169–170° and $[\alpha]_D^{20}$ +47·1° (*c* 2 in ethanol).

N-*Acetyl-L-phenylalanine ethyl ester.* This was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

N-Methanesulphonyl-L-phenylalanine. L-Phenylalanine (24g., 0·145 mole) was dissolved in 5M-NaOH (32ml.). Methanesulphonyl chloride (approx. 12ml., 0·145 mole) and 2M-NaOH (80ml.) were added in alternate portions over 1hr. to the vigorously stirred solution, such that the pH remained above 9. The mixture was stirred for 1hr. after the additions were complete, and then washed with ether, and acidified (to Congo Red) with conc. HCl. The resulting oil was extracted into ether, and the solution was dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The resulting yellow oil, which solidified on standing, was crystallized from benzene to yield white needles (19·6g., 55%), m.p. 106–108°, $[\alpha]_D^{20}$ −22·9° (*c* 1 in ethanol) (Found: C, 49·4; H, 5·2; N, 6·1; S, 13·3. $C_{10}H_{13}NO_4S$ requires: C, 49·4; H, 5·4; N, 5·8; S, 13·2%).

N-Methanesulphonyl-L-phenylalanine methyl ester. N-Methanesulphonyl-L-phenylalanine (4·0g., 16m-moles) was dissolved in dry ether and a slight excess of PCl_5 (3·5g., 17m-moles) was added. The mixture was stirred overnight in a sealed flask at room temperature. The resulting solution was evaporated down under reduced pressure to give a white crystalline solid, which was recrystallized from dry ether–light petroleum (b.p. 40–60°) as white needles (3·4g., 78% yield), m.p. 75–80°. This intermediate acid chloride was not characterized beyond observation of its i.r. spectrum, which contained a peak at 1785cm.$^{-1}$, characteristic of an acid chloride carbonyl group.

N-Methanesulphonyl-L-phenylalanine acid chloride (2g., 7·7m-moles) was dissolved in dry methanol (50ml.) and stirred overnight with dried MgO (1g.) at room temperature. The fine solid was filtered off and washed with methanol, and the combined filtrates were evaporated down to a coloured oil. This oil was taken up in ethyl acetate, and the solution was filtered, washed with 0·5M-HCl and water and dried over $MgSO_4$. Removal of the solvent under reduced pressure yielded a colourless oil that partly solidified. This was recrystallized from chloroform–light petroleum (b.p. 40–60°) as fine white needles (0·95g., 49% yield), m.p. 51–52°, $[\alpha]_D^{20}$ −20·9° (*c* 2 in methanol). The n.m.r. spectrum (in $CDCl_3$) confirmed the structure: methanesulphonyl CH_3 (singlet, τ 7·35); benzylic CH_2 (complex doublet, τ 6·95); ester CH_3 (singlet, τ 6·25); asymmetric CH (multiplet, τ 5·63); amide –NH– (broad doublet, τ 4·95); aromatic protons (singlet, τ 2·77) (Found: C, 51·5; H, 5·4; N, 5·4; S, 12·5. $C_{11}H_{15}NO_4S$ requires: C, 51·4; H, 5·8; N, 5·5; S, 12·5%).

N-Methanesulphonyl-L-phenylalanine methyl thiol ester. N-Methanesulphonyl-L-phenylalanine acid chloride (1g., 3·8m-moles) was dissolved in sodium-dried tetrahydrofuran and stirred at −10° with anhydrous MgO (1g.). A large excess of methanethiol (2ml.) was added and the mixture was stirred at −10° for 3hr. before being left at room temperature overnight. The solution was filtered into an evaporating basin and left to evaporate in the fume cupboard. This produced yellow crystals that were recrystallized from benzene–light petroleum (b.p. 60–80°) to give very pale-yellow needles (0·42g., 40% yield), m.p. 111–112°, $[\alpha]_D^{20}$ −55·2° (*c* 2 in chloroform). The n.m.r. spectrum (in $CDCl_3$) confirmed the structure: methylthio CH_3 (singlet, τ 7·64); methanesulphonyl CH_3 (singlet, τ 7·43); benzylic CH_2 (complex multiplet, τ 6·90); asymmetric CH (multiplet, τ 5·58); amide –NH– (broad doublet, τ 4·69) and aromatic protons (singlet, τ 2·71). The mass spectrum showed the correct molecular ion [*m/e* (intensities relative to 198 peak): 91 (47%), 118 (32%), 119 (36%), 120 (30%), 198 (100%), 226 (0·3%), 245 (2·0%), 273 (0·1%)] (Found: C, 48·2; H, 5·4; N, 5·2; S, 23·1. $C_{11}H_{15}NO_3S_2$ requires: C, 48·3; H, 5·5; N, 5·2; S, 23·5%).

5,5′-Dithiobis-(2-nitrobenzoic acid). This was purchased from Sigma Chemical Co.

NAD$^+$. This was purchased from Sigma Chemical Co.

Yeast alcohol dehydrogenase. This enzyme [crystalline suspension in 2·4M-$(NH_4)_2SO_4$ solution] was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

METHODS

Inhibition constants. These were determined by using the continuous ninhydrin method described by Cornish-Bowden & Knowles (1969).

Rate of appearance of methanethiol. The reaction was carried out under conditions analogous to the inhibition experiments, except that the solutions were made up in 0·01M-HCl rather than 0·1M-citrate buffer. This was done to facilitate the quenching of samples by raising them to pH7·7, the value necessary for the thiol assay method, which utilized 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959). The reagents (substrate: *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine; enzyme: pepsin; inhibitor: *N*-methanesulphonyl-L-phenylalanine methyl thiol ester; solvent: 0·01M-HCl) were mixed in a 10ml. syringe, thermostatically controlled at 37°, to avoid any loss of volatile methanethiol. At 5min. or 10min. intervals 1·0ml. samples were ejected from the syringe into a 10mm.-path-length cuvette (capacity 1·4ml.) containing 0·3ml. of an alkaline phosphate solution, which served both to inactivate the pepsin and to adjust the pH without excessive dilution. This quenching solution was made up from 87ml. of 0·5M-Na_2HPO_4, 13ml. of 0·5M-NaH_2PO_4 and 3·3ml. of 1M-NaOH, such that the samples were effectively converted into 0·12M-phosphate buffer with a measured value of pH7·7 ± 0·05.

Immediately after the contents of the cell were thoroughly mixed, 20μl. of an acetone solution of 5,5′-dithiobis-(2-nitrobenzoic acid) (4mg./ml.) was added, and the E_{412} of the solution, relative to a buffer blank, was measured on a Unicam SP.500 spectrometer. The validity of the Ellman method under these conditions was confirmed by subjecting standard solutions of L-cysteine in 0·01M-HCl to the whole procedure.

Rate of appearance of ethanol. Samples from a reaction mixture containing substrate (*N*-acetyl-L-phenylalanyl-L-phenylalanylglycine), inhibitor (*N*-acetyl-L-phenylalanine ethyl ester) and pepsin in citrate buffer at pH2·0 were assayed for ethanol by the method of Lundquist (1959). Samples (1·1ml.) were added to 2·2ml. of a solution of 20 parts (v/v) of glycine buffer, containing EDTA and semicarbazide hydrochloride, and 1 part (v/v) of NAD$^+$ solution. The pH of this solution was 8·9. The E_{340} was measured. A 3·0ml. portion of this solution was then treated with 10μl. of alcohol dehydrogenase solution. The E_{340} was measured again after the solution had been left for 1hr. The sensitivity of the method was checked with standard solutions of ethanol.

RESULTS

Inhibition constants for the four inhibitors, *N*-acetyl-L-phenylalanine, *N*-acetyl-L-phenylalanine ethyl ester, *N*-methanesulphonyl-L-phenylala-

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

The temperature was 37°; inhibition was tested against *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate.

Inhibitor	K_i (mM)	pH	$[S_0]$ range (mM)	$[I_0]$ (mM)
N-Acetyl-L-phenylalanine	29	2·12	0·345–3·12	8·46
N-Acetyl-L-phenylalanine ethyl ester	14	2·15	0·277–1·11	8·02
N-Methanesulphonyl-L-phenylalanine methyl ester	15	1·97	0·18–1·82	3·42
N-Methanesulphonyl-L-phenylalanine methyl thiol ester	9·8	1·94	0·37–1·88	3·59

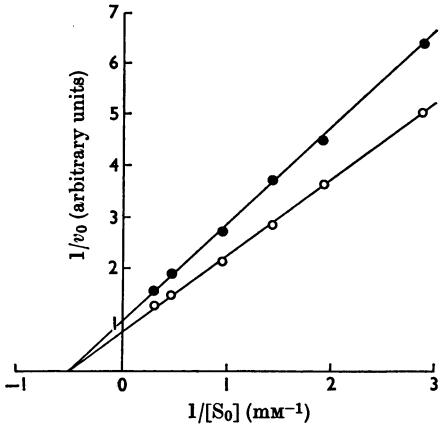

Fig. 1. Lineweaver–Burk plot for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by *N*-acetyl-L-phenylalanine at pH 2·12 at 37°. ○, Uninhibited; ●, inhibited.

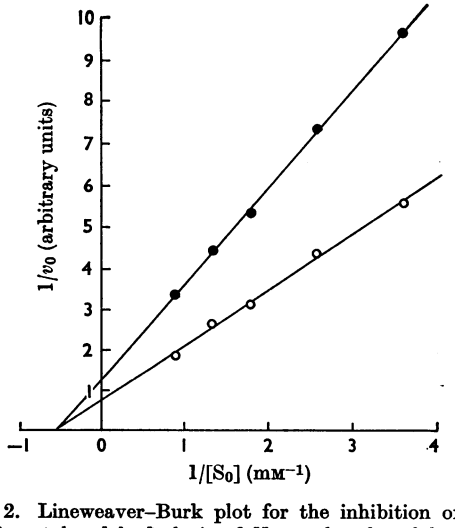

Fig. 2. Lineweaver–Burk plot for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by *N*-acetyl-L-phenylalanine ethyl ester at pH 2·15 at 37°. ○, Uninhibited; ●, inhibited.

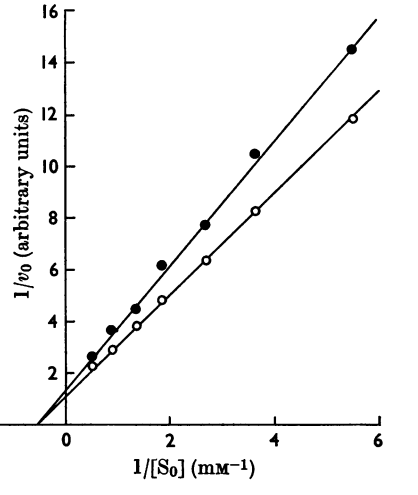

Fig. 3. Lineweaver–Burk plot for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by *N*-methanesulphonyl-L-phenylalanine methyl ester at pH 1·97 at 37°. ○, Uninhibited; ●, inhibited.

nine methyl ester and *N*-methanesulphonyl-L-phenylalanine methyl thiol ester, were determined at 37° against *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate. The values are listed in Table 1. All these inhibitors show non-competitive behaviour (see Figs. 1–4).

The rate of appearance of methanethiol from a mixture of pepsin (0·477 μM), *N*-methanesulphonyl-L-phenylalanine methyl thiol ester (2·24 mM) and substrate (1·66 mM) in 0·01 M-hydrochloric acid, final pH 2·27, was measured at 37°. The rate of thiol production was less than 5% of that expected if the whole of the decrease in rate of appearance of ninhydrin-positive product had been due to transpeptidation.

The rate of appearance of ethanol from a mixture of pepsin (0·488 μM), *N*-acetyl-L-phenylalanine ethyl ester (8·06 mM) and substrate (2·31 mM) in 0·1 M-citrate buffer, pH 2·1, was measured at 37°. The rate of ethanol production was less than 2% of that

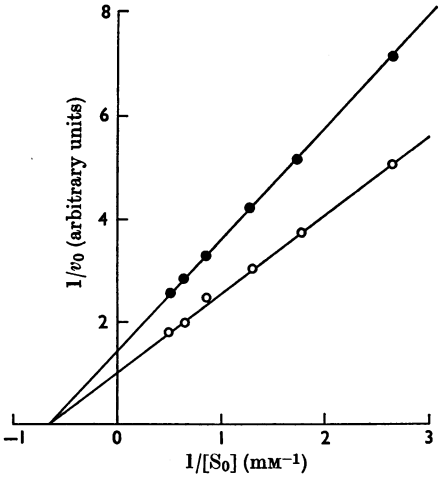

Fig. 4. Lineweaver–Burk plot for the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine by *N*-methanesulphonyl-L-phenylalanine methyl thiol ester at pH 1·94 at 37°. ○, Uninhibited; ●, inhibited.

expected if the whole of the decrease in rate of appearance of ninhydrin-positive product had been due to transpeptidation.

DISCUSSION

Inhibition by N-acetyl-amino acids. Previous work on the inhibition of pepsin-catalysed reactions by acyl-amino acids has been reported by Schlamowitz and his co-workers (Jackson, Schlamowitz & Shaw, 1966; Schlamowitz, Shaw & Jackson, 1968), by Zeffren & Kaiser (1966) and by Inouye & Fruton (1968). [In addition, Baker (1954, 1956) and Silver, Denburg & Steffens (1965) have studied the inhibition of pepsin by *N*-acetyl-L-phenylalanine. However, these workers obtained their data from the time-course of the extensive hydrolysis of substrate. This approach is less reliable than methods involving the measurement of initial velocities in the presence and absence of inhibitor, as has been discussed by Jackson *et al.* (1966).] Inouye & Fruton (1968) studied the inhibition of various *N*-acetyl-amino acids at pH 4·0, and found that the inhibition was very weak, *N*-acetyl-L-phenylalanine having K_i 55 mM. This value largely relates, of course, to the anion of *N*-acetyl-L-phenylalanine, which has pK_a about 3·5, and this study is not as germane to the present discussion as the earlier work quoted, which was done at pH 2·0. Jackson *et al.* (1966) obtained K_i 23 mM for *N*-acetyl-L-phenylalanine, and stated that the inhibition is mixed, with $\alpha = 1{\cdot}9$ (α for non-competitive inhibition is 1, and for competitive inhibition is ∞). By contrast, Zeffren & Kaiser (1966) found that the inhibition of the pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-3,5-dibromo-L-tyrosine by *N*-acetyl-L-phenylalanine is competitive, and has K_i 0·14 mM. Our data are in rather close agreement with those of Jackson *et al.* (1966): inhibition by *N*-acetyl-L-phenylalanine is clearly non-competitive (Fig. 1), with K_i 29 mM (Table 1). We can offer no explanation for the disagreement between the values obtained by us and Jackson *et al.* (1966) and those given by Zeffren & Kaiser (1966).

In our assay method the rate of production of ninhydrin-positive material is measured, and this rate will fall if the breakdown of some intermediate is partially or wholly blocked, e.g. by being diverted in such a way as to decrease the amount of ninhydrin-positive material being released. This will give rise to non-competitive inhibition. In the present experiments added *N*-acetyl-L-phenylalanine can act as an amine acceptor in a transpeptidation process. The effect of this is to increase the rate of the back reaction and decrease the amount of free amino group released. This may be restated in kinetic terms. As discussed by Hsu *et al.* (1966), non-competitive inhibition by a product may arise if there is an ordered release of products, and the product whose inhibition is being studied is the product released first. Thus for the reaction scheme:

$$\mathrm{E}+\mathrm{XY} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{XY} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{X} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{E}+\mathrm{X} \qquad (1)$$
$$+\mathrm{Y}$$

the initial rate (when [X] = 0) is:

$$v_0 = \frac{[\mathrm{E}_0]k_{+1}k_{+2}k_{+3}[\mathrm{XY}]}{k_{-1}k_{+3}+k_{+2}k_{+3}+[\mathrm{XY}](k_{+1}k_{+3}+k_{+1}k_{+2}+k_{+1}k_{-2}[\mathrm{Y}])+k_{-1}k_{-2}[\mathrm{Y}]}$$

A double-reciprocal plot (of $1/v_0$ versus $1/[\mathrm{XY}]$) results in both the slope and the vertical intercept being linearly dependent on [Y], which is the condition (Cleland, 1963) for linear non-competitive inhibition. The present experiments indeed show *N*-acetyl-L-phenylalanine to be a non-competitive inhibitor (though our present data are inadequate to test the linearity of the inhibition). So the non-competitive inhibition by *N*-acetyl-L-phenylalanine is accounted for by the existence of an amino-

enzyme the hydrolytic breakdown of which is diverted into transpeptidation.

Inhibition by analogues of N*-acetyl-amino acids.* The inhibition by *N*-acetyl-L-phenylalanine ethyl ester is also non-competitive (Table 1 and Fig. 2), with both gradient and vertical intercept of the double-reciprocal plot dependent on inhibitor concentration (Cleland, 1963). In principle this material could bind to the amino-enzyme and prevent its hydrolysis (a 'dead-end' inhibitor; Cleland, 1963), or it could act as an amine acceptor in transpeptidation, with release of ethanol. In this case we would have an independent measure of transpeptidation, in that the rate of ethanol release can be measured. However, when a solution containing pepsin, substrate (*N*-acetyl-L-phenylalanyl-L-phenylalanylglycine) and *N*-acetyl-L-phenylalanine ethyl ester was allowed to react under conditions where a significant amount of ethanol would have been formed had the ester acted as a transpeptidation acceptor, no ethanol was detectable. The method of assaying ethanol (with alcohol dehydrogenase–NAD^+) was sensitive enough to have detected less than 2% of the expected amount of ethanol, had the whole decrease in catalytic rate arisen from transpeptidation. Here, therefore, the non-competitive inhibition is most likely to arise simply from the binding of the product analogue, *N*-acetyl-L-phenylalanine ethyl ester, to the amino-enzyme as a dead-end inhibitor, in combination with competitive binding to the free enzyme. If the product or product analogue were to bind only to the amino-enzyme, uncompetitive inhibition would result.

Inhibition by a methyl thiol ester. In its simplest terms the transpeptidation reaction must involve attack by the ·NH· group of the amino-enzyme on the carbonyl carbon of the acceptor carboxylic acid. This process must compete with the hydrolytic breakdown of the amino-enzyme. Since it is known that thiol esters are, relatively to oxygen esters, very much more susceptible to attack by nitrogen nucleophiles (Bruice & Benkovic, 1966), the inhibition of pepsin by a thiol ester and its oxygen analogue was studied. The derivatives chosen were *N*-methanesulphonyl-L-phenylalanine methyl thiol ester and the corresponding oxygen ester. It was expected that if transpeptidation could occur with *N*-acyl-amino acid esters, and if the transpeptidation reaction was analogous to the attack of a nitrogen nucleophile at carbonyl carbon, then the methyl thiol ester would prove to be a very much more effective non-competitive inhibitor than its oxygen analogue. As indicated by Table 1 and Figs. 3 and 4, the thiol ester is a slightly more effective non-competitive inhibitor. The difference is not convincing, and could readily arise from slightly improved binding of the thiol ester to the amino-enzyme. That this is the case was shown by an investigation of the production of methane-thiol in a reaction mixture containing pepsin, substrate and thiol ester inhibitor. No measurable production of methanethiol could be detected during the reaction. The assay method was sensitive enough to detect 5% of the expected amount of methanethiol, had the whole decrease in catalytic rate arisen from transpeptidation. It therefore appears that all the three esters examined act as non-competitive inhibitors simply by virtue of their binding to the amino-enzyme in addition to the free enzymes.

Inhibition by the second product. The method for following the rate of pepsin-catalysed hydrolysis that we have used here does not allow a study of inhibition by the second product, L-phenylalanylglycine, since the necessary concentrations of this material would produce an unacceptable 'blank' ninhydrin colour. In this respect the spectrophotometric method used by Inouye & Fruton (1967) provides the complementary method, and they have indeed used their method to investigate this point (Inouye & Fruton, 1968). Their results are not strictly comparable with ours, since both the pH (4·0) and the substrate (*N*-benzyloxycarbonyl-L-histidyl-*p*-nitro-L-phenylalanyl-L-phenylalanine methyl ester) are different. However, these workers do find, as expected from eqn. (1) for inhibition in the absence of Y but in the presence of X, that linear competitive inhibition is observed. For instance, L-phenylalanine methyl ester (product X) is a linear competitive inhibitor, of K_i 22mM.

In summary, the results reported here on the inhibition of pepsin-catalysed hydrolysis of a peptide substrate by the product expected to be released first and by analogues of it, together with the results of Inouye & Fruton (1968) on the inhibition by the product released last, are nicely accommodated by a mechanistic scheme that demands ordered release of products, and an intermediate amino-enzyme.

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