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An Aspartic Acid Residue at the Active Site of Pepsin

THE ISOLATION AND SEQUENCE OF THE HEPTAPEPTIDE

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Pepsin reacts stoicheiometrically with the active-site-directed irreversible inhibitor *N*-diazoacetyl-L-phenylalanine methyl ester, with concomitant loss of all proteolytic and peptidolytic activity. The reagent esterifies a unique aspartic acid residue in pepsin, which is in the sequence:

Ile-Val-Asp-Thr-Gly-Thr-Ser

On the basis of the pH-dependence of pepsin-catalysed reactions (e.g. Cornish-Bowden & Knowles, 1969), the pH-dependence of the binding of substrate analogues (Knowles, Sharp & Greenwell, 1969) and the inactivation of pepsin by such reagents as diazomethane (Herriott, 1947), di-β-chloroethyl sulphide (Herriott, Anson & Northrop, 1946) and diphenyldiazomethane (Delpierre & Fruton, 1965), it seemed very probable that the active site of pepsin contains one or more carboxyl groups that control, or are directly responsible for, the catalytic activity of this enzyme. Although the reagents mentioned above are all believed to react principally with enzyme carboxyl groups, none of them is specific, in the sense that 1:1 labelling of the enzyme could not be achieved. It was therefore decided to prepare an active-site-directed irreversible inhibitor, in the hope of obtaining a stoicheiometric 1:1 reaction with the active site of the enzyme, and of identifying the site of attachment within the protein. Studies of the pepsin-catalysed hydrolysis of model dipeptide substrates have shown that there is an absolute requirement that the amino acid residues on either side of the bond that is cleaved be of the L-configuration, and of the good substrates that have been reported at least one of these residues is aromatic (Inouye & Fruton, 1967). Accordingly our inhibitor was derived from L-phenylalanine. Of the functional amino acid side chains in proteins, the carboxyl group is one of the most difficult to modify successfully, since it is only a weak nucleophile and the product of modification is in many cases an ester that is undesirably labile. The weak nucleophilicity can, in principal, be overcome by using a modifying agent with a very good leaving group. Two examples of this are diazo compounds, where the leaving group is N_2, and triethyloxonium fluoroborate, where the leaving group is $(C_2H_5)_2O$. Such reagents will, of course, be very reactive, not only towards the carboxyl groups, but also towards other more powerfully nucleophilic centres in the protein molecule and towards water. In the present work we chose to use a diazo compound, namely *N*-diazoacetyl-L-phenylalanine methyl ester, for the specific modification of a single carboxyl group in pepsin. A preliminary report of a part of this work has appeared (Bayliss & Knowles, 1968).

MATERIALS

Pepsin. Pepsin (ex hog-stomach mucosa), three-times crystallized, activity 1:60000, was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks. The concentration of enzyme solutions was determined by measuring E_{280}, assuming $E^{0.1\%}_{280}$ 1·47 for a 10mm. path length (Perlmann, 1959). Pepsin was also prepared for comparative purposes from recrystallized pepsinogen by the method of Rajagopalan, Moore & Stein (1966*b*) (see the Results section).

Pepsinogen. Pepsinogen (ex hog stomach mucosa), crystalline, freeze-dried, was purchased from Koch–Light Laboratories Ltd.

N-Diazoacetyl-L-phenylalanine methyl ester. Glycyl-L-phenylalanine methyl ester hydrobromide was prepared by the debenzyloxycarbonylation of the coupled product from *N*-benzyloxycarbonylglycine and L-phenylalanine methyl ester by standard methods.

Glycyl-L-phenylalanine methyl ester hydrobromide (8·2g.) was dissolved in aq. 2M-sodium acetate (40ml.), and A.R. acetic acid (2·0ml.) was added. The mixture was then cooled in an ice bath and $NaNO_2$ (3·0g.) was added in portions over ½hr. The solution was left for 3hr., during which time some yellow solid was formed. The mixture was then extracted into ice-cold chloroform (3×50ml.), and the chloroform extracts were combined and dried over $MgSO_4$. Light petroleum (b.p. 40–60°) was added until the solution became turbid, and the solution was left overnight. The resulting yellow needles were filtered off and dried (4·4g., 70%). This material had m.p. 126–128° (Found: C, 57·9; H, 5·2; N, 16·9. $C_{12}H_{13}N_3O_3$ requires: C, 58·3; H, 5·3; N, 17·0%). After recrystallization the material

had $[\alpha]_D^{20} + 174°$ (*c* 2 in $CHCl_3$), and after a further recrystallization $[\alpha]_D^{20} + 186°$ (*c* 1 in $CHCl_3$). The i.r. spectrum showed a strong and very sharp peak at 2100 $cm.^{-1}$ (diazo group) and a peak at 1610 $cm.^{-1}$ (carbonyl in diazo ketone). The overall yield on glycine was 21·6%. A second preparation gave an overall yield of 24%.

N-Diazo[1-^{14}C]*acetyl-L-phenylalanine methyl ester.* This compound was prepared by the above reaction sequence starting with 0·5 mc of [1-^{14}C]glycine. This material was diluted with unlabelled glycine before use. The product (857 mg., m.p. 126–128·5°, overall yield on glycine 26%) was compared with the unlabelled compound by t.l.c. on unbaked silica gel G plates eluted with methanol (R_F 0·66, unlabelled compound R_F 0·66) or ethyl acetate (R_F 0·59, unlabelled compound R_F 0·59).

Buffers. All buffers were prepared with deionized water and A.R.-grade reagents.

2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)-benzene. These were obtained from Thorn Electronics Ltd., Tolworth, Surrey.

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

Carboxypeptidase A. Carboxypeptidase A-DFP (carboxypeptidase A treated with di-isopropyl phosphorofluoridate, dialysed and recrystallized) was purchased from Sigma Chemical Co.

Haemoglobin. This was obtained from Sigma Chemical Co.

[1-^{14}C]*Glycine.* This was obtained from The Radiochemical Centre, Amersham, Bucks.

'Polyamide layers'. 'Polyamide layers' for t.l.c. of DNS*-amino acids were obtained from the Cheng-Chin Trading Co. Ltd., Hankow Street, Taipei, Taiwan.

All solvents for chromatography and other operations were redistilled before use.

METHODS

The u.v. spectra were measured on a Unicam SP.800 recording spectrophotometer. Fixed-wavelength measurements were made on a Unicam SP.500 spectrophotometer.

The i.r. spectra were measured on a Unicam SP.200 recording spectrophotometer.

pH values were determined on a Radiometer TTT1c instrument with a pHA630 scale-expander attachment, standardized against standard buffer solutions from British Drug Houses Ltd., Poole, Dorset.

Scintillation counting was initially performed on a 1700 scaler with a 2022 liquid-scintillation head from Isotope Developments Ltd. Later work was performed on an automatic Beckman model DPM100 instrument.

Radiochromatogram scanning was initially performed on an Isotope Developments Ltd. instrument with a 1700 scaler, a 2029 scanner and a 2007 programme and read-out unit. Later work was done on a Packard 7201 gas-flow counter with a recording ratemeter (model 385).

Amino acid analyses were performed on a Technicon automatic amino acid analyser. Later work was done on Beckman–Spinco instruments (model 120C).

Optical rotations were measured on a Perkin–Elmer PE141 polarimeter.

Melting points were determined on a Kofler block and are uncorrected.

*Abbreviation: DNS or dansyl, 1-dimethylaminonaphthalene-5-sulphonyl.

Microanalyses were performed in this Department by Dr Weiler and Dr Strauss.

Protein digestions were performed in a Radiometer pH-stat with the Titrator TTT1c and Titrigraph SBR2c.

Determination of the proteolytic activity of pepsin solutions. The proteolytic activity of pepsin solutions was estimated by a modified form of the method of Anson (1948). Haemoglobin (2·0 g.) was dissolved in about 50 ml. of water, 20 ml. of 0·3 M-HCl was added and the solution was made up to 100 ml. Samples (3·0 ml.) of this solution were pipetted into Pyrex test tubes, which were placed in a thermostatically controlled water bath at 35°. After 5 min. 100 μl. portions of the solution to be assayed were added to the haemoglobin solution, the tubes were inverted twice and the mixture was kept for 10·0 min. at 35°. Then 5·0 ml. portions of aq. 5% (w/v) trichloroacetic acid were added rapidly. The tubes were kept for a further 5 min. in the water bath and were then centrifuged for 10 min. The clear supernatant liquid was decanted into 10 mm. silica cuvettes and the E_{280} was determined.

The background extinction value was determined by adding 100 μl. portions of water to the haemoglobin solution in the place of pepsin solution, and was normally between 0·55 and 0·65. The extinction was obtained by subtracting the background value from the values obtained above. It was shown that the extinction changes linearly with pepsin concentration over the range of enzyme concentration used.

Haemoglobin solutions were not used more than 3 days after being made up, as the background value increased greatly after this period.

The optimum concentration of pepsin was found to be about 0·15 mg./ml. With this concentration 100 μl. portions produced an extinction change of about 1·1 units in the determination of the proteolytic activity described.

Kinetic studies of the inhibition reactions. In a typical experiment 0·7–0·8 mg. of pepsin was dissolved in 5·0 ml. of the required buffer; 1·5 times the required amount of inhibitor was dissolved in 0·3 ml. of methanol. Additional reagents such as cupric acetate either were dissolved in the buffer before the addition of the pepsin, or were added as a small volume (usually 25 μl.) of a concentrated solution.

In experiments involving the addition of reversible inhibitors or other comparatively insoluble compounds, the pepsin solution was made up to 2·5 times the required final concentration, and the inhibitor dissolved in buffer to give a solution 1·67 times the required final concentration. Then 2·0 ml. of the pepsin solution and 3·0 ml. of the inhibitor solution were combined. Then 200 μl. of the methanolic solution of the irreversible inhibitor was added and duplicate 100 μl. portions were immediately removed and their proteolytic activity was determined. At suitable time-intervals further duplicate portions were withdrawn for assay until the reaction had reached completion.

Preparation of inhibited pepsin. A 100 mg. portion of pepsin was dissolved in 55 ml. of 0·05 M-sodium acetate buffer, pH 5·0, and 1·3 ml. of 0·1 M-cupric acetate solution was added. The solution was left for 15 min. and a solution of 25 mg. of *N*-diazoacetyl-L-phenylalanine methyl ester in 1·0 ml. of methanol was added. The solution was swirled and was then left for 15 min. During this time the solution became slightly cloudy and deepened in colour with the formation of a slight brownish sediment. After 15 min. the solution was centrifuged for 3 min. to remove the sediment

and the protein was freed from excess of reagents on a column (3cm. × 30cm.) of Sephadex G-25 (medium grade) with water as eluent. The fractions that contained protein were pooled and used as required.

Reaction of pepsinogen with N-*diazo*[1-^{14}C]*acetyl-*L-*phenylalanine methyl ester.* A 10mg. portion of pepsinogen was dissolved in 5ml. of 0·05M-sodium acetate buffer, pH5·0, and 0·13ml. of 0·1M-cupric acetate solution was added. The solution was then left for 15min. and a solution of 3mg. of *N*-diazo[1-^{14}C]acetyl-L-phenylalanine methyl ester in 0·1ml. of methanol was added. The solution was left for 15min. and was then centrifuged for 3min. The clear suspension was eluted from a column (2·5cm. × 25cm.) of Sephadex G-25 (medium grade) with water as eluent. Fractions were monitored at 280nm., and samples were withdrawn for scintillation counting.

The same reaction was performed in 0·05M-sodium phosphate buffer, pH7·0, with a reaction time of 90min.

Carboxypeptidase digestion of pepsin. A 100mg. portion of pepsin was dissolved in 5ml. of 0·1M-sodium phosphate buffer, pH7·8, and the solution eluted from a column (2cm. × 35cm.) of Sephadex G-25 (medium grade), with the same buffer, to remove low-molecular-weight contaminants. The fractions containing protein were combined and the solution was placed in a thermostatically controlled water bath at 35°. A 10μl. portion of a suspension containing 20mg. of carboxypeptidase A-DFP/ml. was added to 100μl. of 0·5% $NaHCO_3$ solution, and when the protein had completely dissolved the solution was added to the pepsin. At suitable time-intervals 1·5ml. portions from the reaction mixture were withdrawn and 0·5ml. of 1M-HCl was added to each to precipitate the protein. The HCl contained norleucine as an internal standard for the subsequent amino acid analysis. The suspension was then centrifuged for 2min. and 1·5ml. of the supernatant, pH1·1, was withdrawn and applied directly to the column of the amino acid analyser.

Electrophoresis. Electrophoretic separations were performed with the paper vertical, as in the method of Michl (1951), in Shandon 500 Panglas Chromatanks, with cooling by convection of an organic solvent (white spirit) between water-cooled glass coils. Whatman no. 3MC paper (low carboxyl content) and Whatman no. 1 paper was used. In most cases a guide strip a few millimetres wide was cut from the edge of the paper after electrophoresis, and stained with ninhydrin or the ninhydrin–cadmium reagent of Heilmann, Barrollier & Watzke (1957). The buffers used were as follows: pH1·9, formic acid–acetic acid–water (1:4:45, by vol.); pH3·5, pyridine–acetic acid–water (1:10:89, by vol.); pH6·5, pyridine–acetic acid–water (25:1:225, by vol.). The cooling solution in each case was white spirit, but the coolant for the pH6·5 tank had 8% of pyridine added. Maximum loading of the paper was 1mg./cm. for Whatman no. 3MC paper and 0·25mg./cm. for Whatman no. 1 paper.

Paper chromatography. Paper chromatography was performed by the descending method. Whatman no. 1 and Whatman no. 3MC grades of paper were used. Samples were applied to the paper in a straight line to give the same loading as for electrophoresis. In an attempt to sharpen the bands, which were generally much more diffuse than those obtained after electrophoresis, the paper was placed over a pair of glass rods on a sheet of glass with the band of sample between the rods. Solvent was then applied on either side of the sample and allowed to spread inwards as in the method for electrophoresis. The paper was then dried in a current of cool air. Before elution, the paper was allowed to equilibrate with the vapour in the tank for 1–2hr. This was found to improve the speed of elution and reduce tailing of bands, although if the paper was left for too long in the vapour (e.g. overnight) the spots became diffuse. The solvent system finally adopted was: 2-methylpropan-2-ol–butan-2-one–water (2:2:1, by vol.).

Gel filtration. Gel filtrations were performed on Sephadex G-25 and G-50 (Pharmacia Ltd., Uppsala, Sweden). For separation of proteins from low-molecular-weight material medium-grade beads were used; for separation of peptide mixtures fine-grade beads were used. In all cases the beads were allowed to swell as recommended by the manufacturers and the fines were removed by decantation several times. The size of sample was always kept as small as was practicable, but for separation of proteins from low-molecular-weight material up to one-third of the void volume gave adequate separations. For peptide mixtures the sample volume was kept below one-twentieth of the void volume.

Monitoring of column eluates. In preparations involving the separation of proteins from low-molecular-weight material, fractions were monitored at 280nm. to determine the presence of protein material. When digests or mixtures of small peptides were being separated, fractions were monitored at 220nm. Fractions were monitored for radioactivity by withdrawing 50 or 100μl. samples for scintillation counting (see below).

Scintillation counting. Scintillation counting was performed by using a solution made up as follows: 1·75g. of 2,5-diphenyloxazole and 0·06g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene were dissolved in 350ml. of sodium-dried A.R. toluene and left overnight to ensure dissolution. Then 160ml. of Grignard-dry ethanol was added, and the solution was degassed at a water pump and stored in a dark glass bottle. Portions (4ml.) of this solution were used with either 50μl. or 100μl. aqueous portions of sample. Quenching was high, as expected, and external-standard ratio values on the Beckman instrument were about 1–1·5 (approx. 15 is the value for unquenched solutions), but counting efficiency was nevertheless adequate. Counting efficiency could be increased slightly by using a larger amount of scintillant.

Radiochromatogram scanning. For efficient location of radioactive peptides on paper with the Isotope Developments Ltd. instrument it was necessary to have more peptide on the paper than the paper loading requirements permitted. However, with the Packard gas-flow instrument it was possible to locate accurately extremely small amounts (approx. 0·01μmole) of radioactive peptide. Normally chromatograms were scanned at 1cm./min. with a full-scale deflexion on the recorder of 1000 c.p.m. and a time-constant of 10sec.

Elution of peptides from paper. Located spots were marked on the paper and cut out. Elution was achieved with aq. 0·05M-NH_3 unless the peptides were base-labile (as with labelled peptides), in which case they were eluted with water. In all cases elution was allowed to continue overnight at least, even with Whatman no. 3 paper, which allows faster elution than Whatman no. 1 paper. Whenever it was feasible, the peptides were not eluted from the paper, but the section of paper bearing the sample was cut out and sewn on top of a fresh sheet of paper for the next stage of

purification. The paper beneath the sample-bearing section was then cut out so that the eluent or electrophoresis buffer passed through the sample rather than round it. This technique was not used for transference from Whatman no. 3 to Whatman no. 1 paper because of the different thicknesses of the two grades.

Acid hydrolysis of peptides. Complete acid-catalysed hydrolysis of peptides was achieved by using twice-distilled constant-boiling HCl (1 ml./0·1 μmole of peptide) in a sealed tube at 110$\pm$1°. Before the tubes were sealed their contents were subjected to high vacuum and flushed with N_2 three times.

Sequence studies. 'Dansyl–Edman' degradations were performed by the methods described in detail by Gray (1967), the DNS-amino acids being separated and identified by t.l.c. on polyamide layers by the technique described by Woods & Wang (1967). The solvent systems were those numbered 1 and 2 by Woods & Wang (1967), and a system containing ethyl acetate–methanol–acetic acid (20:1:1, by vol.) (Dr B. S. Hartley, personal communication). The solvent systems were run in the order 1, 2 (perpendicular to 1) and finally the third system (in the same direction as 2).

Partial acid hydrolysis of the peptide was performed in an evacuated sealed tube containing 1 M-acetic acid at 100° for 15 hr. Separation of the resultant mixture was achieved electrophoretically on Whatman no. 1 paper at pH 3·5.

The *C*-terminal amino acid was determined by digestion with carboxypeptidase A-DFP. The peptide (approx. 0·01 μmole) was dissolved in 0·02 M-NH_4HCO_3 solution (40 μl.) and 15 μl. of a solution of carboxypeptidase A-DFP (1 mg./ml. in 0·1 M-NH_4HCO_3 solution) was added. Samples of the mixture were incubated at 37° for 6 hr. The reaction was stopped by the addition of acetic acid to bring the pH to approx. 2 before amino acid analysis.

RESULTS

Pepsin homogeneity. In view of the report of Rajagopalan *et al.* (1966*b*) that commercially available preparations of pepsin are heterogeneous, the material used in this work was compared with pepsin freshly prepared from recrystallized pepsinogen by the method recommended by Rajagopalan *et al.* (1966*b*). As a test of the homogeneity of commercially available pepsin, a sample was first eluted from a column of Sephadex G-25 to remove low-molecular-weight contaminants, and the purified enzyme was then treated with carboxypeptidase A. As shown by Fig. 1, 1 mole of alanine/mole was rapidly liberated, but no significant amount of any other amino acid. The *C*-terminal sequence of pepsin is now known to be -Ala-Pro-Val-Ala (Dopheide, Moore & Stein, 1967). We would therefore expect only alanine to be liberated, since carboxypeptidase A does not cleave prolyl bonds. Secondly, the catalytic activity of freshly prepared pepsin was found to be about 1·1 times higher than that of our commercial samples after chromatography on Sephadex G-25. This compares with the factor 1·3 found by Rajagopalan *et al.* (1966*b*) for the analogous comparison. From these findings it

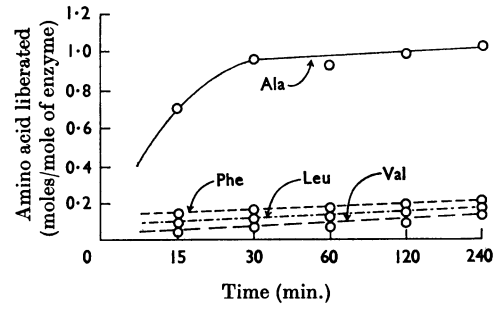

Fig. 1. Liberation of amino acids from commercial pepsin (after chromatography on Sephadex G-25) by digestion with carboxypeptidase A at pH 7·8 at 35°.

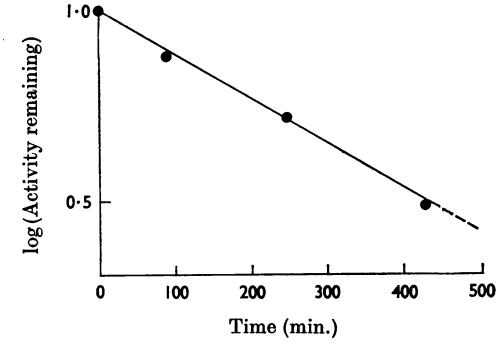

Fig. 2. Inactivation of pepsin by treatment with a 1000-fold molar excess of *N*-diazoacetyl-L-phenylalanine methyl ester at pH 4·5 at 20°.

is apparent that the commercially available pepsin used in this work is less inhomogeneous than the commercial preparations available to Rajagopalan *et al.* (1966*b*), although their method of pepsin preparation from crystalline pepsinogen does result in a more homogeneous material than any obtainable commercially. The prohibitive cost of the pepsin prepared via crystalline pepsinogen, coupled with the encouraging data of Fig. 1, led to the use in this work of our commercial preparation after chromatography on Sephadex G-25.

Inhibition of pepsin by N-diazoacetyl-L-phenylalanine methyl ester. Preliminary experiments showed that *N*-diazoacetyl-L-phenylalanine methyl ester (4 mM) reacts with pepsin (4 μM) at pH 4·5 at 20° with a relatively rapid reaction, the loss of pepsin activity being pseudo-first-order (Fig. 2).

Because of two limiting factors this reaction could only be studied in a narrow pH range. These factors are (*a*) the rapid decomposition of the diazo ketone in aqueous solutions of low pH and (*b*) the

irreversible denaturation of pepsin in solutions of pH higher than 6.

The addition of a 1500-fold molar excess of *N*-diazoacetyl-L-phenylalanine methyl ester to a solution of pepsin at pH 2·0 produced rapid effervescence; there was no detectable loss of activity, even after 20hr. Addition of the same amount of compound to pepsin at pH 3·0 also produced effervescence, although rather more slowly in this case. Again there was no detectable loss of enzymic activity after 20hr. Between pH 4 and pH 6 the diazo ketone was sufficiently stable not to decompose immediately on addition to water, and when pepsin was treated in this pH range with a 1000-fold excess of inhibitor loss of proteolytic activity was observed. In most cases 80–90% of the enzymic activity was lost after 24hr., but sometimes the loss of activity was not so great. This variation is probably due to the presence of trace amounts of heavy-metal ions (see below).

In an attempt to ascertain whether the reaction between *N*-diazoacetyl-L-phenylalanine methyl ester and pepsin takes place at the active site of the enzyme, the rate of loss of activity in the presence of a reversible inhibitor was studied. The rate of loss of proteolytic activity at 0° was followed in the presence and absence of *N*-acetyl-L-phenylalanine ethyl ester, in 0·05M-sodium acetate buffer, pH 5·0. The results are shown in Fig. 3. In the presence of an eight-fold molar excess of reversible inhibitor over irreversible inhibitor, the rate of inactivation of pepsin is decreased 2·4-fold. However, *N*-acetyl-L-phenylalanine ethyl ester is now known to be a non-competitive inhibitor of the enzyme (Greenwell, Knowles & Sharp, 1969) (and not a competitive inhibitor as was erroneously stated in Bayliss & Knowles, 1968), so the slowing of the modification reaction in the presence of *N*-acetyl-L-phenylalanine ethyl ester might simply represent competition with the irreversible inhibitor, which is a very close analogue of the latter.

At this point we learned from Dr W. H. Stein and Dr S. Moore of their studies on the inactivation of pepsin by an analogous diazo compound. These workers found that the presence of Cu^{2+} ion markedly accelerated the inactivation process (Rajagopalan, Stein & Moore, 1966*a*). Fig. 4 shows the effect of added Cu^{2+} ion on the rate of loss of proteolytic activity. The function of the Cu^{2+} ion presumably relates to the well-known effect of copper salts on diazo and diazonium compounds (Zollinger, 1961), and Lundblad & Stein (1969) have proposed the intermediary of a Cu^{2+}–carbene complex in these reactions. Certainly a 100-fold molar excess of cupric acetate over pepsin has no measurable effect on the proteolytic activity of the enzyme determined against haemoglobin as substrate, and the presence of a 165-fold molar excess of cupric chloride lowers the rate of pepsin-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-L-phenylalanine by only 15% at pH 1·49 at 35° (A. J. Cornish–Bowden, unpublished work). However, Cu^{2+} ion apparently does bind to pepsin at pH 5·6, 9·9 moles of copper/mole of pepsin being retained after dialysis against distilled water for 4hr. (Sizer & Fennessey, 1951). This bound copper may possibly be important in the inactivation process.

To determine the excess of inhibitor required to produce total inactivation of the enzyme, a solution of pepsin in 0·05M-sodium acetate buffer was made up in the normal way in the presence of a 15-fold molar excess of cupric acetate, and different amounts of *N*-diazoacetyl-L-phenylalanine methyl ester were added to portions of the pepsin solution. The solutions were left overnight, and the proteolytic activity remaining was then determined. The results are shown in Table 1.

On the basis of the results of this experiment and and previous one, a 30-fold molar excess of inhibitor and a 40-fold molar excess of cupric acetate over

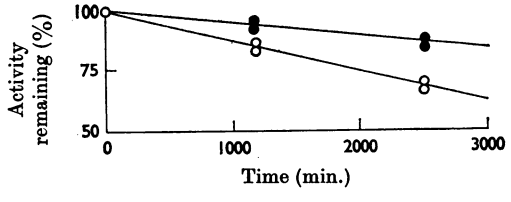

Fig. 3. Inactivation of pepsin (4μM) by *N*-diazoacetyl-L-phenylalanine methyl ester (1·8mM) at pH 5·0 at 0° in the presence (●) and absence (○) of *N*-acetyl-L-phenylalanine ethyl ester (14·4mM).

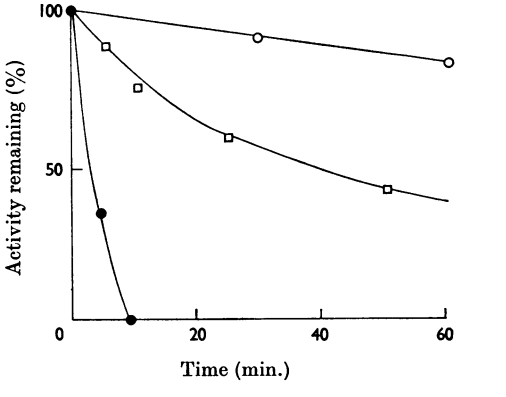

Fig. 4. Inactivation of pepsin (2mg./ml.) by *N*-diazoacetyl-L-phenylalanine methyl ester (250-fold molar excess) at pH 5·0 at 20° with 2% (v/v) methanol in the absence (○) and presence of cupric acetate: □, 20-fold molar excess; ●, 40-fold molar excess.

Table 1. *Effect of [inhibitor]/[enzyme] molar ratio on inactivation of pepsin*

The pepsin concentration was 10mg./ml. The reaction time was 12hr. The cupric acetate concentration was a 15-fold molar excess over pepsin. Inactivation was at pH5·0 and at 20°. The inhibitor was *N*-diazoacetyl-L-phenylalanine methyl ester.

[Inhibitor]/[enzyme]	Activity remaining (%)
0	100
2	46
4	28
8	17
16	2
32	0

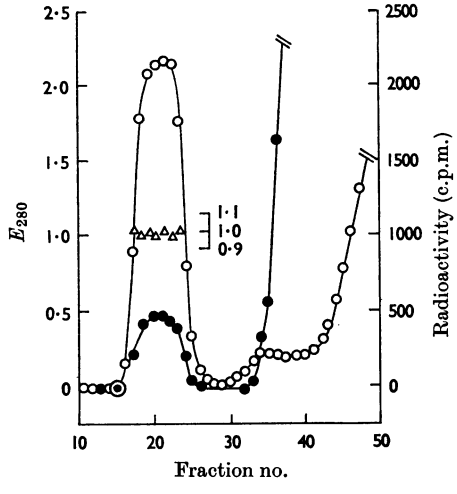

Fig. 5. Separation of inhibited pepsin from excess of inhibitor after reaction with *N*-diazo[1-^{14}C]acetyl-L-phenylalanine methyl ester, and the uptake of inhibitor by the inhibited enzyme (△, in moles/mole: inset scale). ○, E_{280}; ●, radioactivity.

pepsin resulted in complete inactivation of the enzyme (10mg./ml.) after 90min. However, such preparations were found to have incorporated more than 1 mole of inhibitor/mole of enzyme. At a pepsin concentration of 2mg./ml., a reaction time of 15min. and the relative inhibitor and Cu^{2+} ion concentrations as above, the enzyme activity was completely eliminated, and the uptake of inhibitor was 1·00–1·05 moles of inhibitor/mole of enzyme. The homogeneity of the labelling of the protein fraction and the clean separation from excess of inhibitor are shown in Fig. 5.

Reaction with pepsinogen. As a further test of the thesis that reaction between pepsin and *N*-diazoacetyl-L-phenylalanine methyl ester takes place at the active site of the enzyme, pepsinogen was treated with ^{14}C-labelled inhibitor under conditions identical with those described above for preparing inhibited pepsin. After elution from Sephadex, the protein was found to have incorporated radioactivity equivalent to 0·15 mole of inhibitor/mole of zymogen. It was thought possible that this uptake might represent partial activation of the zymogen, which is known to occur slowly in solutions of pH less than 6, followed by reaction of the liberated enzyme. The reaction was therefore repeated at pH7·0, since activation is known not to occur at this pH. After standing for 90min. in the presence of inhibitor it was found that only 0·04 mole of inhibitor had been incorporated/mole of zymogen.

Activity of partially inactivated pepsin. In view of the report of Erlanger, Vratsanos, Wassermann & Cooper (1965) that pepsin that has been inhibited by reaction with *p*-bromophenacyl bromide is totally inactive when assayed with *N*-benzyloxycarbonyl-L-glutamyl-L-phenylalanine, but still possesses 22% activity when assayed by the haemoglobin method, pepsin was partially inactivated by treatment with a limited amount of *N*-diazoacetyl-L-phenylalanine methyl ester in the absence of cupric acetate. This partially inactivated derivative was found to have 26% of the activity of native pepsin by the haemoglobin method. When assayed with *N*-acetyl-L-phenylalanyl-L-phenylalanine amide as the substrate, the derivative was found to have 21% of the activity of native pepsin. Thus, within experimental error, the loss of proteolytic activity here parallels the loss of activity towards small synthetic substrates.

Isolation of the labelled peptide from inactivated pepsin. The procedure finally adopted was as follows. The aqueous solution of inhibited pepsin eluted from Sephadex after inhibition of the enzyme with *N*-diazo[1-^{14}C]acetyl-L-phenylalanine methyl ester was treated with three times its own volume of acetone. The solution was then concentrated on a rotary evaporator with the temperature maintained below 50°, to give a protein concentration of 10–20mg./ml. Native pepsin (5%, w/w, of inhibited enzyme) was then added, and the pH was adjusted to 3·0 by the addition of 1M-hydrochloric acid. The digest was maintained at pH3·0 at 35° in a pH-stat for 12hr. The digest was then centrifuged to remove the remaining solid material and the clear supernatant was used for the further purification steps.

The supernatant was chromatographed on a 1cm. × 140cm. column of Sephadex G-25, with water as eluent. The elution pattern is shown in Fig. 6. The radioactive fragments were eluted in two main peaks, I and II. From its elution volume peak I was assumed to be largely undigested material, and peak II contained 30–50% of the total

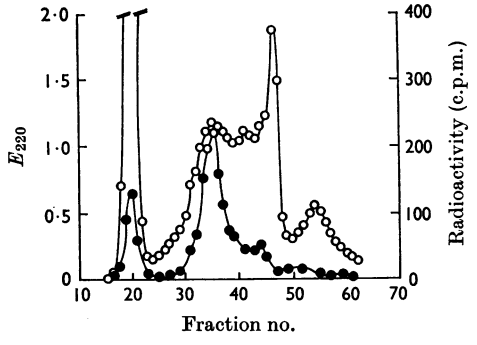

Fig. 6. Elution pattern of pepsin inhibited with ^{14}C-labelled inhibitor after digestion with pepsin (12hr., pH3·0, 35°) from a column of Sephadex G-25 eluted with water. ○, E_{220}; ●, radioactivity.

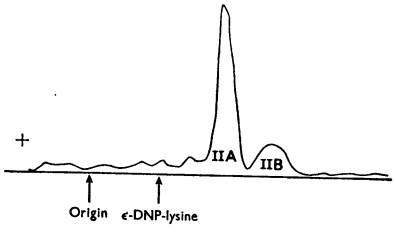

Fig. 7. Radiochromatogram of peak II (Fig. 6, fractions 32–38) after electrophoresis at pH3·5 on Whatman no. 3MC paper.

radioactivity in the inhibited enzyme. The fractions (32–38) comprising peak II were combined and freeze-dried, and the resulting peptide was dissolved in water and subjected to electrophoresis at pH3·5 in pyridine–acetate buffer on Whatman no. 3MC paper. On staining a guide strip with ninhydrin–cadmium reagent a large amount of poorly resolved material was seen, although on strip-scanning (Fig. 7) two radioactive peaks were observed, neither of which was in the position where free inhibitor should appear. ('Free inhibitor' refers to inhibitor that has been liberated from labelled peptide, and is presumably *N*-glycollyl-L-phenylalanine methyl ester.) The peak marked IIA (Fig. 7) contained 80–90% of the radioactivity applied to the paper. The area covered by peak IIA was cut out, the material in it was eluted from the paper with water and this solution was freeze-dried. The material was then taken up in water and submitted to electrophoresis at pH1·9 in formate–acetate buffer on Whatman no. 1 paper. A guide strip was stained with ninhydrin–cadmium reagent and several bands were observed. The single radioactive spot was found to migrate towards the cathode marginally less than ε-DNP-lysine, and corresponded to a spot that slowly developed a red colour with ninhydrin–cadmium reagent. The paper corresponding to the single radioactive spot was located, cut out and sewn into another sheet of Whatman no. 1 paper and subjected to chromatography with 2-methylpropan-2-ol–butan-2-one–water as eluent. The single radioactive spot that was located after this step coincided with a single resolved spot on the guide strip that was slowly stained by ninhydrin–cadmium reagent. The material in this spot was eluted from the paper with water, freeze-dried and subjected to acid-catalysed hydrolysis for 22hr. Amino acid analysis of the hydrolysate showed that threonine, serine, aspartic acid, glycine, valine, isoleucine and phenylalanine were present in non-stoicheiometric amounts, but no other amino acids in detectable quantities. A further portion of peak IIA material was therefore purified by paper chromatography, and the single radioactive component was purified further by electrophoresis at pH1·9. These two steps were performed in the reverse order of the previous preparation because the radioactive spot after electrophoresis tended to be less diffuse than after chromatography. After these two steps the radioactive material was eluted from the paper with water. A small amount of the solution was withdrawn for scintillation counting, and from the radioactivity of this sample it was calculated that there was about 0·05 μmole of labelled peptide in the solution. The solution was then divided into three parts and each portion was freeze-dried. The three portions were then subjected to acid-catalysed hydrolysis for 22, 48 and 94hr. respectively. The results from amino acid analysis are shown in Table 2.

As shown in Table 2, the composition of the peptide is clearly (Asp,Thr_2,Ser,Gly,Val,Ile). No other amino acids were present in integrable amounts. The phenylalanine comes, of course, from the inhibitor. The absolute amount of phenylalanine in the hydrolysates agreed very well with the estimated amount of 0·05 μmole calculated from the radioactivity. The slightly low value for phenylalanine is attributed to slight loss of label in the final purification step. Since labelled and unlabelled peptide migrate at virtually the same rate at pH1·9, loss of label during separation would not affect the rate of migration of the peptide significantly, but would appear as tailing in the radiochromatogram. This effect was observed.

'Diagonal' electrophoresis. Some experiments were performed in which the lability of the peptide–inhibitor bond was put to good purpose. It was found that treatment of the labelled peptide on paper with a saturated aqueous solution of redistilled triethylamine removed the label. A portion

Table 2. *Amino acid analyses of the labelled peptide*

Values are normalized to Asp=1·0. No other amino acids were present in integrable quantity.

		Amino acid composition			
Amino acid	Hydrolysis time ...	22hr.	48hr.	94hr.	Integral values (extrapolated)
Asp		1·0	1·0	1·0	1
Thr		1·75	1·68	1·59	2
Ser		1·08	1·04	0·98	1
Gly		1·15	1·31	1·28	1
Val		0·32	0·73	0·95	1
Ile		0·37	0·73	0·92	1
Phe		0·79	0·87	0·89	1

of peak II material from Sephadex G-25 chromatography was applied to Whatman no. 3MC paper and subjected to electrophoresis at pH 3·5. After the separation, the paper was dried and the strip carrying the peptide material was cut out and treated with a saturated aqueous solution of triethylamine, which was applied to the paper from both sides of the strip in a manner similar to that used for the application of buffer in a normal electrophoretic run. After drying at room temperature, the strip was sewn into a fresh sheet of paper and was resubjected to electrophoresis at pH 3·5 perpendicularly to the original direction. The paper was then dried and developed with ninhydrin–cadmium reagent. Most of the ninhydrin–positive material lay on a diagonal line as expected (see Fig. 8), but one major spot was off this line in a position corresponding to the position of peak IIA material in the original direction. There was another very faint spot closer to the diagonal also corresponding to the position of peak IIA material, and a third spot, which was also very faint, in a position corresponding to that of peak IIB material. All these spots were on the anode side of the diagonal, i.e. they had each acquired a partial negative charge, as expected for peptides in which a carboxyl group had been unmasked on hydrolysis by aqueous triethylamine.

This technique was used for the rapid isolation of peptide for sequence studies. The major off-diagonal peptide was eluted with water from the expected position after electrophoresis in the second dimension.

Sequence studies. 'Dansyl–Edman' degradation on the whole peptide gave Ile-Val-Asp-, but the yields of the subsequent DNS-amino acids were rather low (presumably because of some $\alpha \rightarrow \beta$ rearrangement of the aspartyl peptide link during degradation), and a partial acid hydrolysis was performed. The resulting hydrolysate was separated into its components by paper electrophoresis on Whatman no. 1 paper at pH 3·5. The following

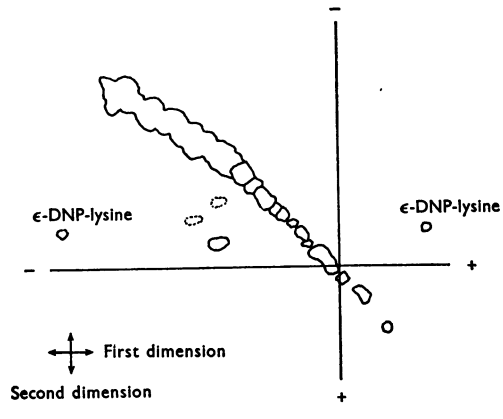

Fig. 8. 'Diagonal' electrophoresis pattern obtained by electrophoresis of material from peak II (Fig. 6) at pH 3·5, where the paper was treated with saturated aqueous triethylamine before electrophoresis in the second dimension.

peptides were eluted from the paper and their sequences determined as: Ile-Val; Thr-Gly-Thr; Ser. The *C*-terminal amino acid of the intact heptapeptide was determined by using a solution of carboxypeptidase A. Amino acid analysis of the resulting digest showed that serine was the *C*-terminal amino acid, with threonine as the penultimate residue.

These data allow the unambiguous assignment of the sequence as:

Ile-Val-Asp-Thr-Gly-Thr-Ser

DISCUSSION

The labile nature of the link between the amino acid side chain of the protein and the radioactive irreversible inhibitor ruled out a number of the

more usual approaches to the problem of isolation of a labelled peptide. It was found that at pH values above 7 in the intact inhibited protein the radioactive label was rapidly lost. Surprisingly, it was shown that the label became more susceptible to basic hydrolytic cleavage on digestion of the protein, and the pH of solutions of resulting peptide mixtures was kept at 5·0 or below in all separation operations. Additionally, it was found that the label was lost from its peptide on freeze-drying solutions (below pH 5) in high salt concentrations. This fact made chromatography on ion-exchangers less attractive. Accordingly column chromatography on Sephadex G-25 with water as eluent and electrophoresis and paper chromatography were used for the purification of the labelled peptide.

To maximize the proteolytic digestion of inhibited enzyme it is necessary first to denature the protein. Although pepsin is irreversibly denatured at pH values above about pH 6, this method was ruled out by the base-lability of the labelled protein. Denaturation at low pH with alcohols was not used, for fear of transesterification and consequent loss of radioactive label, and acetone was finally adopted as the denaturant before digestion. Finally, although pepsin is, compared with trypsin and chymotrypsin, rather an unspecific proteolytic enzyme, the pH optima of the neutral proteases are too high in view of the base-lability of the label, and digestion of the denatured inhibited enzyme was therefore performed with pepsin at pH 3·0.

Two aspects of the present experiments merit discussion. These are: what is the evidence that the site of attachment of the inhibitor is the aspartic acid residue in the sequence Ile-Val-Asp-Thr-Gly-Thr-Ser, and what is the evidence that this aspartic acid residue is part of the active site of pepsin?

The following points lead to the view that the diazo reagent reacts with the β-carboxyl group of aspartic acid: (i) the base-lability of the enzyme–inhibitor bond suggests an ester linkage (see above); (ii) the neutrality of the labelled peptide (judged from its migration electrophoretically) in the pH range 3·5–6·5 indicates that there is only one free carboxyl group; (iii) loss of inhibitor from the labelled peptide results in a peptide with a net negative charge at pH 6·5, and little change in the electrophoretic mobility at pH 1·9; these facts point to the liberation of a carboxyl group on cleavage of the inhibitor–peptide bond, and rule out an attachment to serine or threonine; (iv) the net positive charge of both labelled and unlabelled peptides at pH 1·9 precludes reaction at the terminal amino group.

The question whether the modified aspartic acid residue is actually part of the active site of the enzyme is impossible to answer definitely. On the basis of the stoicheiometric reaction, and the concomitant complete loss of both peptidase and protease activity, we can say that this aspartic acid residue is uniquely reactive towards our reagent, and that it is required free for enzymic activity. Even though the enzyme is partially protected from inactivation by the presence of *N*-acetyl-L-phenylalanine ethyl ester, this latter material is a non-competitive inhibitor of pepsin (Greenwell *et al.* 1969), and is in any case a close analogue of the irreversible inhibitor itself. The low solubilities and relatively high K_i values of dipeptide competitive inhibitors of the enzyme preclude the study of protection by more substrate-like materials. In an analogous study Hamilton, Spona & Crowell (1967) have observed that 'a specific substrate can protect the enzyme from inhibition' (by 1-diazo-4-phenylbutan-2-one), which (since their inhibitor reacts similarly to the one described here; see below) argues for a reaction at or near the active site. Moreover, the finding that pepsinogen will not react with the inhibitor indicates that activation of the zymogen is accompanied by the capacity to react with the specific inhibitor. However, it has been argued (Cornish-Bowden & Knowles, 1969) that the active site of pepsin contains one or two carboxylic acid side chains that are responsible for the catalysis, and that the neighbourhood of the active site has at least one other carboxyl group. Indeed, the observations by Erlanger, Vratsanos, Wassermann & Cooper (1965, 1967) that, whereas *p*-bromophenacyl bromide modifies a unique aspartic acid residue [which is part of a peptide of composition (Gly_2,Asp,Ser,Glu)] with complete loss of peptidase activity and 75–80% loss of protease activity, the diazo analogue (α-diazo-*p*-bromoacetophenone) modifies a different unique residue in pepsin with total loss of both activities. The authors suggest that *p*-bromophenacyl bromide reacts with a carboxylic acid residue that is near the active site but not essential to catalysis, and that the diazo-compound analogue 'reacts with a functional group that participates directly in the catalytic mechanism'. The facts that a virtual substrate (*N*-benzyloxycarbonyl-L-phenylalanine) protects the enzyme against inhibition by *p*-bromophenacyl bromide (Erlanger *et al.* 1967) and that this reagent is believed to react with a catalytically inessential residue stress the difficulties attendant on attempts to implicate catalytically essential residues unequivocally. At the present time, we accept as a working hypothesis that diazo reagents such as that used here react with a unique essential aspartic acid residue at the active site of pepsin.

Work on the inactivation of pepsin by diazo reagents has been reported by Delpierre & Fruton (1965, 1966), Rajagopalan *et al.* (1966*a*), Stepanov, Lobareva & Mal'tsev (1967), Hamilton *et al.* (1967),

Erlanger *et al.* (1967), Ong & Perlmann (1967), Kozlov, Ginodman & Orekhovich (1967) and Bayliss & Knowles (1968). Fry, Kim, Spona & Hamilton (1968) and Stepanov & Vaganova (1968) have reported sequences around the uniquely modified aspartic acid residue of Ile-Val-Asp-Thr and Val-Asp respectively. Our results indicate Ile-Val-Asp-Thr-Gly-Thr-Ser, and it seems likely that the same essential carboxyl group is involved in all three labelling studies.

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REFERENCES

Anson, M.L. (1948). In *Crystalline Enzymes*, p. 305. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.

Bayliss, R. S. & Knowles, J. R. (1968). *Chem. Commun.* p. 196.

Brown, J. R. & Hartley, B. S. (1966). *Biochem. J.* **101**, 214.

Cornish-Bowden, A. J. & Knowles, J. R. (1969). *Biochem. J.* **113**, 353.

Delpierre, G. R. & Fruton, J. S. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 1161.

Delpierre, G. R. & Fruton, J. S. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 1817.

Dopheide, T. A. A., Moore, S. & Stein, W. H. (1967). *J. biol. Chem.* **242**, 1833.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N. & Cooper, A. G. (1965). *J. biol. Chem.* **240**, PC3447.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N. & Cooper, A. G. (1967). *Biochem. biophys. Res. Commun.* **28**, 203.

Fry, K. T., Kim, O-K., Spona, J. & Hamilton, G. A. (1968). *Biochem. biophys. Res. Commun.* **30**, 489.

Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 469. Ed. by Hirs, C. H. W. New York: Academic Press Inc.

Greenwell, P., Knowles, J. R. & Sharp, H. C. (1969). *Biochem. J.* **113**, 363.

Hamilton, G. A., Spona, J. & Crowell, L. A. (1967). *Biochem. biophys. Res. Commun.* **26**, 193.

Heilmann, J., Barrollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.

Herriott, R. M. (1947). *Advanc. Protein Chem.* **3**, 169.

Herriott, R. M., Anson, M. L. & Northrop, J. H. (1946). *J. gen. Physiol.* **30**, 185.

Inouye, K. & Fruton, J. S. (1967). *Biochemistry*, **6**, 1765.

Knowles, J. R., Sharp, H. & Greenwell, P. (1969). *Biochem. J.*. **113**, 343.

Kozlov, L. V., Ginodman, L. M. & Orekhovich, V. N. (1967). *Biokhimiya*, **32**, 1011.

Lundblad, R. L. & Stein, W. H. (1969). *J. biol. Chem.* **244**, 154.

Michl, H. (1951). *Mh. Chem.* **82**, 489.

Ong, E. B. & Perlmann, G. E. (1967). *Nature, Lond.*, **215**, 1492.

Perlmann, G. E. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 915.

Rajagopalan, T. G., Moore, S. & Stein, W. H. (1966*b*). *J. biol. Chem.* **241**, 4940.

Rajagopalan, T. G., Stein, W. H. & Moore, S. (1966*a*). *J. biol. Chem.* **241**, 4925.

Sizer, I. W. & Fennessey, J. F. (1951). *J. biol. Chem.* **188**, 351.

Stepanov, V. M., Lobareva, L. S. & Mal'tsev, N. I. (1967). *Izvest. Akad. Nauk SSSR, Ser. Khim.* p. 1630.

Stepanov, V. M. & Vaganova, T. I. (1968). *Biochem. biophys. Res. Commun.* **31**, 825.

Woods, K. R. & Wang, K.-T. (1967). *Biochim. biophys. Acta*, **133**, 369.

Zollinger, H. (1961). *Diazo and Azo Chemistry*, pp. 116, 164. New York: Interscience Publishers Inc.