The Hydrolysis by Thermolysin of Dipeptide Derivatives that Contain Substituted Cysteine

By A. P. DAMOGLU,* H. LINDLEY AND I. W. STAPLETON
Division of Protein Chemistry, Parkville (Melbourne), Vic. 3052, Australia

(Received 23 November 1970)

1. The preparation of protected dipeptides of the form acetylglycylamino acid amides is described, where the amino acid is phenylalanine, leucine, valine, alanine, *S*-methyleysteine, *S*-ethylcysteine, *S*-benzylcysteine and *S*-phenyleysteine. 2. Kinetic parameters for the thermolytic hydrolysis of these blocked dipeptides are reported. The rate of hydrolysis was fastest when the amino acid was leucine or phenylalanine, slower when it was *S*-methyleysteine, valine or *S*-ethylcysteine, much slower when it was alanine, and negligible for *S*-phenyleysteine or *S*-benzylcysteine. 3. The results are compared with those for similar dipeptide derivatives with benzyloxycarbonyl and furylacryloyl blocking groups, which are hydrolysed faster.

The increase in our knowledge of the specificity and mode of action of proteolytic enzymes has led to the possibility of modifying substrate proteins so as to influence their susceptibility to enzyme attack. This has been especially true of tryptic digestion where the substrate protein has been modified both to inhibit hydrolysis of bonds adjacent to lysine residues (Redfield & Anfinsen, 1956; Anfinsen, Sela & Tritch, 1956; Merigan, Dreyer & Berger, 1962; Goldberger & Anfinsen, 1962) and to permit hydrolysis of bonds adjacent to cysteine and serine residues by forming the lysine analogues, thialysine (2-aminoethylcysteine) (Lindley, 1956) and oxalysine (2-aminoethylserine) (Tesser, Nivard & Gruber, 1964). The thialysine technique has been used extensively but the oxalysine technique has not been used because of the difficulty of amino-ethylation of serine. However, Patchornik, Wilchek & Zioudrou (1969) have overcome this difficulty by devising a technique for the direct conversion of serine residues into thialysine residues.

A search for substituted cysteine derivatives that would serve as substrates for chymotrypsin (Damoglou, Lindley & Stapleton, 1970) has shown that *S*-phenyleysteine peptides are split adjacent to the *S*-phenyleysteine. No satisfactory technique for the *S*-phenylation of cysteine residues in proteins has been found although a theoretical possibility of converting serine into *S*-phenyleysteine is suggested by the work of Patchornik *et al.* (1969).

It seemed worth while to consider other proteolytic enzymes such as thermolysin that are currently in use for sequence work and to examine substituted

cysteine derivatives for their behaviour as substrates. Thermolysin specificity has been studied to a certain extent (Ohta & Ogura, 1965; Matsubara, 1966; Morihara, 1967; Morihara & Ebata, 1967; Morihara & Oka, 1968; Morihara, Tsuzuki & Oka, 1968; Feder & Schuck, 1970), mostly with synthetic peptides of the form *N*-benzyloxycarbonylglycylamino acid amide. Thermolysin preferentially hydrolyses peptide bonds that involve the amino groups of leucine and phenylalanine.

Initially it was intended to obtain the kinetic constants of substituted dipeptides of the form benzyloxycarbonylglycylamino acid amide where the second amino acid was phenylalanine, leucine and valine and to compare them with the constants from similar dipeptide derivatives where the amino acid was *S*-methyleysteine, *S*-ethylcysteine, *S*-benzylcysteine and *S*-phenyleysteine. In preliminary studies, however, it was found that the *S*-substituted cysteine ester dipeptide derivatives were very insoluble in water and since solvents such as acetonitrile, methanol and ethanol inhibit the hydrolysis, reliable kinetic results could not be obtained. Evidence from inhibition experiments indicated that the peptides were binding to the enzyme although this may have been an effect of the benzyloxycarbonyl group because benzyloxycarbonylglycine has been shown (Feder & Schuck, 1970, and this study) to inhibit hydrolysis. Since *N*-acetylglycine did not inhibit hydrolysis and acetyl-peptides were expected to be much more soluble than benzyloxycarbonyl-peptides, dipeptide derivatives of the form acetylglycylamino acid amide were used.

The acetyldipeptide amides were synthesized

* Present address: Biochemistry Division, National Research Council of Canada, Ottawa, Ont., Canada.

by an active ester coupling of acetylglycine either with the amino acid amide (method A) or with the amino acid methyl ester followed by ammonolysis of the resulting peptide ester (method B). The method of choice depended largely on the following factors: method A was used where an acetyl-dipeptide ester proved difficult to purify owing to solubility behaviour and method B was employed where the amino acid amide could not be prepared satisfactorily because of lability of the side chain in basic solution.

EXPERIMENTAL

Microanalyses were carried out by the Australian Microanalytical Service, Melbourne. Melting points are uncorrected. Solvents used were of AR quality.

Amino acids. L-Alanine, L-valine, L-leucine and L-phenylalanine were purchased from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Preparation of cysteine derivatives. *S*-Methyl-L-cysteine, *S*-ethyl-L-cysteine and *S*-benzyl-L-cysteine were prepared by adding methyl iodide (du Vigneaud, Loring & Craft, 1934), ethyl iodide (Armstrong & Lewis, 1951) and benzyl chloride (Wood & du Vigneaud, 1939) respectively to a solution of the disodium salt of cysteine in liquid ammonia. *S*-Phenyl-L-cysteine was prepared by treating the cuprous derivative of cysteine with benzenediazonium sulphate (Zbarsky & Young, 1943). Optical rotations and melting points corresponded closely with the literature values.

Amino acid methyl ester hydrochlorides. Esterifications in methanol were carried out by the usual Fischer (1901) procedure and the ester hydrochlorides were crystallized from warm ethanol by the addition of dry ether. Table I lists the analytical data and physical constants of three *S*-substituted cysteine esters. Physical constants of the methyl ester hydrochlorides of L-alanine (Zahn & Schüssler, 1961), L-valine (Smith, Spackman & Polglase, 1952), L-leucine (Smith & Slouim, 1948), L-phenylalanine (Schrecker & Hartwell, 1957) and *S*-benzyl-L-cysteine (Maclaren, Savage & Swan, 1958) were in accord with the literature values.

Amino acid amide salts. L-Leucine amide (Smith & Slouim, 1948) and L-valine amide (Smith *et al.* 1952) hydrochlorides were prepared by published procedures. *S*-Benzyl-L-cysteine amide hydrobromide was synthesized by the following procedure. *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteine amide (Stapleton & Swan, 1962) (13.1 g) was slurried in acetic acid (15 ml) and treated with a solution (50 ml) of HBr (6M) in acetic acid. After vigorous shaking to bring about rapid solution, the solution was left at room temperature for 20 min. Dry ether (130 ml) was added and the precipitated amide salt filtered by suction on a sintered-glass funnel. The crude amide salt was washed several times with ether and crystallized from hot acetic acid followed by addition of ethyl acetate to give a pure product (9.1 g, 82%) with m.p. 183–184°C, $[\alpha]_D^{20} + 10.3^\circ$ (c 2 in EtOH) (Found: C, 41.4; H, 5.23; N, 9.77; S, 10.9%. Calc. for $C_{10}H_{15}BrN_2OS$: C, 41.2; H, 5.19; N, 9.62; S, 11.0%).

***N*-Acetylglycylamino acid methyl esters (method B).** To a vigorously stirred suspension of amino acid methyl

Table I. Analytical data on amino acid methyl ester hydrochlorides

Amino acid methyl ester hydrochloride	Yield (%)	M.p. (°C)	$[\alpha]_D^{20}$ (c 1 in EtOH)	Formula	Composition							
					Calc.			Found				
					C	H	N	S	C	H	N	S
<i>S</i> -Methyl-L-cysteine	65	142-143*	+11.8°	C ₅ H ₁₂ NO ₂ SCl	32.3	6.52	7.55	17.3	32.6	6.64	7.36	17.3
<i>S</i> -Ethyl-L-cysteine	75	149-150	+11.3°	C ₆ H ₁₄ NO ₂ SCl	36.1	7.07	7.02	16.1	36.3	7.04	6.77	16.3
<i>S</i> -Phenyl-L-cysteine	60	156-157	+99.8°	C ₁₀ H ₁₄ NO ₂ SCl	48.5	5.70	5.66	12.9	48.8	5.90	5.50	12.7

* Sokolovsky, Sadeh & Patchornik (1964) report m.p. 135°C.

ester hydrochloride (0.01 mol) in ethyl acetate (30 ml) was added triethylamine (1.40 ml). After stirring for 1 h a solution of *N*-acetyl glycine *p*-nitrophenyl ester (Zahn & Otten, 1962) (2.40 g) in warm ethyl acetate (20 ml) was added and stirring maintained for 16 h at room temperature. The suspension was filtered and the insoluble triethylamine hydrochloride washed several times with warm ethyl acetate. The filtrate was evaporated to dryness *in vacuo* and the residue triturated with ether followed by decantation. This procedure (which removes *p*-nitrophenol) was repeated several times until the insoluble portion was colourless. Crystallization was effected by the addition of cyclohexane to a solution of the product in ethyl acetate.

With acetyl glycy *S*-ethyl cysteine methyl ester, it was found necessary (because of some solubility of the peptide in ether) to use silica-gel column chromatography to separate the *p*-nitrophenol. The residue from the ethyl acetate filtrate was dissolved in chloroform and passed down a short column. Elution with chloroform was continued until the silica gel was no longer yellow. The peptide was then eluted with methanol-chloroform (1:9, v/v).

N-Acetyl glycy *L*-phenylalanine methyl ester was prepared by a published procedure (Antonovics & Young, 1967) involving a mixed anhydride coupling.

Table 2 lists the melting points, rotations and analytical data of the acetyl dipeptide esters prepared by the above procedure.

N-Acetyl glycy *amino acid amides*. Method A. To a vigorously stirred suspension of amino acid amide hydrohalide (0.01 mol) in ethyl acetate (30 ml) was added triethylamine (1.40 ml). After stirring for 1 h, a solution of *N*-acetyl glycine *p*-nitrophenyl ester (2.40 g) in warm ethyl acetate (20 ml) was added and stirring maintained for 12–16 h. The separated solid, which consisted of product and triethylamine hydrohalide, was filtered by suction and washed several times with ice-cold chloroform. The residual dipeptide amide was crystallized initially from acetonitrile. Final crystallizations were performed in the solvents listed in Table 3, which also includes analytical and other pertinent data.

Method B. A solution of *N*-acetyl glycy amino acid methyl ester (0.01 mol) in dry methanol (25 ml) was surrounded by an ice bath and saturated with ammonia. The flask was enclosed with a spring-loaded stopper and the solution stored for 2 days at room temperature. After removal of excess of NH_3 and solvent *in vacuo*, the residue was purified by the procedure in method A.

Thermolysin. This was isolated from *Bacillus thermo- proteolyticus* and used as bought from Calbiochem (crystalline, cat. no. 58656), Los Angeles, Calif., U.S.A. Total-N determination and amino acid analysis showed the enzyme powder to contain 67.25% protein. The mol.wt. (37500; Ohta, Ogura & Wada, 1966) was used to calculate the enzyme concentration.

Reaction method.

The hydrolysis of the dipeptides by thermolysin produces acetyl glycine and the amino acid amide (X-NH_2). Under the reaction conditions (i.e. pH 7) the carboxyl group of acetyl glycine will be fully dissociated and the

Table 2. Analytical data on acetyl glycy *amino acid methyl esters*

Residue X of Ac-Gly-X-OMe	Yield (%)	M.p. (°C)	[α] _D ²⁰ (c 1 in EtOH)	Formula	Composition							
					Calc.				Found			
					C	H	N	S	C	H	N	S
Alanine	95	117–119	–48.7°	$\text{C}_8\text{H}_{14}\text{N}_2\text{O}_4$	47.5	6.98	13.9	12.9	47.7	7.07	13.7	13.1
<i>S</i> -Methyl cysteine	73	93–94	–23.3°	$\text{C}_9\text{H}_{16}\text{N}_2\text{O}_4\text{S}$	43.5	6.50	11.3	12.9	43.6	6.51	11.5	11.9
<i>S</i> -Ethyl cysteine	45	75–76	–20.3°	$\text{C}_{10}\text{H}_{18}\text{N}_2\text{O}_4\text{S}$	45.8	6.92	10.7	12.2	45.7	7.06	10.8	11.9
<i>S</i> -Phenyl cysteine	63	88–89	+14.8°	$\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_4\text{S}$	54.2	5.85	9.03	10.3	53.8	5.71	8.82	10.0

Table 3. *Analytical data on acetylglycyl amino acid amides*

Crystallization solvents: (a) acetonitrile; (b) ethyl acetate-carbon tetrachloride; (c) ethyl methyl ketone; (d) water; (e) ethanol-benzene; (f) <i>n</i> -propanol; (g) ethanol.				Composition									
Residue X of L-Ac-Gly-X-NH ₂	Yield (%)	M.p. (°C) (solvent)	[α] _D ^b (c, solvent, t° C)	Formula	Calc.				Found				
					C	H	N	S	C	H	N	S	
Alanine	68	256-258 (d)	-36.6° (1, H ₂ O, 25)	C ₇ H ₁₃ N ₃ O ₃	44.9	7.00	22.5		44.9	7.06	22.7		
Valine	77	202-203 (c)	-11.2° (1, EtOH, 20)	C ₉ H ₁₇ N ₃ O ₃	50.2	7.96	19.5		50.5	7.98	19.3		
Leucine	77	180-181 (b)	+30.0° (1, EtOH, 21)	C ₁₀ H ₁₉ N ₃ O ₃	52.4	8.35	18.3		52.2	8.32	18.1		
Phenylalanine	73	174-177 (a)	+9.6° (1, EtOH, 20)	C ₁₃ H ₁₇ N ₃ O ₃	59.3	6.51	16.0		59.2	6.64	16.3		
<i>S</i> -Methylcysteine	78	150-151 (a)	-42.3° (1, EtOH, 20)	C ₈ H ₁₅ N ₃ O ₃ S	41.2	6.48	18.0	13.8	41.3	6.62	18.3	13.8	
<i>S</i> -Ethylcysteine	70	137-138 (e)	-29.2° (1, EtOH, 20)	C ₉ H ₁₇ N ₃ O ₃ S	43.7	6.93	17.0	12.9	44.0	6.80	17.0	12.7	
<i>S</i> -Phenylcysteine	54	176-177 (f)	-54.7° (0.5, EtOH, 20)	C ₁₃ H ₁₇ N ₃ O ₃ S	50.2	5.50	13.5	10.3	52.6	5.60	13.3	10.7	
<i>S</i> -Benzylcysteine	79	180-181 (g)	-43.0° (1, DMF*, 20)	C ₁₄ H ₁₉ N ₃ O ₃ S	54.4	6.19	13.6	10.3	54.5	6.32	13.5	10.3	

* Dimethylformamide.

Table 4. *Substrate and enzyme concentrations used in kinetic experiments*

Substrate	Concn. of substrate (mM)	Concn. of enzyme (μ M)
Ac-Gly-Phe-NH ₂	0.34–3.4	0.38
Leu	0.44–2.2	0.32
Val	1.1–4.5	0.93
Cys(Me)	0.4–4.1	0.38
Cys(Et)	0.75–3.8	0.70

α -amino group of the amino acid amide will only be partly protonated (the amount depending on the pK_a of the amino group and the pH of the solution); hence in unbuffered solutions the pH of the reaction mixture will fall as the reaction proceeds. It can readily be seen that the amount of alkali required to maintain a constant pH during hydrolysis is proportional to the amount of undissociated amino group formed.

The proportion, x , of the amino group that is dissociated is given by the expression:

$$x = \frac{K_a}{K_a + [H^+]} \quad (\text{Lindley, 1960})$$

where K_a is the dissociation constant of the amino group.

Therefore rate of reaction = rate of alkali uptake/ x , and at constant pH the rate of reaction is directly proportional to the rate of alkali uptake.

pK_a determination. The pK_a values of the amino groups were determined with a Radiometer Titrator at 37°C under an atmosphere of nitrogen. Where the amino acid amide was available, the pK_a was determined directly from a titration curve of the compound. Where a pure sample of the amide was not available, a thermolysin digest of the appropriate peptide derivative was allowed to go to completion and a titration curve plotted for the digest. From this the pK_a of the amino acid amide could then readily be derived. Both methods gave identical results.

Reaction kinetics. The hydrolysis of the dipeptides by thermolysin was followed by titration with standard 0.01M-NaOH in a Radiometer pH-stat at pH 7.0, 37°C, and in 1 mM-CaCl₂ under an atmosphere of nitrogen at the substrate and enzyme concentrations shown in Table 4. The substrate was equilibrated at the temperature and pH of the digest and the enzyme solution (0.5–1.0 ml), similarly equilibrated, was added rapidly from a syringe. The initial reaction velocity was found from the graph of alkali addition against time after correction for the pK_a of the amino group and the concentration of alkali. The observed kinetic parameters, k_0 , K_0 , k_0/K_0 , are as defined by Knowles (1965), where $v_0 = k_0[E_0][S_0]/(K_0 + [S_0])$ and k_0/K_0 was called the comparative reactivity, were found by averaging the values obtained for K_0 and $k_0 = V_{\max}/[E]$ from the Lineweaver-Burk plot ($1/v$ against $1/s$), the Eadie and Hofstee plots (s/v against s and v against v/s). In each case the best straight line to fit the experimental points was found by the method of least squares on an Olivetti Programma 101 desk computer.

Table 5. *pK_a values of the amino acid amides at 37°C and correction factor (x) at pH 7*

Amide	pK_a	$x = \frac{K_a}{K_a + [H^+]}$
Phe-NH ₂	6.80	0.61
Leu-NH ₂	7.35	0.31
Val-NH ₂	7.40	0.28
Cys(Me)-NH ₂	6.53	0.74
Cys(Et)-NH ₂	6.53	0.74

Table 6. *Kinetic parameters of protected dipeptides at 37°C, pH 7, in 1 mM-calcium chloride*

Substrate	K_0 (mM)	k_0 (s ⁻¹)	k_0/K_0 (s ⁻¹ ·m ⁻¹)
Ac-Gly-Phe-NH ₂	23.8	6.77	284.5
Ac-Gly-Leu-NH ₂	19.26	7.82	406
Ac-Gly-Val-NH ₂	6.31	0.209	33.05
Ac-Gly-Ala-NH ₂	Hydrolysed too slowly to measure accurately		
Ac-Gly-Cys(Me)-NH ₂	10.56	0.56	53.1
Ac-Gly-Cys(Et)-NH ₂	6.43	0.157	24.4
Ac-Gly-Cys(Ph)-NH ₂	Not hydrolysed		
Ac-Gly-Cys(Bzl)-NH ₂	Not hydrolysed		

RESULTS

The pK_a values of the amino acid amides at 37°C are shown in Table 5 together with the correction factors (x) calculated for the hydrolysis at pH 7.

The hydrolysis rate (corrected by the relevant factor x) showed a pH-dependence for all the substrates very similar to that reported by Feder & Schuck (1970) with a maximum hydrolysis rate at pH 7.

The kinetic parameters found for the hydrolysis of the acetylglycylamino acid amides of phenylalanine, leucine, valine, alanine, *S*-methylcysteine, *S*-ethylcysteine, *S*-benzylcysteine and *S*-phenylcysteine are shown in Table 6.

The inhibition of acetylglycylphenylalanine amide by benzyloxycarbonylglycine gave $K_i = 3.0$ mM (concentration of inhibitor 0.8–4.0 mM), whereas acetylglycine did not inhibit at all at these (or greater) concentrations.

DISCUSSION

The results in Table 6 show that the *S*-alkylcysteines are in fact substrates for thermolysin and although not as good substrates as the leucine and phenylalanine dipeptides, they are on a par with the valine dipeptide and are better substrates than the alanine dipeptide, which in this experiment was hydrolysed too slowly to be measured accurately. Morihara & Oka (1968) have shown that in certain

cases, depending on the amino acid residue before the alanine, the peptide is hydrolysed at the alanine, but not when glycine is the amino acid. The *S*-benzyl- and *S*-phenyl-cysteines are not hydrolysed at all. Comparison with chymotrypsin (Damoglou *et al.* 1970), where *S*-phenylcysteine and phenylalanine are comparable substrates, indicates that thermolysin must have more stringent size and/or shape requirements at the active centre than chymotrypsin.

The values of k_0/K_0 for the acetylglycylphenylalanine amide and acetylglycyl-leucine amide are lower than the values reported (Feder & Schuck, 1970) for the benzyloxycarbonyl-protected dipeptides, which in turn are lower than those for the furylacryloyl-protected dipeptides. This is only to be expected in view of Morihara's finding (Morihara *et al.* 1968) that the activity, measured as percentage hydrolysis of acetylglycyl-leucine amide by thermolysin, was slower than that of benzyloxycarbonylglycyl-leucine amide. The acetyl group is probably too small for the large specificity site of thermolysin (Morihara & Oka, 1968). Inhibition by the benzyloxycarbonyl grouping, possibly due to 'wrong way' binding (Rapp, Niemann & Hein, 1966), may be responsible for the difference between the benzyloxycarbonyl and furylacryloyl protecting groups.

In retrospect it seems that peptides longer than the dipeptides used would have been a better choice as model substrates, since they increase the separation of the acetyl group from the centre of the active site while still retaining their solubility in water. Nevertheless the results have indicated that *S*-methylation of cysteine or conversion of serine into *S*-methyleysteine by the method of Patchornik *et al.* (1969) should provide useful techniques to add extra splitting points for thermolysin when used for sequence studies.

The authors thank Dr F. H. C. Stewart for many helpful discussions about the peptide syntheses.

REFERENCES

- Anfinsen, C. B., Sela, M. & Tritch, H. (1956). *Archs Biochem. Biophys.* **56**, 156.
- Antonovics, I. & Young, G. T. (1967). *J. chem. Soc. C*, p. 595.
- Armstrong, M. D. & Lewis, J. D. (1951). *J. org. Chem.* **16**, 749.
- Damoglou, A. P., Lindley, H. & Stapleton, I. W. (1970). *Biochem. J.* **118**, 553.
- du Vigneaud, V., Loring, H. S. & Craft, H. A. (1934). *J. biol. Chem.* **105**, 481.
- Feder, J. & Schuck, J. M. (1970). *Biochemistry, Easton*, **9**, 2784.
- Fischer, E. (1901). *Chem. Ber.* **34**, 433.
- Goldberger, R. F. & Anfinsen, C. B. (1962). *Biochemistry, Easton*, **1**, 401.
- Knowles, J. R. (1965). *J. theor. Biol.* **9**, 213.
- Lindley, H. (1956). *Nature, Lond.*, **178**, 647.
- Lindley, H. (1960). *Biochem. J.* **74**, 577.
- Maclaren, J. A., Savage, W. E. & Swan, J. M. (1958). *Aust. J. Chem.* **11**, 345.
- Matsubara, H. (1966). *Biochem. biophys. Res. Commun.* **24**, 427.
- Merigan, T. C., Dreyer, W. J. & Berger, A. (1962). *Biochim. biophys. Acta*, **62**, 122.
- Morihara, K. (1967). *Biochem. biophys. Res. Commun.* **26**, 656.
- Morihara, K. & Ebata, M. (1967). *J. Biochem., Tokyo*, **61**, 149.
- Morihara, K. & Oka, T. (1968). *Biochem. biophys. Res. Commun.* **30**, 625.
- Morihara, K., Tsuzuki, H. & Oka, T. (1968). *Archs Biochem. Biophys.* **123**, 572.
- Ohta, Y. & Ogura, Y. (1965). *J. Biochem., Tokyo*, **58**, 607.
- Ohta, Y., Ogura, Y. & Wada, A. (1966). *J. biol. Chem.* **241**, 5919.
- Patchornik, A., Wilchek, M. & Zioudrou, C. (1969). *Israel J. Chem.* **7**, 557.
- Rapp, J. R., Niemann, C. & Hein, G. E. (1966). *Biochemistry, Easton*, **5**, 4100.
- Redfield, R. R. & Anfinsen, C. B. (1956). *J. biol. Chem.* **221**, 385.
- Schrecker, A. W. & Hartwell, J. L. (1957). *J. Am. chem. Soc.* **79**, 3827.
- Smith, E. L. & Slouim, N. B. (1948). *J. biol. Chem.* **176**, 835.
- Smith, E. L., Spackman, D. H. & Polglase, W. J. (1952). *J. biol. Chem.* **199**, 801.
- Sokolovsky, M., Sadeh, T. & Patchornik, A. (1964). *J. Am. chem. Soc.* **86**, 1212.
- Stapleton, I. W. & Swan, J. M. (1962). *Aust. J. Chem.* **15**, 106.
- Tesser, G. I., Nivard, R. J. F. & Gruber, M. (1964). *Biochim. biophys. Acta*, **89**, 303.
- Wood, J. L. & du Vigneaud, V. (1939). *J. biol. Chem.* **130**, 109.
- Zahn, H. & Otten, H. G. (1962). *Justus Liebigs Annln Chem.* **653**, 139.
- Zahn, H. & Schüssler, H. (1961). *Justus Liebigs Annln Chem.* **641**, 176.
- Zbarsky, J. H. & Young, L. (1943). *J. biol. Chem.* **151**, 211.