Degradative Studies on Peptides and Proteins. Part I. A New Method of Stepwise Degradation of Peptides from the End bearing a Free Amino-group, employing N-*Acyldithiocarbamates.*

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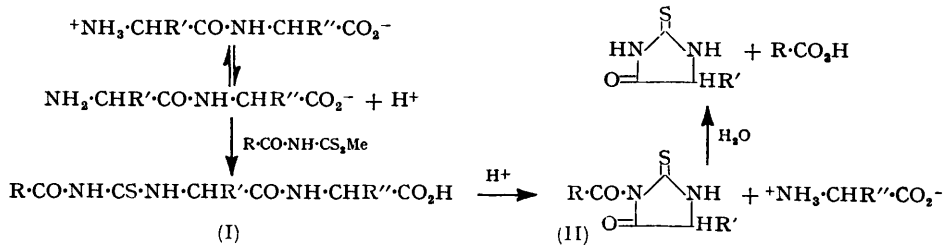
Alkyl *N*-acetyldithiocarbamates react with amino-acids and peptides in aqueous ethanol or aqueous dioxan at pH 7·5—8·5, and with their esters in chloroform, affording *N*-acetylthiocarbamoyl derivatives. Methyl *N*-acetyldithiocarbamate is preferred, since it reacts with amino-acids and peptides in solution at room temperature. *N*-Acetylthiocarbamoyl-peptides and their esters, when submitted to a variety of acidic conditions, undergo cyclisation and degradation to 2-thiohydantoins, liberating acetic acid and a peptide or peptide ester containing one less amino-acid residue. The method has been used on a small scale, the 2-thiohydantoin being identified, without isolation, by paper chromatography. Methyl *N*-benzoyldithiocarbamate has also been used in a few experiments but is less convenient.

One of the current chemical methods of elucidating the sequence of amino-acids in a polypeptide involves the selective removal of either of the terminal amino-acids in the form of a derivative which can be readily separated and characterised. Ideally, the process should result in the cleavage of a controlled number (usually one only) of peptide bonds and should be capable of repetition, so that the polypeptide may be degraded stepwise, with identification of the fission product at each stage. A number of methods for such degradation of peptides from the end bearing a free amino-group have recently become available (Levy, *J.*, 1950, 404; Léonis and Levy, *Bull. Soc. Chim. biol.*, 1951, **33**, 779; Edman, *Acta Chem. Scand.*, 1950, **4**, 283; 1953, **7**, 700; Khorana, *Chem. and Ind.*, 1951, 129; Kenner and Khorana, *J.*, 1952, 2076; Holley and Holley, *J. Amer. Chem. Soc.*, 1952, **74**, 5445; Wessely, Schlögl, and Korger, *Nature*, 1952, **169**, 708; *Monatsh.*, 1952, **83**, 1156; Wessely, Schlögl, and Wawersich, *ibid.*, pp. 1426, 1439). Of these, Edman's method has been most widely employed and various modifications have been proposed (H. Fraenkel-Conrat and J. Fraenkel-Conrat, *Acta Chem. Scand.*, 1951, **5**, 1409; Dahlerup-Peterson, Linderstrøm-Lang, and Ottesen, *ibid.*, 1952, **6**, 1135; Ottesen and Wollenberger, *Nature*, 1952, **170**, 801; Landmann, Drake, and Dillaha, *J. Amer. Chem. Soc.*, 1953, **75**, 3638; Fox, Hurst, and Warner, *ibid.*, 1954, **76**, 1154; Reith and Waldron, *Biochem. J.*, 1954, **56**, 116) in attempts to overcome a number of practical difficulties (for discussions see Rovery and Desnuelle, *Bull. Soc. Chim. biol.*, 1954, **36**, 95; Edman, " The Chemical Structure of Proteins," J. and A. Churchill Ltd., London, 1953, p. 98; H. Fraenkel-Conrat, *op. cit.*, p. 102). In a further effort to provide an adequate technique for the stepwise removal of *N*-terminal amino-acids from peptides and proteins, we have developed the method described herein and reported briefly elsewhere (Elmore and Toseland, *Chem. and Ind.*, 1953, 1227).

Our objective was a reagent which would react under mild conditions with amino-groups of peptides and proteins to the exclusion of thiol and hydroxyl groups, and would be such that wide variations of structure could be used to facilitate the identification of all the amino-acids which might occur as *N*-terminal residues (Kenner and Khorana, *loc. cit.*, had the same object). Moreover, it was essential that the peptide derivative should be cleaved under mild conditions furnishing the *N*-terminal amino-acid in a form which required no further chemical treatment before identification and quantitative determination. Accordingly, we examined *N*-acyldithiocarbamates. It is known (Wheeler, Nicolet, and Johnson, *Amer. Chem. J.*, 1911, **46**, 456) that ethyl *N*-acetyl- or *N*-benzoyl-dithiocarbamate reacts under rather vigorous conditions with amino-acids in water, and more readily with their esters in non-aqueous solution, affording *N*-acylthiocarbamoylamino-acids (I) or their esters. These when heated with strong acid suffered ring-closure and cleavage of

the *N*-acyl group, yielding 2-thiohydantoins. These results were confirmed and it was further demonstrated that the initial condensation with amino-acids and peptides proceeded smoothly at room temperature if the more reactive methyl *N*-acyldithiocarbamates were employed (cf. Kenner and Khorana, *loc. cit.*) in aqueous alcohol or aqueous dioxan at constant pH, the necessary alkali being added by means of an autotitrator. Optimum results were obtained at pH 7·5—8·5; below this, reaction was very slow, and at pH 9 hydrolysis of the *N*-acyldithiocarbamate became noticeable. Methyl *N*-acetyldithiocarbamate proved more satisfactory than methyl *N*-benzoyldithiocarbamate since the



former and its peptide products are the more soluble in the solvents concerned. Other *N*-acyldithiocarbamates, however, have been synthesised and their reactions will be reported later.

N-Acylthiocarbamoyl derivatives of amino-acids, peptides, and their esters have been obtained in yields of usually at least 80% and cyclised and degraded under a variety of conditions of acid catalysis. *N*-Acylthiocarbamoyl derivatives of amino-acids and their esters are more difficult to cyclise than the corresponding peptide and peptide-ester derivatives, and 2-thiohydantoins were not formed under the mild conditions which sufficed for the stepwise degradation of peptides. Many *N*-acylthiocarbamoyl-peptides were degraded in dry nitromethane saturated with dry hydrogen chloride (Edman, *Acta Chem. Scand.*, 1950, **4**, 283; Khorana, *loc. cit.*; Kenner and Khorana, *loc. cit.*) but sometimes they were too insoluble (cf. similar observations by earlier workers). Alternatively, glacial acetic acid saturated with dry hydrogen chloride was employed (Edman, *Acta Chem. Scand.*, 1953, **7**, 700) and this proved more satisfactory. In both systems, however, cyclisation and degradation proceeded stepwise: initially, 3-acyl-2-thiohydantoins (II) were formed and in some cases these have been isolated and characterised; they will be described later. After prolonged degradation the parent 2-thiohydantoins were isolated in reasonable yield. Two variations of this procedure have been investigated. First, after a short treatment with glacial acetic acid saturated with hydrogen chloride, the resultant 3-acyl-2-thiohydantoins could be hydrolysed to 2-thiohydantoins by hot dilute acetic acid. Secondly, degradation to 2-thiohydantoins is faster in glacial acetic acid saturated with hydrogen chloride containing 10% of water (*idem*, *loc. cit*). Finally, degradation was also performed in dilute hydrochloric or sulphuric acids (pH 1) at 65° (Ottesen and Wollenberger, *loc. cit.*) or more slowly at 37°; this procedure always yielded 2-thiohydantoins, cleavage of the 3-acyl group presumably occurring rapidly in aqueous systems, a result not unexpected, since 1 : 3-diacetylhydantoin is readily hydrolysed by boiling water to 1-acetylhydantoin (Siemonsen, *Annalen*, 1904, **333**, 101).

In general, 2-thiohydantoins were identified by analysis, melting points, mixed melting points, and paper chromatography (Edward and Nielsen, *Chem. and Ind.*, 1953, 197). After reaction in a non-aqueous system, the degraded peptide was isolated as its hydrochloride and identified by melting point and paper chromatography. After degradation in dilute hydrochloric acid, however, the product was not isolated but was characterised by chromatography.

In addition, a number of small-scale degradations were carried out. The peptide was first brought into reaction with methyl *N*-acetyldithiocarbamate and the derivative degraded without isolation in dilute hydrochloric acid (pH 1) at 65° for 12—24 hr., the products being separated and then identified by paper chromatography.

Experimental

Methyl and ethyl *N*-acetyl- and *N*-benzoyl-dithiocarbamates were prepared by Wheeler and Merriam's method (*J. Amer. Chem. Soc.*, 1901, **23**, 289).

*Benzyloxycarbonyl-*DL*-norleucylglycylglycine Ethyl Ester.*—(*a*) (With F. B. Falkingham) (cf. Anderson, Blodinger, Young, and Welcher, *J. Amer. Chem. Soc.*, 1952, **74**, 5304.) Glycylglycine ethyl ester hydrochloride (0·98 g.), suspended in anhydrous ether (30 c.c.), was shaken with triethylamine (1·01 g.), followed by diethyl phosphorochloridite (0·78 g.) in anhydrous ether (30 c.c.), then cooled to 0° for 30 min. and kept at room temperature overnight with exclusion of moisture. The solution was filtered, solvent removed under reduced pressure, and toluene (15 c.c.) and benzyloxycarbonyl-DL-norleucine (1·33 g.) were added. After the whole had been heated under reflux for 45 min., toluene was removed under reduced pressure. The brown solid residue was washed once with 10% sodium hydrogen carbonate solution and twice with water; two recrystallisations from aqueous ethanol gave a pure product (1·56 g., 77%), m. p. 118°.

(*b*) (cf. Anderson, Blodinger, and Welcher, *ibid.*, p. 5309.) Benzyloxycarbonyl-DL-norleucine (2·65 g.) and glycylglycine ethyl ester hydrochloride (1·98 g.) were shaken with diethyl hydrogen phosphite (10 c.c.) and tetraethyl pyrophosphite (3·4 g.) with exclusion of moisture. Triethylamine (1·01 g.) was added, the mixture was heated at 90—100° for 1 hr. and then poured into water. The product was extracted with ethyl acetate and washed with 5% sodium hydrogen carbonate solution, N-hydrochloric acid, and water. The solution was dried and evaporated to half its bulk under reduced pressure, and the *product* (2·8 g., 69%; m. p. 118°) caused to crystallise by the addition of light petroleum (b. p. 40—60°) (Found: C, 59·0; H, 7·1; N, 9·9. $C_{20}H_{29}O_6N_3$ requires C, 58·9; H, 7·2; N, 10·3%).

*Benzyloxycarbonyl-*DL*-norleucylglycylglycine.*—The foregoing ester (2·25 g.) was dissolved in 50% aqueous acetone (10 c.c.) and N-sodium hydroxide (9 c.c.) was added. After being kept at room temperature for 15 min., the solution was acidified to pH 1. Acetone was removed under reduced pressure and the residual solution extracted continuously with ethyl acetate. The extract was dried and evaporated to small bulk; the *product* (1·2 g., 57%), precipitated by the addition of light petroleum (b. p. 40—60°) and recrystallised from a small volume of ethyl acetate, had m. p. 144° (Found: C, 56·6; H, 6·7; N, 11·0. $C_{18}H_{25}O_6N_3$ requires C, 57·0; H, 6·6; N, 11·1%).

DL-*Norleucylglycylglycine.*—Benzyloxycarbonyl-DL-norleucylglycylglycine (1 g.) was hydrogenated in methanol containing a few drops of acetic acid with a palladium catalyst. When recrystallised from aqueous ethanol the pure *peptide* (420 mg., 65%) had m. p. 207—210° (decomp.) (Found: C, 48·7; H, 7·5; N, 17·0. $C_{10}H_{19}O_4N_3$ requires C, 48·9; H, 7·8; N, 17·1%). On a paper chromatogram (Whatman No. 1) irrigated with *n*-butanol–acetic acid–water (4 : 1 : 5) it ran as a single spot (R_F 0·55).

N-Acylthiocarbamoylamino-acid Esters.—The procedure of Wheeler, Nicolet, and Johnson (*loc. cit.*) was used to prepare *N*-acetyl- (79%; m. p. 102°) and *N*-benzoyl-thiocarbamoylglycine ethyl ester (65%; m. p. 128—129°), and N-*benzoylthiocarbamoylalanine ethyl ester* (62%; m. p. 120·5°) (Found: C, 56·0; H, 5·6; N, 10·4; S, 11·7. $C_{13}H_{16}O_3N_2S$ requires C, 55·7; H, 5·7; N, 10·0; S, 11·4%).

N-Acetylthiocarbamoylglycylglycine Ethyl Ester.—Glycylglycine ethyl ester hydrochloride (1·9 g.) was suspended in dry chloroform (40 c.c.) and stirred at 0°; triethylamine (1·01 g.) was added dropwise during 15 min. The solution was filtered and ethyl *N*-acetyldithiocarbamate (1·8 g.) added; needles of N-*acetylthiocarbamoylglycylglycine ethyl ester* (2·7 g., 83%), m. p. 112°, were deposited during 2 days. This was recrystallised from ethanol for analysis (Found: C, 41·6; H, 5·9; N, 15·8. $C_9H_{15}O_4N_3S$ requires C, 41·4; H, 5·8; N, 16·1%).

Degradation of this compound (500 mg.) in nitromethane–hydrogen chloride (10 c.c.) for 4 hr. at 0° afforded glycine ethyl ester hydrochloride (105 mg., 39%), m. p. 138—139°. The filtrate was evaporated under reduced pressure, water was added, and the aqueous solution was extracted with ether. After evaporation of the ether crystallisation of the residue from aqueous ethanol gave 2-thiohydantoin (151 mg., 68%), m. p. and mixed m. p. 222—224° (decomp.); paper chromatography (see below) confirmed its identity.

N-Benzoylthiocarbamoylglycylglycine Ethyl Ester.—This *compound* (yield 80%), prepared as above, had m. p. 155° (Found: C, 51·6; H, 5·6; N, 12·9; S, 9·8. $C_{14}H_{17}O_4N_3S$ requires C, 52·0; H, 5·3; N, 13·0; S, 9·9%).

General Method of Preparation of N-Acylthiocarbamoyl Derivatives of Amino-acids and Peptides.—The amino-acid or peptide was dissolved in water (1—2 c.c. per millimol.) and a

solution of methyl *N*-acyldithiocarbamate (2·5—4·0 millimols.) in ethanol or dioxan (2—4 c.c. per millimol. of amino-acid or peptide) added thereto. The ratio of organic solvent to water was varied in individual cases; in certain instances, when the solubilities of *N*-acyldithiocarbamate and peptide were such that both could not be dissolved at one time, it was preferable to keep the latter completely in solution and allow the former to remain in suspension. Reaction was conducted at room temperature and at a constant pH (7·5—8·5) by means of a Beckmann autotitrator, and was usually complete in 1½—3 hr. The solution was then evaporated under reduced pressure to small bulk and diluted with water, and excess of *N*-acyldithiocarbamate was extracted with ether. The aqueous solution was then evaporated to small bulk under reduced pressure and acidified to pH 1, and the product extracted with ethyl acetate. Frequently, the *N*-acylthiocarbamoyl-peptide separated from aqueous solution, but extraction with ethyl acetate was still necessary for maximal yields. The ethyl acetate solution was dried and evaporated to about one-fourth of its bulk, and the product caused to crystallise by the addition of light petroleum (b. p. 40—60°).

N-*Acylthiocarbamoylamino-acids*.—*N*-Acetylthiocarbamoylglycine, prepared by the method of Wheeler, Nicolet, and Johnson (*loc. cit.*) (yield 52%), had m. p. 201—202°, and by the method described above (yield 60%) m. p. 198—200°. *N*-Benzoylthiocarbamoylglycine was similarly derived by the two methods (yields 79% and 75%), m. p. 199—200° and 201—203° respectively.

N-*Acetylthiocarbamoylglycylglycine* (I; R = Me, R′ = R″ = H) was obtained in 71% yield by the general procedure outlined above. Recrystallised from ethyl acetate–light petroleum (b. p. 40—60°) it had m. p. 214—216° (Found: C, 35·9; H, 4·7; N, 17·9. $C_7H_{11}O_4N_3S$ requires C, 36·0; H, 4·8; N, 18·0%).

Degradative studies were carried out as follows:

(i) Dry hydrogen chloride was bubbled through a suspension of *N*-acetylthiocarbamoylglycylglycine (450 mg.) in anhydrous nitromethane (4 c.c.), at room temperature; the whole was shaken for 8 hr. and then left overnight at 0°. Glycine hydrochloride (128 mg., 60%), m. p. 176—178°, was collected, the filtrate evaporated to dryness, and the residual oil warmed with 2N-acetic acid (5 c.c.) for 2 hr. On cooling, crystals of 2-thiohydantoin [121 mg., 54%; m. p. and mixed m. p. 222—224° (decomp.)] separated. Both products were further characterised by paper chromatography.

(ii) *N*-Acetylthiocarbamoylglycylglycine (500 mg.) was added to glacial acetic acid (7 c.c.) previously saturated at room temperature with dry hydrogen chloride. The solution was shaken for 5 hr. and set aside overnight. Glycine hydrochloride (140 mg., 59%), m. p. 179—180°, was collected and the filtrate was evaporated under reduced pressure to dryness. The residue crystallised from 2N-acetic acid, yielding 2-thiohydantoin (128 mg., 52%), m. p. and mixed m. p. 224—226° (decomp.).

(iii) *N*-Acetylthiocarbamoylglycylglycine (54 mg.), in acetic acid saturated with hydrogen chloride and then diluted with 10% v/v of water (2 c.c.), was kept overnight at room temperature. Solvent was removed, and the residue crystallised from water, giving 2-thiohydantoin (16 mg., 59%), m. p. 220—222° (decomp.).

(iv) A solution of *N*-acetylthiocarbamoylglycylglycine (400 mg.) in dilute hydrochloric acid (7 c.c.) at pH 1 was kept for 24 hr. at 65°. After continuous extraction with ether for 4 hr. followed by concentration of the extract to dryness, 2-thiohydantoin [105 mg., 53%; m. p. and mixed m. p. 220—223° (decomp.)] crystallised from 2N-acetic acid.

N-*Acetylthiocarbamoyl*-DL-*alanylglycine* (I; R = R′ = Me, R″ = H) was obtained in nearly quantitative yield from DL-alanylglycine. Recrystallised from ethyl acetate–light petroleum (b. p. 40—60°), it had m. p. 206° (Found: C, 38·7; H, 5·4; N, 16·8. $C_8H_{13}O_4N_3S$ requires C, 38·9; H, 5·3; N, 17·0%). Degradation in glacial acetic acid saturated with hydrogen chloride for 40 hr. afforded glycine hydrochloride (70%) and 5-methyl-2-thiohydantoin (64%), m. p. 157° (Found: C, 36·6; H, 4·5; N, 21·2. Calc. for $C_4H_6ON_2S$: C, 36·9; H, 4·6; N, 21·5%). Degradation in dilute hydrochloric acid (pH 1) at 65° gave only 48% of the expected yield of 5-methyl-2-thiohydantoin.

N-*Acetylthiocarbamoylglycyl*-DL-*alanine* (I; R = R″ = Me, R′ = H) was prepared in the usual manner in 80% yield and, recrystallised from ethyl acetate–light petroleum (b. p. 40—60°), had m. p. 192° (Found: C, 38·6; H, 5·2; N, 16·9. $C_8H_{13}O_4N_3S$ requires C, 38·9; H, 5·3; N, 17·0%). When degraded in glacial acetic acid saturated with hydrogen chloride (40 hr.), it gave DL-alanine hydrochloride (72%), m. p. 199—201°, and 2-thiohydantoin (60%), m. p. 220—223° (decomp.).

N-*Acetylthiocarbamoylglycyl*-DL-*valine* (I; R = Me, R′ = H, R″ = Pr^i), obtained (83%) in the usual manner and recrystallised from ethyl acetate–light petroleum (b. p. 40—60°), had

m. p. 173—174° (Found : C, 43·4; H, 6·2; N, 14·9. $C_{10}H_{17}O_4N_3S$ requires C, 43·6; H, 6·2; N, 15·3%). In dilute hydrochloric acid (pH 1) at 65° for 12 hr., it yielded 2-thiohydantoin (41%), m. p. 222—224° (decomp.).

N-*Acetylthiocarbamoyl*-DL-*phenylalanylglycine* (I; R = Me, R′ = Ph·CH_2, R″ = H) was obtained (80%) as an oil which slowly crystallised from ethyl acetate–light petroleum (b. p. 40—60°), then having m. p. 184—185° (Found : C, 52·2; H, 5·4; N, 12·9. $C_{14}H_{17}O_4N_3S$ requires C, 52·0; H, 5·3; N, 13·0%). Degradation in dilute hydrochloric acid (pH 1) at 37° for 48 hr. gave 5-benzyl-2-thiohydantoin (45%), m. p. 176° (Found : C, 58·0; H, 4·8; N, 13·6. Calc. for $C_{10}H_{10}ON_2S$: C, 58·2; H, 4·9; N, 13·6%). Swan (*Austral. J. Sci. Res.*, 1952, *A*, **5**, 734) gives m. p. 180—182° but a sample prepared according to his directions, in our hands, had m. p. 175°, undepressed by admixture with the specimen obtained from degradation; Kenner, Khorana, and Stedman (*J.*, 1953, 673) found that 5-benzyl-2-thiohydantoin, obtained by stepwise removal of the *C*-terminal amino-acid from *N*-toluene-*p*-sulphonylglycylphenylalanine had m. p. 175—182°.

N-Acetylthiocarbamoyl-DL-norleucylglycylglycine was obtained only as an oil, in 65% yield, from DL-norleucylglycylglycine. It was degraded in dilute sulphuric acid (pH 1) at 37° for 40 hr. and the resultant solution was extracted continuously with ether for 8 hr. After evaporation of the ether extract to dryness, the residue was warmed with 2N-acetic acid on the steam-bath for 1 hr. On cooling, 5-*n*-butyl-2-thiohydantoin (42%), m. p. 122—124°, crystallised. It was recrystallised twice from aqueous ethanol and then had m. p. 131°, undepressed on admixture with an authentic specimen (Jackman, Klenk, Fishburn, Tullar and Archer, *J. Amer. Chem. Soc.*, 1948, **70**, 2884) (Found : C, 48·4; H, 7·0; N, 16·5. Calc. for $C_7H_{12}ON_2S$: C, 48·8; H, 7·0; N, 16·3%). The aqueous extract after removal of 5-*n*-butyl-2-thiohydantoin was evaporated under reduced pressure to 5 c.c., the pH value adjusted to 8·0, and reaction with methyl *N*-acetyldithiocarbamate conducted in the usual manner. *N*-Acetylthiocarbamoylglycylglycine (10% based on original peptide), m. p. 208—210°, was isolated, identical with that obtained from glycylglycine.

N-*Benzoylthiocarbamoylglycylglycine* (I; R = Ph, R′ = R″ = H), prepared in the usual manner in 79% yield, had m. p. 227° (Found : C, 48·6; H, 4·8; N, 13·8. $C_{12}H_{13}O_4N_3S$ requires C, 48·8; H, 4·4; N, 14·2%). In dilute sulphuric acid (pH 1) at 37° for 3 days, it afforded 2-thiohydantoin (14%), m. p. 219—224° (decomp.), mixed m. p. 222°.

Small-scale Stepwise Degradation of Peptides.—Glycyl-DL-valine, DL-phenylalanylglycine, or DL-norleucylglycylglycine (10 mg.) was treated with methyl *N*-acetyldithiocarbamate in the usual way. The solution was then acidified to pH 1 and kept at 65° for 12—24 hr. The resultant 2-thiohydantoin derivative was extracted into ether and this as well as the degraded peptide were identified by paper chromatrography. Satisfactory results were in general obtained, although in the case of DL-norleucylglycylglycine the peptide resulting from degradation was contaminated with DL-norleucine which presumably arose from the slow hydrolysis of 5-*n*-butyl-2-thiohydantoin in acid solution.

Paper Chromatographic Techniques.—2-Thiohydantoins were identified on descending paper chromatograms (Whatman No. 1) irrigated with *n*-butanol saturated with water (Edward and Nielsen (*loc. cit.*), markers being run in every case. 3-Acyl-2-thiohydantoins, when present, were thus readily differentiated from the parent 2-thiohydantoin. Chromatograms were viewed, or photographed on Ilford Reflex Document Paper No. 50, in ultra-violet light (2537 Å) (cf. Holiday and Johnson, *Nature*, 1949, **163**, 216; Markham and Smith, *ibid.*, p. 250).

Amino-acids and peptides arising from degradations were identified by chromatography in *n*-butanol–acetic acid–water (4 : 1 : 5). Markers were run and spots were located by spraying with 0·1% ninhydrin in acetone.

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