530. *The Stepwise Degradation of Peptides.*

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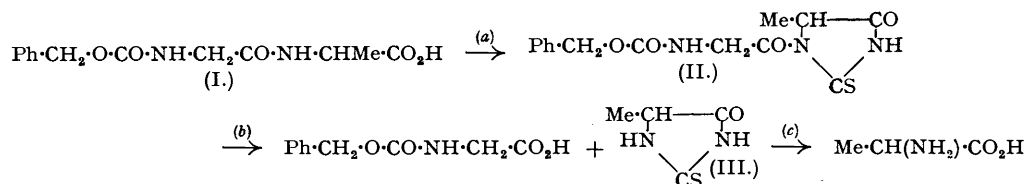
A method for the elucidation of the amino-acid sequence in peptides by conversion of the residue bearing a terminal carboxyl group into a thiohydantoin (Schlack and Kumpf, *Z. physiol. Chem.*, 1926, **154**, 125) has been further investigated. By use of paper chromatography it has been found advantageous to identify the terminal residue as the amino-acid. Application of the method to insulin has revealed alanine as a terminal amino-acid.

Several methods for the stepwise degradation of peptides have been described recently which are concerned with the amino-acid bearing a terminal amino-group (Khorana, *Chem. and Ind.*, 1951, 129; Fox, *Adv. Protein Chem.*, 1945, **2**, 155; Edman, *Acta Chem. Scand.*, 1950, **4**, 283; Levy, *J.*, 1950, 404). Fewer methods, however, are available for the carboxyl end of the peptide chain (Fox, *loc. cit.*); the most promising utilises the formation of a thiohydantoin (Schlack and Kumpf, *loc. cit.*) : this method has been applied to glutathione (Nicolet, *J. Biol. Chem.*, 1930.

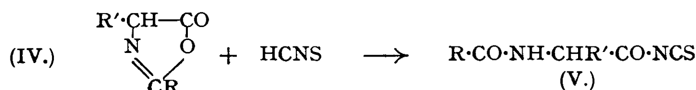
88, 389). The peptide is heated with acetic anhydride and ammonium thiocyanate, and the acyl-thiohydantoin, *e.g.*, (II), is then hydrolysed with dilute alkali.



Hitherto the terminal residue has been identified by isolation of the thiohydantoin, *e.g.*, (III); it is, however, more convenient to convert the thiohydantoin into the amino-acid, which can be purified and identified by paper chromatography. In this way, only small amounts of the peptide are needed, and, by Boissonas's method (*Helv. Chim. Acta*, 1950, **33**, 1975), the amount of amino-acid on the chromatogram can be determined.

Stage (a). Experiments carried out with carbobenzyloxyglycyl-DL-alanine (I) gave 1-carbobenzyloxylglycyl-5-methyl-2-thiohydantoin (II) in 73% yield under the conditions employed by Schlack and Kumpf (*loc. cit.*). Since proteins and peptides are not soluble in acetic anhydride alone, the cyclisation was investigated in various mixtures of acetic anhydride with formic acid, formamide, dimethylformamide, and dichloroacetic acid. Thiohydantoin formation occurred in each case, but the yields were low, except in acetic anhydride containing 20% of dichloroacetic acid.

An alternative method of thiohydantoin formation was also investigated. The formation of thiohydantoins by the action of acetic anhydride and ammonium thiocyanate on acylamino-acids proceeds through the oxazolone (IV), which probably reacts with thiocyanic acid to form the acyl *iso*thiocyanate (V) (Johnson and Scott, *J. Amer. Chem. Soc.*, 1913, **35**, 1136). If the



latter could be formed directly from the acylamino-acid, side reactions which may be occasioned by the labile oxazolone (IV) would be avoided. Therefore the action of benzoyl *iso*thiocyanate on the peptide (I) was examined (cf. the preparation of acid chlorides by use of benzoyl chloride; Brown, *J. Amer. Chem. Soc.*, 1938, **60**, 1325). Cyclisation only occurred under such vigorous conditions (heating the reactants in the absence of a solvent at 120°) that this modification does not seem suitable for use on peptides.

Stage (b). The hydrolytic fission of the 1-acyl-2-thiohydantoins was carried out by Schlack and Kumpf (*loc. cit.*) with N-alkali. We found that these conditions were unnecessarily vigorous: 1-acetyl-5-methyl-2-thiohydantoin lost the acetyl group in only a few minutes in 0·1N-alkali at room temperature, and in about an hour in 0·01N-alkali. The thiohydantoins are such weak acids [*e.g.*, (III), p*K* = 9·1; du Vigneaud and Melville, "The Chemistry of Penicillin," Princeton Univ. Press, 1949, p. 288] that they can be extracted from neutral solution with organic solvents.

Stage (c). The thiohydantoin (III) was converted into alanine by hydrobromic acid at 155° in 6 hours: the yield was 90% and was not changed by increasing the time of heating to 20 hours. All yields are based on the colorimetric estimation of alanine after paper chromatography by Boissonas's method (*loc. cit.*). The overall recovery of alanine from the acylthiohydantoin (II) in stages (*b*) and (*c*) was 84%.

Since the method worked smoothly with the simple peptide (I), we applied it to insulin. In the preliminary experiments reported here, the protein was not oxidised to break the linkages between the chains (Sanger, *Biochem. J.*, 1949, **44**, 126) but was acetylated to protect the amino- and hydroxy-groups and then heated with acetic anhydride and ammonium thiocyanate. After stages (*b*) and (*c*) had been carried through as described above, paper chromatography of the product (development with phenol) showed the presence of alanine and a trace of material with the same R_F as aspartic acid. Although no accurate measurement was made of the amount of alanine formed in this experiment, the yield was approximately 1 μg. from 1 mg. of insulin. Lens (*Biochim. et Biophys. Acta*, 1949, **3**, 367), using carboxy-peptidase, has shown that there may be one, two, or three terminal alanine residues with free carboxyl groups in the

insulin sub-unit of molecular weight 12,000.* Thus, at least 7·4 μg. of alanine should be obtained from 1 mg. of insulin.

Since the low yield might be partly attributed to the low solubility of the acetylated insulin in the acetic anhydride, the reaction was carried out in dichloroacetic acid–acetic anhydride mixtures in which the protein dissolved. In this case, however, as well as the alanine, more of the slower-moving amino-acid (probably aspartic acid) appeared, and also a faster-moving amino-acid (possibly phenylalanine); when a blank experiment was undertaken in which the ammonium thiocyanate was omitted, small amounts of these slower and faster amino-acids were found, but no alanine was detectable. Thus, although side reactions occur to a greater extent in the presence of dichloroacetic acid, they do not furnish any alanine. This modification, moreover, did not improve the yield of alanine, which was only 0·5 μg. per mg. of insulin. This result suggested that the losses might occur in the isolation of the thiohydantoin (III) from solutions containing insulin. This was confirmed when it was found that the recovery of (III) from a solution containing acetylated insulin was only 17%. There appears to be some interaction between the thiohydantoin and the protein which prevents the extraction of the thiohydantoin from neutral or acid solutions.

It is, of course, essential that any method for the stepwise degradation of polypeptides should give high yields in the removal of each residue. Although from our experiments with the simple peptide (I) the thiohydantoin method seemed promising, if the preliminary experiments on insulin described above are typical, there are considerable difficulties in its application to proteins. For the more limited objective of determining the residues bearing a terminal carboxyl group the method may be more useful.

EXPERIMENTAL.

1-*Carbobenzyloxy*-5-*methyl*-2-*thiohydantoin* (II).—(1) Carbobenzyloxyglycyl-DL-alanine (0·28 g.), ammonium thiocyanate (0·14 g.), and acetic anhydride containing 10% of acetic acid (1 c.c.) were heated at 100° for 20 minutes. The *thiohydantoin* (0·235 g.; m. p. 192°) was collected and washed with acetic anhydride, then ether–light petroleum. The m. p. was not raised by recrystallisation from aqueous ethanol (Found: C, 52·2; H, 4·8; N, 12·6; S, 10·0. $C_{14}H_{15}O_4N_3S$ requires C, 52·3; H, 4·7; N, 13·1; S, 10·0%). The thiohydantoin is sparingly soluble in water and soluble in most organic solvents; it is sparingly soluble in acetic acid–sodium acetate buffer (pH 5), and can thus be differentiated from the petide (I) which readily dissolves in the buffer.

(2) Ammonium thiocyanate (0·25 g.) and acetic anhydride (11·25 c.c.) were added to carbobenzyloxyglycylalanine (0·25 g.) in warm formic acid (1·25 c.c.). After 48 hours the reaction mixture was diluted with water, and the thiohydantoin collected (yield, 0·13 g.; m. p. 192°).

(3) Ammonium thiocyanate (0·05 g.) and acetic anhydride containing 10% of acetic acid (0·3 c.c.) were added to carbobenzyloxyglycylanine (0·144 g.) in formamide (0·38 c.c.). After being kept overnight, the thiohydantoin was isolated from the diluted reaction mixture and recrystallised from 75% ethanol; the yield was 0·04 g., and the m. p. 188—191°.

(4) Ammonium thiocyanate (0·066 g.) and acetic anhydride containing 33% of dichloroacetic acid (0·7 c.c.) were added to carbobenzyloxyglycylalanine (0·13 g.). After 13 minutes at 100°, ice and 2 c.c. of 50% sodium acetate solution were added; the crude product (0·123 g.; m. p. 164—168°) gave 0·039 g. of thiohydantoin after recrystallisation. If heating were carried out for only 4 minutes, the crude product (0·1 g.; m. p. 178—182°) was less impure. Similar results were obtained after 1¾ hours at 50°.

(5) Carbobenzyloxyglycylalanine (0·219 g.) and ammonium thiocyanate (0·12 g.) were heated in acetic anhydride containing 20% of dichloroacetic acid at 100° for 10 minutes. The thiohydantoin (m. p. 192°) separated in a yield of 0·156 g.

(6) Carbobenzyloxyglycylalanine (0·208 g.) was heated with benzoyl *iso*thiocyanate (0·5 c.c.) at 100° for 1 hour and then at 120° for 2 hours. The reaction mixture was diluted with benzene. The product, recrystallised twice from 75% ethanol, had m. p. and mixed m. p. with an authentic sample of thiohydantoin, 192°.

Conversion of 5-*Methyl*-2-*thiohydantoin into Alanine*.—5-Methyl-2-thiohydantoin (4·9 mg.) was heated with constant-boiling hydrobromic acid (0·1 c.c.) at 150° for 6 hours. After dilution with water to 2 c.c., 0·005 c.c. was transferred to Whatman number 54 filter paper. The same volumes of several solutions of alanine of known concentration were also put on the same paper, and after equilibration overnight the chromatogram was developed with phenol–water (4 : 1). The yield of alanine was estimated by Boissonas's method (*loc. cit.*) to be 90%. In a similar experiment in which the time of heating was extended to 22 hours the yield was 88%.

Conversion of 1-*Carbobenzyloxyglycyl*-5-*methyl*-2-*thiohydantoin into Alanine*.—The thiohydantoin (7 mg.) in 0·1N-sodium hydroxide (0·7 c.c.) was kept for 20 minutes at 0°, then brought to pH 8 by passing in carbon dioxide and extracted thrice with ethyl acetate. After drying, the solvent was distlled, and the residue heated with constant-boiling hydrobromic acid (0·2 c.c.) overnight at 155°. The amount

* [*Added in proof.*] Chibnall and Rees (*Biochem. J.*, 1951, **48**, xlvii) and Fromageot, Jutisz, Meyer, and Pénasse (*Compt. rend.*, 1950, **230**, 1905) have also shown alanine to be a terminal amino-acid in insulin.

of alanine in the hydrolysate (which was free from glycine) was estimated, as described above, to be 82%. In a second experiment, the yield was 86%.

After the extraction with ethyl acetate, the aqueous layer was acidified to pH 2 and again extracted with ethyl acetate. When treated as above, glycine, but no alanine, was detected in the hydrolysate.

The Action of Acetic Anhydride and Ammonium Thiocyanate on Insulin.—(1) Insulin was acetylated by cautious addition of acetic anhydride to the protein dissolved in aqueous potassium hydrogen carbonate; the acetylated material separated from the acid solution. The dried product (18 mg.) was heated at 100° for 30 minutes with ammonium thiocyanate (3·5 mg.) and acetic anhydride (0·5 c.c.). The acetic anhydride was then distilled off *in vacuo*, and the last traces were removed by dissolving the residue in water, neutralising with ammonia, and drying in a vacuum-desiccator over phosphoric oxide and sodium hydroxide. The residue was treated with 0·1N-barium hydroxide (0·8 c.c.) for 15 minutes, and the solution neutralised with carbon dioxide and extracted with ethyl acetate. The solvent was distilled from the dried extract, and the residue hydrolysed with constant-boiling hydrobromic acid for 3 hours at 155°. The diluted hydrolysate was filtered and evaporated; analysis by chromatography on Whatman No. 4 paper, phenol–water being used as solvent, revealed the presence of alanine and possibly a small amount of aspartic acid. Comparison of the size of the alanine spot with spots from solutions of known concentrations indicated a yield of approximately 20 μg. of alanine.

Recovery of 5-Methyl-2-thiohydantoin in Presence of Insulin.—5-Methyl-2-thiohydantoin (0·19 mg.) in 0·1N-sodium hydroxide (0·5 c.c.) was added to acetylated insulin (12·6 mg.) in 0·1N-sodium hydroxide (0·5 c.c.). After 30 minutes the mixture was neutralised with carbon dioxide and extracted with ethyl acetate. The yield of alanine after hydrolysis with hydrobromic acid was 17% based on the amount of 5-methyl-2-thiohydantoin present.

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