274. N-*Substituted Amino-acids. Part III. The Reductive Alkylation of Some Di- and Tri-peptides. A New Method of Determining the "End Amino-acid" in Polypeptides.*

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On reductive condensation with aliphatic aldehydes, polypeptides undergo alkylation only at the terminal amino-group. Hydrolysis of the alkylated peptide, followed by isolation of the resulting alkylamino-acid, furnishes a new method for the identification of the "end" amino-acid in a polypeptide.

Previous investigations on the alkylation of peptides have been confined to methylations by methyl iodide or diazomethane and have yielded mixtures of the corresponding betaines and their esters (Abderhalden and Kautzsch, *Z. physiol. Chem.*, 1911, **72**, 44; Imsi, *ibid.*, 1924, **136**, 192; Abderhalden and Sickel, *ibid.*, 1926, **153**, 16).

In view of the readiness with which the simple amino-acids may be converted by reductive alkylation with aliphatic aldehydes into their *NN*-dialkyl or *N*-monoalkyl derivatives (Parts I and II), it seemed probable that this method could also be employed for the alkylation of peptides:

$$2R{\cdot}CHO + NH_2{\cdot}\underset{R'}{CH}{\cdot}CO{\cdot}NH{\cdot}\underset{R''}{CH}{\cdot}CO{\cdot}NH{\cdot}\underset{R'''}{CH}{\cdot}CO- \longrightarrow \begin{matrix} R{\cdot}CH_2 \\ R{\cdot}CH_2 \end{matrix}\!\!>\!N{\cdot}\underset{R'}{CH}{\cdot}CO{\cdot}NH{\cdot}\underset{R''}{CH}{\cdot}CO{\cdot}NH{\cdot}\underset{R'''}{CH}{\cdot}CO-$$

It has already been found that hippuric acid does not undergo alkylation under the conditions usually employed and it was therefore expected that the peptide linkage would remain intact and that only the terminal primary amino-groups of the polypeptide would be alkylated; this has been found to be the case.

Thus crystalline NN-*dimethylglycylglycine*, NN-*dimethylalanylglycine*, and NN-*dimethylglycylglycylglycine* were obtained from the appropriate peptide by catalytic reduction with formaldehyde in aqueous solution at room temperature in the presence of palladised charcoal. The purification of these methylated peptides presented some difficulty since steam-distillation could not be used for removal of paraformaldehyde without risk of hydrolysis. Methylation of peptides derived from optically active amino-acids has also been accomplished. Thus glycyl-L-tyrosine has been converted into (+)-NN-*dimethylglycyl-L-tyrosine*; (+)-NN-*dimethyl-L-leucyl-L-tyrosine* and (+)-NN-*dimethyl-L-leucylglycylglycine* have also been prepared.

This methylation of the terminal amino-group paves the way for the development of a new method for the identification of the "end" amino-group in a polypeptide. A peptide (I) would give rise to the dimethyl derivative (II) which could be hydrolysed to the dimethylamino-

$$H_2N{\cdot}CHR{\cdot}CO{\cdot}NH{\cdot}CHR'{\cdot}CO{\cdot}NH{\cdot}CHR''{\cdot}CO_2H \quad (I.)$$

$$\downarrow$$

$$Me_2N{\cdot}CHR{\cdot}CO{\cdot}NH{\cdot}CHR'{\cdot}CO{\cdot}NH{\cdot}CHR''{\cdot}CO_2H \quad (II.)$$

$$\swarrow \quad \downarrow \quad \searrow$$

$$\underset{(III.)}{Me_2N{\cdot}CHR{\cdot}CO_2H} + \underset{(IV.)}{H_2N{\cdot}CHR'{\cdot}CO_2H} + \underset{(V.)}{H_2N{\cdot}CHR''{\cdot}CO_2H}$$

acid (III) and the simple amino-acids (IV) and (V). Dimethylamino-acids differ from the unmethylated acids in that they are easily soluble in ethanol and can therefore be readily separated.

In order to test these conclusions, (+)-*NN*-dimethylglycyl-L-tyrosine was hydrolysed with boiling hydrochloric acid and the resulting amino-acids, after isolation in the free condition *via* the silver salts, were separated by extraction with ethanol. The residue consisted of

(−)-L-tyrosine having the full activity, whilst pure *NN*-dimethylglycine was readily obtained from the ethanolic extract. In a similar manner, (+)-*NN*-dimethylglycyl-L-leucine yielded *NN*-dimethylglycine and leucine, whilst (+)-*NN*-dimethyl-L-leucylglycylglycine gave (+)-*NN*-dimethyl-L-leucine and glycine.

Attention was next directed to the alkylation of peptides by means of higher aldehydes. Although such alkylations proceed fairly readily, it was not possible, except in one case, to obtain the alkylated peptides in a crystalline condition. The products were all contaminated with aldehyde polymerides and formed viscid gums which, on prolonged storage, were transformed into hard glasses. The crude alkylated peptides were, however, submitted to hydrolysis with hydrochloric acid and, in all cases, terminal alkylation was demonstrated by the isolation of the " end " amino-acid in the form of its alkyl derivative. Thus, the products resulting from the *n*-butylation and *n*-heptylation of glycylglycine furnished *NN*-di-*n*-butylglycine and *NN*-di-*n*-heptylglycine which were readily separated from the accompanying glycine by extraction with ethanol. Similarly, the products obtained from the tripeptide, diglycylglycine, by *n*-propylation or *iso*butylation, followed by hydrolysis, were separated into *NN*-di-*n*-propylglycine and *NN*-di*iso*butylglycine, respectively, in addition to glycine itself.

It was considered of interest to extend the observations to peptides containing a terminal amino-acid such as leucine which had already been shown to furnish monoalkylderivatives. D-Leucylglycine was accordingly submitted to reductive butylation in absolute ethanol; absorption of hydrogen proceeded rapidly until the stage corresponding to monoalkylation was reached, and then ceased abruptly. Pure N-n-*butyl*-D-*leucylglycine* was isolated and on hydrolysis furnished, in quantitative yield, a mixture of glycine and (−)-N-n-*butyl*-D-*leucine* having $[\alpha]_D^{20}$ −28·7°, $[\alpha]_{5461}^{20}$ −31·7°, which were separated by taking advantage of the sparing solubility of the butyl derivative in water. Butylation of L-leucylglycylglycine gave a non-crystalline butyl derivative, which by hydrolysis furnished (+)-N-n-*butyl*-L-*leucine*, $[\alpha]_D^{20}$ +29·1°, $[\alpha]_{5461}^{20}$ +31·4°. Since the rotation is almost identical with that found for the optical isomer, it would appear that alkylation and hydrolysis of peptides are not accompanied by appreciable racemisation. The separation of the products of hydrolysis in the last two cases was rendered possible by the small solubility of the butyl-leucines in water, but the separation of *N*-monoalkylamino-acids from a mixture containing unsubstituted amino-acids would usually present considerable difficulties.

In view of the possibility of monoalkylation occurring and the difficulties of separation, the use of aldehydes other than formaldehyde, cannot be recommended for identifying the " end " amino-acid in a polypeptide chain; the use of formaldehyde is free from these objections.

Experimental.

Methylation of Peptides.—A solution of the peptide (2 g.) in water (100 ml.) containing aqueous formaldehyde (6 ml. of 40%) was stirred with palladised charcoal (3 g. of 10%) in an atmosphere of hydrogen at room temperature until the absorption of gas ceased. The aqueous solution was filtered, the catalyst was washed with water (20 ml.), and the combined filtrates were then evaporated to dryness under reduced pressure. The residual methylated peptide was kept *in vacuo* over sulphuric acid, in order to remove water and formaldehyde polymers as far as possible, and was then crystallised from a suitable solvent.

Hydrolysis of the methylated peptides and isolation of the resulting amino-acids were carried out as follows: the peptide was heated under reflux for 12 hours with ten times its weight of hydrochloric acid (6N.), and the solution, after dilution with water, was filtered and evaporated to dryness. The resulting mixture of hydrochlorides was dried over potassium hydroxide *in vacuo* and then dissolved in water (50 ml.), and the solution shaken vigorously with twice the theoretical amount of freshly precipitated silver hydroxide necessary to remove the halogen acid. After filtration, the solution was saturated with hydrogen sulphide, and the silver sulphide precipitated was removed by filtration and washed with boiling water. Evaporation of the filtrate to dryness furnished a mixture of amino-acids from which the methylated amino-acid was extracted with boiling ethanol.

Methylation of (a) *Glycyl-L-tyrosine.*—The colourless gum obtained as described above, on treatment with boiling ethanol, furnished (+)-NN-*dimethylglycyl*-L-*tyrosine* (1 g.) which separated from aqueous ethanol (70%) in white prisms, m. p. 159°, $[\alpha]_D^{20}$ +23·7°, $[\alpha]_{5461}^{20}$ +29·0° ($l = 1$; c, 3·040 in water) (Found: N, 10·2. $C_{13}H_{18}O_4N_2$ requires N, 10·5%).

The methylated peptide (0·3 g.) was hydrolysed and the resulting amino-acids were separated by extraction with boiling ethanol. The residue consisted of (−)-L-tyrosine (0·13 g.), m. p. 307—309°, $[\alpha]_D^{20}$ −8·1 ($l = 1$; c, 0·681 in 2% hydrochloric acid). Evaporation of the filtrate gave *NN*-dimethylglycine (0·15 g.) which, after purification by sublimation *in vacuo*, melted at 180—183°.

(b) *Glycyl-L-leucine.* Treatment of the crude reaction product with ethanolic acetone furnished *NN*-dimethylglycyl-L-leucine (0·4 g.) in slender needles, m. p. *ca.* 90°, which were very hygroscopic and rapidly liquefied on exposure to air. Extraction of the amino-acids (0·3 g.), obtained from the hydrolysis of the peptide (0·4 g.) with ethanol (10 ml.), yielded a residue consisting of leucine (0·12 g.), m. p. 269°; evaporation of the ethanolic extract yielded *NN*-dimethylglycine, m. p. 178°.

(c) L-*Leucylglycylglycine.* The colourless gum resulting from the methylation, readily crystallised from boiling ethanol and gave (+)-NN-*dimethyl*-L-*leucylglycylglycine* as colourless prisms, m. p. 172°, $[\alpha]_D^{20}$ +59·0°, $[\alpha]_{5461}^{20}$ +67·7° (*l* = 1; *c*, 3·564 in water) (Found: N, 15·5. $C_{12}H_{23}O_4N_3$ requires N, 15·4%).

A specimen of the methylated tripeptide (0·65 g.) was hydrolysed and the resulting amino-acids (0·731 g.; theory, 0·736 g.) were separated by extraction with boiling ethanol. Glycine (0·28 g.) remained undissolved, whilst (+)-*NN*-dimethyl-L-leucine was isolated from the filtrate as an extremely hygroscopic vitreous resin which, after sublimation *in vacuo* (bath, 180—190°/0·6 mm.), formed colourless crystals, $[\alpha]_D^{20}$ +30·5° (*l* = 1; *c*, 2·230 in ethanol) [cf. −35·8° in ethanol for the (−)-form; Part I].

(d) L-*Leucyl*-L-*tyrosine.* Evaporation of the combined aqueous extracts furnished (+)-NN-*dimethyl*-L-*leucyl*-L-*tyrosine* (1·45 g.) which crystallised from boiling aqueous ethanol in colourless, elongated triangular prisms, m. p. 158°, $[\alpha]_D^{20}$ +7·3°, $[\alpha]_{5461}^{20}$ +6·3° (*l* = 1; *c*, 2·856 in 0·1N-hydrochloric acid) (Found: N, 9·0. $C_{17}H_{26}O_4N_2$ requires N, 8·7%).

(e) *Diglycylglycine.* The crude reaction product separated from ethanolic-acetone as a white crystalline powder, m. p. 172°. NN-*Dimethylglycylglycylglycine* thus obtained was readily soluble in ethanol but, after remaining in a desiccator over calcium chloride for 4 years, was no longer soluble in this solvent but dissolved readily on the addition of a trace of water and then separated in clusters of colourless prisms, m. p. 172° (Found: N, 19·1. $C_8H_{15}O_4N_3$ requires N, 19·3%).

(f) *Alanylglycine.* The colourless gum, prepared according to the standard procedure, crystallised from ethanolic acetone in small white prisms (0·42 g.), m. p. 164° (Found: N, 16·7. $C_7H_{14}O_3N_2$ requires N, 16·1%).

Alkylation of Peptides with Higher Aldehydes.—Unless otherwise stated, the following general procedure was employed: A mixture of the peptide (2 g.), the aldehyde (3 mols.), catalyst (4 g.), and aqueous ethanol (100 ml. of 50%) was stirred in an atmosphere of hydrogen at room temperature until the theoretical amount of hydrogen had been absorbed. The ethanolic solution was then filtered and the catalyst washed with a further portion of the solvent. Evaporation of the combined extracts to dryness under reduced pressure furnished the alkylated peptides in the form of yellowish-brown gums. Attempts to isolate these peptides in a crystalline condition by converting them into their hydrochlorides, hydrobromides, and picrates, and also by chromatography, were unsuccessful. The crude products were therefore hydrolysed and the resulting amino-acids isolated as described for the methylated peptides.

Glycylglycine was submitted to reductive condensation with *n*-butanal and *n*-heptanal, and the mixtures of amino-acids obtained by hydrolysis were separated by extraction with hot ethanol. The residue consisted, in each case of pure glycine, m. p. 237°. Evaporation of the ethanolic extracts furnished *NN*-di-*n*-butylglycine and *NN*-di-*n*-heptylglycine, which crystallised from acetone in needles, m. p. 134° and 131°, respectively, undepressed on admixture with authentic samples.

Diglycylglycine was also alkylated by means of propaldehyde and *iso*butanal. The products were hydrolysed and then separated as in the previous experiments into glycine and *NN*-di-*n*-propylglycine [extremely hygroscopic prisms from acetone–light petroleum (b. p. 40—60°)], m. p. 130°, and *NN*-di*iso*-butylglycine [needles from boiling light petroleum (b. p. 40—60°)], m. p. 92—93°, both m. p.s undepressed by authentic specimens.

Butylation of D-*Leucylglycine.*—A mixture of the peptide (1·88 g.), *n*-butanal (2·7 ml.), catalyst (2 g.), and ethanol (75 ml.) was reduced in the usual manner. The reduction proceeded very slowly at room temperature but at 50° became more rapid and finally ceased when a total of 260 ml. of hydrogen had been absorbed (theory for monoalkylation, 237 ml. at 15°). After filtration, the residue consisting of catalyst and the butylpeptide was extracted with hot ethanol (25 ml.) containing hydrochloric acid (1·5 ml. of 10N.). Addition of a slight excess of pyridine to the acid extract failed to precipitate the peptide which was therefore recovered from the solution by evaporation to dryness and freed from the accompanying pyridine hydrochloride by successive treatment with silver hydroxide and hydrogen sulphide as described above. Final evaporation of the resulting aqueous solution furnished N-n-*butyl*-D-*leucylglycine* (0·61 g.) as a slightly brown powder, m. p. 232° (decomp.) (Found: N, 11·4. $C_{12}H_{24}O_3N_2$ requires N, 11·5%).

Hydrolysis of the peptide (0·42 g.) gave, in almost quantitative yeild (0·455 g.), a mixture of amino-acids which by extraction with cold water (5 ml.) gave (−)-N-n-*butyl*-D-*leucine* (undissolved) as white prisms, m. p. 275°, $[\alpha]_D^{20}$ −28·7°, $[\alpha]_{5461}^{20}$ −31·7° (*l* = 1; *c*, 2·893 in 0·2N-hydrochloric acid) (Found: N, 7·6. $C_{10}H_{21}O_2N$ requires N, 7·5%). The aqueous solution furnished, on evaporation, glycine (0·15 g.), m. p. 235°.

Butylation of L-*Leucylglycylglycine.*—The tripeptide (1·22 g.) was stirred in hydrogen at 52° with *n*-butanal (3 ml.) and ethanol (75 ml.) in the presence of palladised charcoal (2 g.). The reaction was interrupted after 9 hours when 200 ml. of hydrogen had been absorbed (theory for dialkylation, 224 ml. at N.T.P.). The butylated peptide was isolated from the ethanol solution as a yellow gum which was hydrolysed in the usual manner. Extraction of the resulting amino-acids (0·45 g.) with boiling acetone (10 ml.), followed by crystallisation of the residue from water (2 ml.), furnished (+)N-n-*butyl*-L-*leucine* (0·11 g.) as prisms, m. p. 262°, having $[\alpha]_D^{20}$ +29·1°, $[\alpha]_{5461}^{20}$ +31·4° (*l* = 1; *c*, 2·200 in 0·2N-hydrochloric acid) (Found: N, 7·3. $C_{10}H_{21}O_2N$ requires N, 7·5%). The aqueous solution was evaporated to dryness and the glycine so obtained crystallised from aqueous ethanol; it had m. p. and mixed m. p. 235°.

The author of this and the preceding paper acknowledges the help and encouragement received from Dr. F. Barrow under whose direction these researches were carried out.

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