A METHOD FOR THE STEPWISE DEGRADATION OF POLYPEPTIDES

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In the analysis of proteins one of the problems encountered is the determination of the arrangement of the amino acid residues in polypeptides. A solution to this problem has been sought by protein chemists for a long time. Fischer and Abderhalden (1) hydrolyzed dipeptides in which the free amino group was substituted by means of a naphthalenesulfonyl residue; the amino acid bearing the free amino group in the dipeptide was isolated from the hydrolysate as the naphthalenesulfonyl derivative. Several authors (2, 3) later used analogous methods in order to label a terminal amino acid in a peptide and to isolate its derivative following hydrolysis. The phenyl isocyanate method described by Bergmann, Miekeley, and Kann (3) was further developed and applied by Abderhalden and Brockman (4) in order to determine the order of amino acids in the tripeptide *dl*-alanylglycyl-*dl*-leucine.

In what follows, a method is described whereby each successive amino acid in polypeptides may be characterized and the order of amino acids in the polypeptide chain determined. The method involves a combination of the azide degradation of Curtius with our carbobenzoxy method (5). The general principle of the method was recently described (6), the phenyl isocyanate

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compound of the tripeptide glycyl-*l*-alanyl-*l*-leucine being used. Fischer and Waibel (7) found the same carbobenzoxy degradation in experiments with pyrrolecarboxylic acids.

It was found during the further development of the peptide degradation that the best results were obtained by employing benzoylated peptides. The method will therefore be described for the benzoyl derivative of the tetrapeptide glycyl-*l*-alanyl-*l*-leucyl-*l*-glutamic acid. As an introduction, there is described the carbobenzoxy degradation of the benzoyl derivatives of *l*-leucine, *dl*-phenylalanine, and *l*-glutamic acid.

When an amino acid (I) is to be degraded according to the carbobenzoxy method, it is first benzoylated at the α-amino group (II) and converted into the azide (III) through the methyl ester and hydrazide. On slight heating with benzyl alcohol, this azide forms the carbobenzoxy compound, benzylurethane (IV), which may be catalytically hydrogenated in a hydrochloric acid solution to the hydrochloride of the amine (V). This type of compound belongs to the derivatives of doubly aminated aldehydes. They are sufficiently stable to be isolated in a well crystallized form and therefore deserve closer investigation.

For the purpose of this work it was important that on warming with water these derivatives of doubly aminated aldehydes are split to benzamide, ammonium chloride, and the aldehyde (VI) and can easily be characterized in the form of derivatives.

I. $R \cdot CH(NH_2) \cdot COOH$
II. $R \cdot CH(NH \cdot Bz) \cdot COOH$
III. $R \cdot CH(NH \cdot Bz) \cdot CON_3$
IV. $R \cdot CH(NH \cdot Bz) \cdot NH \cdot CO \cdot O \cdot CH_2 \cdot C_6H_5$
V. $R \cdot CH(NH \cdot Bz) \cdot NH_2$
VI. $R \cdot CHO$

$Bz = C_6H_5 \cdot CO$

By this method isovaleraldehyde is obtained from *l*-leucine, while phenylalanine yields phenylacetaldehyde.

As is to be expected, aminodicarboxylic acids are degraded in two positions. Benzoylglutamic acid (VII) gives, for example, the diazide (VIII) and then the dicarbobenzoxy compound (IX). The last named compound is hydrogenated to (X), which on splitting yields β-aminopropionaldehyde (XI). It was found that

this aldehyde may well be characterized as the dimethone derivative.

VII. $HO \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot Bz) \cdot COOH$
VIII. $N_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot Bz) \cdot CO \cdot N_3$
IX. $C_6H_5 \cdot CH_2 \cdot O \cdot CO \cdot NH \cdot CH_2 \cdot CH_2(NH \cdot Bz) \cdot NH \cdot CO \cdot O \cdot CH_2 \cdot C_6H_5$
X. $NH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot Bz) \cdot NH_2$
XI. $NH_2 \cdot CH_2 \cdot CH_2 \cdot CHO$

Degradation experiments with diaminocarboxylic acids have not yet been performed. These should, however, also give amino aldehydes.

It should be pointed out that in the case of optically active amino acids all the reaction products up to (V) inclusive, and similarly (VII to X), retain the optical activity. This corresponds to the finding of Wallis (8) that optically active carboxylic acids give optically active amines on degradation.

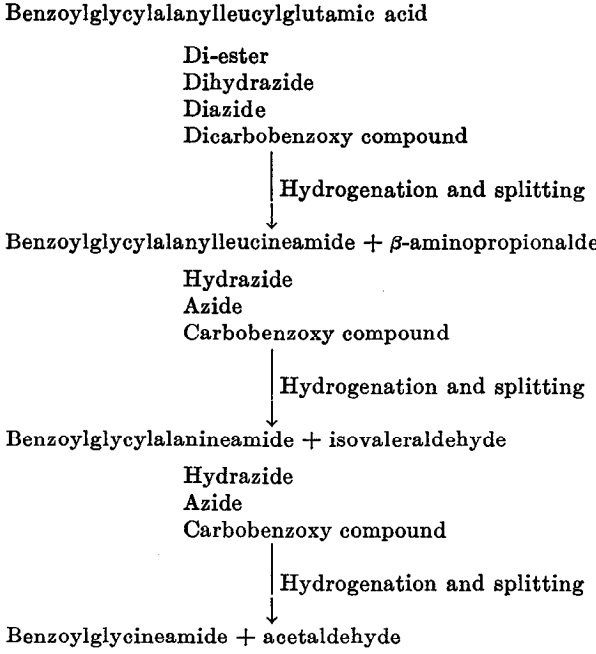
Curtius (9) had already attempted the degradation of benzoylated amino acids but was forced to split the urethanes, obtained with methyl or ethyl alcohol (analogous to (IV)), by energetic treatment with acids. The resulting aldehydes and acid amides were usually decomposed under these conditions. Therefore, the original Curtius method could not be used in peptide chemistry. The carbobenzoxy degradation will also be useful for the degradation of other carboxylic acids, when it is necessary to isolate labile degradation products under mild conditions.

In order to perform the carbobenzoxy degradation on a tetrapeptide, glycyl-*l*-alanyl-*l*-leucyl-*l*-glutamic acid was synthesized by the carbobenzoxy method and then the individual amino acids were successively split off and identified in the form of the corresponding aldehydes with 1 carbon atom less. In this degradation the intermediate steps shown in the accompanying diagram were involved.

The conversion of the intermediate amide into the hydrazide was performed by the direct action of hydrazine.[1]

In its present form the carbobenzoxy degradation should be applicable to the identification of every α-amino acid in peptides.

[1] This reaction usually involves losses. It will therefore be attempted in the future to transform the amides into the carboxylic acids with nitrous acid, and to secure the hydrazide through the ester.



Further special experiments are necessary on the behavior of proline and hydroxyproline.

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EXPERIMENTAL

Degradation of l-Leucine

N-Benzoyl-l-Leucine Methyl Ester—10 gm. of *l*-leucine were suspended in 50 cc. of dry methanol which was then saturated with HCl without cooling. After evaporation, the esterification was repeated. From the hydrochloride an ethereal solution of the free ester was prepared in the usual manner, and to the latter there were added, at 0°, 6 cc. of benzoyl chloride in 50 cc. of ethyl acetate. Then 6 cc. more of benzoyl chloride and 100 cc. of 10 per cent sodium carbonate solution were added at 0° in several portions, with shaking. The ether-ethyl acetate solution was

washed in turn with pyridine, water, hydrochloric acid, and potassium bicarbonate, then dried and evaporated down, yielding crystals which were transferred to the filter with cold ether. A second crystallization was obtained by precipitation with petroleum ether. Yield, 10.6 gm. After recrystallization from ether the melting point was 104°.

$C_{14}H_{19}O_3N$ (249.2). Calculated, N 5.6; found, 5.5[2]

Benzoyl-l-Leucine Hydrazide—8.5 gm. of the above ester were dissolved in 15 cc. of hot alcohol and 2.5 cc. of hydrazine hydrate, and allowed to stand 24 hours at room temperature. The needles which separated out were washed with ice-cold water. Yield, 7.5 gm. On recrystallization from alcohol, the melting point was 153°.

$C_{13}H_{19}O_2N_3$ (249.2). Calculated, N 16.9; found, N 17.0

1-Benzamido-1-Carbobenzoxyamido-3-Methylbutane—7 gm. of the above hydrazide were dissolved in a mixture of 10 cc. of 5 N hydrochloric acid and 40 cc. of 50 per cent acetic acid; the solution was diluted with 150 cc. of water, cooled to 0°, and an aqueous solution of 2.2 gm. of sodium nitrite was added within 2 minutes. The sirupy precipitate of azide was extracted with a quantity of ether, the ether solution was washed four times with ice-cold water, then with bicarbonate, and again with water, filtered, dried over sodium sulfate, and 10 cc. of benzyl alcohol were added. The ether was evaporated *in vacuo* and the resulting solution heated to 70–80° (nitrogen generated). The crystals which separated out were washed with ether and filtered. Yield, 3 gm. The substance was recrystallized from glacial acetic acid. Needles; m.p., 178°.

$C_{20}H_{24}O_3N_2$.	Calculated.	C 70.6, H 7.1, N 8.2
340.2	Found.	" 70.6, " 7.2, " 8.0

1-Benzoylamino-1-Amino-3-Methylbutane—1 gm. of the carbobenzoxy compound was suspended in methanol which contained 1.1 moles of 5 N aqueous hydrochloric acid, and hydrogenated with palladium catalyst in an open vessel. The hydrogenation

[2] The nitrogen determinations reported in this paper were carried out by the micro-Dumas method except in the two instances in which the micro-Kjeldahl method is designated.

and carbon dioxide regeneration were complete after about 20 minutes. On evaporation under diminished pressure (bath temperature 35°), flat needles of the hydrochloride were obtained, which were transferred to the filter with acetone-ether. Yield, 0.6 gm.

$[\alpha]_D^{22} = -47°$ (5 per cent in methanol)

$C_{12}H_{19}ON_2Cl$.	Calculated.	C 59.4, H 7.9, N 11.5, Cl 14.6
242.6	Found.	" 59.7, " 7.9, " 11.4, " 14.7

The base can be liberated from the hydrochloride with sodium hydroxide and can be taken up in ether. On passing hydrochloric acid gas into the ethereal solution, the hydrochloride separates out immediately. On boiling the hydrochloride with methanol there crystallized out after a short time needles (in good yield) which contained no chlorine and which had a melting point of 210°. On recrystallization from methanol no elevation of the melting point was observed. The analysis indicates that the substance is the dibenzamido derivative of isovaleraldehyde.

$(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5)_2$

$C_{19}H_{22}O_2N_2$.	Calculated.	C 73.5, H 7.2, N 9.0
310.2	Found.	" 73.3, " 7.2, " 9.0

Splitting of the Hydrochloride—0.3 gm. of the above hydrochloride was dissolved in 5 cc. of water and the solution distilled in 5 minutes from a bath of 120° into an ice-cooled receiving flask which contained 0.3 gm. of *p*-nitrophenylhydrazine in 5 cc. of 50 per cent acetic acid. In the receiving flask there separated out the *p*-nitrophenylhydrazone of isovaleraldehyde (after recrystallization 0.26 gm. of needles of melting point 113° was obtained (10); calculated, N 19.0; found, N 19.2). In the distilling flask benzamide (0.1 gm., m.p., 127°; mixed m.p. with commercial benzamide, 127°) separated out, while the ammonium chloride remained in solution.

Degradation of dl-Phenylalanine

Benzoyl-dl-Phenylalanine Methyl Ester—From 10 gm. of phenylalanine there were obtained 14 gm. of benzoyl ester by the same method employed for leucine. On recrystallization from ether the melting point was 90°.

$C_{17}H_{17}O_3N$ (283.1). Calculated, N 4.9; found, N 5.0

Benzoyl-dl-Phenylalanine Hydrazide—A solution of 14 gm. of the above ester in 30 cc. of warm alcohol was treated overnight with 4 cc. of hydrazine hydrate at room temperature. On working up the solution, 12.5 gm. of recrystallized hydrazide (m.p., 192°) were obtained.

$C_{16}H_{17}O_2N_3$ (283.1). Calculated, N 14.8; found, N 15.1

1-Carbobenzoxyamino-1-Benzamino-2-Phenylethane—8 gm. of the above hydrazide were dissolved in 50 cc. of 50 per cent acetic acid and 5 cc. of 5 N hydrochloric acid, the solution was diluted with 150 cc. of water and cooled to 0°, and an aqueous solution of 2 gm. of sodium nitrite added within 2 minutes. After about 3 minutes, the precipitated azide was taken up in ethyl acetate, washed three or four times with ice-cold water, quickly with bicarbonate, and again with water, filtered, and dried over sodium sulfate. 20 cc. of benzyl alcohol were then added and the ethyl acetate was removed under reduced pressure at 50°; then the solution was heated on the steam bath for ½ hour. The crystals which separated out were treated with glacial acetic acid and filtered with suction. Yield, 5.9 gm. On recrystallization from glacial acetic acid, rods with a melting point of 196° were obtained.

$C_{23}H_{22}O_3N_2$.	Calculated.	C 73.7, H 5.9, N 7.5
374.2	Found.	" 73.6, " 6.1, " 7.4

Hydrogenation to 1-Amino-1-Benzamido-2-Phenylethane—5.5 gm. of carbobenzoxy compound were suspended in 100 cc. of methanol and 5 cc. of 5 N HCl, and hydrogenated in the usual manner with palladium (about 2 gm.) as catalyst. After hydrogenation, the filtrate was evaporated down at 35° and the residue transferred to the filter with acetone-ether. A spongy mass of needles was obtained; yield, 3 gm.

$C_{15}H_{17}ON_2Cl$.	Calculated.	C 65.1, H 6.2, N 10.1, Cl 12.8
276.6	Found.	" 64.8, " 6.0, " 10.2, " 13.0

If the hydrochloride is dissolved in water and sodium hydroxide or ammonium hydroxide is added, the free base separates out in nearly quantitative yield. Needles; m.p., 156° (after browning and sintering).

$C_{15}H_{16}ON_2$.	Calculated.	C 75.0, H 6.7, N 11.7
240.1	Found.	" 75.0, " 6.7, " 11.5

Deamination of the Amine—0.5 gm. of the hydrochloride was dissolved in water containing several drops of acetic acid. On addition of an aqueous solution of 0.18 gm. of sodium nitrite, there began nitrogen generation which ended after a short time. The precipitated needles (0.35 gm.) were recrystallized from methanol-water. M.p., 128°.

$C_{15}H_{15}O_2N$.	Calculated.	C 74.7, H 6.3, N 5.8
241.1	Found.	" 74.9, " 6.3, " 5.9

The substance is not affected by boiling water.

Splitting of the Amine—0.8 gm. of the above hydrochloride was dissolved in 10 cc. of water and after the addition of 0.6 gm. of sodium acetate and 0.2 gm. of hydroxylamine hydrochloride, heated to 100° for 15 minutes. On cooling, 0.3 gm. of phenylacetaldehyde oxime was obtained. Needles with a melting point of 103° (11) resulted after recrystallization from ether-petroleum ether.

C_8H_9ON (135.1). Calculated, N 10.4; found, N 10.4

On concentrating the mother liquor *in vacuo*, 0.2 gm. of benzamide was obtained as plates (m.p., 127°). The mixed melting point with commercial benzamide showed no depression.

Degradation of l-Glutamic Acid

Benzoyl-l-Glutamic Acid Dimethyl Ester—This was prepared from 14.7 gm. of glutamic acid in the same way as for leucine (instead of soda, potassium bicarbonate was employed). Yield, 25 gm. On recrystallization from ether, needles, m.p. 83°, were obtained.

$C_{14}H_{17}O_5N$ (279.1). Calculated, N 5.0; found, N 4.8 (micro-Kjeldahl)

Benzoyl-l-Glutamic Acid Dihydrazide—From the solution of 25 gm. of the dimethyl ester in 75 cc. of absolute alcohol to which 11 cc. of hydrazine hydrate were added, there separated out on standing overnight 20 gm. of dihydrazide. The substance was recrystallized from alcohol. Needles; m.p., 216°.

$C_{12}H_{17}O_3N_5$ (279.2). Calculated, N 25.1; found, N 24.9

Benzoyl-l-Glutamic Acid Diazide—8 gm. of dihydrazide were dissolved in 130 cc. of hot water, 4.3 gm. of sodium nitrite were added, and the solution was cooled to 0°; this was followed by the addition of 31 cc. of 2 N HCl within 1 minute. After about 3 to 5 minutes at 0°, the supernatant solution was discarded and the semisolid precipitate treated with about 2 cc. of ether, whereupon it crystallized. It was transferred to the filter with a little cold water, dried on a porous plate, and then over sulfuric acid *in vacuo*. Yield, 5.6 gm. of crystals which effervesced around 75°.

1,3-Biscarbobenzoxyamino-1-Benzaminopropane—4.8 gm. of diazide were added to 20 cc. of xylene and 12 cc. of benzyl alcohol, and heated slowly in a paraffin bath. Nitrogen generation began around 60–70°. The reaction mixture was kept at 70–80° until the nitrogen formation had slowed down, whereupon the temperature was raised to the boiling point of xylene for 15 minutes. On adding ether, 4.2 gm. of a spongy crystalline substance were obtained. It was dissolved in 30 cc. of hot 50 per cent acetic acid and allowed to cool slowly. The yield is decreased by one-half. Hair-like needles were obtained; m.p., 174°.

$C_{26}H_{27}O_6N_3$.	Calculated.	C 67.6, H 5.9, N 9.1
461.2	Found.	" 67.5, " 6.1, " 9.2

Hydrogenation of the Dicarbobenzoxy Compound—2 gm. of the dibenzylurethane were suspended in absolute methanol which contained 2.1 moles of hydrogen chloride. The hydrogenation in the presence of 0.5 gm. of palladium catalyst was completed in 15 minutes. On evaporation *in vacuo* at 30°, the crystalline hydrochloride remained behind. Yield, 0.8 gm. Recrystallization from cold methanol-ether gave rods which melted at 158° with sintering. $[\alpha]_D^{22} = -50.7°$ (6.9 per cent in water). Owing to the instability of the dihydrochloride, the analysis was performed on the dipicrate of the diamino compound.

The dipicrate can be obtained in quantitative yield from an aqueous solution of the hydrochloride by adding sodium picrate. It crystallizes from hot water containing picric acid in long rods which decompose slowly at 100°.

$C_{22}H_{21}O_{15}N_9 \cdot H_2O$.	Calculated.	C 39.4, H 3.5, N 18.8
669.2	Found.	" 39.4, " 3.4, " 18.7

The instability of the substance on heating prevented a determination of the water of crystallization.

Splitting of the Diamine—0.4 gm. of dimethone was dissolved in the required volume of water, and 0.4 gm. of the dihydrochloride added to the solution, which was then heated to boiling for 5 minutes. On cooling, the solution was extracted with a 1:1 mixture of ethyl acetate-ether, followed by ether extraction. The extracts were washed with a small volume of dilute sodium carbonate solution (to remove free dimethone) and concentrated down. 0.22 gm. of the benzamide in the form of square plates (m.p., 127° after recrystallization from water) was obtained. This showed no melting point depression with commercial benzamide. The aqueous solution (see above) contained the dimethone compound of β-aminopropionaldehyde as the hydrochloride. This substance was precipitated by making the solution slightly alkaline with sodium hydroxide and acidifying again with 1 or 2 drops of acetic acid. On standing overnight, 0.25 gm. of the dimethone anhydride of β-aminopropionaldehyde was obtained, which after recrystallization from aqueous alcohol and then from absolute alcohol melted at 208–209°.

$C_{19}H_{27}O_3N$.	Calculated.	C 71.9, H 8.6, N 4.5
317.2	Found.	" 72.1, " 8.3, " 4.4

Synthesis of Glycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid

Carbobenzoxyglycyl-l-Alanine Ethyl Ester—To a solution of *l*-alanine ethyl ester in ethyl acetate (prepared from 15 gm. of the hydrochloride) there were added, in four portions with cooling, 11 gm. of carbobenzoxyglycyl chloride. With shaking, 11.5 gm. more of carbobenzoxyglycyl chloride were added in five portions, each of which was followed by 10 cc. of a 10 per cent sodium carbonate solution. After addition of about 100 cc. of water, the reaction mixture was shaken for a short time with 20 cc. of saturated bicarbonate solution. The aqueous layer was removed, the unchanged chloride decomposed with pyridine, and the ethyl acetate layer washed with water, dilute hydrochloric acid, and again with water, dried over sodium sulfate, and evaporated down. Upon scratching, the resulting sirup crystallized in long prisms. These were recrystallized from ether-petroleum ether. Yield,

22.5 gm.; m.p., 59° after two recrystallizations from ether-petroleum ether.

$C_{15}H_{20}O_5N_2$ (308.2). Calculated, N 9.1; found, N 9.2

Carbobenzoxyglycyl-l-Alanine Hydrazide—22 gm. of the above ester were dissolved in 30 cc. of absolute alcohol and refluxed with 6 gm. of hydrazine hydrate for 1 hour. On cooling, a crystalline mass was obtained which was washed with a little absolute alcohol and a quantity of ether. Yield, 16.5 gm.; m.p., 133° after recrystallization from alcohol.

$C_{13}H_{18}O_4N_4$ (294.2). Calculated, N 19.0; found, N 18.8

Carbobenzoxyglycyl-l-Alanine Azide—7.5 gm. of the above hydrazide were dissolved in 150 cc. of water and 17 cc. of 3.6 N HCl. The solution was cooled to 0° and there were added solutions of 4.8 gm. of sodium acetate (containing water of crystallization) in 10 cc. of water and 1.9 gm. of sodium nitrite in 10 cc. of water, the latter being added dropwise. The resulting sirup was taken up in ether, leaving a small portion undissolved. The ethereal solution was washed often with ice-cold water, then with bicarbonate solution, and again with water, and dried over sodium sulfate. This solution was used immediately for coupling.

Carbobenzoxyglycyl-l-Alanyl-l-Leucine Methyl Ester—To an ethereal solution of *l*-leucine methyl ester prepared from 14 gm. of the hydrochloride there was added the above ethereal solution of azide prepared from 20 gm. of the hydrazide. After 12 hours, 8 gm. of the reaction product had separated out in needles. The filtrate was concentrated to 100 cc., freed of crystals (0.5 gm.) which had separated out, washed with dilute hydrochloric acid, water, and bicarbonate, dried over sodium sulfate, and the substance precipitated with petroleum ether. Total yield, 15 gm.; m.p., 112° after recrystallization from ethyl acetate-petroleum ether.

$C_{20}H_{29}O_6N_3$ (407.2). Calculated, N 10.3; found, N 10.4

Carbobenzoxyglycyl-l-Alanyl-l-Leucyl Hydrazide—5.7 gm. of the above tripeptide ester were dissolved in about 12 cc. of warm absolute alcohol, 0.8 cc. of hydrazine hydrate was added, and the solution allowed to stand overnight at room temperature. The

needles which separated out were washed with a little alcohol. Yield, 3.3 gm.; m.p., 186° after recrystallization from alcohol.

$C_{19}H_{29}O_5N_5$ (407.2). Calculated, N 17.2; found, N 17.5

Carbobenzoxyglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid Dimethyl Ester—9 gm. of the hydrazide described above were dissolved in 225 cc. of water, 15 cc. of glacial acetic acid, and 22.5 cc. of N HCl, and the solution was cooled to 0°. Then an aqueous solution of 3 gm. of sodium acetate (with water of crystallization) was added, followed by the dropwise addition (within 2 minutes) of an aqueous solution of 1.65 gm. of sodium nitrite. The resulting azide was taken up immediately in about 300 cc. of ethyl acetate, the azide solution washed three times with ice-cold water, followed by bicarbonate solution and again by water, filtered, and dried quickly over a quantity of sodium sulfate. It was then coupled with an ethereal solution of *l*-glutamic acid dimethyl ester (prepared from about 9 gm. of glutamic acid). On concentrating the mixture and allowing it to stand overnight, needles were obtained. On further concentration of the mother liquor, a second crystallization resulted. Total yield, 7.2 gm.; m.p., 149° after recrystallization from ethyl acetate.

$C_{26}H_{38}O_9N_4$ (550.3). Calculated, N 10.2; found, N 10.0 (micro-Kjeldahl)

Glycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid—2.7 gm. of the tetrapeptide ester were suspended in 10 cc. of methanol, and with occasional cooling and shaking saponified with 11 cc. of N sodium hydroxide within 15 minutes. After 15 minutes more, the solution was made slightly acid to Congo red, the methanol removed under reduced pressure, and the resulting sirup dissolved in a large volume of ethyl acetate. The ethyl acetate layer was extracted with bicarbonate. The bicarbonate extract was then acidified, and the carbobenzoxytetrapeptide was again taken up in ethyl acetate. After washing with water, the solution was evaporated down, the residue dissolved in aqueous methanol and 0.3 cc. of glacial acetic acid, and catalytically hydrogenated. The solution was filtered, the catalyst washed frequently with water, and the combined filtrate evaporated under reduced pressure. The crystals (rhombic) were transferred to the filter with alcohol. Yield,

1.5 gm. On recrystallization from water the tetrapeptide forms long prisms which are transformed into rhombic crystals.

$C_{16}H_{23}O_7N_4 \cdot 1\frac{1}{2}H_2O$.	Calculated.	C 46.2, H 7.5, N 13.5, H_2O 6.5
415.2	Found.	" 45.9, " 7.6, " 13.3, " 6.3

3.77 mg. required 1.9 cc. of 0.01 KOH in 90 per cent alcohol, thymolphthalein being used as indicator. Calculated, 1.8 cc.

Degradation of the Tetrapeptide

Benzoylglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid—1 gm. of the free tetrapeptide was dissolved in a solution of 1.2 gm. of potassium bicarbonate in 10 cc. of water. Within 20 minutes there was added with shaking 0.35 cc. of benzoyl chloride in four portions. Each addition was preceded by cooling in ice. The shaking was performed at room temperature. On acidifying, the benzoyl compound separated out at first as a sirup; on scratching, however, it crystallized. After drying, the substance was boiled up with ether. Yield, 1.05 gm. Needles; m.p., 215° after recrystallization from aqueous alcohol.

$C_{23}H_{32}O_8N_4$.	Calculated.	C 56.1, H 6.5, N 11.4
492.3	Found.	" 56.2, " 6.5, " 11.6

Benzoylglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid Dimethyl Ester—0.5 gm. of benzoyltetrapeptide was suspended in methanol and esterified with an excess of an ethereal solution of diazomethane. The residue obtained on evaporation was transferred to the filter with ethyl acetate-ether and recrystallized from ethyl acetate. Needles; m.p., 178°; yield, 0.4 gm.

The compound was also more simply prepared by the hydrogenation of the carbobenzoxytetrapeptide dimethyl ester in the presence of 1 mole of methyl alcoholic hydrogen chloride, followed by the benzoylation of the free ester in the usual manner.

$C_{25}H_{36}O_8N_4$ (520.3). Calculated, N 10.8; found, N 10.7

Benzoylglycyl-l-Alanyl-l-Leucyl-l-Glutamic Acid Dihydrazide—3.6 gm. of the above dimethyl ester were dissolved in hot absolute alcohol, 0.9 cc. of the hydrazine hydrate was added, and the solution allowed to stand overnight at room temperature. The

spongy mass which separated out was washed well with alcohol. Yield, 3.5 gm.; m.p., 253° after recrystallization from alcohol.

$C_{23}H_{36}O_6N_8$ (520.3). Calculated, N 21.5; found, N 21.1

Repeated recrystallizations did not improve the analysis.

Benzoylglycyl-l-Alanyl-l-Leucyl Derivative of 1-Amino-1, 3-Dicarbobenzoxypropane—3.3 gm. of the above dihydrazide were dissolved in 25 cc. of water and 12.7 cc. of N hydrochloric acid, the solution was cooled to 0°, and to it was added within 1 minute an aqueous solution of 0.95 gm. of sodium nitrite. The precipitate was separated from the supernatant liquid and washed with cold water containing 6 cc. of N hydrochloric acid, treated with a small volume of ether, filtered, washed with water and ether, and dried well over phosphorus pentoxide in a desiccator. Yield of diazide, 2.4 gm. The dry azide was added in small portions within 3 to 4 minutes to a solution of 20 cc. of xylene and 5 cc. of benzyl alcohol which had been warmed to 90°. After completion of the nitrogenation, the solution was heated for 5 minutes at the boiling point of xylene. On cooling, a spongy mass separated out, the precipitation being made more complete by the addition of ether. Yield, 1.7 gm.; m.p., 201° after recrystallization from 50 per cent acetic acid and washing with ethyl acetate.

$C_{37}H_{46}O_8N_6$.	Calculated.	C 63.2, H 6.6, N 12.0
702.4	Found.	" 63.4, " 6.7, " 12.1

Hydrogenation and Splitting of the Dicarbobenzoxy Compound—1.4 gm. of the dicarbobenzoxy compound were suspended in methanol and 3 cc. of 2 N hydrochloric acid, and hydrogenated in the presence of 0.5 gm. of fresh palladium black. The hydrogenation was completed in about ½ hour and the filtered solution was evaporated to dryness at 30–35°, the residue dissolved in a small volume of water, the solution filtered from traces of undissolved material, neutralized to litmus with sodium hydroxide, and added to a hot saturated aqueous solution of 0.6 gm. of dimethone. The mixture was kept at 100° for about 5 minutes, the unchanged dimethone removed by ether extraction, and the aqueous solution concentrated *in vacuo* at 35° to 4 to 5 cc. On standing overnight, crystals of benzoylglycyl-*l*-alanyl-*l*-leucylamide separated out, which were contaminated with a slight amount of

sirup. They were filtered off, washed with ether, and recrystallized from methanol-water. Yield, 0.4 gm.; m.p., 186°.

$C_{18}H_{26}O_4N_4$.	Calculated.	C 59.6, H 7.2, N 15.5
362.2	Found.	" 59.8, " 7.3, " 15.3

The mother liquor was made slightly alkaline with sodium hydroxide and acidified slightly with acetic acid. On standing overnight, plates of the dimethone derivative of β-aminopropionaldehyde (0.3 gm.) separated out which, after recrystallization from alcohol, melted at 208°. The mixed melting point with the dimethone obtained from the degradation product of benzoylglutamic acid was 208°.

$C_{19}H_{27}O_3N$ (317.2). Calculated, N 4.4; found, N 4.6

Benzoylglycyl-l-Alanyl-l-Leucyl Hydrazide—1 gm. of the amide obtained by the above degradation was refluxed for 2 hours in 3 cc. of ethanol and 0.5 cc. of hydrazine hydrate, and then heated for 1.2 hours without refluxing; whereupon ammonia was liberated. On standing overnight, 0.45 gm. of the hydrazide separated out in needles bunched in spheres. M.p., 230° after recrystallization from ethanol.

$C_{18}H_{27}O_4N_5$ (377.2). Calculated, N 18.6; found, N 18.3

Benzoylglycyl-l-Alanyl Compound of 1-Amino-1-Carbobenzoxyamino-3-Methylbutane—2.5 gm. of the above hydrazide were dissolved with heating in 7 cc. of glacial acetic acid and 40 cc. of water, the solution was cooled to 0°, and to it was added dropwise an ice-cold aqueous solution of 0.5 gm. of sodium nitrite. After about 10 minutes at 0°, the azide was taken up in about 70 cc. of ethyl acetate, the solution washed repeatedly with ice-cold water, bicarbonate, and again water, and dried over sodium sulfate. To the solution 6 cc. of benzyl alcohol were added. The ethyl acetate was removed *in vacuo* at 35–40°, and then the residue was heated to 80° for ½ hour. On cooling, the urethane separated out in fine needles, which were redissolved by adding benzene and heating. On cooling, 1.1 gm. of substance formed; from the mother liquor 0.2 gm. was obtained. Recrystallization from glacial acetic acid-water gave long needles with a melting point of 212°.

$C_{25}H_{32}O_5N_4$ (468.3). Calculated, N 12.0; found, N 11.8

Hydrogenation and Splitting of the Carbobenzoxy Compound—1.1 gm. of the carbobenzoxy compound were suspended in methanol which contained 1.1 moles of hydrogen chloride, and hydrogenated in the usual manner. The residue obtained on evaporation *in vacuo* at 35° was dissolved in 10 cc. of water, and the solution heated to boiling in a bath at 125° for 5 to 8 minutes. The distillate was collected in a receiving flask which contained 0.4 gm. of *p*-nitrophenylhydrazine in 10 cc. of 50 per cent acetic acid. In the receiving flask 0.35 gm. of the nitrophenylhydrazone of isovaleraldehyde separated out. After recrystallization it melted at 113°.

$C_{11}H_{15}O_2N_3$ (221.1). Calculated, N 19.0; found, N 19.3

On allowing the filtered aqueous solution to stand in the ice box, 0.4 gm. of benzoylglycyl-*l*-alanineamide was obtained. After recrystallization from a small volume of methanol, the melting point was 192°.

$C_{12}H_{15}O_3N_3$.	Calculated.	C 57.8, H 6.1, N 16.9
249.1	Found.	" 57.7, " 5.9, " 16.9

Benzoylglycyl-l-Alanine Hydrazide—0.5 gm. of the above benzoylglycyl-*l*-alanineamide was refluxed for 2 hours in 5 cc. of alcohol and 0.4 gm. of hydrazine hydrate, and then heated without refluxing for ½ hour on the steam bath, whereupon ammonia was liberated. On cooling, 0.4 gm. of crystals separated out, which were recrystallized from alcohol. M.p., 212°.

$C_{12}H_{16}O_3N_4$ (264.1). Calculated, N 21.2; found, N 21.1

Benzoylglycyl Derivative of 1-Amino-1-Carbobenzoxyaminoethane—2.2 gm. of the preceding hydrazide were dissolved in 80 cc. of hot water and 3 cc. of glacial acetic acid, the solution was cooled to 0°, and an aqueous solution of 0.7 gm. of sodium nitrite added dropwise. On scratching, crystals (needles) of the azide were obtained. These were filtered off after ¼ hour, washed, and dried on a porous plate in a desiccator over phosphorus pentoxide. Yield, 2 gm. of azide. This was added in small portions (within 10 minutes) to a mixture of 15 cc. of xylene and 5 cc. of benzyl alcohol which had been heated in a bath at 150°. After the nitrogen generation had stopped, ether was added and the spongy

precipitate dissolved in 5 cc. of hot acetic acid. Water was added to cloudiness, and on slow cooling 0.35 gm. of needle-like crystals, with a melting point of 207°, was obtained.

$C_{19}H_{21}O_4N_3$ (355.2). Calculated, N 11.8; found, N 12.1

Hydrogenation and Splitting of the Carbobenzoxy Compound—0.3 gm. of the carbobenzoxy compound was dissolved in methanol and 0.1 cc. of 10 per cent hydrochloric acid, and catalytically hydrogenated in the usual way. After completion of the hydrogenation, the solution was evaporated down *in vacuo* at 35°, the residue dissolved in about 8 cc. of water, and the solution filtered and heated to boiling for 5 minutes. The vapors were led into an ice-cooled receiving flask which contained a saturated aqueous solution of 0.3 gm. of dimethone. On standing overnight, 0.2 gm. of ethylidene dimethone (m.p., 143°) separated out in the receiving flask. The mixed melting point with the acetaldehyde dimethone obtained with paraldehyde (12) was 143°.

$C_{18}H_{26}O_4$.	Calculated.	C 70.5, H 8.5
306.2	Found.	" 70.4, " 8.3

The original aqueous solution was concentrated *in vacuo* to 2 cc. On standing overnight, crystals (0.1 gm.) of hippurylamide separated out, which had a melting point of 185° after recrystallization from water. The mixed melting point with hippurylamide prepared according to Fischer (13) showed no depression.

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