On the Hydrazinolysis of Proteins and Peptides: A Method for the Characterization of Carboxyl-terminal Amino Acids in Proteins

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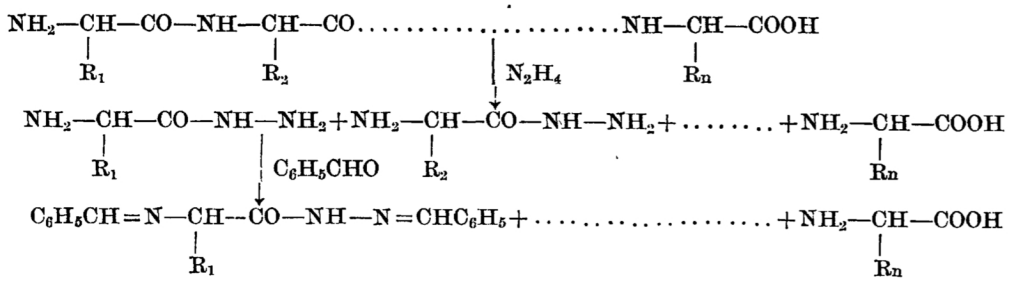
The problem of determining the amino acid sequence in proteins has attracted the interest of many investigators because of its bearing on protein structures. Although several chemical methods[1-5] have been published heretofore for the characterization of carboxyl-terminal amino acids in peptides, most of them are not entirely suitable to be applied for proteins practically. C. Fromageot *et al.*[6] have reported the most eminent method, in which insulin was treated with lithium aluminium hydride and only carboxyl-terminal amino acids were reduced to amino alcohols. However, it seems to be worth-while to study some other methods, because the applicability of the methods cited is for practical reasons restricted.

The present authors have investigated the hydrazinolysis of peptides for the purpose of finding a simple method of the characterization of carboxyl-terminal amino acids in proteins. It is well known that acylamino compounds are easily decomposed by hydrazine to acylhydrazines and amines. But, so far as the present authors are aware of hydrazinolysis of protein has never been studied, except that A. Stoll *et al.*[7] have studied the degradation of ergot alkaloids which contain peptide linkages.

When proteins are treated with anhydrous hydrazine under certain conditions, only carboxyl-terminal amino acids are liberated as free amino acids and other amino acid residues are converted to amino acid hydrazides. On treating with benzaldehyde the latter residues condense with it to form water-insoluble dibenzal compounds while the former remain in solution and can be identified by paper chromatography as amino acids. The reactions are shown in the following scheme:

(1) P. Schlack and W. Kumpf, *Z. physiol. Chem.*, **154**, 125 (1926).
(2) F. Bettzieche, *Z. physiol. Chem.*, **161**, 178 (1926).
(3) E. Abderhalden and H. Brockmann, *Biochem. Z.*, **225**, 386 (1930).
(4) M. Bergmann and L. Zervas., *J. Biol. Chem.*, **113**, 351 (1936).
(5) N. Lichtenstein, *J. Am. Chem. Soc.*, **60**, 560 (1938).
(6) C. Fromageot, N. Jutisz D. Meyer and L. Penasse, *Biochim. Biophys. Acta*, **6**, 238 (1950); *Compt. rend.*, **230**, 1905 (1950).
(7) A. Stoll, T. petrzilka and B. Becker, *Helv. Chim. Acta.*, **33**, 57 (1950).



Amino acid hydrazides can also be detected on a paper chromatogram by their reducing power against ammoniacal silver nitrate solution as described in the experimental part.

Experimental

(I) Syntheses of Amino Acid Hydrazides and Their Dibenzal Compounds.—Amino acid hydrazides have not yet been synthesized except glycine-,[8] aspartic acid-[9] and tyrosine-hydrazides.[10] The authors synthesized, therefore, several new hydrazides as described below:

DL-Valine Hydrazide.—To DL-valine ethyl ester, which had been prepared as usual, a small excess of anhydrous hydrazine was added and heated in a water-bath for 10 minutes. On storing the reaction mixture *in vacuo* over sulfuric acid overnight, fine white needle valine hydrazide crystallized out. This was dissolved in hot chloroform and precipitated by addition of 3–4 volumes of ether. Thus, white crystalline DL-valine hydrazide, m. p. 96–7°C. (uncorrected), was obtained. This is very soluble in cold water (somewhat hygroscopic), ethanol and hot chloroform but insoluble in ether.

Analysis (Micro Dumas): N found, 31.06 %;* calcd. for $C_5H_{13}ON_3$, 32.05 %.

DL-Phenylalanine Hydrazide.—This was synthesized in the same manner as DL-valine hydrazide from DL-phenylalanine ethyl ester. After recrystallization from ethanol it melted at 123–4°C. (uncorrected).

Analysis: N found, 22.53%;* calcd. for $C_9H_{13}ON_3$, 23.45 %.

L-Leucine Hydrazide Dihydrochloride.—L-Leucine ethyl ester was treated with anhydrous hydrazine as usual and white plate crystals were obtained. But free hydrazide was so hygroscopic that conc. hydrochloric acid was added to it and heated for a few minutes. Leucine hydrazide dihydrochloride thus crystallized out, which, after evaporation of excess hydrochloric acid *in vacuo* over potassium hydroxide, was recrystallized from absolute ethanol, m.p. 275°C. (uncorrected), fine needles.

$$[\alpha]_D^{15} = +30.6°. \text{ (5\% in distil. water).}$$

Analysis: N found, 19.16%; calcd. for $C_6H_{17}ON_3Cl_2$ (as 2 HCl), 19.26%.

Other amino acid hydrazides synthesized in the same manner are cited in Table 1. Glycine-, alanine- and tryptophan-hydrazides were obtained not in crystalline but in syrupy forms, although these were sufficiently pure for paper chromatography. L-Tyrosine hydrazide[10] and L-glutamic acid-γ-hydrazide[11] were obtained in crystalline forms.

Amino acid hydrazides react with ninhydrin to give reddish purple color, with ammoniacal silver nitrate solution to give dark brown color, and reduce iodine and permaganate solutions. For the color reagent for paper chromatography, n-butanol solution saturated with ammoniacal silver nitrate aqueous solution is preferable. The chromatograms

Table 1

RF Values of Several Amino Acid Hydrazides and Corresponding Amino Acids. Solvent for Paper Chromatography is Pyridine Base-aniline-water (9 : 1 : 4)

Amino acid hydrazides	RF	Amino acids	RF
Glycine hydrazide	0.26	Glycine	0.17
L-Alanine hydrazide	0.36	L-Alanine	0.24
DL-Valine hydrazide	0.56	DL-Valine	0.40
L-Leucine hydrazide	0.65	L-Leucine	0.51
DL-Phenylalanine hydrazide	0.71	DL-Phenylalnine	0.52
L-Tyrosine hydrazide	0.71	L-Tyrosine	0.55
L-Tryptophan hydrazide	0.70	L-Tryptophan	0.50
L-Glutamic acid-γ-hydrazide	0.15	L-Glutamic acid	0.11

(8) T. Curtius, *J. prakt. Chem.*, (2) **76**, 102 (1904).
(9) T. Curtius, *ibid.*, (2) **95**, 354 (1917).
(10) T. Curtius, *ibid.*, (2) **95**, 330 (1917).
* As there is a long time lag between the syntheses and analyses of these hydrazides, low values of found nitrogen must be due to their decomposition during their storage.
(11) S. Akabori and K. Narita, to be published shortly.

thus obtained can be stored after dipping them into thiosulfate solution, washing with water and drying. As the developing solvent for paper chromatography, pyridine base-aniline-water (9 : 1 : 4 : by volume) is found to be best. The "pyridine base" used is a high boiling fraction (b.p. 130–150°C.) of pyridines, *i. e.* a mixture of picolines and lutidines.

Amino acid hydrazides can be quantitatively estimated by oxidative titration using bromate or iodine standard solution. For example, we carried out the titration of leucine hydrazide dihydrochloride according to I. M. Kolthoff's analytical method for hydrazine.[12]

(1) **Potassium bromate method.**—Leucine hydrazide dihydrochloride (49 mg.) was dissolved in 2 ml. distilled water, added 2 ml. conc. hydrochloric acid and titrated with 0.100 N $KBrO_3$ using indigo as indicator. The sample required 2.22 ml. of the bromate. The calculated value for the next equation is 2.20 ml.: $RCH(NH_2)CONHNH_2 + 2/3KBrO_3 \rightarrow RCH(NH_2)COOH + N_2 + H_2O + 2/3KBr$

(2) **Iodine method.**—Leucine hydrazide dihydrochloride (18.8 mg.) was dissolved in 10 ml. distilled water containing 0.4 g. sodium bicarbonate and titrated with 0.1057 N iodine solution. The sample required 3.20 ml. of the iodine solution. The calculated value for the next equation is 3.25 ml.: $RCH(NH_2)CONHNH_2 + 2I_2 + H_2O \rightarrow RCH(NH_2)COOH + N_2 + 4HI$

In both cases the solution after titration was found to contain only free leucine by paper chromatography.

Dibenzal Compounds of Amino Acid Hydrazides.—Amino acid hydrazides condense with benzaldehyde in neutral aqueous solution to form white cold-water-insoluble dibenzal compounds, which develop no color by either ninhydrin or silver nitrate reagents. These benzal compounds, however, decompose to hydrazides and benzaldehyde in strong acidic solution. We synthesized dibenzal compounds of L-leucine- and L-alanine-hydrazides.

Dibenzal L-Leucine Hydrazide.—Crystalline L-leucine hydrazide dihydrochloride was dissolved in a small amount of water and an excess of benzaldehyde was added under cooling and stirring. As no precipitation occurred, the solution was neutralized with dil. NaOH until the precipitate separated out (*p*H 7.5). After standing over night in an ice box, the precipitate was collected on a filter and recrystallized from absolute ethanol. Thus white needle crystals, m.p. 149–50°C. (uncorrected), were obtained.

Analysis (Micro Dumas): N found, 13.20%; calcd. for $C_{20}H_{23}ON_3$, 13.10%.

Dibenzal Alanine Hydrazide.—Syrupy alanine hydrazide was dissolved in a small portion of water and an excess of benzaldehyde was added under cooling and stirring. On standing over night in an ice box, the white precipitate formed was collected on a filter and recrystallized from a large amount of water, whereby dibenzal L-alanine hydrazide was deposited in needles, m.p. 110–1°C. (uncorrected)

Analysis (Micro Dumas): N found, 15.06%; calcd. for $C_{17}H_{17}ON_3$, 15.05%.

Curtius[8] reported that he had obtained monobenzal compound of glycine hydrazide from acidic aqueous solution of the hydrazide, and that it was soluble in water, but for the present purpose it is preferable to precipitate hydrazides as dibenzal compounds. To accomplish this precipitation care must be taken not to shift the *p*H of the solution to acidic.

(II) **Hydrazinolyses of Peptides.**—As preliminary experiments we carried out the hydrazinolysis of several synthetic di- and tri-peptides. Peptides examined were as follows:

Dipeptides: glycyl-L-leucine, glycyl-L-tyrosine, glycyl-L-aspartic acid and L-leucyl-glycine.

Tripeptides: glycyl-glycyl-L-leucine and glutathione (SH form). All of these experiments gave successful results with ease. For example, two of them are described below.

Hydrazinolysis of Glycyl-L-Leucine.—Glycyl-L-leucine (73 mg.) was dissolved in 1 g. anhydrous hydrazine and heated under reflux at 125°C. (oil bath) for 1 hour. All glass apparatus was preferred in this treatment. After cooling the hydrazinolysate was poured into a watch glass and excess hydrazine was evaporated as perfectly as possible *in vacuo* over conc. sulfuric acid. The residue was dissolved in water and examined by paper chromatography. Using ammoniacal silver nitrate solution as color reagent, only glycine hydrazide developed the black spot, whereas on spraying ninhydrin solution two spots corresponding to glycine hydrazide and free leucine appeared. Therefore the carboxyl-terminal amino acid in this peptide was confirmed to be leucine.

Hydrazinolysis of Glutathione.—Glutathione (reduced form) (26 mg.) was dissolved in 1 g. anhydrous hydrazine and treated in the same manner. The paper chromatogram of the hydrazinolysate showed four spots indicating hydrazine, glutamic acid-γ-hydrazide, cysteine hydrazide and free glycine. The terminal amino acid, glycine, was confirmed further by treating as follows: The hydrazinolysate was dissolved in 1 cc. water and an excess of benzaldehyde was dropped into it under cooling and stirring, whereupon hydrazides condensed with benzaldehyde to form pale yellow amorphous precipitate (the yellow color is due to the formation of benzalazine from remaining hydrazine), and free glycine remained in the solution. After filtering the precipitate off, the colorless filtrate was examined by paper chromatography. Only one spot corresponding to glycine was found on the chromatogram, and no spot which reduced the silver nitrate solution was found. Fig. 1 shows the chromatograms of the hydrazinolysate and benzaldehyde-treated hydrazinolysate.

(III) **Hydrazinolyses of Proteins.**—In the case

(12) I. M. Kolthoff, *J. Am. Chem. Soc*, **46**, 2009 (1924).

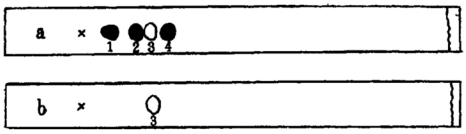


Fig. 1—a-Hydrazinolysate and b-benzaldehyde -treated hydrazinolysate of glutathione. Black spots, silver nitrate positive; white spots, ninhydrin positive. 1, cysteine hydrazide, 2, glutamic acid-γ-hydrazide; 3, glycine, and 4, hydrazine.

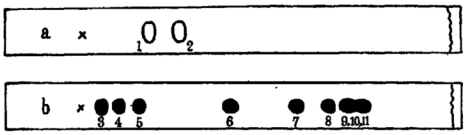


Fig. 2—a-Terminal amino acids of insulin: 1, glycine; 2, alanine. b-Hydrazinolysate of tyrocidin. Spots are hydrazides of ornithine (3), aspartic acid (4), glutamic acid (5), prolin (6), valine (7), leucine (8), tryptophan (9), tyrosine (10) and phenylalanine (11).

of lower peptides, the hydrazinolysis was completed in 1 hour, but in the case of proteins it was suspected that the time was not enough to hydrazinolyse proteins completely, and it was proved to be the case in a preliminary experiment. Hereby we hydrazinolysed glycyl-L-leucine for 10 hours and confirmed the fact that the other by-reaction such as hydrazidation of free amino acids did not occur in such a time. Therefore we decided to treat proteins with anhydrous hydrazine for 10 hours. This time of hydrazinolysis was enough to degrade proteins into amino acid hydrazides and free amino acids.

Hydrazinolysis of Insulin.—C. Fromageot *et al.*[6] reported by lithium aluminium hydride method that the carboxyl-terminal amino acids in beef insulin are glycine and alanine. On the other hand A. C. Chibnall and M. W. Rees[13] showed recently by means of the lithium boron hydride method that they are two alanines, one glycine and one as yet undetermined amino acid. We applied the hydrazinolysis to beef insulin, kindly supplied by Dr. J. Lens, N. V. Organon, Oss (the Netherlands), to compare its result with those of investigators mentioned above.

Crystalline insulin (55 mg.) was dissolved in 1 g. anhydrous hydrazine, heated to 125°C. for 10 hours, and after cooling, the excess hydrazine was evaporated *in vacuo* over sulfuric acid. The residue was taken up in water, an excess of benzaldehyde was added in small portions, and precipitates thus formed were filtered off. If hydrazides could not be completely precipitated, the *p*H of the solution was to be adjusted and made neutral by addition of dilute alkali. The mother liquor was concentrated *in vacuo* and free amino acids in it were identified by paper chromatography. Only glycine and alanine were detected on chromatogram (Fig. 2 a) showing good agreement with the result of Fromageot *et al.* Although we did not determine the result quantitatively, the intensity of the color and the area of the spots indicated that both amino acids existed in the protein in equal portions. From this result we reconfirmed the validity of this hydrazine treatment.

Hydrazinolysis of Tyrocidin.—Tyrocidin hydrochloride (200 mg.), which was kindly provided by Prof. Ohtani, Osaka Municipal University, was treated with 2 g. hydrazine for 9 hours. The hydrazinolysate gave, after evaporation of excess hydrazine, silver nitrate- and ninhydrin-positive 8 spots on paper chromatogram (Fig. 2 b), which corresponded to the hydrazides of amino acids composing tyrocidin. The chromatogram of the benzaldehyde-treated mother liquor gave no spots by spraying either ninhydrin or silver nitrate solutions. Therefore, tyrocidin was proved to be a cyclic peptide as reported heretofore.

Discussion

The present method has two excellent points: one of which is that anhydrous hydrazine is a good solvent for proteins. All proteins and peptides can be dissolved even at room temperature. The other point is that, being identified as amino acids themselves in respect to the terminal amino acids, there is no need to synthesize authentic amino acid derivatives and, if necessary, the quantitative estimations can be made by some suitable methods for microdetermination of amino acids published heretofore. Further, this method is very simple and can be carried out easily.

Besides the above mentioned experiments, the completion of the hydrazinolysis of proteins in 10 hours, on which we shall report elsewhere with details, was confirmed, in the following way: the benzaldehyde treated with mother liquor of protein hydrazinolysate was found to undergo no change in its amino acid either by further treatment with anhydrous hydrazine for 10 hours or by hydrolysis with 6 N HCl.

The applications of the hydrazinolysis to several crystalline proteins shall be reported in near future.

Summary

(1) A brief method for the characterization of carboxyl-terminal amino acids in peptides and proteins was investigated.

(2) Several new amino acid hydrazides were synthesized.

(3) Soveral di- and tri-peptides were hydrazinolyzed and identified their carboxyl-

(13) A. C. Chibnall and M. W. Rees, *Biochem. J.*, **48**, xlvii (1951).

terminal amino acid.

(4) The method was applied to beef insulin and tyrocidin for the demonstration of its applicability.

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