Synthesis of Aspartic and Glutamic Hydrazides and their Chromatographic and Ionophoretic Separation. C-Terminal Groups of Whale Insulin[1]

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In the hydrazinolysis of peptides and proteins[2], non-C-terminal acidic amino acids or their amides residues in the polypeptide chain will be converted into α-mono- (derived from aspartyl and glutamyl residues) or di-hydrazides (derived from asparaginyl and glutaminyl residues). C-Terminal aspartic and glutamic acids or their amides will be found as free amino acids or as β- or γ-monohydrazides in the hydrazinolysates of proteins, respectively[3].

By quantitative determination of these hydrazides in the hydrazinolysates of proteins, the number of aspartyl, glutamyl, asparaginyl and glutaminyl residues in the original proteins could be estimated[3]. For that purpose, it is desirable to prepare standard substances for the identification and recovery determination by chromatography or ionophoresis.

Glutamic α- and γ-monohydrazides have previously been prepared[4,5], but the other hydrazides of the acidic amino acids have not heretofore been prepared, as far as we are aware. In the present paper, the synthesis of DL-aspartic α-hydrazide from phthaloyl-DL-aspartic α-methylester, of the isomeric β-hydrazide from L-asparagine, and of the dihydrazides of aspartic and glutamic acids from their diesters will be described. The present contribution also includes separation of these hydrazides by chromatography or ionophoresis, and its application to the hydrazinolysates of beef and whale insulins. The separation methods, however, were not satisfactory, since it was observed that appreciable amounts of some hydrazides were decomposed during the operation.

Experimental

Synthesis of DL-Aspartic α-Hydrazide.—A suspension of 14 g. of phthaloyl-DL-aspartic anhydride[6] in 120 ml. of absolute methanol was refluxed for 30 min. and a small amount of insoluble material was filtered off while it was warm. After being kept overnight, phthaloyl-DL-aspartic α-methyl ester crystallized as prisms. It was recrystallized from methanol; yield, 7.9 g., 43.8%, m.p. 181°C (reported[7] m.p. 182°C). The crude β-methyl ester obtained upon evaporation of the filtrate was recrystallized from water; yield, 3.9 g., 24.8%, m.p. 151~152°C (reported[7] m.p. 147~148°C).

A mixture of 4 g. of the α-ester, 50 ml. of methanol and 2.5 g. of 80% hydrazine hydrate was refluxed for 30 min. The precipitate of phthaloylhydrazine which resulted was filtered off and was washed with hot water. The combined solution of the filtrate and the washings

1) Some parts of this paper were presented at the 9th Annual Meeting of the Chemical Society of Japan, held at Kyoto in April, 1956.

2) S. Akabori, K. Ohno and K. Narita, This Bulletin, **25**, 214 (1952).

3) K. Ohno, *J. Biochem.*, **41**, 345 (1954).

4) S. Akabori and K. Narita, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **74**, 829 (1953).

5) K. Narita, ibid., **74**, 832 (1953).

6) F. E. King and D.A.A. Kidd, *J. Chem. Soc.*, **1951**, 2976.

7) F. E. King, J. W. Clark-Lewis and G. R. Smith, ibid., **1954**, 1046.

were adjusted to pH 3.5 with 1 N sulfuric acid. Enough amounts of Amberlite XE-58 (weak anion exchanger, free form) were added to the filtrate to remove sulfate ion, and the resin was filtered off after standing for 30 min. and was washed with water. The combined solution was concentrated in vacuo and two volumes of ethanol were added. An oily material solidified after being kept in a refrigerator and it was recrystallized from dilute ethanol; yield, 2.1 g., m.p. 143°C (with decomp.). It was found by paper chromatography and paper ionophoresis that this material was contaminated with a small amount of free aspartic acid. The aqueous solution of the crude material was again treated with Amberlite XE-58 in a similar manner as mentioned above. On evaporation of the filtrate to a small volume in vacuo, DL-aspartic α-hydrazide crystallized as needles. After addition of ethanol to the filtrate, a second crop of the hydrazide was obtained. The two crops combined were recrystallized from water-ethanol; yield, 1.1 g., 44%, m.p. 180°C (with decomp.).

Anal. Found: N, 27.7. Calcd. for $C_4H_9O_3N_3$: N, 28.6%.

Synthesis of L-Aspartic β-Hydrazide.—A mixture of 3 g. of L-asparagine, 2 ml. of ethanol and 2 g. of 80% hydrazine hydrate was refluxed for 30 min. and held overnight at room temperature. Ethanol was added to the syrup which was obtained on evaporation of the reaction mixture in vacuo and the β-hydrazide crystallized upon scratching of the wall of the vessel under ice cooling. The product was recrystallized from water-ethanol; yield, 2.8 g., 74.5%, m. p. 186°C. When the material was dried over phosphorous pentoxide at 100°C in vacuo, analysis indicated loss of 1.5 mol. water of hydration.

Anal. Found: N, 27.9. Calcd. for $C_4H_9O_3N_3$: N, 28.6%.

Synthesis of L-Aspartic and L-Glutamic Dihydrazides.—L-Aspartic and L-glutamic acids were converted into diethyl ester hydrochlorides, respectively, the free ester was extracted with ether as usual, and the solvent was removed after drying over sodium sulfate. Two and a half moles of hydrazine hydrate were added to the ethanolic solution of the ester, the mixture was held overnight at room temperature and the dihydrazide crystallized in needles. Recrystallization from aqueous ethanol gave about 60% yield in both cases. L-Aspartic α,β-dihydrazide, m.p. 164~165°C (with decomp.).

Anal. Found: N, 42.0. Calcd. for $C_4H_{11}O_2N_5$: N, 43.5%.

L-Glutamic α,γ-dihydrazide, m. p. 134~135°C (with decomp.).

Anal. Found: N, 39.2. Calcd. for $C_5H_{13}O_2N_5$: N, 40.0%.

Paper Chromatography.—Chromatography was performed by an ascending method using lutidine-water mixture (5 : 3, vol.) and Toyo No. 3 filter paper. In the case of multiple development the same solvent was repeatedly used. For the detection of both amino acid and hydrazide, 0.2% ninhydrin *n*-butanol solution (saturated with water) was used and for hydrazide only the ammoniacal silver nitrate solution was sprayed[8].

Paper Ionophoresis.—Toyo No. 3 filter paper (18×40 cm.) was generally used. For the separation of amino acids and hydrazides into three groups, namely, acidic, neutral and basic, neutral buffer of pH 6.4 was prepared in a similar manner as suggested by Lockhart and Abraham[9], except lutidine was used in place of collidine. For the mutual separation of neutral substances, 0.5 M acetic acid (pH 2.64) was used. Potential of 500 V. (12.5 V./cm.) was applied in each case. The current was 6 milliamp. in the case of neutral buffer and 2 milliamp. in dilute acetic acid. The ionophoretic rates of the hydrazides varied from experiment to experiment but the order of the rates did not change. Consequently, in the case of the measurements of relative rates of various hydrazides and the corresponding amides, those on the same paper sheet were compared.

Ion Exchange Chromatography.—Elution chromatography on a Dowex 50−X8 (200~400 mesh) column was tested to separate and estimate amino acid monohydrazides according to the procedure of Moore and Stein[10]. C-Terminal amino acids with the exception of terminal basic amino acids must be present as neutral or acidic substances in the hydrazinolysate of proteins. Since basic materials were adsorbed on the top of the resin column which equilibrated with 0.1 or 0.2 M citrate buffer of pH 3.4, neutral substances were eluted with citrate buffers of several pH's from a short column (0.9×15 cm. or 0.9×30 cm.). In the present experiments, the behaviors of alanine, aspartic β- and glutamic α-hydrazides were mainly studied with regard to the study on insulin.

Hydrazinolysis of Insulin and Treatment of the Product.—Beef and whale insulins studied were kindly supplied by Dr. J. Lens, Organon, Oss, and Shimizu Pharmaceutical Co., respectively. Anhydrous hydrazine was prepared in a similar manner as suggested by Ohno[3]. About 30 mg. of insulin was dissolved in 0.5 ml. of anhydrous hydrazine and heated at 100°C for 8 hr. The hydrazinolysate was taken into dryness over calcium chloride in vacuo at room temperature. In paper ionophoresis, the dried residue was dissolved in a minimum volume of water, an aliquot was applied to Toyo No. 50 paper, thick paper (18×40 cm.), along the starting line, and a potential gradient of 500 V. was applied at pH 6.4 in lutidine-acetic acid buffer. After ionophoresis and air drying, strips from both sides of the paper sheet were cut off and treated with the silver nitrate reagent to detect hydrazides. Using these as a guide, the remainder of the sheet containing neutral substances was cut and they were eluted with water by the technique of Sanger and Tuppy[11]. The wetting top of the cut

8) A mixture of one part of 0.1 N $AgNO_3$ and one part of 0.5 N NH_4OH solution.

9) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, 58, 633 (1954).

10) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

11) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

paper strip was applied to another paper sheet (Toyo No. 3) and subjected to ionophoresis at pH 2.64 or one- or two-dimensional chromatography.

For the characterization of C-terminal amino acid with the exception of the basic one, asparagine and glutamine, the following technique is also applicable. After ionophoresis of the hydrazinolysate at pH 6.4, the area of hydrazides which traveled to cathode was cut off and the remaining area which contained C-terminal amino acid and monohydrazides of acidic amino acids was then developed with phenol and lutidine two-dimensionally. In this case, it is preferable to develop them first with phenol and then lutidine. During the first development, most of the monohydrazides were destroyed and several very faint unidentified spots could be observed near the phenol front. This procedure might be useful to characterize the C-terminal group of polypeptide of a small molecule, and was successfully used for several cytochromes[12] and for insulin shown in Fig. 1.

In the quantitative determination of the C-terminal groups of insulin, the dried hydrazinolysate was dissolved in 0.5 ml. of 0.05~0.1 N hydrochloric acid 0.5 ml. of 0.2 M citrate buffer (pH 3.5) was added and any insoluble material was centrifuged off, if necessary. A half milliliter of the supernatant was applied to the Dowex 50 column (0.9×15 cm.) equilibrated with 0.2 M citrate of pH 4.23 and elution was started.

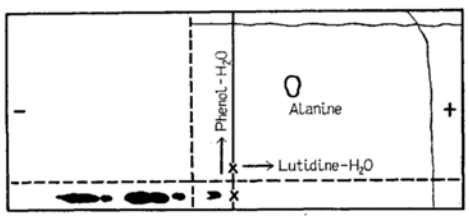


Fig. 1. Ionophoresis at pH 6.40 and subsequent chromatography of the hydrazinolysate of whale insulin. Mark X and broken line indicate original spot and line to be cut before chromatography, respectively. Black and white spots indicate ammoniacal silver nitrate and ninhydrin positive, respectively.

Results and Discussion

Paper Chromatography. — Multiple development technique was used in lutidine-water, since the difference between *Rf* values of the hydrazides was slight in a single development. However, no remarkable effect in resolution of the spots resulted by repeating the development with the same solvent more than twice. *Rf* values of the hydrzaides of acidic amino acids were recorded in Table I.

TABLE I. *Rf* VALUES OF ASPARTIC AND GLUTAMIC HYDRAZIDES*

Number of development	1	2	3
Aspartic α-hydrazide	0.23	0.41	0.48
Aspartic β-hydrazide	0.15	0.33	0.39
Aspartic α,β-dihydrazide	—	0.36	—
Glutamic α-hydrazide	0.24	0.43	0.50
Glutamic γ-hydrazide	0.18	0.37	0.45
Glutamic α,γ-dihydrazide	—	0.44	—

* Lutidine-water (5 : 3, vol.) and the ammoniacal silver nitrate solution were used as the solvent and the reagent, respectively.

Paper Ionophoresis.—Acidic, neutral and basic substances were separated during ionophoresis at pH 6.4, but the acidic amino acid monohydrazides and neutral amino acids could not be separated from each other in this medium. Mutual separation of neutral amino acids has usually been performed in either an acidic or alkaline medium based on the difference of the dissociation constants of their carboxyl or amino group. In the present experiments, acidic pH was used, since amino acid hydrazide seemed to be labile in alkaline medium. The ionophoretic rates of aspartic and glutamic hydrazides to cathode on a paper at pH 2.64 were in the following order:

Glutamic α,γ-dihydrazide>
Aspartic α,β-dihydrazide>
Glutamic α-hydrazide>
Aspartic α-hydrazide>
Glutamic γ-hydrazide>
Aspartic β-hydrazide

The pK'_{COOH} values of aspartic and glutamic monohydrazides are unknown but those of the corresponding amides have been reported[13]. Therefore, the distance that the monohydrazides traveled to cathode during ionophoresis on the

TABLE II. DISTANCES TRAVELED TO CATHODE DURING IONOPHORESIS OF ASPARTIC AND GLUTAMIC HYDRAZIDES AND THE CORRESPONDING AMIDES AT pH 2.64*

	Hydrazid	Amide	
	Distance	Distance	pK'_{COOH}[13]
Aspartic β-	6.1 cm.	4.4 cm.	2.02
Glutamic γ-	6.7	4.8	2.17
Aspartic α-	7.5	—	2.97
Glutamic α-	8.8	8.3	3.81

* Toyo No. 3 filter paper (18×40 cm.), 0.5 M acetic acid, 500 V., 2 milliamp. and 120 min. were used.

12) K. Titani and H. Ishikura, *J. Biochem.*, **44**, 499 (1957).

13) D. M. Greenberg, "Amino Acids and Proteins", Charles C. Thomas Publishers, Springfield (1951), p. 430.

same paper sheet in acidic medium were compared with those of the corresponding amides, to find relationship between the dissociation constant and behavior on the paper (Table II).

The parallel relationship between the ionophoretic rate and the dissociation constant of the amides was observed. Comparing the rates of the amides with those of the corresponding hydrazides, it is apparent that the dissociation constant of the latter is slightly higher than that of the former, and that the difference between the dissociation constant of the hydrazide of α-position and that of ω-position is smaller than that of the corresponding amides. In the ionophoresis performed under the conditions used, the hydrazides in the following pairs were barely separated from one another: Aspartic α-hydrazide and glutamic γ-hydrazide, and glutamic γ-hydrazide and aspartic β-hydrazide. Futhermore, it was observed that the dihydrazides were partially decomposed into both the isomers of the monohydrazides liberating hydrazine in acidic medium.

Ion Exchange Chromatography.—In the ion exchange chromatography, no hydrazides eluted with 0.1 M citrate of pH 3.45 from the 30 cm. column, although aspartic and glutamic acids and alanine eluted each forming a single sharp peak, respectively. When the pH of the buffer was changed to 4.34, leucine, aspartic β- and glutamic γ-hydrazides successively came out from the column. The recoveries of the hydrazides in effluent were only a small percentage using the ninhydrin color yields of the Table III, in which the ratio of ninhydrin color value per mol. hydrazide to that of leucine is listed[10].

TABLE III. NINHYDRIN COLOR YIELDS OF ACIDIC AMINO ACID HYDRAZIDES

Hydrazide	Color yield
Leucine	1.00
Aspartic α-hydrazide	0.93
Aspartic β-hydrazide	1.05
Aspartic α, β-dihydrazide	0.79
Glutamic α-hydrazide	0.85
Glutamic γ-hydrazide	0.69
Glutamic α, γ-dihydrazide	0.48

It was observed that the recoveries of the hydrazides became lower the more acidic the resin surface and the longer the time of contact with the resin. In order to elute hydrazides more rapidly, the length of the column was halved and the ionic strength of the buffer was doubled. Concerning insulin, alanine, aspartic β- and gultamic α-hydrazides could be separated from one another, but aspartic β- and glutamic γ-hydrazides and leucine overlapped in such a column system. Recoveries of the hydrazides were still low under such conditions and further unknown degradation products of the hydrazides eluted gradually and the base line between the peaks of aspartic β- and glutamic α-hydrazides was relatively high. In the quantitative estimation, the base line behind the peak of glutamic α-hydrazide was used.

C-Terminal Groups of Whale and Beef Insulins.—Characterizations of C-terminal amino acids and free carboxyl groups of whale insulin were carried out by means of hydrazinolysis-ionophoresis method described in the experimental section. Alanine, aspartic β- and glutamic α-hydrazides could be identified. These results indicate that the C-terminal groups of the hormone must be alanine and asparagine and that all of the non-C-terminal carboxyl groups belong to glutamic acid residues.

Quantitative estimation of these groups was done by chromatography on a Dowex 50 column. For this purpose it is necessary to determine to what extent known amounts of amino acids could be recovered after treatment with hydrazine and chromatography. A mixture of alanine, asparagine and isoglutamine was heated with anhydrous hydrazine for 8 hr. at 100°C and the reaction mixture was treated according to the procedures described in the experimental section. The recoveries for those are recorded in Table IV. A typical elution curve of the hydrazinolysate of whale insulin is shown in Fig. 2

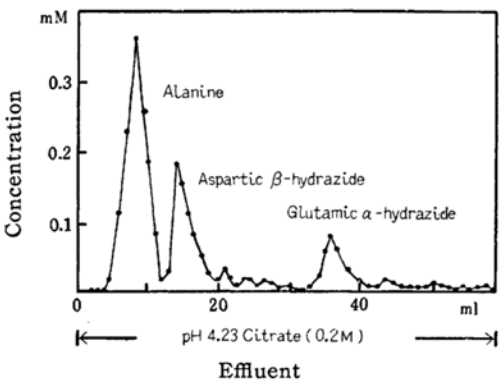


Fig. 2. Elution curve of the hydrazinolysate of whale insulin from Dowex 50 column (0.9×15 cm.).

TABLE IV. RECOVERIES OF AMINO ACIDS AFTER HYDRAZINOLYSIS AND CHROMATOGRAPHY

Material	Product after hydrazinolysis	Recovery after hydrazinolysis and chromatography	Recovery after chromatography	Recovery calculated for hydrazinolysis
Alanine	Alanine	0.48	1.0	0.48
Asparagine	Aspartic β-hydrazide	0.25	0.39	0.64
Isoglutamine	Glutamic α-hydrazide	0.088	0.31	0.29

TABLE V. C-TERMINAL GROUPS AND GLUTAMYL RESIDUES IN WHALE AND BEEF INSULINS*

Insulin		Observed value (Average)	Observed value (Range)	Corrected value
Whale**	Alanine	0.54	0.41～0.65	1.1
	Aspartic β-hydrazide	0.26	0.22～0.29	1.0
	Glutamic α-hydrazide	0.25	0.20～0.29	2.8
Beef***	Alanine	0.46		1.0
	Aspartic β-hydrazide	0.28		1.1
	Glutamic α-hydrazide	0.14		1.6

* Figures are expressed as mol. per mol. insulin of which molecular weight is assumed to be 6,000.

** Average value for four experiments.

*** Value for single experiment.

and the analytical data of whale and beef insulins are summarized in Table V.

It appears probable that the C-terminal groups of both the insulins (assumed molecular weight, 6,000) are each one mole alanine and asparagine which are consistent with the results of Sanger et al.[14], Harris et at.[15,16] and Chibnall et al.[17] but differ from our previous results[2], in which we reported an additional C-terminal glycine but missed asparagine which escaped from the detection in the earlier method. The main reason which caused such a difference may be attributed to the drastic conditions used in the previous experiments and non-C-terminal glycine might be erroneously identified.

To confirm the singleness of the peak of alanine in Fig. 2, an aliquot of the hydrazinolysate of whale insulin was chromatographed on a Dowex 50 column (0.9×15 cm.) which equilibrated with 0.1 M citrate of pH 3.46. As is shown in Fig. 3, a small peak appeared before alanine and it corresponded to aspartic acid which might be derived from the hydrazide during chromatography. The amount of alanine found in this system was 0.8 mol. per mol. insulin.

In connection with the number of free

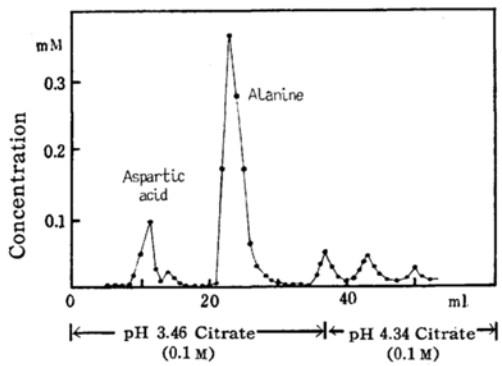
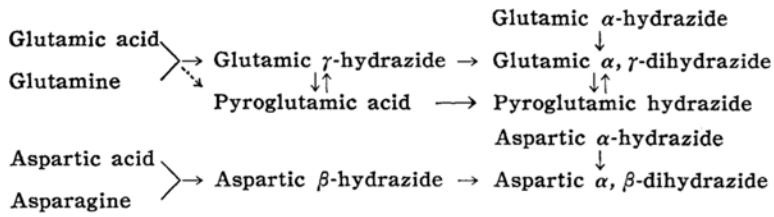


Fig. 3. Chromatogram of the hydrazinolsate of whale insulin on Dowex 50 column (0.9×15 cm.). Concentration and pH of the buffer differ with those in Fig. 2.

carboxyl groups, the present results appear to be of little value, since significant differences were obsereved between the values derived by reduction method and by hydrazinolysis have been obtained with lysozyne[3,18] and the protein of tobacco mosaic virus[19]. As is well known, recoveries of some amino acids, especially aspartic and glutamic acids, after hydrazine treatment are pretty low[20,21]. It was found that one of the main decomposition products was the corresponding hydrazide,

14) F. Sanger, E. O. P. Thompson and R. Kitai, *Biochem. J.*, **59**, 509 (1955).
15) J. I. Harris and C. H. Li, *J. Am. Chem. Soc.*, **74**, 2944 (1952).
16) J. I. Harris, F. Sanger and M. A. Naughton, *Arch. Biochem. Biophys.*, **65**, 427 (1956).
17) A. C. Chibnall and M. W. Rees, *Biochem. J.*, **68**, 105 (1958).
18) A. C. Chibnall, C. H. Haselbach, J. L. Mangan and M. W. Rees, ibid., **68**, 122 (1958).
19) L. K. Ramachandran and K. Narita, *Biochim. et Biophys. Acta*, **30**, 616 (1958).
20) R. H. Locker, ibid., **14**, 533 (1954).
21) C.-I. Niu and H. Fraenkel-Conrat, *J. Am. Chem. Soc.*, **77**, 5882 (1955).

which was identified by paper chromatography. The situation of aspartic and glutamic acids is quite complicated. When glutamic acid alone, for example, was heated with hydrazine and the reaction products were analyzed by chromatography, glutamic acid, glutamic γ-hydrazide, glutamic α, γ-dihydrazide, pyroglutamic acid, pyroglutamic hydrazide and an unknown product were identified. The reaction products of glutamine, glutamic α- and glutamic γ-hydrazides, aspartic acid, asparagine, aspartic α- and aspartic β-hydrazides with hydrazine were analyzed, respectively. Summarizing these results which were obtained, the following reaction scheme could be deduced.



Since free acidic amino acid has never been detected in the reaction mixture of hydrazine with the amide of acidic amino acid, C-terminal asparagine and glutamine can be estimated by hydrazinolysis method by the use of appropriated recovery factors. However, it appears difficult to determine the numbers of non-C-terminal acidic amino acids or their amides residues by hydrazinolysis, owing to the fact that both of them produce the same product, dihydrazide.

In the quantitative determination of C-terminal asparagine or glutamine, elution chromatography described in the present paper seems not to be suitable, since hydrazide derived from the terminal group is decomposed in significant quantity during operation. The dinitrophenyl derivatives of aspartic β- and glutamic γ-hydrazides from the hydrazinolysate of protein by the method III of Akabori et al.[22] or slightly modified method[19], can be separated from one another on two-dimensional paper chromatogram[23,24] developed with Levy's solvent system[25] and this method appears to be more suitable than the present one.

Summary

Aspartic α- and β-monohydrazides and aspartic α, β- and glutamic α, γ-dihydrazides were prepared and their qualitative separation has been performed by means of paper ionophoresis and chromatography. During ionophoresis in acidic medium, the dihydrazide partially decomposed into the isomers of monohydrazides liberating hydrazine. The hydrazinolysis products of whale and beef insulins were analyzed by the aid of elution chromatography on a Dowex 50 column and it was observed that the C-terminal positions were occupied by each one mole of alanine and asparagine in both cases. Furthermore, the present experiments suggested that all of the non-terminal carboxyl groups of the hormone belonged to only glutamic acid residues. Complicated reaction of the acidic amino acids or their amides with hydrazine was discussed.

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22) S. Akabori, K. Ohno, T. Ikenaka, Y. Okada, H. Hanafusa, I. Haruna, A. Tsugita, K. Sugae and T. Matsushima, This Bulletin, **29**, 507 (1956).
23) K. Narita, unpublished experiments.
24) Y. Kawanishi, K. Iwai and T. Ando, Symposium on Protein Structure, held at Osaka in November, 1958.
25) A. L. Levy, "Methods of Biochemical Analysis", Vol. II, Interscience Publishers, New York (1955), p. 397.