*Hydrazinolysis of Peptides and Proteins. II. Fundamental Studies on the Determination of the Carboxyl-ends of Proteins**

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(Received January 17, 1956)

Since it was first proposed by Akabori, Ohno and Narita in 1952[1] the hydrazinolysis method has been applied by the same authors and others for the characterization of carboxyl-terminal amino acids in various proteins. On heating the protein in anhydrous hydrazine, peptide bonds are split with the formation of amino acid hydrazides, while the C-terminal residues only are liberated as free amino acids. In earlier works[1,2] hydrazides were removed by condensing with benzaldehyde to give insoluble dibenzal derivatives and the remaining amino acids were identified by paper chromatography. Although it has been applied to several proteins and peptides[1–6], this procedure was rather troublesome, and some less soluble amino acids might be lost in the treatment. Later we have improved the method in the following way: dinitrophenylating the hydrazinolysates and separating the C-terminal DNP-amino acids from di-DNP-amino acid hydrazides, the former thus extracted were characterized and estimated quantitatively by silica gel chromatography[7,8]. Further a method of pre-treatment with isovaleraldehyde followed by dinitrophenylation was also reported briefly[7]. These improved methods were applied to lysozyme[7,8], Taka-amylase A[7], ovalbumin[7], α-chymotrypsinogen[9], α-chymotrypsin[9], tobacco mosaic virus[10,11], and protease hydrolysates of lysozyme[12]. Another application of this method to characterize the mode of linkage of acidic amino acids in the protein was also reported[13].

Another technique to separate C-terminal amino acids from hydrazides using ion-exchangers was also proposed[14].

On the other hand, criticism on the hydrazinolysis method (chiefly on the hydrazinolysis-benzaldehyde method) was proposed in which it was said that the results obtained

* The present paper was written before the Symposium on Protein Structure held on November 15, 1954 at Osaka University.

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

2) S. Akabori and K. Ohno, IIe Congres Internationale de Biochimie, Paris, 1952, Resume des communications, p. 47.

3) K. Schlögl and E. Wawersich, *Naturwiss.*, 41, 38 (1954).

4) R.H. Locker, *Biochim. Biophys. Acta*, 14, 533 (1954).

5) K. Satake, K. Kusama and H. Ozawa, Symposium on Protein Structure, Osaka, 1954.

5a) N. Ohta and M. Yamasaki, Symposium on Protein Structure, Osaka, 1954.

6) K. Narita, *J. Chem. Soc. Japan*, 75, 487 (1954).

7) S. Akabori, K. Ohno, T. Ikenaka, A. Nagata and I. Haruna. *Proc. Japan Acad.*, 29, 561 (1953).

8) K. Ohno, *J. Biochem.*, 40, 621 (1953).

9) N. Sakota, S. Kubo, Y. Okada and S. Akabori, *Symposia on Enzyme Chemistry*, 10, 155 (1954).

10) G. Braunitzer, *Z. Naturforsch.*, 9b, 675 (1954).

11) C.I. Niu and H. Fraenkel-Conrat, *Biochim. Biophys. Acta*, 16, 597 (1955).

12) K. Okunuki, B. Hagihara and H. Matsubara, Symposium on Protein Structure, Osaka, 1954.

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

14) A.J. Ultée, Personal communication.

by this procedure were not in line with those obtained by carboxypeptidase method[15,16] and that the hydrazine is not an entirely satisfactory reagent for this purpose[16].

The present investigation was designed to reinspect this method according to the results obtained by various fundamental experiments, to establish the best method applicable to various proteins, and to consider the scope of it. Experiments reported in this paper include those on (a) the decomposition of free amino acids, (b) the hydrolysis of amino acid hydrazides to amino acids during the treatment, (c) the separation procedure of amino acids from hydrazides, and (d) the rate of hydrazinolysis of peptide bonds. From the results obtained here it can be concluded that the hydrazinolysis-DNP method gives mostly correct results with the exception of the case where tyrosine, cystine, histidine and tryptophan are situated at the C-terminal. These amino acids can be characterized by adopting the modified method described below.

Materials

Anhydrous hydrazine. — Anhydrous hydrazine was prepared by the method of Stähler[17]. Fifty grams of 80 per cent hydrazine hydrate and 175 g. of calcium oxide were mixed in a copper flask and gently heated in an oil bath. The distillate was led through a glass tube and a cooler and collected into a flask, which was attached by a calcium chloride tube. All cork fittings were covered with tin foil. After a few ml. of forerun were discarded, the main distillate was run at a bath-temperature range of 130–160°C. This was immediately kept in sealed tubes, each containing a 1 ml. portion. Yield 15–18 g. Purity was more than 99 per cent.

Amino acid hydrazides.—Leucine hydrazide dihydrochloride is the same one as reported previously[1]. Glycine-, alanine- and serine-hydrazides were prepared as follows: amino acids were esterified as usual, and hydrochlorides of amino acid esters were treated with computed amounts of dry chloroform saturated with dry ammonia. After filteration, the solution was concentrated under diminished pressure to give a syrup. The computed amount of anhydrous hydrazine was added to the syrup under cooling, and after standing overnight at room temperature, the reaction mixture was concentrated in vacuo. In all the treatments care was taken to avoid moisture. Thus glycine- and serine-hydrazides were obtained as crystals, but alanine hydrazide was crystallized as hydrochloride by adding dry alcohol saturated with HCl gas to the syrup of hydrazide until the pH of the solution reached to pH 6 and was concentrated in vacuo. The former two hydrazides were recrystallized three to five times from ethanol-ether, and the latter hydrazide hydrochloride from hot methanol.

Glycine hydrazide. m.p. 84–85°C (Found: N, 47.16. Calcd: 46.91%)

Serine hydrazide. m.p. 99–100°C (Found: N, 35.28. Calcd: 35.25%)

Alanine hydrazide dihydrochloride. m.p. 222–223°C (Found: N, 23.84. Calcd.: 23.87%)

15) A.C. Chibnall, "Les protéines", (1953) p. 126.
16) A. C. Chibnall and J.L. Bailey, "CIBA Symposium" (1952) p. 148, 149.
17) A. Stähler, *Ber.*, **42**, 3018 (1909).

Experiments and Results

(1) Decomposition of Amino Acids during Hydrazinolysis

It has been observed that C-terminal amino acids liberated during hydrazinolysis are partly decomposed. Locker[4] reported that common amino acids were recoverable in amounts exceeding 85 per cent with the exception of glutamic and aspartic acids, tryptaphan, arginine, cystine, cysteine and asparagine after heating them for eight hours at 100°C in a sealed tube. We have, therefore, estimated recoveries of about fifteen amino acids during hydrazinolysis, and we have studied briefly the decomposition products.

(a) Recoveries.—About 1 mg. of each amino acid was heated at 100°C with 0.5 ml. anhydrous hydrazine for five and ten hours. After excess hydrazine was evaporated in vacuo over sulphuric acid, the residue was dinitrophenylated, extracted, chromatographed and the recoveries of DNP-amino acids were estimated in the same way as in the case of proteins[7]. The procedure is similar to that shown in Table VIc. On the other hand, free amino acids were treated, without heating with hydrazine, in the same way to find control values of this procedure. The control values were mostly found to be more than 85 per cent. The recoveries cited in Table I are corrected by these control values. Histidine is dinitrophenylated not quantitatively—to an extent of only 30 per cent of theory—and was estimated, therefore, as the free amino acid by column chromatography using Dowex-50. Cystine and proline were also not fully dinitrophenylated. As DNP-derivatives of tyrosine and tryptophan are extracted by sodium bicarbonate solution not completely from ethyl acetate solution but completely from ether solution.

Table I indicates that the recoveries of amino acids during the hydrazinolysis of ten hours range from 90 per cent (leucine) to 27 per cent (cystine) and most of them are 50 to 70 per cent. Arginine decomposes completely in ten hours' hydrazinolysis, but is recoverable as ornithine. Contrary to the report of Locker[4], methionine does not change to sulfoxide, but remains about 80 per cent unchanged in ten hours. The reason of discrepancies between the results of Locker and ours is not clear. Some experiments were done in a dark room, but the effect of light was not remarkable.

It is not known whether C-terminal amino acid residues in protein decompose or not in the same way as free amino acids in the course of hydrazi-

TABLE I
RECOVERY OF AMINO ACIDS

Amino acid	Blank	After heating with anhydrous hydrazine for 5 hr.	10 hr.	Locker[4]
Glycine	87%	85%	52, 53**%	85
Alanine	84	81	48, 55**	85
Valine	100*		85**	85
Leucine	100*		90	85
Serine	86	75	57	85
Proline	71		65	
Tyrosine	100	92	74	85
Tryptophan	94**	57**	47**	40
Histidine	89	73	62	85
Arginine	83	36	0	0
(Ornithine)	85*	39	45	
Aspartic acid	85*		50**	20
Glutamic acid	85*		46**	40
Cystine	60	38	27	—
Methionine	91		80	sulphoxide

* Assumed values.
** Treated under shutting from direct light.

nolysis, but, being recovered 80 to 90 per cent of theory in the case of protein[7,13], C-terminal amino acid seems to be recoverable not less than free amino acids (Table I) as discussed later.

(b) Decomposition Product.—It is not yet known how amino acids decompose by the treatment with hydrazine. They did not change to hydrazides principally. Decomposition products gave no DNP-compounds which might be apprehensive for confusion with DNP-amino acids with the exception of cystine. Cystine changes partially to three sorts of by-products, two of which, on dinitrophenylating, changes to DNP-compounds which have nearly the same R values of DNP-glycine and -alanine, respectively, but on treating with isovaleraldehyde before dinitrophenylation, these compounds disappear almost completely.

In the case of arginine, a trace of DNP-compounds other than DNP-ornithine was detectable on silica gel columns, but on dissolving in acetic acid the yellow colour disappeared.

The degree of decomposition of amino acids in hot hydrazine seems to depend on the purity of anhydrous hydrazine. Anhydrous hydrazine of lower concentration (about 95 per cent) might cause more decomposition and undersirable by-products as reported previously[8].

(2) Hydrolysis of Hydrazides

Amino acid hydrazides which are produced in the course of hydrazinolysis might partially change to other substances by side-reactions. If free amino acids were produced during hydrazinolysis and/or after-treatment, they might be mistaken for C-terminals. Therefore the degrees of hydrolysis of hydrazides in aqueous solution and in hot hydrazine were determined.

(a) Estimation Method of Hydrolysis.—(i) Estimation as DNP-derivatives of Amino Acids produced by Hydrolysis.—The mixture of amino acid hydrazides and free amino acids, the latter resulting from the former by treating with hydrazine, were together dinitrophenylated, and DNP-amino acids were extracted fractionally by the same procedure as in the case of C-terminal, chromatographed on silica gel and estimated colorimetrically.

(ii) Estimation of Hydrazine liberated by Hydrolysis.—Preliminary experiments indicated that the hydrazine reacted with piperonal quantitatively to give a condensation product and that the product in acidic solutions showed a specific absorption curve which is distinctly different from those of amino acid hydrazides with the aldehyde in the range of the wave lengths of 365–380 mμ as shown in Fig. 1. The optical density

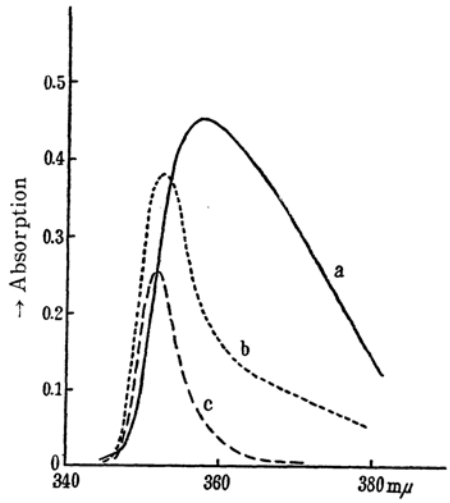


Fig. 1. Absorption spectra of condensation products of piperonal with hydrazine and amino acid hydrazides.
(a) Condensation product of piperonal with hydrazine (0.08 μM).
(b) Condensation product of piperonal with leucine hydrazide (4.36 μM).
(c) Condensation product of piperonal with serine hydrazide (4.87 μM).

reached a constant value within thirty minutes and was stable at least one to two hours. The colour intensity obeyed Beer's law. Basing on the results the following standard method to estimate microgram quantities of hydrazine was devised: to one ml. of sample solution containing about 5 to 10 μg. of hydrazine 2 ml. of propanol containing 34 mg. piperonal and 1 ml. of glacial acetic acid were added. After standing one hour at room temperature, the reaction mixture was subjected to colorimetric estimation by Beckman's spectrophotometer at the wave lengths of 363–375 mμ.

(b) Hydrolysis of Hydrazides in Aqueous Solution.—Serine hydrazide was kept at room temperature for various times in aqueous solution, in dilute acetic acid (pH 4) and in 1% sodium bicarbonate solution. The degrees of hydrolysis under these conditions were estimated by the method **(ii)**. As shown in Fig. 2 serine hydrazide

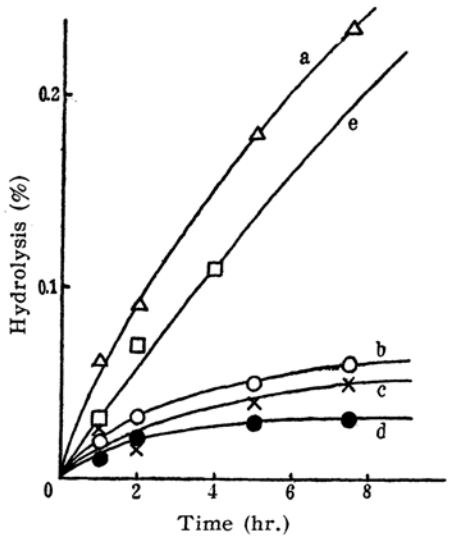


Fig. 2. Hydrolysis of amino acid hydrazides in aqueous solutions at room temperature
(a) Serine hydrazide in 1% sodium bicarbonate solution.
(b) Serine hydrazide in 66% alcoholic solution of sodium bicarbonate (medium for dinitrophenylation).
(c) Serine hydrazide in water.
(d) Serine hydrazide in dilute acetic acid (pH 4).
(e) Leucine hydrazide in 1% sodium bicarbonate solution.

is hydrolysed 0.05% in 7.5 hours in aqueous solution, 0.03% in acetic acid solution of pH 4, and 0.25% in 1% sodium bicarbonate solution. Further results obtained by the method (i) with glycine-, alanine- and leucine-hydrazides (Table II) indicate that the extent of hydrolysis of these hydrazides in neutral solution is less than 0.1 per cent in eighteen hours at room temperature.

From these results it is concluded that the amino acid hydrazides are stable in neutral and slight acidic media but somewhat unstable in alkaline medium. Therefore, one must avoid standing the hydrazides in solutions, especially in alkaline, for a long time. Consequently, it might be better to avoid treating the hydrazinolysates with strong basic and acidic ion-exchange resins.

The extent of hydrolysis of hydrazides by dinitrophenylation are shown in Table II, that is, 0.1 to 0.5 per cent of hydrazides are hydrolysed by the dinitrophenylation for two hours. The longer the time of dinitrophenylation, the greater the extent of hydrolysis.

To examine the hydrolysis during the extraction of C-terminal amino acids according to the above-mentioned procedure ethyl acetate solution of di-DNP-serine hydrazide was shaken with 2% sodium bicarbonate solution for three and five hours and the extent of hydrolysis of the DNP-hydrazide was estimated, the results of which are shown in Table II.

From these results, it can be concluded that the hydrazinolysates must be given a preliminary treatment with aldehyde to remove the bulk of the hydrazides, and all the treatment must be carried out as rapidly as possible.

(c) Hydrolysis of Hydrazides during Hydrazinolysis.—Amino acid hydrazides, such as serine-, glycine-, alanine- and leucine-hydrazides were heated with anhydrous hydrazine in the same conditions as in the case of proteins, and free amino acids liberated by this treatment were estimated. The results shown in Table II indicate that serine-, glycine-, alanine- and leucine-hydrazides are hydrolysed by 1.2, 1.7, 0.4 and 0.5 per cent, respectively, which are considerably higher than in aqueous solutions. Of course, these data do not indicate directly the percentage of free amino acids of non-C-terminal liberated during hydrazinolyses of proteins, as discussed later, and it might be preferable to compare results of hydrazinolysis of various reaction time, when C-terminal amino acids of proteins are intended to be characterized.

(3) Hydrazinolyses of Peptides

It is desirable to know the rates of hydrazinolysis of various peptides and to have some informations about the extent of hydrolysis of non-C-terminal peptide bonds during the hydrazinolysis. Experiments have been carried out on three peptides, L-leucyl-L-alanine, glycyl-L-alanine and glycyl-L-leucine. The results are cited in Table III, in which it is remarkable that as much as 30 percent of leucyl-alanine remains intact by hydrazinolysis for ten hours.

TABLE II
HYDROLYSIS OF AMINO ACID HYDRAZIDES IN VARIOUS CONDITIONS

	Serine hydrazide	Glycine hydrazide	Alanine hydrazide	Leucine hydrazide
Dinitrophenylation in 2 hr.	0.1%	0.5%	0.1%	0.2%
Dinitrophenylation in 7 hr.	0.7			
Shaking for 3 hr.*	0.15			
Shaking for 5 hr.*	0.3			
Heating with N_2H_4 for 5 hr.	0.5	0.7	0.2	0.2
Heating with N_2H_4 for 10 hr.	1.1	1.2	0.3	0.3
Standing in solution (pH 7.2) for 18 hr. at room temp.	0.06	0.02	0.04	0.08

* Hydrazides were dissolved in ethyl acetate and shaken with 2% sodium bicarbonate solution (to test the hydrolysis in extractive process).

TABLE III

HYDRAZINOLYSIS OF PEPTIDES

Peptide	Time of hydrazinolysis	Residual peptide	C-terminal liberated	Free amino acid non-C-terminal
L-Leucyl-L-alanine	5 hr.	60%	33%	
	10	30	45	
Glycyl-L-alanine	10	0	60	
Glycyl-L-leucine	3	10	83	
	5	Trace	87	Trace
	10	0	90	Trace

(4) Separation between Amino Acids and their Hydrazides

(a) Treatment with Aldehydes.—From the data cited in Table III, it is preferable to treat hydrazinolysates with aldehyde prior to the dinitrophenylation in order to remove the bulk of the hydrazides. We in our earlier works[1,2] and other investigators[3,4] used benzaldehyde, but it must be used as a freshly distilled preparation because it is apt to produce peroxide, which might oxidize hydrazides to form free amino acids. Thus we used piperonal and isovaleraldehyde in place of benzaldehyde. As the former did not give very good results, the latter was employed throughout this investigation. The procedure, which is nearly the same as reported before[7], is shown in Table VIa. Care was taken to avoid the hydrolysis of hydrazides by treating in shorter time under mild conditions.

(b) Fractional Extraction of C-terminal Amino Acids as DNP-derivatives.—The solution obtained by the above treatment contains C-terminal amino acids and residual hydrazides which could not be removed. After dinitrophenylation, the C-terminal DNP-amino acids must be separated from di-DNP-amino acid hydrazides. To separate the DNP-amino acids we have used the procedure cited in Table VIa, in which ethyl acetate was employed as organic solvent. Although nearly all sorts of DNP-amino acids were extracted quantitatively by dilute sodium bicarbonate solution from ethyl acetate solution while di-DNP-hydrazides remained in the latter, all of the di-DNP-tyrosine, and a part of the di-DNP-histidine and DNP-tryptophan were found to remain in the organic phase. Accordingly, if these amino acids are situated at C-terminal, they are fully or partly lost and cannot be characterized as DNP-derivatives.

This is the reason why contrary to the result by carboxypeptidase method, tyrosine has not been found as C-terminal of chymotrypsin by our earlier method.

To take measures on this problem, ethyl acetate was replaced by ethyl ether or isopropyl ether, or using ethyl acetate as organic solvent a special treatment for the three amino acids was established. These procedures are shown in Table VIb.

Of these procedures the ethyl acetate method gives the most clear-cut separation between DNP-amino acids and di-DNP-hydrazides. By the other methods, di-DNP-hydrazides contaminate the C-terminal fraction, so that it is necessary to treat hydrazinolysate with aldehyde prior to the dinitrophenylation. Applying these methods to lysozyme hydrazinolysates plus tyrosine, results shown in Table IV were obtained, i.e. about 90 per cent of leucine (C-terminal of lysozyme) and tyrosine added were recovered.

TABLE IV

RESULTS OBTAINED BY STANDARD METHODS WITH LYSOZYME PLUS TYROSINE

Sample	Method	Results
Lysozyme* tyrosine**	I	Leucine, 88%; tyrosine 90%
Lysozyme* tyrosine**	II (ether)	Leucine, 94%; tyrosine 88%
Lysozyme* tyrosine**	II (isopropyl ether)	Leucine, 90%; tyrosine 86%
Lysozyme*	III	Leucine, 90%

* Lysozyme was hydrazinolysed for ten hours at 100°C.
** Tyrosine was added to the hydrazinolysate of lysozyme.

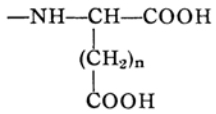
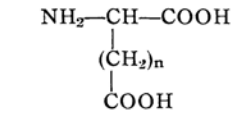
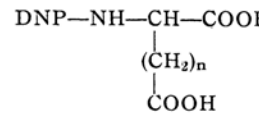
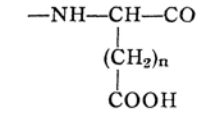
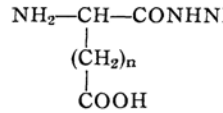
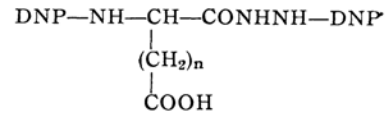
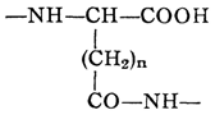
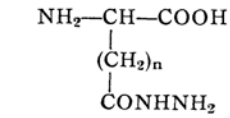
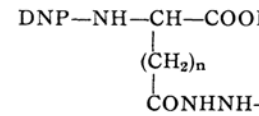
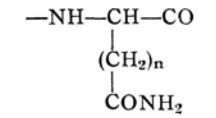
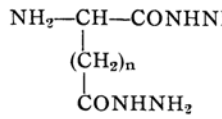
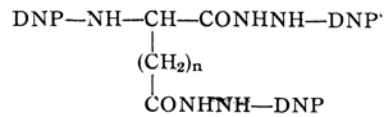
Being not dinitrophenylated quantitatively histidine might be better estimated by other methods, such as ion-exchanger chromatography etc., after treating the hydrazinolysates with aldehyde.

(5) Characterization of Aspartyl and Glutamyl Residues in Chain and C-terminal Asparagine and Glutamine

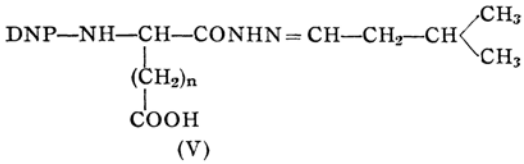
Contrary to the case of determination of N-terminal by DNP method, in which lysine residue in chain is also detected as DNP-derivative, by the hydrazinolysis method aspartic and glutamic acid residues bearing free carboxyl group in the chain and C-terminal asparagine and glutamine are extracted into acidic fraction together with other C-terminals. The possible products throughout this method are shown in Table V.

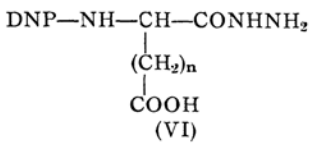
TABLE V

POSSIBLE PRODUCTS OF HYDRAZINOLYSIS AND DINITROPHENYLATION DERIVED FROM ASPARTIC AND GLUTAMIC RESIDUES

Dicarboxylic acid residue	Products of hydrazinolysis	Corresponding DNP-derivatives
 (Ia)	 (Ib)	 (Ic)
 (IIa)	 (IIb)	 (IIc)
 (IIIa)	 (IIIb)	 (IIIc)
 (IVa)	 (IVb)	 (IVc)

By the treatment with isovaleraldehyde followed by dinitrophenylation, other derivative such as (V) and/or (VI) might be formed from dicarboxylic acids bearing one free carboxyl group.


(V)


(VI)

In the fractional extractions by the procedure of Table VIa, (I) and (IV) are distributed as sharply as other DNP-amino acids and di-DNP-hydrazides, respectively, and (II) and (III) are distributed less sharply but finaly all extracted to alkaline solution from ethyl acetate, but (V) and (VI) behave somewhat irregularly and are distributed to all the fractions by the procedure. These facts seem to disturb the characterization of C-terminal amino acids of proteins, although no appreciable difficulties were experienced in the case of the proteins which we have investigated. For the quantitative estimation of C-terminal amino acids, however, it is convenient to estimate DNP-aspartic and/or DNP-glutamic acids, if they exist, and then to estimate other DNP-amino acids after hydrolysing the C-terminal fraction with 6 N hydrochloric acid at 100°C for several hours, whereby (II), (III) or (V), (VI) are hydrolysed to DNP-aspartic and glutamic acids. Thus a clear-cut estimation of all the C-terminal amino acids and aspartyl and glutamyl residues in chain are attained.

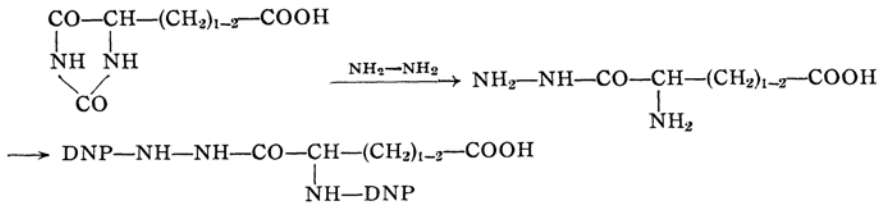
In spite of the complexity of these dicarboxylic acid derivatives, we can make use of the fact described above, to characterize the mode of linkage of these acidic amino acids in peptide chain and also to determine the C-terminal glutamine and asparagine. This advantage can not be achieved by the original benzaldehyde method. Thus lysozyme was proved to have one aspartyl, 12 asparaginyl and 4 glutaminyl residues in the molecule[13]. The characterization of a free carboxyl group in peptide chain have been reported by Jollès and Fromageot[18] and the characterization of the amide group was reported by Chibnall and Rees[19].

Preliminary experiments to distinguish four types of derivatives of dicarboxylic acids were done as described hereinafter.

Di-DNP-aspartic acid-α-hydrazide was prepared as follows: Hydantoin acetic acid was hydrazinolysed for ten hours at 100°C, whereby it converted partly to aspartic acid-α-hydrazide, which was purified as di-DNP-derivative through silica gel chromatography. Di-DNP-glutamic acid-α-hydrazide was prepared in the same manner from hydantoin propionic acid, which was identical with that obtained by the hydrazinolysis of poly-α-glutamic acid*. The syntheses through hydantoin

18) T. Jollès and C. Fromageot, *Biochim. Biophys. Acta*, 9, 287, 416 (1952).

19) A.C. Chibnall and M.W. Rees, *Biochem. J.*, 48, xlvii 1951); ibid., 52, iii (1952).



derivatives were rather poor in yields and many other by-products were also detected as DNP-derivatives. Di-DNP-glutamic acid-γ-hydrazide was obtained by dinitrophenylating the hydrazide*.

These four DNP-derivatives can be detected together with other DNP-amino acids, on suitable silica gel columns. Fig. 3 is an example indicating that all the DNP-derivatives can be separated distinctly. Moreover, di-DNP-aspartic and glutamic acid monohydrazides can be distinguished easily from DNP-amino acids as they develop brown red colour on dissolving in dilute bicarbonate solution.

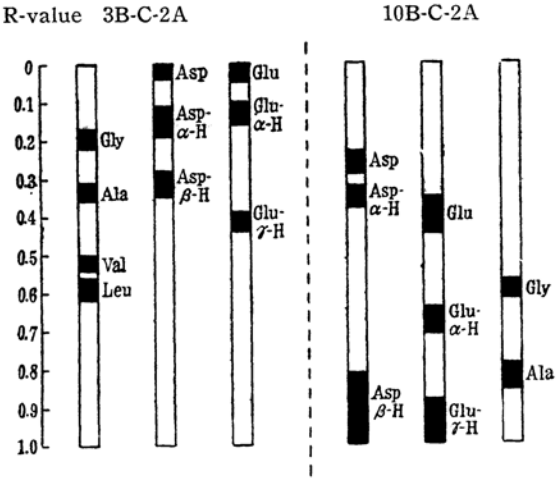


Fig. 3. Chromatograms of DNP-derivatives of aspartic and glutamic acid monohydrazides and DNP-amino acids on silica gel chromatograms.
Solvent: 3B-C-2A: n-BuOH: $CHCl_3$: 2% acetic acid=3: 97: 100
10B-C-2A: n-BuOH: $CHCl_3$: 2% acetic acid=10: 90: 100

Standard Methods

Considering the results mentioned above, three standard methods shown in Table VIa, VIb and VIc are established to characterize C-terminal amino acids and aspartyl and glutamyl residues in chain. In most cases all methods are available to give correct results, but differ in some points described below, so that the suitable method(s) may be chosen for the protein investigated.

The methods described here were applied succesfully to the proteins, but the volumes or weights of reagents described in Table VI may be modified according to the proteins investigated. As the C-terminal fraction is contaminated with DNP-derivatives of acidic amino acid monohydrazides and some C-terminal peptides, each component separated on chromatograms is better to be identified after hydrolysis with hydrochloric acid. Hydrazinolysis must be done twice or more varying the time of hydrazinolysis to estimate the amount of C-terminals, where the time of hydrazinolysis must not be longer than fifteen hours, as non-C-terminal amino acids appear and C-terminal amino acids decompose considerably for the longer hydrazinolysis.

By the three methods described below almost all amino acids can be characterized almost entirely quantitatively, but care must be taken when tyrosine, tryptophan, arginine, histidine, cystine, glutamine and asparagine are at the C-ends, among which histidine, cystine and amides may be characterized less quantitatively. The mode of linkage of acidic amino acids in peptide chain may be characterized, too, but less quantitatively.

Standard Method I.—This is a slightly modified method reported previously[7] and may be applied for general protein except the case where histidine and tryptophan are at C-ends and when one desires to seek terminal glutamine and asparagine or glutamyl and aspartyl residues in chain. If tyrosine is situated at C-terminal, the ethyl acetate fraction asterisked of Table VIa must be treated further, where all DNP-tyrosine and a part of DNP-histidine and DNP-tryptophan which are not included in the C-terminal fraction are detected.

Standard Method II.—While method I gives very clear-cut separation of DNP-amino acids from di-DNP-amino acid hydrazides, method II, in which ethyl ether or isopropyl ether is used in place of ethyl acetate, has more advantages than the method I in the case where tyrosine, histidine and tryptophan are at C-terminals. However, separation of DNP-amino acids from di-DNP-hydrazides is rather dull, so that the pretreatment of the hydrazinolysates with isovaleraldehyde is necessary.

Standard Method III.—This method which is nearly the same as reported previously[7,8] is established for the purpose of determining C-terminal glutamine and asparagine or glutamyl or aspartyl residues in chain. Of course other C-terminal amino acids can be characterized as in the case of method I, but small amounts of non-C-terminal amino acids would also be detected, though they would be almost negligible. Ethyl-

* Poly-α-glutamic acid was available through the kindness of Mr. H. Yuki, Laboratory of Fibre Research of this University, to whom the authors' thanks are due.
* Glutamic acid-γ and α-hydrazides and aspartic acid-β-hydrazide were also supplied by Mr. K. Narita, to whom we are grateful.

TABLE VIa

STANDARD METHOD I

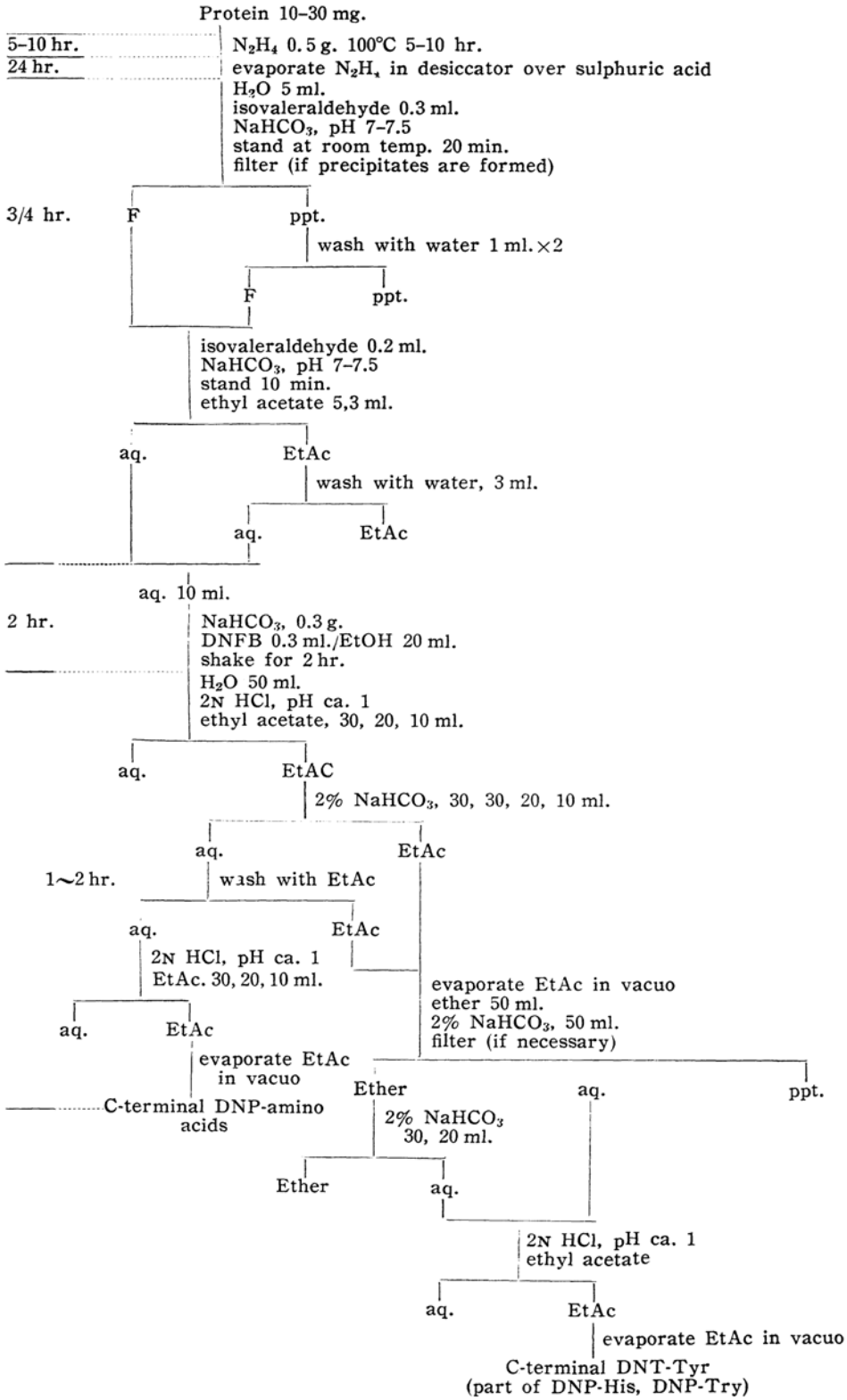
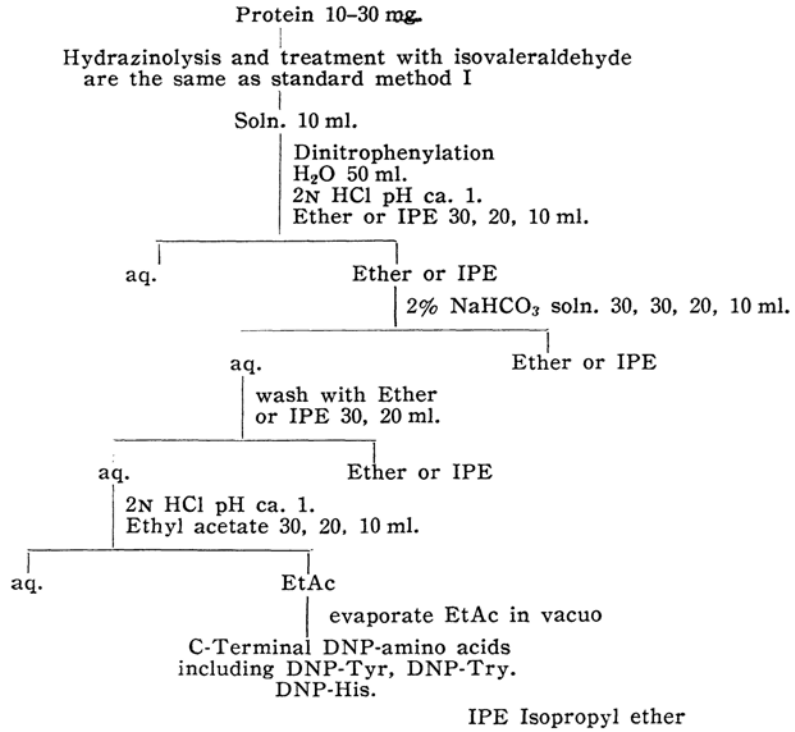


TABLE VIb

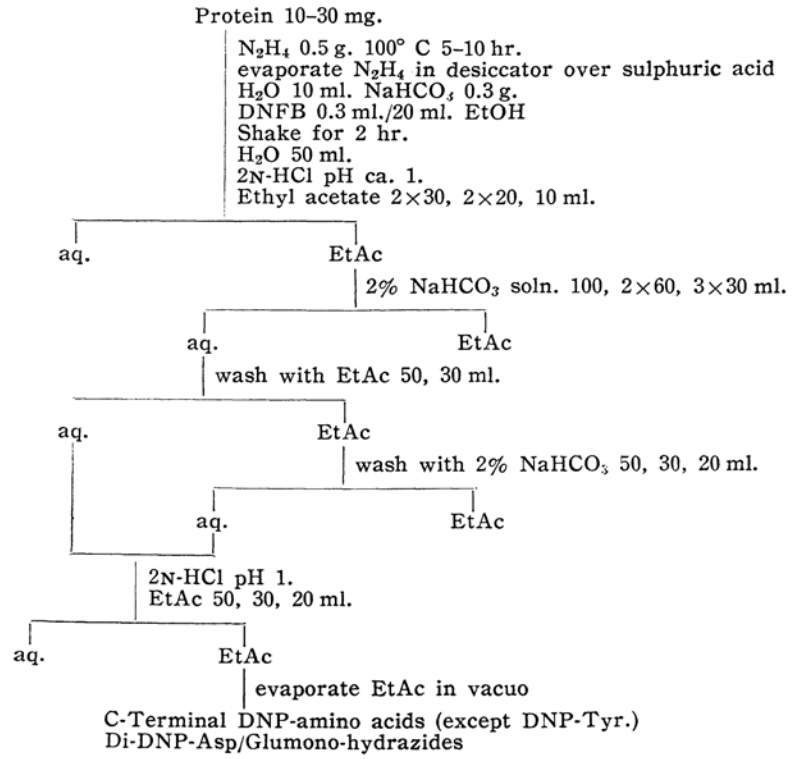
STANDARD METHOD II



IPE Isopropyl ether

TABLE VIc

STANDARD METHOD III

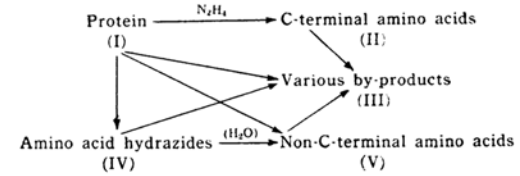


acetate cannot be replaced by ethyl ether or isopropyl ether in this case.

Discussion

As a matter of course, various data obtained by the present fundamental experiments cannot be applicable directly in the case of proteins; for example, it was really confirmed that about one per cent of amino acids were liberated during the ten hours' "hydrazinolysis" of amino acid hydrazides, but in the case of proteins, it takes several hours to produce amino acid hydrazides which, in turn, were subjected to hydrolysis partially, thus the percentages obtained in fundamental experiments indicate only the maximum values. About the decomposition of amino acids during the "hydrazinolysis", too, it is uncertain whether the amino acids bound at the C-end decompose similarly to the results cited in Table II. Therefore, we shall discuss below how to interpret the results obtained in the hydrazinolysis of real proteins.

First of all, let us consider all possible reactions involved in the hydrazinolysis. These may be expressed by the following scheme. The principal reaction is, of course,



the splitting of the peptide bonds with formation of amino acid hydrazides (I → IV) and the liberation of C-terminal residues as free amino acids (I → II). Yet the hydrazides thus formed liberate, though very slightly, free amino acids (IV → V), and free amino acids are also partly decomposed to unknown by-products (II → III, V → III). Being not yet comfirmed directly, following other side reactions might be possible: splitting of peptide bond under formation of free amino acids (I → V); decomposition of amino acid residues in peptide and at C-terminal before their liberation by hydrazinolysis (I → III); and decomposition of amino acid hydrazides directly to some by-products (IV → III).

On applying the hydrazinolysis to a protein, participations of all side reactions depend on the sorts of C-terminal amino acids and compositions and sequences of amino acids in the protein, so that it cannot be decided summarily which is predominant among these reactions. However, we have estimated almost one mole of each C-terminal, but no appreciable other amino acids applying this method to two proteins (lysozyme, and Taka-amylase A). If we are allowed to generalize the results from these few examples, we might be able to consider that results really obtained on proteins are mainly due to the desired principal reaction, no matter how many side reactions are possible theoretically. It can be admitted, at least, to speak generally, that if more than 0.5 mol. of free amino acids are obtained by the hydrazinolysis of ten hours, they can be regarded as C-terminals, but if only less than 0.2 mol. of them are detected they may be artifact, and it needs further study if 0.2 to 0.5 mol. appears.

The supposition described above was justified also by some calculations from the fundamental data obtained. We shall calculate for example, the amount of non-C-terminal amino acid formed through the reactions $I \xrightarrow{k_1} IV \xrightarrow{k_2} V$.

Assuming that the each step of the reactions is the first order reaction and the step of V → III is negligible, the concentration of V at time t is given by equation (1).

$$[V]_t=[I]_0+\frac{[I]_0}{k_1-k_2}(k_2e^{-k_1t}-k_1e^{-k_2t}) \qquad (1)$$

where $[I]_0$ and $[V]_t$ are the concentration of I and V at time 0 and t respectively, k_1 and k_2 are the rate constants of each step.

If the conversion of I → IV is 90 per cent in eight hours' hydrazinolysis and IV → V is one per cent in ten hours, the amount of V at ten hours is calculated as only 0.67 per cent.

The similar calculation can be applied to the reactions $I \xrightarrow{k_3} II \xrightarrow{k_4} III$. Assuming that, here again, each step of the reactions is the first order, the concentration of II at time t is

$$[II]_t=\frac{k_3[I]_0}{k_3-k_4}(e^{-k_4t}-e^{-k_3t}) \qquad (2)$$

where $[I]_0$ is the initial concentration of I and $[II]_t$ is the concentrations of II at time t, k_3 and k_4 are the rate constants of each step.

If the conversion I → II is 90 per cent in ten hours and II → III is 10 per cent in ten hours the following result is obtained.

Time (hr.)	2	5	10	14
Mol. of II from 1 mol. of I	0.37	0.74	0.84	0.87 (max.)

This value is rather coincident with the observed value in the case of liberation of leucine from lysozyme[7].

Further if the conversion of I → II is 90 per cent in twenty hours and II → III is 45 per cent in ten hours (decomposition of alanine, Table I), the calculated value of II is as follows:

Time (hr.)	5	10	12
Mol. of II from 1 mol. of I	0.37	0.49	0.50 (max.)
Mol. of alanine from 1 mol. of leucyl-alanine (observed)	0.33	0.45	

Since these calculations involve some assumption and simplification of complex reactions, one must avoid attaching too much importance to the result derived from these calculations. However, it is obvious from these considerations that liberation of C-terminal is a function of time, which has one maximum value, so that it may be necessary to determine C-terminal at various time intervals.

On Taka-amylase A, we have other data not mentioned before. Even if the amylase solution in hydrazine was brought to dryness in vacuo at room temperature directly after the addition of hydrazine 0.3 mol. of serine and 0.1 mol. of glycine were detected as C-terminals. On the other hand, 8.955 mg. (47.7 μM.) of glycyl-L-leucine, the content of glycine of which is about the same as that of Taka-amylase[20], were added to 51.5 mg. (1.1 μM,) of Taka-amylase A followed by hydrazinolysis, whereby 0.068 mg. (0.9 μM.) and 0.082 mg. (1.1 μM.) of glycine were found, respectively, by the hydrazinolysis of five and ten hours. These results indicate that only 0.4 per cent of glycyl residue was liberated as free amino acid from added glycyl-leucine in ten hours' hydrazinolysis. From these two facts it can be concluded that glycine and serine are really C-terminals of this protein. On another terminal, alanine, there might be some doubt because of its slow liberation by hydrazinolysis[7], but on considering that the rate of hydrazinolysis of L-leucyl-L-alanine is slow as in case of this protein (see Table IV), it is more probable that alanine is bound at a C-terminal by some hard-hydrazinolysable bond.

On the C-terminal of ovalbumin, Steinberg[21] reported the liberation of alanine by carboxypeptidase but later[22] found this due to a DFP-sensitive, contaminated enzyme. Locker[4] also reported that the protein was not attacked by DFP-treated carboxypeptidase. Turner and Schmerzler[23], however, have found alanine to be a C-terminal by the thiohydantoin method. Although some of us[7] also reported the presence of one alanine at the C-terminal, Dr. Fraenkel-Conrat[24] informed us recently that the C-terminal of this protein is valyl-seryl-proline. Thus we reinvestigated on the protein and found that we had taken proline for alanine. The DNP-derivative of the C-terminal amino acid was proved to be proline and not alanine by variety of chromatography and its absorption curve. The detail of this account shall be published later by one of the authors.

Although carboxypeptidase method has been applied successfully to many proteins, it has several distinct restrictions by nature. For example, it is not applicable to insoluble proteins such as wool keratin[14] and silk fibroin[5]; its action depends not only on the nature of the C-terminal but also on the adjacent amino acid residues; when a protein has more than two C-terminals, it is difficult to characterize it distinctly; and so on. The hydrazinolysis method has, at least, the advantages in these cases. The result on ovalbumin might be the best example of the success of the hydrazinolysis method, becauce proline situated at C-terminal is liberated neither by carboxypeptidase nor by the thiohydantoin method.

Summary

1. Fundamental experiments on the hydrazinolysis method, a method for characterization of C-terminal amino acids of proteins, were carried out.

2. Amino acids were found to decompose to various extents by heating with anhydrous hydrazine.

3. Amino acid hydrazides were hydrolysed about 1% or less by heating with anhydrous hydrazine (ca. 99%) for ten hours. Hydrolyses of them in neutral and slightly acidic solutions at room temperature are negligible, but in alkaline solution the extent of hydrolyses are somewhat greater though less than 0.2% in five hours in 1% bicarbonate solution.

4. Procedures for separating free amino acids from amino acid hydrazides as DNP-derivatives with or without treatment of iso-valeraldehyde prior to dinitrophenylation were reinvestigated thoroughly.

5. Some experiments on the hydrazinolyses of peptides were done.

6. Fundamental investigations of the characterization of the mode of linkage

20) S. Akabori, T. Ikenaka, H. Hanafusa and Y. Okada, *J. Biochem.*, **41**, 803 (1954).

21) D. Steinberg, *J. Am. Chem. Soc.*, **74**, 4217 (1952).

22) D. Steinberg, *J. Am. Chem. Soc.*, **75**, 4875 (1953).

23) R.A. Turner and G. Schmerzler, *Biochim. Biophys. Acta*, **11**, 586 (1953).

24) H. Fraenkel-Conrat, Personal communication. The authors are grateful to him for his information prior to his publication, *J. Am. Chem. Soc.*, **77**, 5882 (1955).

of monoaminodicarboxylic acids in peptide chains by the hydrazinolysis method indicate its availability.

7. Standard methods for quantitative estimation of C-terminal amino acids or proteins are established based upon the results obtained by the present investigation.

8. Brief consideration on the mechanism of the hydrazinolysis was described and the scope of this method was discussed in connection with that of the carboxypeptidase method.

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