The Participation of Aspartyl Residues in the Hydroxylamine- or Hydrazine-Sensitive Bonds of Collagen*

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Received March 26, 1962

Certain bonds of soluble collagens (previously referred to as "ester-like") can be preferentially cleaved with aqueous 1 M hydrazine or hydroxylamine at *p*H values of 8–10. Kinetic studies indicate that approximately 6 bonds of this nature occur for each 1000 amino acid residues. Attempts have been made to isolate peptides containing acid hydrazide groups resulting from the reaction of hydrazine with ichthyocol or calfskin collagen, and to identify those acyl groups which participate in the sensitive linkages. Toward this end collagen was treated with hydrazine and the modified protein digested with collagenase. A small number of peptides containing acid hydrazide groups were then isolated in an aggregate fraction by use of phosphocellulose columns and selective reaction with *o*-benzaldehyde sodium sulfonate. Amino acid composition, hydrazide contents, and terminal amino acid analyses of these peptides suggest that they originate from termini of polypeptide chains in the collagen molecule. On the average, each peptide can be considered to contain ten amino acid residues of which two derive from aspartic acid and bear hydrazone functions. In collagen treated with hydroxylamine, half of the resulting hydroxamic acid groups are labile and, in this respect, resemble model α-hydroxamic acids. Lossen rearrangement of gelatin-dinitrophenylhydroxamate gave approximately equal molar quantities of α,β-diaminopropionic acid and an aliphatic aldehyde, suggesting the presence of both α- and β-aspartyl functions in the hydroxylamine-sensitive groups of the original protein. Treatment of ichthyocol with lithium borohydride gave, among other products, homoserine and β-aminobutyrolactone; the former would be expected to originate from reduction of an aspartyl residue in β-linkage as an ester or imide, and the latter from an aspartyl residue in α-linkage also as an ester or imide. The evidence suggests that those bonds in collagen which are sensitive to nucleophilic agents and selected reducing agents involve the participation of equal numbers of α- and β-aspartyl carboxyl groups. In collagen, sequences probably occur which bear, in close proximity, one α- and one β-aspartyl ester or imide group.

Gallop *et al.* (1959) reported that treatment of soluble collagens with hydroxylamine or hydrazine in each case resulted in cleavage of the protein molecule to units of molecular weight of approximately 20,000. Concomitantly it was found that the modified protein contained respectively hydroxamic acid or acid hydrazide groups. The authors considered the possible types of linkages which would be sensitive to nucleophilic attack of this nature, and cautiously adopted the phrase "ester-like" to describe the bonds which are cleaved in collagen. Bello (1960) and Hörmann (1960) confirmed the reaction of hydroxylamine with gelatin or collagen, and the latter investigator indicated further that insoluble collagens became soluble in the presence of hydroxylamine and urea, each agent alone having no effect. The studies of Hörmann (1960) suggested that "ester-like" bonds are involved in intermolecular as well as intramolecular cross-linkages.

* This work was supported by grants from the National Institutes of Health, United States Public Health Service (H-4762, A-5821, H-3838, M-2562, A-2965).

† Holder of a Postdoctoral Fellowship from the Arthritis and Rheumatism Foundation, 1960–1962.

The present communication defines further the nature of the hydroxylamine- or hydrazine-sensitive bonds in collagen, and establishes that the acyl participants are aspartyl residues in the protein.

EXPERIMENTAL SECTION

Materials

Ichthyocol was prepared from the tunics of carp swim bladders and calf procollagen from calf skin by the method described by Gallop (1955). The parent gelatin of each was prepared by suspending the dry material in water and heating for 10 minutes at 60°. The warm gelatin solutions were clarified by centrifugation in a Spinco Model L centrifuge at 100,000 × *g* at 40° for 20 minutes. The concentration of protein in a solution was computed from Kjeldahl nitrogen determination. (Corrected for moisture and ash, ichthyocol used in these experiments contained 18.97% of nitrogen and calf procollagen contained 18.70% of nitrogen.)

Collagenase was prepared from culture filtrates of *Clostridium histolyticum* as described previously (Seifter *et al.*, 1959).

Hydrazine (95%), hydroxylamine hydrochloride, and *p*-dimethylaminobenzaldehyde were purchased from Matheson, Coleman and Bell; *o*-benzaldehyde sodium sulfonate from Eastman Distillation Products; lithium borohydride from Metal Hydrides, Inc.; α,β-diaminopropionic acid hydrobromide and homoserine from Mann Biochemical Co.; α,γ-diaminobutyric acid hydrochloride and phosphocellulose (cellex-P) from California Corporation for Biochemical Research; and 3-methyl-2-benzthiazolone hydrazone hydrochloride from the Aldrich Chemical Co.

Pyridine was redistilled and residual ninhydrin-reacting material was removed by treatment with permutit. Polyanhydroaspartic acid was a gift of Dr. Joseph Kovacs, and had been prepared as described by that investigator and his co-workers (1953).

N - benzoyl - β - amino - γ - hydroxybutyrylamide[1] was prepared as follows: *N*-benzoylasparagine ethyl ester (400 mg) was refluxed for 30 minutes with 110 mg of lithium borohydride in 10 ml of dry tetrahydrofuran; after the mixture had cooled to room temperature, 6 N HCl was added dropwise until excess lithium borohydride had decomposed, and the *p*H of the solution was approximately 3. The precipitate was removed by filtration. The filtrate was evaporated *in vacuo*, and a gummy white material resulted; on addition of water, crystals appeared. The crystals were collected, washed with water, and dried. Yield, 100 mg (30%); m.p., 178–179° (uncorrected).

Anal. Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.41%; H, 6.34%; N, 12.66%. Found: C, 58.88%; H, 6.16%; N, 12.49%.

Methods

Treatment of Gelatin with Hydrazine.—Gelatin was dissolved in water and treated with hydrazine at *p*H 10.0 for 90 minutes at 40°; the concentration of protein was 2 to 4% and the final concentration of hydrazine was always 1 M. The *p*H of the solution was then adjusted to 4 by addition of HCl, and the material was dialyzed at 4° against distilled water until all free hydrazine was removed; this process usually required 48 hours. The quantity of acid hydrazide attached to the protein was determined by the method of Seifter *et al.* (1960) and was expressed as μmoles of acid hydrazide per 100 mg of protein (determined from Kjeldahl N) or as residues of acid hydrazide per 1000 amino acid residues.[2]

[1] The same compound had been prepared by Chibnall *et al.* (1958) from *N*-benzoylasparagine methyl ester and lithium aluminum hydride as an uncharacterized intermediate in the synthesis of β-aminobutyrolactone.

[2] Since the average weight of a residue in collagen is approximately 92, a value of 10 μmoles of acid hydrazide per 100 mg of protein, for example, would be equivalent to 9.2 residues of acid hydrazide per 1000 amino acid residues.

Treatment of Gelatin with Hydroxylamine.—A 2–4% aqueous solution of gelatin was mixed with an equal volume of 2 M hydroxylamine hydrochloride previously adjusted to *p*H between 9 and 10; the mixture was then kept for 90 minutes at 40°. Next the solution was brought to *p*H 4.0 by addition of HCl and dialyzed against distilled water at 4° until all free hydroxylamine was removed. The quantity of hydroxamic acid attached to the protein was determined by a modification of the method of Hill (1947) as described by Seifter *et al.* (1960); in some cases the method of Bergmann and Segal (1956) was used. In some experiments hydroxylamine was not removed by dialysis, and the amount of hydroxamic acid was then determined by the method of Lipmann and Tuttle (1945).

Hydrolysis of Proteins and Analysis of Amino Acids.—Unless otherwise indicated, protein and polymer preparations were hydrolyzed in 6 N HCl at 105° for 22 hours. Excess HCl was then removed by evaporation *in vacuo*. Amino acids were determined by chromatography on IR 120 by the automatic technique of Spackman *et al.* (1958).

Lossen Rearrangement of Gelatin-Hydroxamic Acids.—A 1–2% solution in water of hydroxylamine-treated gelatin was mixed with an equal volume of 1% ethanolic fluorodinitrobenzene; the mixture was maintained at *p*H 8.0 by continuous titration with NaOH, and after 5 minutes no additional alkali was required, indicating that the reaction had been completed. The mixture was then extracted three times with ether, and Lossen rearrangement was carried out by heating the protein-dinitrophenylhydroxamate for 2 minutes at 100° in 0.1 N NaOH (Gallop *et al.*, 1960).

In later experiments, as will be indicated, the time of heating was extended to 10 minutes.

Identification of α,β-Diaminopropionic Acid in Acid Hydrolysates Prepared After Lossen Rearrangement.—Protein (100 to 150 mg), after conversion to the dinitrophenylhydroxamate derivative and Lossen rearrangement, was hydrolyzed in 6 N HCl under reflux, and amino acid analyses were performed respectively after 22, 48, and 72 hours.

The respective positions of α,β-diaminopropionic acid and α,γ-diaminobutyric acid standards were established for chromatograms obtained on a 50-cm column of Amberlite IR 120; the column was operated at 30° and the eluting buffer was 0.38 N (for sodium ion) sodium citrate buffer of *p*H 3.8.

As noted previously (Gallop *et al.*, 1960), the color yield in the ninhydrin reaction for α,β-diaminopropionic acid is 33% of the yield for leucine. Because of this relatively low value and the small quantity of α,β-diaminopropionic acid anticipated, large amounts (10–70 mg) of the protein hydrolysate were employed in analysis. Because both α,β-diaminopropionic acid and α,γ-diaminobutyric acid emerge close to the ammonia peak, it was necessary to reduce the concentration of ammonia in a sample prior to chromatography.

This was accomplished by adjusting the hydrolysate, after removal of HCl, to *p*H 12 and exposing the solution to concentrated sulfuric acid in a vacuum desiccator overnight. The solution was then adjusted to *p*H 2 and applied to the resin.

Determination of Aldehyde after Lossen Rearrangement.—The specific and sensitive method of Sawicki *et al.* (1961) for aldehydes was applied to the protein solution after Lossen rearrangement. For analysis, 0.8 ml of the solution (containing approximately 2 mg of protein) was mixed with 0.2 ml of 1% 3-methyl-2-benzthiazolone hydrazone hydrochloride and heated at 100° for 3 minutes. After the solution had been cooled to room temperature, 2.5 ml of 0.2% ferric chloride solution was added and, after 5 minutes, 6.5 ml of acetone. The solutions were examined at a wave length of 670 mμ in a Coleman Junior Spectrophotometer and compared with standards containing from 0.02 to 0.10 μmoles of acetaldehyde. For a control we used a sample of hydroxylamine-treated gelatin which had not been dinitrophenylated but nevertheless had been kept for 5 minutes at *p*H 8, extracted with ether, and then heated with 0.1 N NaOH at 100° for 10 minutes.[3] By use of the control gelatin, a base line of aldehyde was measured. Then, with use of the dinitrophenylated derivative of the hydroxylamine-treated gelatin, an aldehyde determination was made after heating in alkali. The difference in aldehyde values between the two samples represented aldehyde due to Lossen rearrangement.

Preparation and Reduction of Esterified α,β-Polyaspartic Acid.—Nucleophilic attack by hydroxylamine causes the imides of polyanhydroaspartic acid to open preferentially at α-carboxyl linkages, giving rise to a new polymer containing approximately twice as many β- as α-peptide bonds (Gallop *et al.*, 1960). In the same manner, treatment of polyanhydroaspartic acid with alkali results in scission of the imide rings and production of a polypeptide containing the two types of peptide linkages (Kovacs *et al.*, 1961). For the purposes of the present experiments, LiOH was used as the alkali.

Polyanhydroaspartic acid (2.0 g) was dissolved in 10 ml of 2 N LiOH and stirred for 20 minutes at room temperature. The solution was then acidified to *p*H 2 and the resulting polyaspartic acid (designated hereafter as "α,β-polyaspartic acid") precipitated with ethanol. The ethyl ester was prepared from 800 mg of this material by treatment with 30 ml of ethanolic HCl (0.5 M) at room temperature for 48 hours, a procedure which resulted in solution of the polymer. Most of the solvent was removed by evaporation, and the esterified polymer was precipitated by addition of ether; the product was washed with ether and dried. Titration in ethanol with standard sodium methoxide solution with thymol blue as indicator revealed that 92% of the polyaspartic acid had been esterified. α,β-Polyaspartic acid ethyl ester (300 mg) was dissolved in 20 ml of dry tetrahydrofuran and the solution refluxed with lithium borohydride (300 mg, 88% assay) for 1 hour with stirring. The lithium-boron complex of the reduced polymer was filtered and suspended in 10 ml of ether; it was then decomposed by dropwise addition of 6 N HCl until evolution of gas subsided. The material was washed successively with ethanol and ether, collected by filtration, and dissolved in a small volume of water. The reduced polymer was then precipitated with ethanol. Yield, 150 mg; Kjeldahl N, 11.3%.

Reduction of Ichthyocol.—A sample of ichthyocol was washed with dry tetrahydrofuran and dried thoroughly; 700 mg was then suspended in 100 ml of dry tetrahydrofuran and 1 g of lithium borohydride was added. The mixture was stirred vigorously and simultaneously refluxed for 12 hours. The protein was filtered, suspended in 10 ml of ether, and treated with 6 N HCl to decompose the lithium-boron complex. The reduced protein was filtered, washed with dioxane and ethanol, and air-dried. Yield, 400 mg.

Identification of Homoserine and β-Amino-γ-hydroxybutyric Acid and Their Respective Lactones.—These compounds were separated and estimated by column chromatography with IR 120 as indicated in Table I. Chibnall *et al.* (1958) and Gundlach *et al.* (1959) had shown that γ-hydroxyamino acids undergo cyclization to lactones during hydrolysis with acids; and we established that homoserine, subjected to conditions used for acid hydrolysis of proteins, was converted to α-aminobutyrolactone to the extent of 78%. We also found that the model compound, *N*-benzoyl-β-amino-γ-hydroxybutyrylamide, was converted to β-aminobutyrolactone under these conditions. Since both lactones and free hydroxyacids could be present in acid hydrolysates of the reduced polymers and collagens under study, it was necessary to determine their color yields in the assay with ninhydrin. In a given experiment, saponification of a hydrolysate by treatment for 5 minutes with 0.01 N NaOH at 100° would provide a measure of total hydroxyacid, and from a measure of the color obtained with ninhydrin before saponification and the established color yields given in Table I, one could calculate the quantities of lactone and open form of the hydroxyacid.

[3] Using the reagent of Sawicki *et al.* (1961), we have shown that gelatin from ichthyocol or calf skin procollagen, preheated at 100° in the presence of 0.1 N NaOH, contains a variable amount of aldehyde depending on time of treatment. A maximum value of 3 to 5 μmoles of aldehyde (as compared with a standard of acetaldehyde) per 100 mg of protein is attained after 10 minutes' heating in alkali. Since the Lossen rearrangement is carried out in the same concentration of NaOH at 100°, it was necessary to determine the quantity of aldehyde occurring or released independently of the rearrangement—that is, to establish a base line. Thus the condition of Lossen reaction with respect to heating was changed from 2 to 10 minutes.

TABLE I

CHROMATOGRAPHY ON IR 120 AND COLOR YIELDS WITH NINHYDRIN OF HOMOSERINE, β-AMINO-γ-HYDROXYBUTYRIC ACID, AND THEIR RESPECTIVE LACTONES

Starting Compound	Treatment	Compounds Found After Treatment	Color Yields of Compounds Found[a]	Position of Emergence (ml effluent) of Compound on IR 120 Columns of: 15 cm[b]	50 cm[c]	150 cm[d]
Homoserine	None	Homoserine	0.80			210
	6 N HCl, 22 hours, 105°	Homoserine	0.17			210
		α-Aminobutyrolactone	0.43	95	345	
	6 N HCl, 22 hours, 105°, then 0.01 N NaOH, 5 min., 100°	Homoserine	0.80			210
Benzoyl-β-amino-γ-hydroxybutyryl-amide	6 N HCl, 22 hours, 105°	β-Amino-γ-hydroxybutyric acid	0.035			470
		β-Aminobutyrolactone	0.22	108	380	
	6 N HCl, 22 hours, 105°, then 0.01 N NaOH, 5 min., 100°	β-Amino-γ-hydroxybutyric acid	0.087			470

[a] Compared to leucine = 1.0. [b] Eluting buffer of *p*H 5.28, 0.38 N Na citrate; temperature, 50°. [c] Eluting buffer of *p*H 5.28, 0.38 N Na citrate; temperature, 30°. [d] Eluting buffer at start, *p*H 3.0, 0.2 N Na citrate, changed after 315 ml to *p*H 4.25, 0.2 N Na citrate; temperature, 50°.

Table I also shows the relative positions of the hydroxyacids and their lactones in chromatograms obtained with columns of IR 120 in lengths of 15, 50, and 150 cm. The position of a given compound is expressed in terms of the milliliters of eluting buffer required for its emergence.

Digestion by Collagenase of Hydrazine-Treated Gelatin from Ichthyocol.—Hydrazine-treated gelatin (800 mg) was dissolved in 14 ml of 0.005 M calcium chloride and digested at 25° with 50 units of collagenase. The *p*H of the reaction mixture was maintained at 8.0 by continuous titration with 0.1 N NaOH. After 10 hours, proteolysis was shown to be virtually complete by determination of amino groups by the method of Rosen (1957). The analysis revealed that approximately 200 peptide bonds per unit of 100,000 molecular weight had been hydrolyzed, and that the average molecular weight of peptides in the digest was 500. Since analysis of the digest showed that 95% of the acid hydrazide groups of the initial hydrazine-treated gelatin were intact, it could be calculated that approximately four to six of the peptides in the digest would contain acid hydrazide.

Chromatography of Hydrazide-Containing Peptides on Phosphocellulose.—A column of phosphocellulose (0.9 × 30 cm) was equilibrated with 0.01 M pyridine-acetate buffer of *p*H 3.7. A 5-ml aliquot of collagenase digest of hydrazine-treated gelatin, corresponding to 100–150 mg of protein, was used for chromatography. Effluent was collected as 2-ml fractions at the rate of 10 ml per hour; elution was started with 0.01 M pyridine-acetate buffer of *p*H 3.7 and, after delivery of approximately 400 ml, continued with a gradient of 0.40 M pyridine-acetate buffer of *p*H 4.7. An aliquot of each fraction was analyzed for amino groups by the ninhydrin method, and a second aliquot for content of acid hydrazide. Suitable fractions were then pooled and solvent evaporated *in vacuo*.

Reaction of Peptides Containing Acid Hydrazide with o-Benzaldehyde Sodium Sulfonate, and Rechromatography on Phosphocellulose.—An aliquot (5 ml) of the combined fractions containing the peptides bearing acid hydrazide groups was treated with *o*-benzaldehyde sodium sulfonate (20- to 30-fold molar excess referent to acid hydrazide) at 80° for 30 minutes. This procedure resulted in formation of hydrazones carrying negatively charged groups, so that the peptides bearing these functions could then be separated from other peptides in the fraction.

The reaction mixture containing the hydrazones was then diluted with water to yield a sodium ion concentration corresponding to 0.01 M, the *p*H was adjusted to 3.7, and the final solution was placed on a column of phosphocellulose. Conditions of elution were the same as applied for the whole digest except that a different gradient of pyridine-acetate buffer was used (this is seen in Figure 5).

Determination of the Amino-Terminal Residues in the Isolated Peptide Fraction Containing Hydrazone Functions.—An aliquot (3 ml) of the combined fractions of peptides containing hydrazone groups, corresponding to 0.72 μmoles of acid hydrazide, was adjusted to *p*H 9.0 with NaOH and treated with 3 ml of 1% (v/v) 1-fluoro-2,4-

dinitrobenzene in ethanol for 3 minutes. The solution was extracted three times with ether and then refluxed in 6 N HCl in order to hydrolyze the peptides. After 4 hours the solution was extracted with ether and the aqueous layer was refluxed for an additional 16 hours. The ether extract was dried over sodium sulfate and concentrated by evaporation *in vacuo*. Paper chromatography for identification of dinitrophenyl amino acids was performed in one dimension with a developing solvent of butanol–acetic acid–water (4:1:5), and again in two dimensions with developing systems of *tert*-amyl alcohol-phthalate buffer, *p*H 6.0, and 1.5 M phosphate buffer, *p*H 6.0. The acid hydrolysate (*i.e.*, the aqueous layer) was dried *in vacuo* and then analyzed for amino acid contents.

Results

Reactions of Hydrazine and Hydroxylamine with Gelatins.—The results of kinetic studies of the reaction of hydrazine with gelatin of ichthyocol or calf skin collagen are plotted in Figures 1 and 2. It can be seen that the characteristic rapid phase of the reaction is virtually complete in one hour and, at this time, the fish gelatin contains 6.6 μmoles of acid hydrazide per 100 mg of protein and the calf skin gelatin 6.0 μmoles (corresponding, respectively, to 6.1 and 5.5 acid hydrazide groups per 1000 amino acid residues). Determination of amino groups by the ninhydrin method was also performed in order to discover whether large-scale splitting of peptide bonds had occurred, and the results are also shown in Figures 1 and 2. Because of the significant background of ninhydrin reactivity due to ε-amino groups of lysyl residues in the protein, a small increase in amino groups cannot be measured with precision. Nevertheless the results show that few peptides or amide bonds were split by hydrazine during the time in which acid hydrazide groups appeared, and that only after approximately 4 hours did such scission become significant.

Similar results were observed when gelatins were treated with hydroxylamine. After reaction for 90 minutes each 100 mg of gelatin derived from ichthyocol contained 6.0 μmoles of hydroxamic acid. In this experiment the hydroxamic acid content was measured in the presence of hydroxylamine, so that it was not possible to determine increase in reactivity with ninhydrin. However, previous studies (Gallop *et al.*, 1959) in which the reaction mixture was dialyzed showed no increase, during the rapid phase of the reaction, of amino groups relative to Kjeldahl nitrogen.

Stability of Hydroxamic Acid Groups in Modified Proteins.—Gallop *et al.* (1960) provided evidence that the α-hydroxamic acid groups of α,β-polyaspartylhydroxamic acid were unstable to dialysis against distilled water, whereas the β-hydroxamic acid groups were stable. In contrast, both α- and β-acid hydrazide groups were

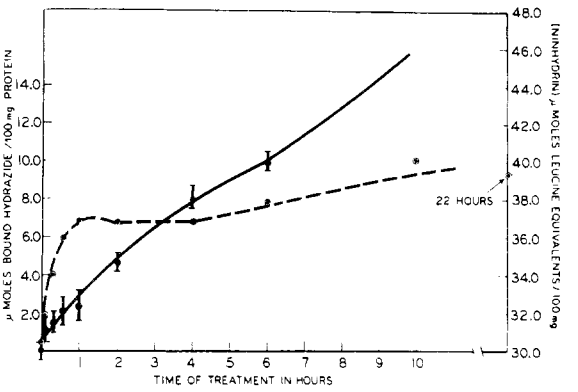


FIG. 1.—Kinetics of the reaction of ichthyocol with hydrazine. 1% ichthyocol, 1 M hydrazine, at 40°

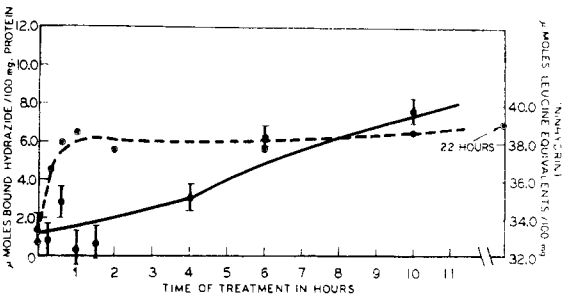


FIG. 2.—Kinetics of the reaction of calf procollagen with hydrazine. 1% calf procollagen, 1 M hydrazine, at 40°.

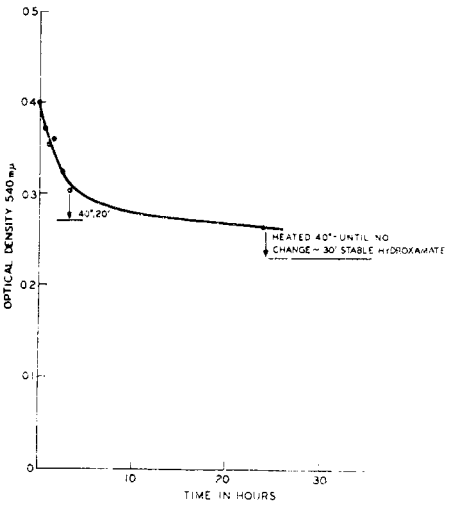


FIG. 3.—Stability of α,β-polyaspartyl hydroxamate at *p*H 8.0, 25°

stable to dialysis under similar conditions. In Figure 3 are shown the results of an experiment in which α,β-polyaspartylhydroxamic acid was maintained at 25° at *p*H 8.0, and hydroxamic acid contents were determined at various periods of time. It is seen that a large fraction of the

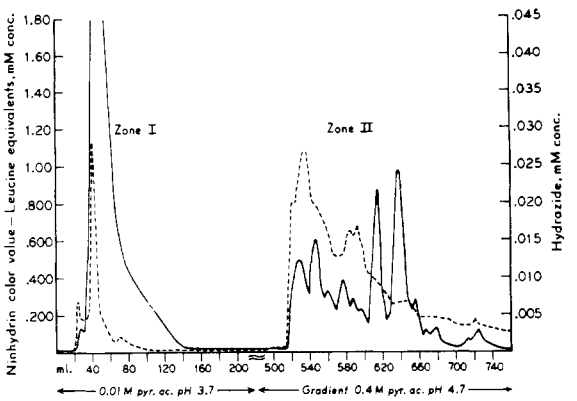


FIG. 4.—Chromatography of collagenase digest of hydrazine-treated gelatin from ichthyocol on a 0.9 × 30 cm phosphocellulose column at 25°; (—) ninhydrin, (- - - -) hydrazide. The quantity of peptides placed on the column corresponded to 100 mg of protein.

total hydroxamic acid is labile under these conditions, and that another fraction is stable at a temperature of 40° even after 30 hours. (The last two points in Figure 3, in contrast to the other points, represent values obtained at 40°.)

In Table II are shown results of experiments in which five different preparations of hydroxylamine-treated gelatin from ichthyocol were dialyzed under various conditions. It can be seen that approximately one-half of the total hydroxamic acid groups (*i.e.*, approximately 3 of 6 per 1000 amino acid residues) are labile to extended dialysis against distilled water at 25°. That these groups represent hydroxamic acid groups attached to α-aspartyl functions will be considered in the discussion.

TABLE II
LABILITY OF HYDROXAMIC ACIDS IN FIVE PREPARATIONS OF HYDROXYLAMINE-TREATED GELATIN FROM ICHTHYOCOL[a]

Dialysis	μmoles Hydroxamic Acid per 100 mg Protein
None	6.0
72 hr. at 5°	5.3
24 hr. at 8°	4.5
35 hr. at 25°	3.4
35 hr. at 25°	3.2

[a] Treated at 40° for 90 minutes with 1 M hydroxylamine at *p*H 10.0.

The Nature of the Peptide Fraction Containing Acid Hydrazide Groups.—Figure 4 shows the elution pattern obtained when a collagenase digest of hydrazine-treated gelatin from ichthyocol was chromatographed on phosphocellulose. With respect to location of acid hydrazide groups, one discerns two zones of peptides; that designated as zone II contains approximately 86% of the total acid hydrazide in association with peptide. The aggregate peptides of zone II were then converted to the strongly anionic hydrazones of *o*-benzaldehyde sulfonic acid, and chromatography of these derivatives yielded the elution pattern seen in Figure 5. It can be seen that the peptides containing hydrazone groups were eluted selectively with the buffer of lower *p*H value and ionic strength, so that they were obtained separate from peptides which did not contain the hydrazone function. That such a separation indeed had occurred was demonstrated by a glycine-hydrazone ratio of 1.9:1 compared to a glycine–acid hydrazide ratio of 69:1 found in the initial mixture of peptides (see Table III).

TABLE III
COMPOSITION OF HYDRAZONE-CONTAINING PEPTIDES FROM ICHTHYOCOL COMPARED WITH THAT OF A HYDRAZINE-TREATED CONTROL OF ICHTHYOCOL GELATIN

Component	Hydrazone-Containing Peptides from Ichthyocol (Residues per 100 residues)	Hydrazine-Treated Control
Glycine	38.0	34.1
Proline	8.8	11.8
Hydroxyproline	0	6.7
Alanine	12.0	12.8
Aspartic acid	20.2	4.6
Glutamic acid	4.6	7.1
Threonine	1.8	2.7
Serine	3.6	3.2
Isoleucine	0.9	1.1
Leucine	3.2	2.1
Valine	4.5	1.6
Phenylalanine	0	1.4
Tyrosine	0	0.3
Methionine	0	1.2
Lysine	2.3	2.8
Arginine	0	4.5
Histidine	0	0.4
Hydroxylysine	0	0.7
Hydrazone or hydrazide	19.9	0.5

The amino acid contents of the aggregate fraction of peptides bearing hydrazone functions (approximately 4 to 6 peptides) are shown in Table III. It can be seen that the molar equivalents of aspartic acid (20.2) are almost equal to the number of hydrazone groups (19.9). An almost identical result was obtained in other experiments with three separate preparations of hydrazone-containing peptides. In contrast it is seen that the content of glutamic acid in these peptides is considerably less. These findings suggest strongly that the acyl functions of the hydrazine-sensitive groups in ichthyocol, from which the isolated hydrazones ultimately were derived, are aspartyl residues.

Further examination of Table III shows that aspartic acid (or hydrazone) occurs in a ratio of

approximately 2 with respect to proline. The significance of this observation will be dealt with in the discussion.

A similar analysis was performed with respect to hydrazone-containing peptides derived from gelatin of calf skin procollagen carried through identical procedures. The results, shown in Table IV, in general are similar to those obtained with fish gelatin. Strikingly, the ratio of aspartic acid to hydrazone is 1, again suggesting that aspartyl residues provide the acyl functions of the hydrazine-sensitive groups. The aspartic acid to proline ratio is approximately 2.5 instead of the 2 found with ichthyocol. The glutamic acid and threonine contents are higher than in the case of fish gelatin peptides, and small quantities of histidine and arginine are present. Whether these differences between fish and calf arise from experimental conditions or represent actual species differences cannot be said at present. What is most striking, however, is the close similarity of the over-all analyses obtained in the two cases.

TABLE IV
COMPOSITION OF HYDRAZONE-CONTAINING PEPTIDES FROM CALF SKIN GELATIN COMPARED WITH COMPOSITION OF A HYDRAZINE-TREATED CONTROL OF CALF SKIN GELATIN

Component	Hydrazone-Containing Peptides from Calf Skin Gelatin (Residues per 100 residues)	Hydrazine-Treated Control
Glycine	36.0	35.0
Proline	6.8	14.0
Alanine	12.0	12.0
Aspartic acid	17.6	4.68
Glutamic acid	6.7	7.78
Threonine	1.7	1.64
Serine	7.4	3.53
Isoleucine	1.1	1.1
Leucine	3.6	2.6
Phenylalanine	1.0	1.32
Lysine	3.1	2.81
Arginine	0.7	5.0
Histidine	1.1	0.73
Hydrazone or hydrazide	17.4	0.6

A note should be added concerning quantitative aspects of the preparation and chromatography of the peptides which contain hydrazone functions. Because of the number of steps in these procedures, and because elution of the peptides from phosphocellulose is apparently incomplete, final yields were of the order of 20 to 30% when calculated on the basis of initial equivalents of acid hydrazide. Further, in the final elution patterns obtained by chromatography of the hydrazone-containing peptides, a significant quantity of ninhydrin-positive material, subsequently identified as homoserine and/or α-aminobutyrolactone,

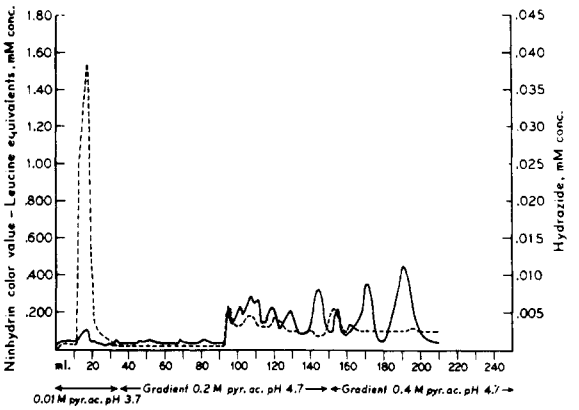
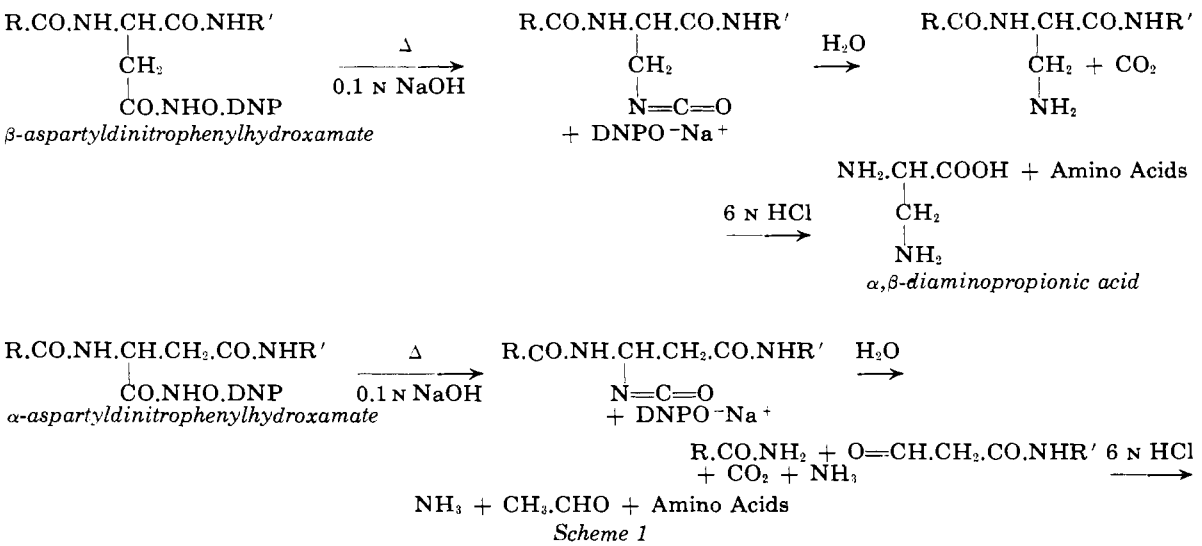


FIG. 5.—Rechromatography of zone II on 0.9 × 30 cm phosphocellulose column at 25° after reaction with o-benzaldehyde sodium sulfonate; (——) ninhydrin, (- - - -) hydrazone.

was found. The appearance of this amino acid conceivably could be related to the diminished yields of peptides, although a suitable mechanism is not apparent for conversion to homoserine of aspartyl residues bearing the particular hydrazone function.

The Nature and Amount of Amino-Terminal Groups in the Aggregate Peptides Containing Hydrazone Groups.—After treatment of this peptide fraction with 1-fluoro-2,4-dinitrobenzene, glycine was the only amino acid found as a dinitrophenyl derivative. This result is consistent with the established specificity of collagenase, the action of which results in peptides containing only amino-terminal glycine. However, with respect to the small number of peptides bearing the hydrazone function, this finding and the fact that the parent ichthyocol does not contain amino-terminal glycine indicate that the separated peptides originate either from carboxyl-termini of polypeptide chains in the protein or from more centrally located regions of the chains.

Analysis of the aggregate dinitrophenylated hydrazone-containing peptides, compared with analysis of a suitable control of hydrazone-containing peptides which had not been dinitrophenylated, showed that glycine decreased from 37.7 to 27.3 residues per 100 total residues, and further that this was the only amino acid to decrease. The extent of disappearance of glycine (10.4 residues) was approximately equal to the proline content (11.6 residues); this observation becomes significant when one considers that the specificity of collagenase requires that proline residues be penultimate to amino-terminal glycine residues in peptides resulting from its action. Thus the peptides probably originated from carboxyl-termini of polypeptide chains in the protein or most likely they would have contained additional proline residues. Also, from the decrease in glycine after dinitrophenylation one can calculate that the average chain length of a pep-



Scheme 1

tide in the fraction containing hydrazones was 10 amino acid residues.

Identification, by Lossen Rearrangement, of the Acyl Function of the Hydroxylamine-Sensitive Bonds in Ichthyocol.—As just discussed, amino acid analysis of the aggregate hydrazone-containing peptides strongly suggested that the acyl functions of the hydrazine-sensitive groups in gelatin are aspartyl residues. Independent evidence in support of this suggestion was then sought by application of the Lossen rearrangement to hydroxylamine-treated gelatin.

Earlier studies (Gallop *et al.*, 1960) had shown that, after Lossen rearrangement, each residue of β - aspartyldinitrophenylhydroxamate characteristically yields one residue of α,β-diaminopropionic acid. After Lossen rearrangement of α-aspartyldinitrophenylhydroxamate residues in a polypeptide one would find peptide-bound residues of malonic semialdehyde; after hydrolysis and decarboxylation by acid, each residue of semialdehyde appears as a molecule of acetaldehyde. These reactions may be summarized as shown in Scheme 1.

If the γ-carboxyl groupo f glutamic acid residues provided the acyl function of the hydroxamic acid, characteristically α,γ-diaminobutyric acid would be formed after Lossen rearrangement and acid hydrolysis. If the α-carboxyl group of glutamic acid were involved, then succinic semialdehyde would be the characteristic aldehyde. In those reactions in which an aldehyde appears, two equivalents of ammonia would also be found.

Hydroxylamine-treated gelatin from ichthyocol was treated further with 1-fluoro-2,4-dinitrobenzene and the isolated dinitrophenylhydroxamate subjected to Lossen rearrangement. Complete amino acid analysis, including analysis for α,β-diaminopropionic acid and α,γ-diaminobutyric acid, was performed. These results are summarized in Table V, and the elution pattern of α,β-diaminopropionic acid is shown in Figure 6.

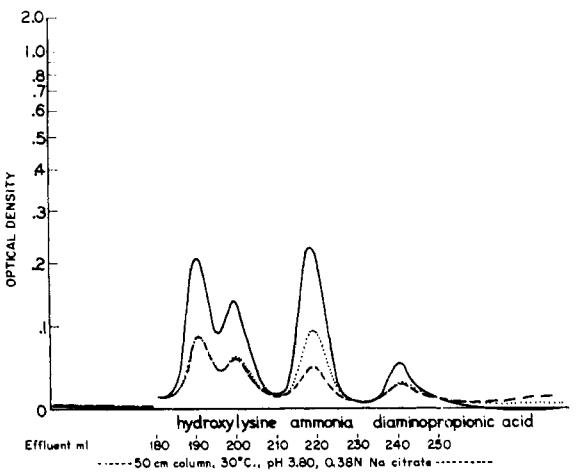


FIG. 6.—Chromatography of α,β-diaminopropionic acid from a 22-hour acid hydrolysate of hydroxylamine-treated gelatin from ichthyocol subjected to Lossen rearrangement. The quantity of hydrolysate used in this analysis corresponded to 13.2 mg of protein.

The data for products obtained shows the presence of α,β-diaminopropionic acid and an aldehyde as determined by the reaction of Sawicki *et al.* (1961). The sum of these equals approximately the aspartic acid which disappeared. These results indicate that both α- and β-carboxyl groups of aspartyl residues were present as hydroxamic acids and were, therefore, originally involved in the hydroxylamine-sensitive bonds of the protein. The data also indicate that the sensitive bonds are probably distributed equally between α- and β-carboxyl groups of aspartic acid residues. Since no α,γ-diaminobutyric acid was found and no disappearance of glutamic acid was observed, the participation of glutamic acid residues in the hydroxylamine-sensitive linkages can be excluded.

TABLE V

ANALYSIS OF MATERIAL OBTAINED AFTER LOSSEN REARRANGEMENT OF THE DINITROPHENYLHYDROXAMATE DERIVATIVE OF GELATIN FROM ICHTHYOCOL COMPARED WITH THE ANALYSIS OF A SUITABLE CONTROL GELATIN[a]

(All values expressed as residues or groups per 1000 total residues of amino acids)

Component	A. Control Gelatin	B. Treated Gelatin	Difference (B − A)
Hydroxamic Acid	0	4.9	4.9
Glutamic Acid	71.0[b]	71.4[b]	0.4
DAB	0	0	0
Aspartic Acid	46.0[c]	41.5[c]	−4.5
DAP	0	2.6	2.6
Aldehyde[d]	4.9	6.7	1.8

[a] If one adds the figure in the last column for difference in aldehyde to the difference in α,β-diaminopropionic acid a value of 4.4 is obtained. This is to be compared with the figure in the last column for disappearance of aspartic acid. It can be seen that the two figures are almost equal. [b] An average of 4 analyses. [c] An average of 6 analyses. [d] This value, expressed in terms of acetaldehyde equivalents, is obtained after the sample has been heated at 100° for 10 minutes in 0.1 N NaOH. The reasons for this procedure are discussed in footnote 3.

Identification of Products After Reduction of α,β-Polyaspartic Acid Ethyl Ester.—The third method employed for characterization of the acyl functions in the sensitive bonds of collagen was reduction by lithium borohydride. However, model studies were conducted first with the ethyl ester of a preparation of α,β-polyaspartic acid. In the experimental section of this paper we already have described the reduction of this compound with lithium borohydride.

The reduced polymer was hydrolyzed with 6 N HCl at 105° for 22 hours and a second sample with 1 N NaOH at 105° for 2 hours. The expected products (homoserine, β-amino-γ-hydroxybutyric acid, and their respective lactones) were separated by chromatography on Amberlite IR 120 as seen in the elution patterns in Figures 7b and 8b. In Table VI are presented the relative molar quantities of these compounds estimated by use of the data for color yields given in Table I.

Although 80% of the total nitrogen of the polymer was recovered, recovery of nitrogen in the form of expected products was approximately 40%. Ammonia and several as yet unidentified materials appeared in the chromatograms obtained on 15 and 50 cm columns. One can conclude that both α- and β-aspartyl esters had been present in the polymer before reduction. The ratios obtained for homoserine (and its lactone) to β - amino - γ - hydroxybutyric acid (and its lactone) indicate a preponderance of α-aspartyl esters in the polymer. This observation is consistent with the manner in which the original polyaspartic acid had been prepared from polyanhydroaspartic acid—hydrolytic opening of the imide rings by LiOH, which, like other nucleophilic agents, apparently causes preferential hydrolysis of imide bonds adjacent to an α-carboxyl function. The finding, in these experiments, of large amounts of ammonia indicates that reductive cleavage of N—C bonds probably occurred with formation of amide groups; the amides, on acid hydrolysis, would provide some of the ammonia.[4]

TABLE VI

PRODUCTS RECOVERED AFTER HYDROLYSIS WITH ACID OR ALKALI OF REDUCED α,β-POLYASPARTIC ACID ETHYL ESTER

	Experiment I Hydrolysis with Acid[a]	Experiment II Hydrolysis with Acid[b]	Experiment III Hydrolysis with Alkali[c]
(1) Aspartic acid, μmoles	1.31	0.34	0.52
(2) Homoserine, μmoles	0.84		0.61
(3) α-Aminobutyrolactone, μmoles		0.63	
(4) β-Amino-γ-hydroxybutyric acid, μmoles	2.94		2.32
(5) β-Aminobutyrolactone, μmoles		1.85	
Total N of polymer recovered as N of (1), (2), (3), (4), and (5) above, %[d]	41	42	33
Ratio, β-ester/α-ester	0.28	0.34	0.26

[a] 11.5 μmoles of polymer (calculated, assuming one N per residue, from N content) were treated with 6 N HCl for 22 hours at 105°; 150-cm column used for chromatography. [b] 6.7 μmoles of polymer were treated with 6 N HCl for 22 hours at 105°; 50-cm column used for chromatography. [c] 10.0 μmoles of polymer were treated with 1 N NaOH for 2 hours at 105°; 150-cm column used for chromatography. [d] The low recoveries in terms of the compounds listed is due to the fact that approximately 40% of the total N of the polymer appears as N of ammonia; the ammonia arises from amides formed by reductive cleavage of N—C bonds in the prior reduction with lithium borohydride.

Identification of the Acyl Functions of the Sensitive Bonds of Ichthyocol by Reduction with Lithium Borohydride.—Ichthyocol was reduced by lithium borohydride as described in the experimental section of this paper. The products obtained after hydrolysis with acid were chromatographed, before and after saponification with dilute alkali, on IR 120 columns. Elution patterns plotted in Figures 7c and 8c were obtained. These may be compared with elution patterns for

[4] Experiments in which *N*-benzoyl-α-aminosuccinimide was treated with lithium borohydride revealed that, to a large extent, reduction of the N—C bond occurred, and that benzamide was one of the products.

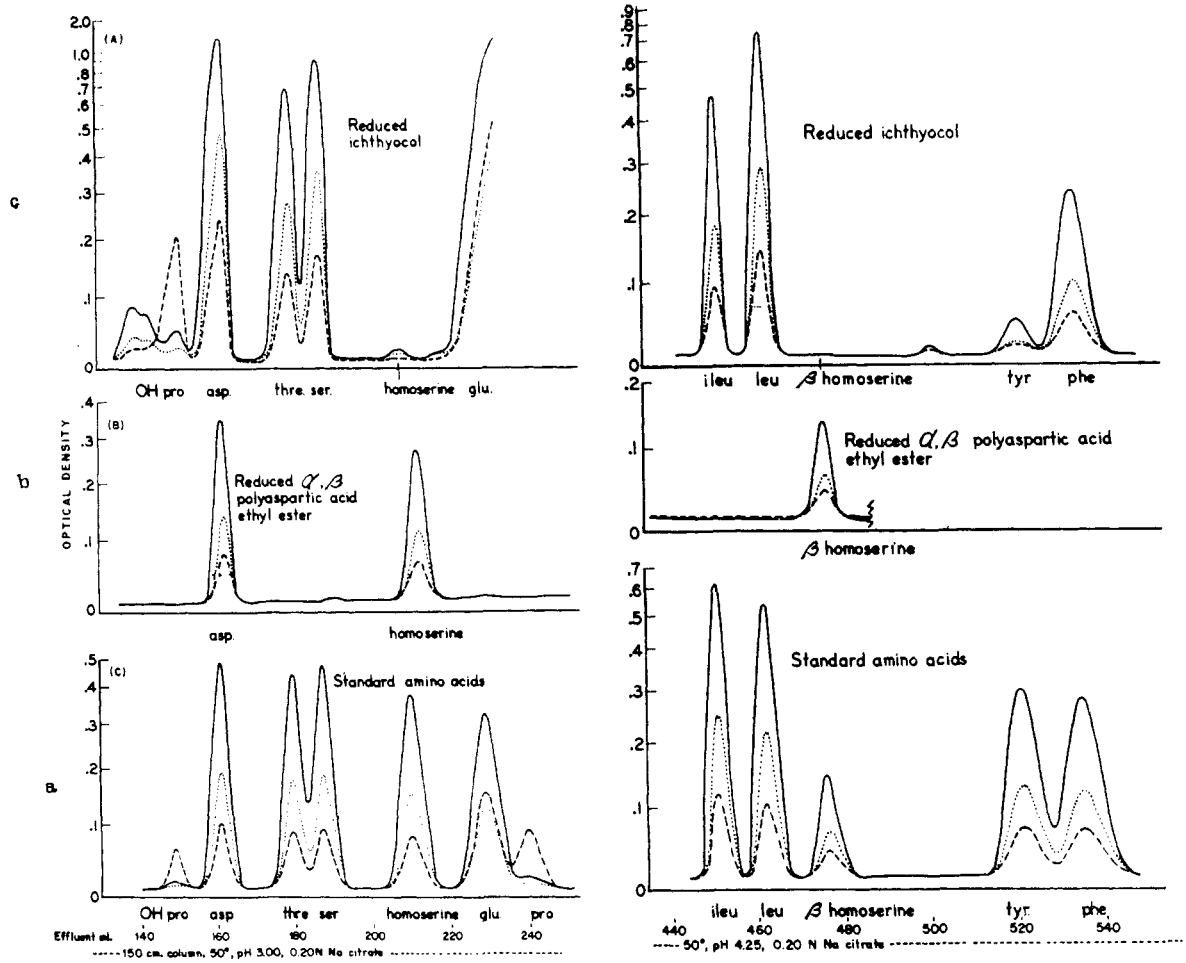


FIG. 7.—Amino acid analysis of (*a*) standard amino acids, (*b*) reduced α,β-polyaspartic acid ethyl ester, (*c*) reduced ichthyocol. (*b*) and (*c*) hydrolyzed in 6 N HCl for 22 hours at 105°, then treated with 0.01 N NaOH for 5 minutes at 100°. The quantity of hydrolysate used corresponded to 1.2 mg of reduced polymer and to 4.7 mg of reduced ichthyocol.

products obtained after reduction of polyaspartic acid ethyl ester and for standards of homoserine, β-amino-γ-hydroxybutyric acid, and their respective lactones.

It is clear from Figures 7 and 8 that homoserine and β-aminobutyrolactone were obtained after reduction of ichthyocol. Because of the small quantity of product which could possibly be obtained after reduction of 6 ester or imide groups per 1000 amino acid residues, and because these products would have low color yields in the ninhydrin assay, relatively large quantities of hydrolysate of reduced ichthyocol were used for chromatography.

Using a 15-cm Amberlite IR 120 column, one observes that α-aminobutyrolactone emerges very close to the peak of ammonia, characteristic effluent volumes being 84 and 95 ml respectively. In the elution pattern shown a protein hydrolysate corresponding to 14 mg of protein was used; this contained 7 μmoles of ammonia, and necessitated the introduction of ninhydrin into the apparatus after the passage of 85 ml of effluent. Thus it was not possible to distinguish the α-aminobutyrolactone peak from the ammonia tail. However, β-aminobutyrolactone, which emerges at an effluent volume of 108 ml, is seen clearly, and corresponds to 0.80 residues per 1000 total amino acid residues.

Chromatography, on a 150-cm column, of the products after saponification of the lactones revealed the presence of 0.68 residues of homoserine per 1000 total amino acid residues. Since the color yield with ninhydrin of β-amino-γ-hydroxybutyric acid is very low (0.087 as seen in Table I), one should not expect to observe a peak corresponding to this compound in the elution pattern unless much greater quantities of protein hydrolysate were employed.

Thus the yields of products after reduction are lower than expected from theoretical considerations. This may be due to incomplete reduction of the protein, intervention of side-reactions during reduction, or both. (It should be noted

that the amino acid analysis of the reduced ichthyocol did not differ significantly from that of the untreated ichthyocol.) However, trace quantities of ornithine and α,γ-diaminobutyric acid, originating probably from reduction of glutamine and asparagine, were observed in elution patterns obtained with a 50-cm column; several unidentified components also were observed, and these are under investigation presently. Despite failure to obtain quantitative results, appearance of homoserine and β-aminobutyrolactone after hydrolysis of reduced ichthyocol is strong supportive evidence that both α- and β-aspartyl residues contribute the acyl functions of the sensitive bonds in ichthyocol.

Discussion

Gallop *et al.* (1959) established the existence in collagen of "ester-like" bonds. These were defined by a special sensitivity, under relatively mild conditions of *p*H and temperature, to attack by nucleophilic agents such as hydroxylamine or hydrazine. The reactions of these agents produce smaller units of the collagen molecule which then bear characteristic hydroxamic acid or acid hydrazide groups, indicating that acyl functions are involved in the sensitive bonds. Since it is possible that the nucleophile-sensitive bonds are concerned with intramolecular crosslinking of collagen, it is important to determine their precise nature, to identify the acyl functions involved, and, if indeed they are esters, to identify the components bearing the alcohol function.

In this communication we have presented several types of evidence indicating that the acyl functions of the hydroxylamine- or hydrazine-sensitive bonds are aspartyl residues. The first body of evidence was obtained by separation, from a collagenase digest of hydrazine-treated gelatin, of an aggregate of peptides containing acid hydrazide groups. By conversion of these to corresponding hydrazones of *o*-benzaldehyde sulfonic acid, strongly anionic peptides were obtained which were easily separated, as a group, by chromatography on phosphocellulose. As yet no determined effort has been made to isolate individual peptides in this aggregate, but studies have been conducted on the whole fraction consisting of a mixture of approximately four to six peptides. Results of amino acid analysis of this fraction can be explained by assigning an average chain length of 10 residues to each of the constituent peptides, and by considering each to contain an amino-terminal residue of glycine followed by a penultimate residue of proline (only one proline residue being in the peptide). Further, each peptide can be considered to contain two residues of aspartic acid and two hydrazone groups. Because of the low contents of glutamic acid and the close correspondence of aspartic acid and hydrazone contents, it would appear that the acyl functions of the hydrazine-sensitive groups of collagen are aspartyl residues.

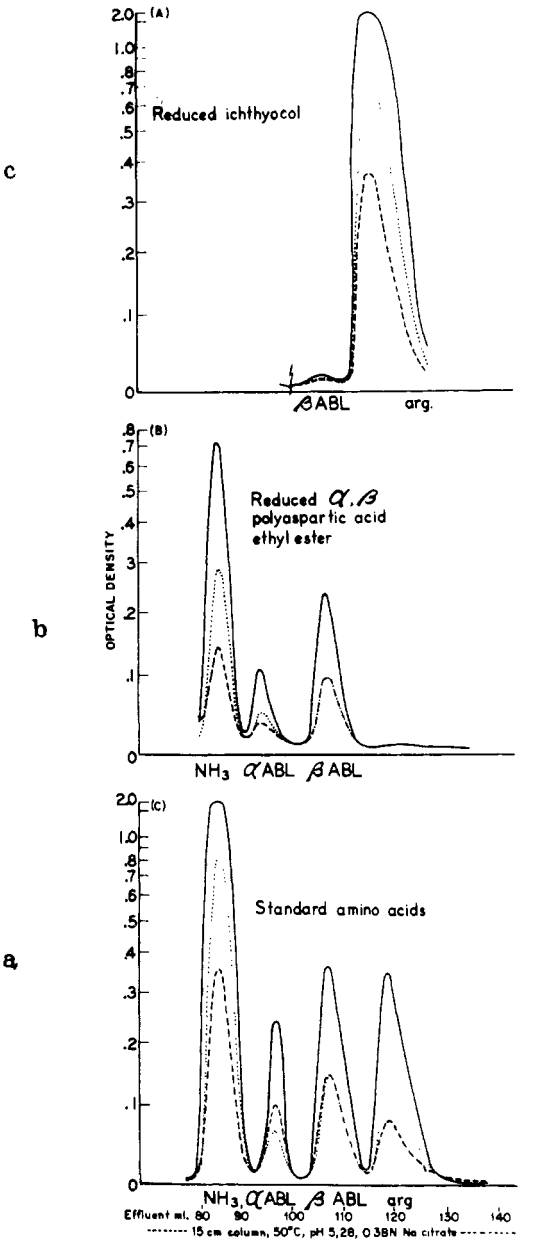


FIG. 8.—Chromatography of lactones obtained from 22-hour hydrolysates of (*a*) standard amino acids, (*b*) reduced α,β-polyaspartic acid ethyl ester, (*c*) reduced ichthyocol. The quantity of hydrolysate used corresponded to 1.1 mg for (*b*) and to 14.5 mg for (*c*).

The second line of evidence for participation of aspartyl residues in the sensitive bonds is derived from results of Lossen rearrangement of dinitrophenylhydroxamate derivatives of hydroxylamine-treated gelatin. Such experiments show that an acid hydrolysate of the Lossen-rearranged protein contains both α,β-diaminopropionic acid and an aldehyde, which in sum are equivalent to the moles of aspartic acid disappearing. Since α,β-

diaminopropionic acid can originate only from β-aspartyldinitrophenylhydroxamate residues, and since no α,γ-diaminobutyric acid (which characteristically would arise from γ-glutamyldinitrophenylhydroxamate residues) was found, it again was evident that aspartic acid residues provided the acyl functions of the nucleophile-sensitive linkages.

The third line of evidence implicating aspartyl residues results from analyses of acid hydrolysates of collagen previously reduced by treatment with lithium borohydride. In these experiments both homoserine and β-aminobutyrolactone were isolated, and these must have originated from reduction of the acyl functions of aspartyl residues in ester or imide linkages.

Of the three methods used to demonstrate the participation of aspartyl functions in the ester or imide linkages, the Lossen rearrangement is quantitative in the sense that one may obtain an absolute balance of reactants and products. The fact that the results secured by the other two methods are completely consistent, in a qualitative way, with those obtained by Lossen rearrangement attests further to the validity of all three methods.

In the experiments involving Lossen rearrangement, one starts with a cleavage of certain sensitive bonds by hydroxylamine. In the experiments in which hydrazone-containing peptides are ultimately separated, one begins with a scission of sensitive bonds by hydrazine. Finally, in the reductive method one cleaves bonds sensitive to reduction by lithium borohydride. Yet in each instance subsequent analyses lead to the same conclusion that aspartyl residues participate in those linkages which are sensitive to attack by nucleophilic agents and selective reducing agents. The evidence is cogent that all three methods result in cleavage of the same bonds. This belief is strengthened by the fact that treatment of gelatin with hydroxylamine results in the same number of hydroxamic acid groups as treatment with hydrazine yields acid hydrazide groups. Thus, the circumstance that neither the method involving separation of hydrazone-containing peptides nor the procedure with lithium borohydride was quantitative does not alter the conclusion that aspartic acid residues participate in the sensitive bonds.

There were also three independent experimental demonstrations that the aspartyl residues in the sensitive linkages are divided between α- and β-aspartyl groups. First, it was shown that approximately half of the hydroxamic acid groups appearing in hydroxylamine-treated gelatin were unstable to extended dialysis against distilled water—a reactivity shown, in model systems, to be characteristic of α-aspartylhydroxamic acid groups. Second, in the experiments with Lossen rearrangement of dinitrophenylhydroxamates of gelatin, both α,β-diaminopropionic acid and an aldehyde appeared in the acid hydrolysates; the former would originate from a residue of aspartic acid in combination through its β-carboxyl group with the dinitrophenylhydroxamate function, and the latter from a residue of aspartic acid in similar combination through its α-carboxyl function. Third, in the experiments in which collagen was reduced with lithium borohydride, the demonstration in acid hydrolysates of both homoserine and β-amino-γ-hydroxybutyric acid (or their respective lactones) indicates that both α- and β-aspartyl functions are involved in the nucleophile-sensitive linkages. All of the studies, in particular those dealing with the lability of the hydroxamic acids, suggest that half of the sensitive linkages occur through α-aspartyl residues and half through β-aspartyl residues.

Some inferences can also be drawn concerning the location of the sensitive bonds in collagen. From the evidence that half occur through α-acyl groups of aspartic acid residues, one must postulate that these residues are terminal functions or alternatively, occur in β-peptide linkage if they are located in regions remote from the termini of polypeptide chains. There is no evidence that aspartic acid occurs in collagen in β-peptide linkage (Franzblau, 1962), and it would seem more likely that the 3 α-aspartyl sensitive linkages mark the termini of three separate polypeptide chains in the protein. A second aspartyl residue bearing a nucleophile-sensitive group on its β-carboxyl function must then be located in the terminal amino acid sequence of each of these chains no farther removed from the first aspartyl residue than a distance of seven amino acid residues.

The isolated hydrazone-containing peptides each appear to contain an amino-terminal glycine residue, a single proline residue, and no hydroxyproline residues. From the established specificity of collagenase, the enzyme used in obtaining these peptides, one might anticipate that each peptide, if it originated from a region remote from the carboxyl-terminus of a polypeptide chain, should contain a second imino acid penultimate to its terminal carboxyl residue. Clearly, the isolated peptides do not fulfill this expectation. Since it is these same peptides that contain the two sensitive groups in close proximity, one may reasonably conclude that each peptide probably originates from the carboxyl-terminus of a polypeptide chain in collagen.

Thus far the evidence strongly indicates that the acyl functions of the nucleophile-sensitive linkages are provided equally by α- and β-aspartyl residues. However, the precise definition of the linkages is still not possible. One may state with some assurance that the sensitive bonds are either esters or imides, but none of the methods used sufficiently distinguishes between the two. Nucleophilic agents employed under mild conditions can rupture both ester and imide bonds, and lithium borohydride may cause reductive cleavage of both types of linkage.

Imides could be of two types: cyclic imides involving a single terminal aspartyl residue; and crosslinking imides involving the side-chain carboxyl groups either of terminal or internally located aspartyl residues on separate polypeptide chains. From the standpoint of crosslinking in collagen, interchain imide or ester bonds could serve equally well.

ACKNOWLEDGMENT

The authors are deeply indebted to Professor Sam Seifter for his invaluable advice and help.

REFERENCES

Bello, J. (1960), *Nature 185*, 241.
Bergmann, F., and Segal, R. (1956), *Biochem. J. 62*, 542.
Chibnall, A. C., Haselbach, D., Mangan, J. L., and Rees, M. W. (1958), *Biochem. J. 68*, 123.
Franzblau, C. (1962), Ph.D. Dissertation, Albert Einstein College of Medicine, Yeshiva University, New York.
Gallop, P. M. (1955), *Arch. Biochem. Biophys. 54*, 486.
Gallop, P. M., Siefter, S., Lukin, M., and Meilman, E. (1960), *J. Biol. Chem. 235*, 2619.
Gallop, P. M., Seifter, S., and Meilman, E. (1959), *Nature 183*, 1659.
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.
Hill, U. T. (1947), *Ind. Eng. Chem., Anal. Ed. 19*, 932.
Hörmann, H. (1960), *Das Leder 11*, 173.
Kovacs, J., Könyves, I., and Pusztai, A. (1953), *Experientia 9*, 459.
Kovacs, J., Kovacs, H., Könyves, I., Csaszar, J., Vajda, T., and Mix, H. (1961), *J. Org. Chem. 26*, 1084.
Lipmann, F., and Tuttle, L. C. (1945), *J. Biol. Chem. 159*, 21.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Sawicki, E., Hansen, T. R., Stanley, T. W., and Elbert, W. (1961), *Anal. Chem. 33*, 93.
Seifter, S., Gallop, P. M., Klein, L., and Meilman, E. (1959), *J. Biol. Chem. 234*, 285.
Seifter, S., Gallop, P. M., Michaels, S., and Meilman, E. (1960), *J. Biol. Chem. 235*, 2613.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Studies on the Structure of α_1-Acid Glycoprotein. III. Polymorphism of α_1-Acid Glycoprotein and the Partial Resolution and Characterization of Its Variants*

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Received January 15, 1962

α_1-Acid glycoprotein isolated from pooled normal human plasma was resolved on starch gel electrophoresis between *p*H 2 and 4 into seven variants. The best separation was obtained at *p*H 2.9, *i.e.* near the isoelectric point of this protein. It was demonstrated that this resolution is a consequence of the polymorphism of α_1-acid glycoprotein. The sialic acid–free form of pooled α_1-acid glycoprotein was separated into three zones by starch gel electrophoresis near its isoelectric point (*p*H 5.0). A large-scale method is described for the partial resolution of the variants of human α_1-acid glycoprotein by chromatography on diethylaminoethyl cellulose at *p*H 5.0. The obtained variants exhibited chemical and physical-chemical similarities and differences.

α_1-Acid glycoprotein (Schmid, 1953; Weimer *et al.*, 1950), as judged by classical criteria of purity, is one of the most highly purified proteins of normal human plasma. Thus, homogeneity of this protein was observed by free electrophoresis and ultracentrifugation over the *p*H range from 1 to 13, chromatography on Amberlite IRC-50, immunochemical and terminal amino acid analyses (Schmid, 1963). Moreover, monodispersity was also observed on starch gel electrophoresis at *p*H 8.6 (Smithies, 1959).

In the present paper a systematic starch gel electrophoretic investigation over the *p*H range from 1.4 to 8.6 is described which led to the discovery of the polymorphism of α_1-acid glycoprotein (Schmid and Binette, 1961). This observation suggested a study of the differences and similarities of the variants of this plasma protein.

* This is publication No. 321 of the Robert W. Lovett Memorial for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston. This study was aided by grants from The Lilly Research Laboratories, Eli Lilly and Company, the National Science Foundation (G-16253), and the National Institutes of Health, United States Public Health Service (A-3564[C2]).

† This work was carried out during the tenure of an Established Investigatorship from The Helen Hay Whitney Foundation, New York.