Isolation and Identification of α-Amino Aldehydes in Collagen*

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ABSTRACT: Six α-aminoaldehydes, namely glycinal, α-alaninal, α-aspartal, lysinal, serinal, and threoninal, are demonstrated to be present as components of various tropocollagens. They have been isolated after reduction to their corresponding α-amino alcohols with tritiated sodium borohydride. Considerable effort has been extended to demonstrate that they are onefold reduced, arising from a one-stage aldehyde to alcohol reduction, and are not artifactually derived by peptide or ester bond reductions to alcohols. Furthermore, direct treatment of the protein with 2,4-dinitrophenylhydrazine led to isolation of osazones which correspond to the products expected from at least four of the α-amino aldehydes by the Fischer α-amino aldehyde or α-acylamino aldehyde to osazone reaction. Much attention is directed to describing methodology leading to isolation and characterization of 2,4-dinitrophenylamino alcohols and 2,4-dinitrophenylhydrazones and -osazones, particularly by mass spectrometry, chromatography, and comparisons with authentic compounds. The syntheses of many such model compounds are described. There are a total of at least 12 α-amino aldehydes/tropocollagen molecule. It is suggested that they arise biosynthetically by carboxyl reductions of subunit C-terminal or branched C-terminal amino acid residues and that these α-amino aldehydes are involved in collagen and connective tissue maturation. They may participate in subunit, α chain, or tropocollagen attachment or cross-linking sites, or as intermediates in the further biosynthesis of cross-linking compounds. In the case of lysinal, which has already been shown to arise from lysine, its biosynthetic relationship to ω-amino aldehydes already partially characterized and also briefly noted in this paper is apparent. It appears certain that tropocollagens contain a variety of biosynthetic aldehyde intermediates, derived from some of their constituent amino acids in pathways leading to the maturation of connective tissue.

Over the past few years it has become increasingly apparent that covalently bound aldehydes are present in collagen (Gallop, 1964; Rojkind *et al.*, 1964, 1966; Bornstein *et al.*, 1966; Blumenfeld and Gallop, 1966; Bornstein and Piez, 1966; Piez *et al.*, 1966; Tanzer *et al.*, 1966; Schneider *et al.*, 1967). Although some of these aldehydes may be involved in cross-linking, details of their chemical nature, location, and attachment in the protein remain to be elucidated. This paper deals with investigations concerning a group of these compounds which are α-amino aldehydes. Some of these studies have been reported in a preliminary way (Blumenfeld and Gallop, 1966; Schneider *et al.*, 1967). Lysinal,[1] one of the family of α-amino aldehydes, already has been shown to be synthesized biologically, in rat skin tropocollagen, from lysine (Schneider *et al.*, 1967).

Evidence is presented to show that these α-amino aldehydes are bound to tropocollagen through their α-amino group as α-acylated amino aldehydes. The amino aldehydes can be reduced to radioactively labeled amino alcohols by treatment of the protein with an aqueous solution of tritiated sodium borohydride. In fact, the derived amino alcohols isolated after acid hydrolysis of the protein have been used to identify the corresponding parent aldehydes. Furthermore, additional evidence for the presence of these α-acylamino aldehydes is the production, as described, of corresponding DNP-osazones (bis-DNP-hydrazones) upon treatment of otherwise untreated tropocollagen with 2,4-dinitrophenylhydrazine.

Materials

Ichthyocol was prepared from the tissues of carp swim bladder and calf tropocollagen from calf skin

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: $DNPNHNH_2$, 2,4-dinitrophenylhydrazine; FDNB, 1-fluoro-2,4-dinitrobenzene; glycinol, ethanolamine; glycinal, aminoethanal; α-alaninol, 2-aminopropanol; α-alaninal, 2-aminopropanal; lysinol, 2,6-diaminohexanol; lysinal, 2,6-diaminohexanal; α-aspartol, 3-amino-4-hydroxybutyric acid (isohomoserine); α-aspartol (lactone), β-aminobutyrolactone; α-aspartal, 3-aminosuccinic semialdehyde; serinol, 1,3-dihydroxy-2-aminopropane; serinal, 3-hydroxy-2-aminopropanal; threoninol, 1,3-dihydroxy-2-aminobutane; threoninal, 3-hydroxy-2-aminobutanal; valinol, 1-hydroxy-2-amino-3-methylbutane; leucinol, 1-hydroxy-2-amino-4-methylbutane; phenylalaninol, 1-hydroxy-2-amino-2-benzylethane; enosoline, 6-aminohexenol; enosaline, 6-aminohexenal; dienosoline, di-(6-aminohexenol); dienosaline, di-(6-aminohexenal).

(Gallop and Seifter, 1963). Rat skin collagen was prepared from skins of rats previously administered lysine-^{14}C (Schneider *et al.*, 1967). Heat denaturation was achieved by suspending dried tropocollagen in water and heating for 10 min at 60°. The warm gelatin solutions were clarified by centrifugation in a Spinco Model L centrifuge at 100,000*g* at 40° for 20 min. The protein concentration in a solution was computed from a Kjeldahl nitrogen determination using the value of 18.9% N.

Tritiated sodium borohydride and lysine-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. 2-Acetamido-3-butanone, α-amino-γ-butyrolactone hydrobromide, 4-aminobutyraldehyde diethyl acetal, ethanolamine, DL-4-amino-3-hydroxybutyric acid, and 2-piperidinecarboxylic acid were purchased from Aldrich Chemical Co., Milwaukee, Wis. DL-α-Alaninol and purified DL-alanine ethyl ester hydrochloride were obtained from Mann Research Laboratories, New York, N. Y.

δ-Aminolevulinic acid hydrochloride was obtained from Calbiochem, Los Angeles, Calif., glycine ethyl ester hydrochloride and serinol from Nutritional Biochemical Corp., Cleveland, Ohio, and valinol, leucinol, and phenylalaninol from Research Organic Chemical Co., Sun Valley, Calif. Silicic acid, Bio-Sil A, was purchased from Bio-Rad Laboratories, Richmond, Calif., and the thin-layer chromatabs (Eastman Chromagram sheet (6061)) from Eastman Kodak Co., Rochester, N. Y. The spectroquality solvents were obtained from Matheson Coleman and Bell, Norwood (Cincinnati), Ohio. ε-Hydroxynorleucine was prepared from δ-bromobutylhydantoin (Eastman Kodak Co., Rochester, N. Y.) following the procedure of Gaudry (1948). Lysinonorleucine was a gift from Dr. Carl Franzblau of Boston University.

Methods

Calibration of Tritiated Sodium Borohydride. REDUCTION OF MODEL COMPOUNDS WITH TRITIATED SODIUM BOROHYDRIDE. $NaBH_4$-^{3}H (19 mg) (specific activity 200 mCi/mmole) was added to 1.4 g of carrier $NaBH_4$ and thoroughly ground in an agate mortar. This sodium borohydride preparation was calibrated by reduction of a ketone, aldehyde, or a specially reactive ester compound. The former two types of compounds involve a one-stage and esters a two-stage reduction. Since the carbonyl compounds of interest in this investigation are amino compounds, and the presence of an amino group is useful for the subsequent determination of specific radioactivity, the following compounds were used for calibration of the reagent: 2-acetamido-3-ketobutane, γ-DNP-aminobutyraldehyde, δ-amino-γ-ketovaleric acid, glycine ethyl ester, and DL-alanine ethyl ester. The reduction of the last three of these compounds for calibration of $NaBH_4$ was carried out as follows. $NaBH_4$-^{3}H (222 mg) was dissolved in 30 ml of water; 5 ml of this solution, containing 37 mg of $NaBH_4$-^{3}H or 1000 μmoles, was added to 250 μmoles of the standard compound dissolved in 5 ml of water. The reaction mixture was stirred at room temperature for about 20 hr. The pH of the solution finally reached a value of about 10, and then, to destroy excess $NaBH_4$, was lowered dropwise to about 2 with HCl.

DINITROPHENYLATION OF REDUCED MODEL COMPOUNDS. The solutions were evaporated down to dryness and treated at room temperature, overnight, with FDNB in the presence of 0.5 M carbonate buffer (pH 9.0) in 50% ethanol–water. The solutions were filtered, the precipitate containing mainly DNP-ethyl ether was washed with 50% ethanol–H_2O, and the washings were added to the filtrate. The DNP-amino derivatives of glycinol and alaninol were obtained by extraction of the alkaline filtrate with ethyl acetate (three times). The combined ethyl acetate extracts were washed several times with saturated $NaHCO_3$ and then water to remove dinitrophenol and any DNP-amino acids if present, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*.[2]

The DNP-amino derivative of δ-amino-γ-hydroxyvaleric acid was extracted into ether after acidification of the aqueous layer. The ether phase was dried over anhydrous Na_2SO_4, and the ether was then evaporated *in vacuo*. The residue was dissolved in ethyl acetate and subjected to vacuum sublimation in a cold finger to remove excess dinitrophenol.

PURIFICATION OF REDUCED DINITROPHENYLATED COMPOUNDS. The DNP derivatives of the model compounds were purified from DNP-ethyl ether and dinitroaniline by chromatography on a 0.9 × 17 cm column of silicic acid (Bio-Sil A, 30–60 μ) using benzene–ethyl acetate (4:1) as eluent. Fractions (1 ml) were collected; each was evaporated to dryness and redissolved in a suitable volume of ethyl acetate. For each fraction the absorbance at 348 mμ and the radioactivity were determined. It was found that some of these compounds on chromatography exhibited an isotope effect in that the absorbance peak always slightly preceded the radioactivity peak (see Figure 3 for isotope effect of similar compounds isolated from collagen). The lag of radioactivity varied with the compound and usually was not more than one to two tubes. However, unless a correction was used, this lag could cause very significant errors in the calculation of specific activity of a given compound. The correction was made by plotting radioactivity and absorbance of consecutive fractions on two different semilog plots and superimposing the curves, now of similar shape, to determine the number of fractions involved in the lag. The specific activity of the component contained in individual fractions could then be correctly computed by juxtaposing the corresponding tubes of absorbance and radioactivity. Often, for purposes of calculation of specific activity, pooling of the total peaks emerging was found more expedient. The purity of each peak was checked further by chromatography on thin layers of silicic acid where a single yellow and radioactive spot was present. To determine radioactivity the strip of the thin-layer plate was cut

[2] The use of stopcock grease was avoided during the course of this investigation since contamination with trace amounts occasionally showed in the background of spectra obtained in the mass spectrometer.

into 0.5-cm sections, the silicic acid in those sections was scraped off and transferred to a small tube, and 1 ml of ethanol or ethyl acetate was added. The material was then eluted by heating the silica suspension to 60°, with shaking. The suspension was centrifuged, and an aliquot of the supernatant solution was used for determination of radioactivity.

The absorption spectra of the *N*-dinitrophenylated amino alcohols in ethyl acetate showed a peak at 348 mμ, a shoulder at 405 mμ, and a trough at 293 mμ. The purity of each compound could be assessed from the ratio of the absorption at 348 mμ to that at 293 mμ, which in a pure compound is about 10:1. After examining about 20 model compounds which varied in their extinction coefficients from 16 to 18 $\times$ 10^3 and from 346 mμ to about 350 mμ in their maximal absorption, the average extinction coefficient of pure compounds was taken as 17,000.

DETERMINATION OF SPECIFIC ACTIVITY OF REDUCED DINITROPHENYLATED MODEL COMPOUNDS. Specific activity of a compound of this type was determined from the measure of its radioactivity in relation to its absorbancy at 348 mμ. All samples were counted in a Packard Tri-Carb scintillation counter using 10 ml of scintillation fluid consisting of 200 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 8 g of 2,5-diphenyloxazole in 2 l. of toluene. A small aliquot of tritiated water dissolved in ethanol, of known disintegrations per minute, was used as a standard for calculation of efficiency. In all cases, aliquots of different magnitude were used to establish that no quenching of counts due to ultraviolet absorption of the DNP group occurred. Because of the high specific activities employed, such quenching was negligible with the aliquots measured.

Other model carbonyl compounds were used during the course of this investigation for the calibration of $NaBH_4$-^3H. The calibration with 2-acetamido-3-ketobutane is described in a previous publication (Blumenfeld and Gallop, 1966).

DNP-γ-aminobutyraldehyde can also be used for calibration. In this instance, the reduction is carried out in ethanol–water (1:1). At the end of the reaction time the solution is evaporated to dryness, washed several times with ethanol, and then dried. This procedure facilitates complete removal of all exchangeable counts. The reduced and dinitrophenylated derivative (4-DNP-aminobutanol) is then chromatographed on a silicic acid column and its specific activity determined as described above.

Reduction of Various Acid-Soluble Tropocollagens. To 500 ml of a 2% solution of heat-denatured acid-soluble collagen, adjusted to pH 8, were added 10 ml of 1% Versene and about 600 mg of radioactive precalibrated $NaBH_4$. This represented approximately a 200-fold M excess of $NaBH_4$/mole of α chain, or roughly a 300-fold M excess/mole of directly measureable aldehyde as determined by the colorimetric and spectrophotomeric procedures (Paz *et al.*, 1965) using *N*-methylbenzothiazolone hydrazone. The reaction mixture was maintained at pH 8–9, by addition of small amounts of 1 N HCl, and stirred at room temperature for about 1 hr. The reduction was terminated by lowering the pH to about 4 to destroy excess $NaBH_4$, and the solution was then dialyzed in the cold against many changes of distilled water, until constant specific activity (disintegrations per minute per milligram of protein) was achieved. As expected, the colorimetric test for free aldehydes in the $NaBH_4$-reduced protein was negative.

Isolation of Amino Alcohols from Sodium Borohydride Treated Tropocollagen. Reduced tropocollagen of constant specific radioactivity was hydrolyzed for 22 hr in 6 N HCl, under reflux. The HCl was removed *in vacuo;* all the radioactivity was recovered in the residue which, in addition to the constituent amino acids and ammonia, contained the tritium-labeled alcohols. No significant difference in amino acid composition could be determined between reduced and unreduced tropocollagens.

Since all the radioactivity of the residue of the hydrolysate was retained on Dowex 50-X8 columns and could be eluted subsequently with concentrated buffers of increasing pH, it was concluded that the tritium-containing alcohols had a basic group, most likely an amino group. This was confirmed in the following way. The residue of the hydrolysate was dissolved in distilled water, and an aliquot was treated at 100° for 5 min with an excess of ninhydrin reagent in order to deaminate any amino compounds present. The radioactivity of the treated residue, in contrast to that of the nontreated, was not retained on Dowex 50-X8 columns with water elution, proving that the basic group indeed was of an amine nature.

The tritiated amino alcohols were separated from the bulk of amino acids and ammonia by chromatography on Dowex 1-X8 (200–400 mesh, OH^- form) and IR 120 (Spinco automatic amino acid analyzer) (Spackman *et al.*, 1958). A Dowex 1 column, 1.6 $\times$ 100 cm, was generally employed for an aliquot of hydrolysate corresponding to about 1 g of reduced protein determined either by a Kjeldahl nitrogen determination or by its radioactivity. (To determine the radioactivity of aqueous samples, 3 ml of Hyamine was used with 10 ml of scintillation fluid.) The residue of the hydrolysate was dissolved in water and the solution was adjusted to pH 7–8 before application to the column. A basic function (B) was eluted with water (about 200 ml), and then a neutral fraction (N) was eluted with 0.4 N acetic acid. Radioactivity and a ninhydrin determination (Rosen, 1957) were carried out on aliquots of each fraction. For each radioactive peak, tubes were pooled and an amino acid analysis was performed on an aliquot using the automatic technique of Spackman *et al.* (1958). Occasionally, with a Dowex 1 (OH^-) column, bubbles appeared in the resin after the introduction of acetic acid. When this occurred, the resin bed was withdrawn from the column and the neutral peak was eluted in batch form. The basic and neutral fractions were each concentrated to a small volume, and further fractionations were carried out in several repeated operations on an IR 120 column, using the Spinco automatic amino acid analyzer Model 120B, equipped with the split-stream attachment.

The basic fraction, B, was resolved into four radio-

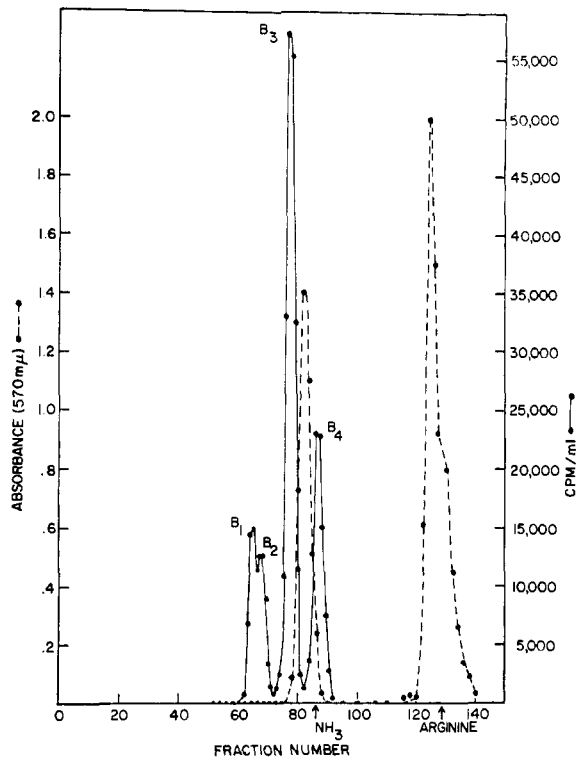


FIGURE 1: Elution pattern of the basic fraction B on a 10-cm column of IR 120 using 0.35 N citrate buffer (pH 5.28) at 50°; (- - -) ninhydrin; (——) counts per minute.

active peaks on the 10-cm column using 0.35 N sodium citrate buffer (pH 5.28) at 50°, but one component, subsequently shown to be lysinol, was retained on this column and could be eluted with 0.5 N NaOH. The elution pattern obtained is shown in Figure 1. The neutral fraction, N, was resolved into six radioactive peaks on the 54-cm column using 0.2 N sodium citrate buffer (pH 3.05) for the elution of the first four small peaks, N_1a–d, and 0.2 N sodium citrate buffer (pH 4.25) for the elution of the other two sharp peaks, N_2 and N_3. The elution pattern obtained is shown in Figure 2. A very low background of radioactivity was present in all the fractions corresponding to some non-specific tritiation of amino acids most likely by very radioactive tritium gas formed by destruction of $NaBH_4$-^3H with acid.

Isolation of Pure Amino Alcohols as Their N-Dinitrophenylated Derivatives. DINITROPHENYLATION OF THE B FRACTION. In separate experiments, the entire basic peak obtained from chromatography on Dowex 1, or each of the individual peaks combined from repeated split-stream chromatographic separations on the 10-cm column of IR 120, was treated overnight, at room temperature, with an excess of FDNB in 0.5 M sodium carbonate buffer (pH 9) in 50% ethanol–water. The mixture was filtered and extracted three times with ethyl acetate. The ethyl acetate layer was washed several times with saturated $NaHCO_3$ and water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was dissolved in a small volume of ethyl acetate and the radioactivity was determined. Recovery of radioactivity from the whole B fraction was 50–60%,

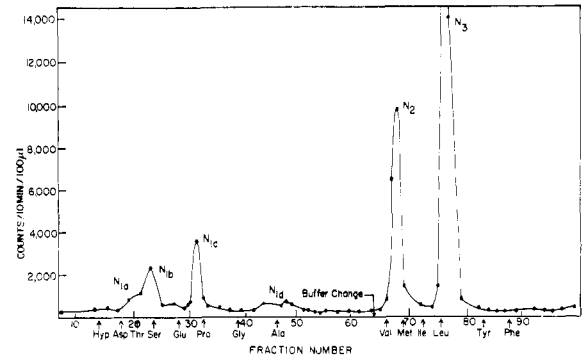


FIGURE 2: Elution pattern of the radioactive compounds of the neutral fraction N on a 54-cm column of IR 120 using 0.2 N citrate buffer of pH 3.05, followed by 0.2 N citrate buffer (pH 4.25) at 50°. The locations of the peaks of the amino acids which would be found in an acid hydrolysate of collagen are indicated.

some losses occurring during the washing procedures; however, some radioactive material did remain in the aqueous phase. The latter radioactivity could be extracted into butanol, and the nature of the responsible compounds will be discussed in a subsequent report.

DINITROPHENYLATION OF THE N FRACTION. The radioactive peaks N_2 and N_3 obtained from several (at least ten) chromatographic experiments on the 54-cm column of IR 120 were dinitrophenylated with excess FDNB in 50% ethanol–water at room temperature and for 3.5 hr; pH was maintained at 8.5–9.0 by addition of small amounts of NaOH. The alkaline solution was extracted with ethyl acetate and after acidification to pH 2 all radioactivity was extracted with ether or ethyl acetate. The ether or ethyl acetate layer was washed with dilute HCl and water and dried in a cold finger which also sublimed traces of 2,4-dinitrophenol.[3] The residue was dissolved in a small volume of ethyl acetate and radioactivity determined.

Since peak N_3 emerged from the IR 120 column after appearance of leucine and before tyrosine (see Figure 2), the product obtained following its dinitrophenylation would contain only traces of DNP-leucine, and indeed, pure "DNP-N_3" could readily be obtained by chromatography on silicic acid columns as shown below. Peak N_2, however, was eluted on columns of IR 120 together with valine, isoleucine, and leucine, and its dinitrophenylated product contained relatively large amounts of the DNP derivatives of these amino acids. Since, as shown subsequently, compound DNP-N_3 was present in the form of a lactone after sublimation, it was assumed that DNP-N_2 might also be present as a lactone after anhydrous heating, an assumption which proved correct. Therefore, the dinitrophenylated product of fraction N_2 was dissolved in ethyl acetate and extracted several times with dilute $NaHCO_3$. The radioactivity did not appear in the aqueous bicarbonate phase. Thus, the ethyl acetate

[3] Subsequently this step in the procedure was shown to facilitate lactonization of the DNP derivatives of N_2 and N_3, both of which contain a free carboxyl group as well as an alcohol function.

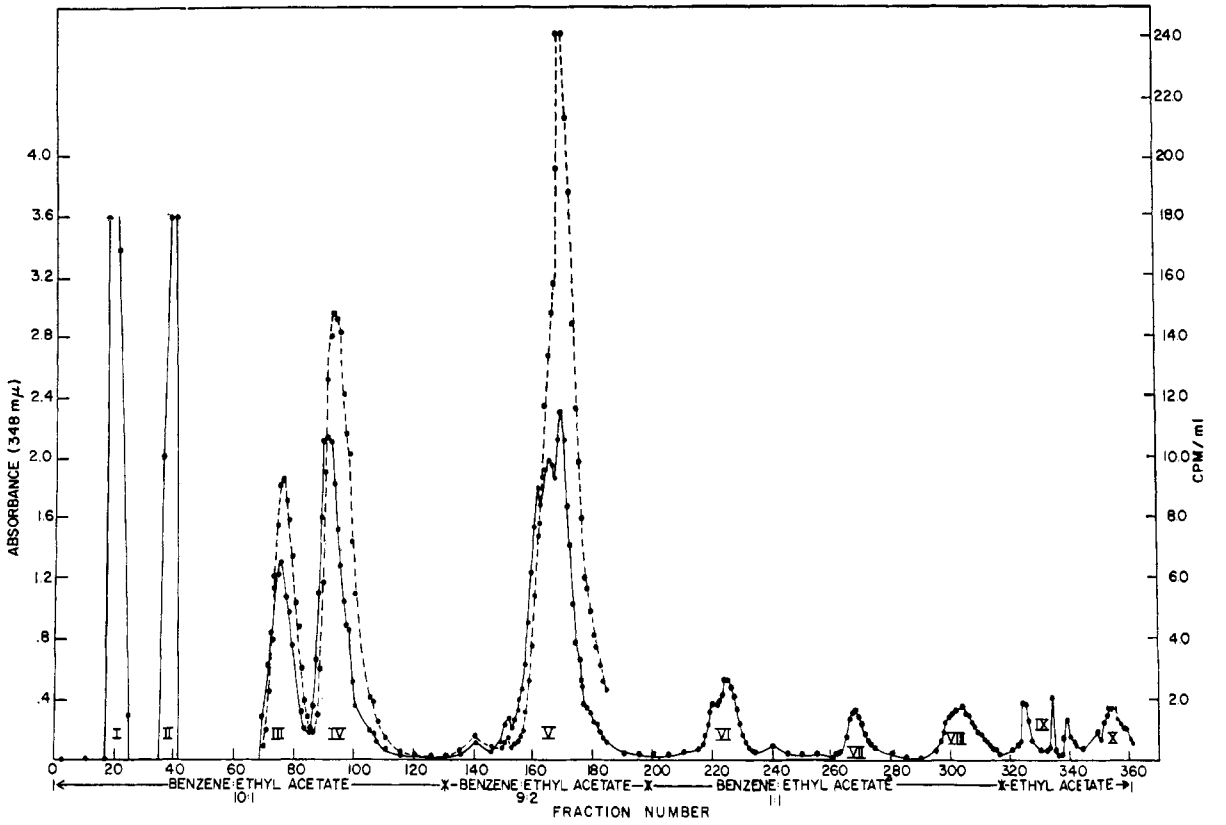


FIGURE 3: Elution pattern of dinitrophenyl compounds of fraction B on a 1.1 × 50 cm silicic acid column; (——) absorbance at 348 mμ; (- - -) counts per minute per milliliter. Fractions VI–X were radioactive but were counted as the pooled peaks.

layer, now containing only traces of the DNP-amino acids, retained the radioactivity; it was then concentrated to a small volume, dried over Na_2SO_4, and then purified with use of columns of silicic acid.

Preparation and isolation of the DNP derivatives of peaks N_1 have not yet been undertaken.

ISOLATION OF PURE *N*-DNP-AMINO ALCOHOLS. Chromatography on silicic acid columns (Bio-Sil A, 10–30 μ, without binder) and two-dimensional chromatography on thin layers of silicic acid were used.

For isolation of the DNP-amino alcohols of the basic fraction from a sample with radioactivity equivalent to that of about 100 mg of the parent reduced protein, a column 1.1 × 50 cm was used. The radioactive dinitrophenylated mixture was usually dissolved in a small volume of the solvent of the same composition as the initial solvent used for elution. Solvents composed of benzene and increasing amounts of ethyl acetate and finally ethyl acetate or ethanol were used as indicated in Figure 3. In some experiments, optical density at 348 mμ was read on every other fraction and in other experiments radioactivity was determined as well. Since, as with model compounds, an isotope effect was usually present, the entire peaks of the basic fraction were pooled. In early experiments, each peak was rechromatographed to constant specific activity using smaller columns of silicic acid. It was subsequently found that other compounds, in lesser amounts, were associated with some of these peaks. This was particularly true in preparative experiments in which large amounts of the basic fraction were used. Some of the compounds present were di-DNP derivatives, both of lysinol, present as a *N,N'*-DNP derivative, and of some of the amino alcohols, which, under the conditions used for dinitrophenylation, had formed *O,N*-di-DNP derivatives. In these instances, the following procedure of purification was required and was subsequently routinely used for all peaks. Pooled peak fractions were subjected to two-dimensional thin-layer chromatography on silicic acid. Solvents containing benzene–ethyl acetate (7:3) were used for the first dimension, and distilled water for the second dimension. The water system was found to be selective for separation of di-DNP-lysinol and other di-DNP compounds from most other DNP-amino alcohols. For the fractions which progressively were more polar, increasing amounts of ethyl acetate were used with benzene in the first dimension. This is shown in Figure 4 for a pooled fraction obtained from a basic DNP fraction from ichthyocol, eluted from a silicic acid column with benzene–ethyl acetate (10:1). The yellow spots from several thin-layer chromatograms were removed by scraping and those marking identical locations pooled. The pure DNP derivatives were eluted from the silicic acid by adding ethyl acetate, warming the contents, and centrifuging to remove silicic acid.

A solvent consisting of benzene, pyridine, and glacial acetic acid (80:20:2) was employed for separation of neutral fractions N_2 and N_3, either for column or thin-layer chromatography. The radioactive peaks of these

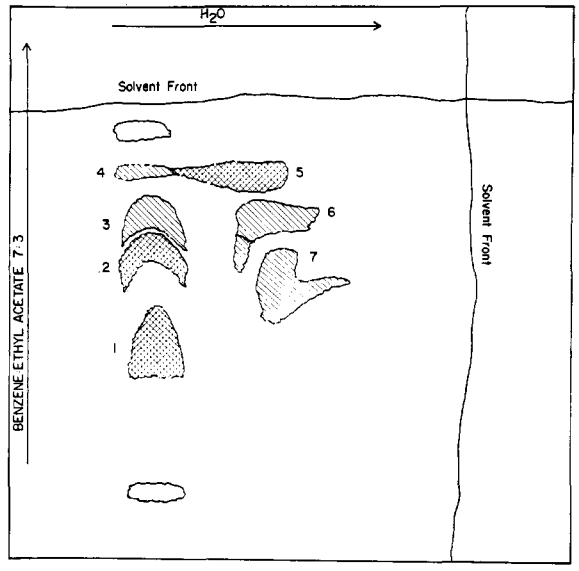


FIGURE 4: Two-dimensional chromatography on a thin layer of silicic acid of a pooled peak obtained from dinitrophenylated fraction B (from ichthyocol preparation E). This peak was eluted from a silicic acid column (2 × 25 cm) using benzene–ethyl acetate (10:1). Position of elution was between 63 and 90 ml. The first dimension was benzene–ethyl acetate (7:3); second dimension, water. Different spots were identified as follows: spot 1, *N,N'*-di-DNP-lysinol; spot 2, *O,N*-di-DNP-glycinol; spot 3, *O,N*-di-DNP-α-alaninol; spot 6, *N*-DNP-α-alaninol; spot 7, *N*-DNP-glycinol. Spots 4 and 5 contained negligible amounts of radioactivity and are in part dinitrophenyl ethyl ether and DNP-aniline.

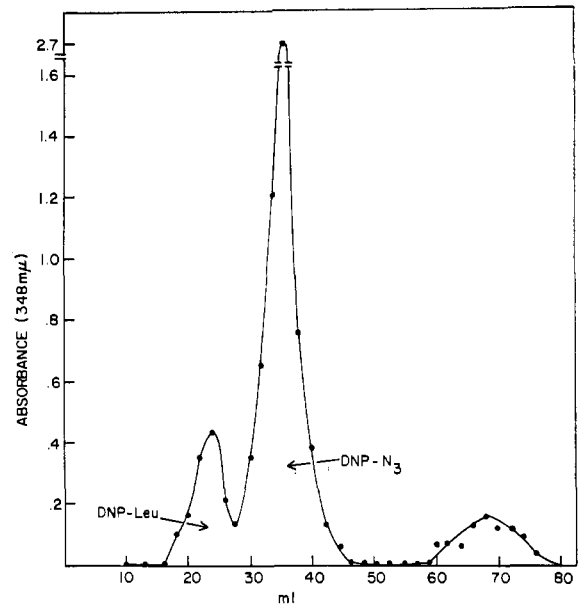


FIGURE 5: Elution pattern of dinitrophenylated fraction N_3 on a 1 × 15 cm silicic acid column, using benzene–pyridine–glacial acetic acid (80:20:2). DNP-N_3 was radioactive and DNP-leucine was not.

fractions as obtained from chromatography on silicic acid columns were usually free of impurities. (See Figure 5 for chromatography of DNP-N_3.) Whenever thin-layer chromatography of a fraction indicated the presence of impurities or more than one component, the peak contents were rechromatographed on smaller columns of silicic acid, usually 1 × 12 cm, using benzene–pyridine–glacial acetic acid (80:20:2). Identical rechromatographed peaks were pooled and again checked for purity.

The specific radioactivity of a homogeneous (pure) peak was determined as described previously for model compounds. This then was compared with the specific activities of products obtained, in the calibration procedure, by reduction of model compounds with the same lot of sodium borohydride which had been used in the reduction of collagen from which the compound in question was isolated. This comparison established whether the isolated compound originated as a result of a one- or twofold reduction and was essential for the conclusion that it was derived from a parent aldehyde or ketone component of the protein rather than from reduction of a peptide bond.

Identification of DNP-amino Alcohols. Since the amounts of pure materials obtained from various reduced collagens were very small, mass spectrometry was used to facilitate their identification. The measurements were performed in some cases on an Associated Electrical Ind. MS-9 double-focusing mass spectrometer and in other cases on the Hitachi RMU6 using the direct sample insertion technique. Spectra were taken at several consecutive intervals of temperature from 70° to more than 300°.

The pure *N*-dinitrophenylated compounds obtained from silicic acid columns and thin-layer chromatography were used for analysis in the mass spectrometer. After evaporation of solvent, the residues of pure material were washed free of most solvent impurities with petroleum ether (bp 37.4–50.7°) and dissolved in a small volume of spectrophotometrically pure dichloromethane for transfer to the small glass crucibles or ceramic rod employed with the direct inlet system of the spectrometer. After evaporation of the solvent, the sample was again washed with petroleum ether and dried *in vacuo*.

The mass spectra of several known *N*-dinitrophenylated amino alcohols, such as DNP-glycinol, DNP-alaninol, DNP-α,β-aminobutyrolactones, *N,N'*-di-DNP-lysinol, and many others, were determined for use as reference compounds. The properties and mass spectra of these compounds are described in Table I in the Appendix and in previous publications (Blumenfeld and Gallop, 1966; Schneider *et al.*, 1967).

In most cases the identity of the amino alcohols from the protein as obtained from mass spectrometry was confirmed by cochromatography with established authentic compounds. Chromatography on thin layers of silicic acid using the solvents previously described was used for the *N*-DNP derivatives of the compounds. The underivatized compound, N_3, which was identified as α-aspartol, was in addition cochromatographed with an authentic sample of the compound on the 54-cm column of IR 120 using the split-stream attachment. The correspondence of the radioactivity of the unknown compound and the ninhydrin color equivalent of the authentic compound was determined. Whenever pos-

TABLE I: Elution Positions and Color Yields with Ninhydrin of Standard Amino Alcohols upon Chromatography on IR 120 Columns, at 50°.

Compound	Column Size (cm)	Buffer Used[a]	Std Ref Compd	Position of Elution Rel to Std Ref Compd	Color Yield Compared with Leucine = 1
Glycinol	10[b]	3	NH_3	0.92	0.80
α-Alaninol	20[c]	3	NH_3	1.00	0.10
β-Alaninol	10[b]	3	NH_3	0.84	
3-Amino-2-hydroxy-butane	10[b]	3	NH_3	1.14	0.85
5-Amino-1-hydroxy-pentane	23[c]	3	NH_3	1.54	1.0
Serinol	23[c]	3	NH_3	0.76	0.27
Lysinonorleucine	25[c]	3	NH_3	0.43	0.25
Lysinol	20[c]	3	NH_3	Retained (eluted with NaOH)	0.60
β-Aminobutyrolactone	20[c]	3	NH_3	1.32	0.002[d]
ε-Hydroxynorleucinol[e]	15[b]	3	NH_3	1.47	0.30
β-Amino-γ-hydroxy-butyric acid	150[c]	1 + 2	Leucine	1.05	0.54[f]
ε-Hydroxynorleucine	54[b]	1	Glycine	1.10	1.0
ε-Hydroxynorleucine[g] (after acid hydrolysis)	54[b]	1	Glycine	(a) 1.10	0.54
		1 + 2	Leucine	(b) 1.22	0.46
ε-Hydroxynorleucine[g] (base treated)	54[b]	1	Glycine	(a) 1.10	0.50

[a] Buffers used: 1 = 0.2 N sodium citrate (pH 3.0), 2 = 0.2 N sodium citrate (pH 4.24), and 3 = 0.38 N sodium citrate (pH 5.28). [b] Beckman PA-27. [c] Beckman PA-35 resins. [d] β-Amino-γ-hydroxybutyric acid obtained from chromatography on Dowex 1 (lactonized by refluxing 1 hr in 6 N HCl). [e] See footnote 5 in text. [f] β-Amino-γ-hydroxybutyric acid obtained from chromatography on Dowex 1 (neutral fraction). [g] Acid hydrolyzed: 6 N HCl, 24 hr, 105° *in vacuo;* base hydrolysis: pH 13, 5 min, 100°. There is 54% hydroxynorleucine (a) and 46% ε-chlorononorleucine (b). After base treatment the latter compound is not seen in that it is converted in part into pipecolic acid with a low color yield. See footnote 5 in text.

sible the basic amino alcohols were cochromatographed, in a similar manner, with authentic samples of these compounds on 10-cm columns of IR 120. Additional model compounds such as ε-hydroxynorleucine were also studied for possible relationship to the separated amino alcohols under investigation. The elution positions of these standard amino alcohols are included in Table I.

Action of Periodate on the Intact $NaBH_4$-Reduced Protein and on the Amino Alcohols Isolated from Its Acid Hydrolysate. Reduced ichthyocol (34 mg) was treated overnight, at room temperature in the dark, in 0.17 N acetic acid with 1.5 ml of 0.1 M sodium periodate. The total volume was 5.4 ml. The reaction mixture was dialyzed against distilled water; a bag, open at one end so that samples could easily be withdrawn during dialysis, was used. A control sample of reduced ichthyocol of the same radioactivity was dissolved in 0.17 N acetic acid and similarly dialyzed. At defined time intervals, small aliquots were removed from both solutions and radioactivity was determined. To establish that the periodate oxidation had been effective, the absence of hydroxylysine in the amino acid hydrolysate of the periodate-treated protein was ascertained (Blumenfeld *et al.*, 1963).

The effect of periodate oxidation on the basic amino alcohols obtained after acid hydrolysis of the reduced protein was determined in the following way. An aliquot of the basic fraction obtained from chromatography on Dowex 1-X8 was treated with a 100-fold M excess of sodium periodate at room temperature in the dark for 24 hr. The pH of the solution was 5.3 and the volume 2.5 ml. The quantity of amino alcohols in the aliquot used was estimated from the radioactivity and the calibration of the sodium borohydride used for the reduction and was approximately 1 μmole. A control sample containing 1 μmole each of standard glycinol and alaninol was treated similarly. An aliquot of the reaction mixture was then chromatographed on a 10-cm column of IR 120 using a split-stream attachment, so that radioactivity could be determined in the effluent fractions. Aliquots of the control amino alcohols were

also chromatographed on the same column of IR 120, and their elution pattern was obtained using the ninhydrin reaction.

Preparation of Model 2,4-DNP-hydrazones and -osazones and Their Chromatographic Separation. General procedure for formation of 2,4-DNP-hydrazones or -osazones of aliphatic, unsaturated, α-hydroxy or α-amino aldehydes or ketones. A 0.2% stock solution was prepared by adding 2,4-dinitrophenylhydrazine to 2 N HCl, heating to boiling, and filtering through glass wool. To 2 mequiv of the carbonyl compound in about 5 ml of water was added 8 mequiv of 2,4-dinitrophenylhydrazine in 2 N HCl. The reaction mixture was heated at 100° for 5 min, cooled, and allowed to stand overnight at room temperature. The solution was then filtered and the precipitate was washed several times with 0.1 N HCl. If the carbonyl compound contains an amino group, as in the case of the lysinal models or with compounds such as 4-aminobutyraldehyde, the bulk of its hydrazone or osazone is soluble in 2 N HCl as the amine hydrochloride. In these cases the following procedure was used to obtain derivatives: to the filtrate from the reaction described above, an excess of acetone (a few milliliters) was added, converting the excess dinitrophenylhydrazine into acetone DNP-hydrazone. The acid solution was exhaustively extracted with ether to remove the acetone DNP-hydrazone. This procedure was repeated twice until the ether extract was colorless. The aqueous yellow phase was concentrated to dryness by rotary evaporation. The residue contained the DNP-hydrazones or -osazones of the various amino aldehydes in the form of amine hydrochlorides.

Preparation of model carbonyl compounds from α-amino alcohols. Chloramine T (1 mequiv) dissolved in 5 ml of water was added to 2 mequiv of an α-amino alcohol in 5 ml of water, adjusted to pH 8. The reaction mixture was stirred overnight at room temperature. Unreacted chloramine T was removed by adding drop by drop a solution of aqueous hydrazine until a starch–KI test was negative. The solution was filtered to remove toluenesulfonamide, and the filtrate was used directly for the formation of DNP-hydrazones or -osazones as described above. The following α-amino alcohols were used: glycinol, α-alaninol, 4-amino-3-hydroxybutyric acid, valinol, leucinol, phenylalaninol, serinol, α-aminobutanol, and lysinol obtained by hydrolysis of dibenzoyllysinol (see Schneider *et al.*, 1967, for synthesis). (See Table II for reactions of some of these compounds.)

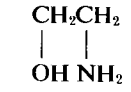
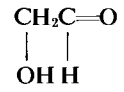
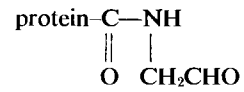
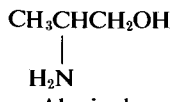
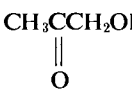
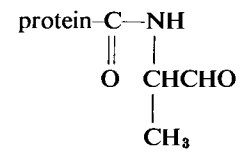
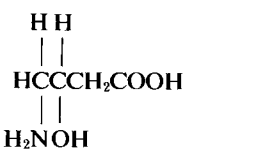
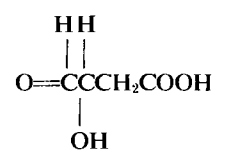
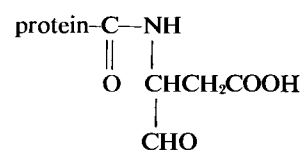
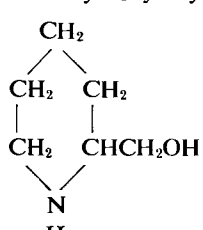
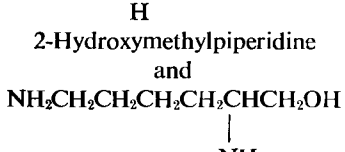
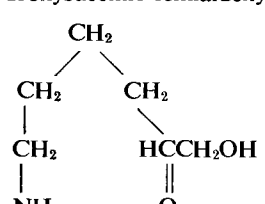
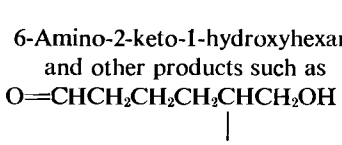
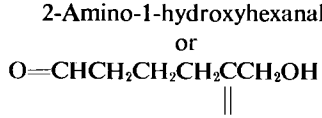
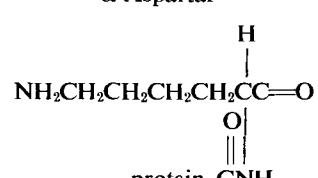
Preparation of lysinal DNP-hydrazones and -osazones from 2-piperidinecarboxylic acid. 2-Piperidinecarboxylic acid was esterified with ethanol and dry HCl (mp 212–214°, as the hydrochloride, checked by mass spectrometry, molecular ion, *m/e* (−HCl) 157 (19.4%);[4] base ion, *m/e* 84 (100%)). 2-Hydroxymethylpiperidine hydrochloride was prepared by treating 1.9 g (10 mequiv) of the above ethyl ester at pH 9–10, room temperature for 48 hr, with 1.6 g of sodium borohydride in 10 ml of water. Excess borohydride was destroyed by lowering the pH to 5 with HCl. The solution was then made alkaline (pH 10) and evaporated to dryness by rotary evaporation. The residue was extracted with hot ethyl acetate, the ethyl acetate was evaporated *in vacuo*, and the compound was recrystallized from alcohol and ethyl acetate after conversion into the hydrochloride by treatment with dry HCl gas (mp 129–130°, checked by mass spectrometry, molecular ions *m/e* at 151 (2.9%) and 153 (1.9%) as the hydrochloride, and at 115 (−HCl); base ion at 84 (100%)). 2-Hydroxymethylpiperidine was then oxidized with chloramine T as described above for the oxidation of α-amino alcohols (see Table II). Two products could arise from this oxidation depending on the position of cleavage of the piperidine ring: 6-amino-2-keto-1-hydroxyhexane and 2-amino-1-hydroxyhexanal. In some cases 2 mequiv of chloramine T was used/mequiv of 2-hydroxymethylpiperidine to obtain 1-hydroxy-2-keto-hexanal. The reaction mixtures were treated with excess dinitrophenylhydrazine as described above, and the products were separated by chromatography.

Chromatographic separation of DNP-hydrazones and -osazones of model compounds. Chromatography on thin layers (chromatabs) and columns of silicic acid was used. After development of thin-layer chromatograms, DNP-hydrazones and -osazones were distinguished by application of a small amount of 5% methanolic KOH to the yellow spot; the hydrazones give a characteristic brown-orange coloration whereas the osazones yield a stable blue to magenta color. Silicic acid columns (10–30 or 30–60 μ with no binder), usually 1 × 15 cm, were used for about 15–20 mg of material. The absorbance of consecutive fractions was determined at 370 mμ, the peak fractions were evaporated to dryness, and the residue was dissolved in a small volume of methanol. The spectrum of each peak was determined from 300 to 650 mμ in a Cary spectrophotometer in methanol and in 5% methanolic–KOH.

The precipitate or extracted filtrate obtained after reaction with dinitrophenylhydrazine was mixed with a few milligrams of the silicic acid and applied to the column in dry form. This step occasionally was found necessary and then adopted as a routine procedure, since no single solvent was found which both was able to dissolve all of the encountered products of reaction with dinitrophenylhydrazine and also was suitable for starting the elution from the column. For purposes of thin-layer chromatography, the material was dissolved in a small volume of either benzene, ethanol, ethyl acetate, or suitable combination of these, and applied to the silicic acid. Resolution on thin-layer chromatograms was accomplished either by two-dimensional procedures or by repeated one-dimensional chromatography on the same sheets using solvents of different composition. For example, in a mixture of DNP-hydrazones and -osazones, certain components moved closely to the front if the developing solvents contained large proportions of benzene; other components with lesser mobility in benzene-containing solvents could

[4] Numbers in parentheses are the relative intensity to the base ion taken as 100%.

TABLE II: Reaction of Dinitrophenylhydrazine with Carbonyl Compounds Used as Models of Aldehydes in Collagen.

Starting Compound	Compd Obtained *in Situ* after Reaction with Chloramine T	Model for Aldehyde in Collagen in Reaction with Dinitrophenylhydrazine	2,4 Dinitrophenylhydrazone and/or -Osazone Obtained
 Glycinol	 Glycolaldehyde	 Glycinal	Glyoxal osazone
 α-Alaninol	 Hydroxyacetone	 α-Alaninal	Methylglyoxal osazone
 DL-4-Amino-3-hydroxybutyric acid	 3-Hydroxysuccinic semialdehyde	 α-Aspartal	Osazones of carboxymethylglyoxal, methylglyoxal (by decarboxylation), and various ring forms
 2-Hydroxymethylpiperidine and  Lysinol	 6-Amino-2-keto-1-hydroxyhexane and other products such as  2-Amino-1-hydroxyhexanal or  1-Hydroxy-2-ketohexanal	 Lysinal	δ-Aminobutylglyoxal osazone 2-Amino-1-hydroxyhexanal hydrazone 1,2,6-Tri-DNP-hydrazone of 1,2,6-trioxohexane (osazone hydrazone)

then be resolved on the same chromatogram sheet using more polar developing solvents. This procedure was found very useful for indicating the presence in a DNP-osazone or-hydrazone of additional functional groups, such as carboxyl or amino groups. The chromatographic properties of these compounds could be altered characteristically by exposure of the sheets to dry HCl gas or triethylamine vapor.

Resolution was accomplished with solvents as follows. Benzene–ethyl acetate (50:1) was used for resolution of DNP-osazones of glyoxal, methylglyoxal, ethylglyoxal, isobutylglyoxal, isopropylglyoxal, and benzylglyoxal. This solvent was also used as the first in a series of solvents for all other compounds and purified fractions. Hydroxymethylglyoxal DNP-osazone obtained from glyceraldehyde was separated with benzene–ethyl acetate (10:1). In addition, various combinations of benzene and ethyl acetate or acetonitrile in proportions ranging from 10:0 to 1:1 were used. Compounds that showed no mobility in benzene–acetonitrile (1:1) were chromatographed by use of ethyl acetate, ethanol, or pyridine.

Resolution on silicic acid columns was accomplished by a discrete gradient of benzene–ethyl acetate (50:1, 10:1, and 1:1) followed successively by pure ethyl acetate or acetonitrile, pyridine, ethanol, and formic acid. A criterion for change of solvent was an uncolored zone (5–7 cm) on the column. When experiments were conducted to compare products of reaction of the protein with those of standards, an identical elution schedule was followed on columns of identical size.

IDENTIFICATION OF DNP-HYDRAZONES AND -OSAZONES OF MODEL COMPOUNDS AND OF OTHER PRODUCTS RESULTING FROM THEIR REACTION WITH DINITROPHENYLHYDRAZINE. Many of the carbonyl compounds studied after reaction with dinitrophenylhydrazine yielded pure DNP-osazones with properties which agreed well with those reported in the literature (Wells, 1966). Other model compounds, for example, 3-hydroxysuccinic semialdehyde, gave several reaction products (see Table II). These were characterized by their behavior on columns and thin layers of silicic acid and by their spectral properties. As expected, the main product was methylglyoxal osazone, resulting from β decarboxylation of the parent carboxymethylglyoxal osazone. Although carboxymethylglyoxal osazone was not positively identified, a carboxy-containing osazone was found and assumed to be this product because under acidic conditions it was unstable and gave rise to methylglyoxal osazone. Other model osazones were identified by means of mass spectrometry (see Appendix).

Reaction of Various Tropocollagens with Dinitrophenylhydrazine; Isolation and Identification of Several DNP-hydrazones and -osazones. A PARALLEL REACTION OF α-METHYL GLUCOSIDE, GALACTOSE, OR LACTOSE WITH DINITROPHENYLHYDRAZINE. To 50 ml of a 2% solution of heat-denatured ichthyocol or rat skin collagen was added 50 ml of 0.6% dinitrophenylhydrazine in 4 N HCl. In parallel experiments, control solutions containing 30 μmoles of α-methyl glucoside, galactose, or lactose in 50 ml of water were identically treated. The reaction mixtures were heated 30 min at 60°, cooled, and allowed to stand overnight at room temperature. The solutions were then carefully filtered on a Büchner funnel through a small filter paper (1 cm in diameter) on which was layered a thin pad of washed Celite. The precipitate obtained with collagen (there was none after these periods of heating of the control compounds) was washed several times with 0.1 N HCl (precipitate I). The filtrate was heated for an additional 10 min at 90° and allowed to cool and stand for 48–72 hr at room temperature, and the precipitate that formed was filtered as above (precipitate II). The filtrate was treated as described above for model compounds, to obtain the acid-soluble DNP-osazones and -hydrazones in solid form.

Precipitates I and II and the solid acid-soluble hydrazones and osazones were chromatographed on silicic acid columns following the exact procedure described for model compounds (see above). Each peak fraction obtained from the columns was examined by thin-layer chromatography in a manner similar to that used for model compounds.

The compounds in the several peak fractions were identified by comparison with model compounds in chromatographic behavior on columns and on thin layers of silicic acid and in their absorption spectra in methanol and in 50% methanolic KOH.

Mass spectrometry also was used to obtain the structure of several DNP-osazones obtained from the protein when they could be volatilized before decomposition. This was the case for DNP-osazones of the simple aliphatic osazones (see Appendix).

Results

Calibration of Sodium Borohydride and One-Stage and Two-Stage Reduction of Model Compounds. It is obvious that in the reduction of a carbonyl group to an alcohol, one atom of nonexchangeable hydrogen is incorporated in the formation of a $>$CH bond, whereas in the reduction of an ester or a peptide bond, where the carbon is at the carboxyl level of oxidation, two nonexchangeable atoms of hydrogen are incorporated in the formation of $>CH_2$ from a $>C{=}O$. Thus, in the calibration of tritium-labeled sodium borohydride the incorporation of one atom of tritium per mole of model compound was expected upon reduction of carbonyl-containing model compounds, and two atoms of tritium per mole where esterified model compounds were reduced to the alcohol level. It should be noted that the ester compounds employed contained a free α-amino group. As recently reported by Seki *et al.* (1965) the presence of an α-amino group facilitates ester reductions, and they can occur under mild aqueous conditions using sodium borohydride. Under such conditions most other types of esters are not usually reduced. Similarly, peptide bonds are not reduced under these conditions.

The results obtained with one typical preparation of tritiated sodium borohydride are shown in Table III. It can be seen that, as expected, the specific activity of

reduced, esterified model compounds is two times that obtained from the reduction of the carbonyl compound.

Each lot of sodium borohydride used in these studies was calibrated by reduction of at least one model compound. The specific activity of different batches of $NaBH_4$ used varied from about 2 to 6 $\times$ 10^6 dpm/μmole as calibrated for a onefold reduction. On repeated calibrations of the same lot it was found that if the tritiated borohydride was stored in a vacuum desiccator, its specific activity remained constant for several months.

From the manner in which the specific activity of model compounds is calculated, it is self-evident that absolute purity of the dinitrophenylated compounds is essential.

As mentioned above, radioactive model dinitrophenylated amino alcohols exhibited isotope effects in the course of their purification on silicic acid columns. Similar derivatives obtained from collagen also showed these effects as depicted in Figure 3. Calculation of specific activity of individual fractions of the peaks requires that correction be made for the slight retardation of the tritiated compound relative to the carrier. The procedure for correction was described under Methods. However, if small fractions (1 ml) were collected and if the emerging peak were well resolved, all the fractions of the peak may be pooled, allowing for a lag of about two to three fractions, and the specific activity of the pool measured. However, the purity of the pooled material should be ascertained by chromatography on thin layers of silicic acid.

Reduction of Various Collagens with Tritium-Labeled Sodium Borohydride. From the calibration value of the sodium borohydride used in the reduction of a particular collagen preparation, the specific activity of the treated preparation can be computed, and thereby an estimate made of the number of groups reduced. For various preparations used, if onefold reductions were assumed, it was found that between 4 and 7 μmoles of groups were reduced per 100 mg of protein, or approximately that number per mole of an α chain. As will be shown below and also as has been observed during reduction of hemoglobin (R. Bookchin, unpublished data), this variation is partly due to a low background of nondialyzable radioactivity present in most of the amino acids of the treated protein, most likely resulting from low-level tritiation of the amino acid residues by tritium gas during the decomposition of the tritiated $NaBH_4$. In most instances this background was equivalent to about 20% of total radioactivity incorporated into the protein. No significant differences in the uptake of radioactivity were found upon reduction of the tropocollagen of fish, calf skin, and rat skin.

The reduction of the protein was carried out under relatively mild conditions, *i.e.*, 1 hr at pH 8–9 and 25°. These conditions, chosen primarily because they were favorable for subsequent isolation and characterization of reduced products, also avoided occurrence of possible side reactions. However, had reduction been carried out under more drastic conditions or for a longer time, a somewhat larger quantity of carbonyl compounds most likely would have been reduced and consequently more radioactivity would have been incorporated into the collagen. As shown previously (Gallop, 1964) collagens contain so-called "indirectly reactive" aldehydes which react slowly with carbonyl reagents and the carbonyl functions of which are probably involved in covalent linkage. Apparently, these indirectly reactive aldehydes are reduced by sodium borohydride but at a slower rate than the free, or "directly reactive," aldehydes. Possibly, reduction of the indirectly reactive aldehydes may not proceed to completion under the conditions employed for reduction. Our best estimate, using a combination of methods, indicates that there are at least a total of 12 α-amino aldehydes/tropocollagen molecule (mol wt 300,000), of which 7–8 are reduced under these mild conditions.

TABLE III: Calibration of Tritiated Sodium Borohydride (preparation V).

Starting Compound	Reduced Compound	Sp Act. of Reduced Dinitrophenylated Model Compd (dpm/μmole)
δ-Amino-γ-ketovaleric acid	δ-Amino-γ-hydroxyvaleric acid or its lactone	2.04 $\times$ 10^6
Glycine ethyl ester hydrochloride	Glycinol	4.1 $\times$ 10^6
DL-Alanine ethyl ester hydrochloride	DL-Alaninol	3.9 $\times$ 10^6

Isolation of Tritium-Labeled Amino Alcohols from Reduced Collagens. Acid hydrolysis of the reduced protein followed by evaporation of HCl *in vacuo* resulted in no loss of radioactivity. One may conclude that tritium-labeled alcohols of low volatility were not present in the hydrolysate.

As described above, the alcohols present were shown to contain amino groups as well, since all their radioactivity (present in the CH_2OH groups) could be retained on Dowex 50 resins but could not be so retained subsequent to deamination by ninhydrin. Moreover, dinitrophenyl or other characteristic derivatives of the amino groups of the alcohol compounds could be prepared readily.

Chromatography of the acid hydrolysate on Dowex 1-X8 (OH^-) columns yielded a basic fraction eluted with water and a neutral fraction that eluted with acetic acid. The basic fraction contained about 50–60% of the total radioactivity; the remaining radioactivity was present in the neutral fraction. Analysis of the basic fraction on the amino acid analyzer revealed the

presence of ammonia and only one amino acid, nonradioactive arginine. Four radioactive peaks were present; two were eluted before ammonia (B_1 and B_2), and the other (B_3 and B_4) emerged at about the same position as did ammonia (see Figure 1). Some radioactivity was retained on the column and could be removed with NaOH. The relative amounts of radioactivity present in these peaks were, respectively: $B_1 + B_2 = 19\%$, $B_3 = 58\%$, $B_4 = 23\%$, for calf skin collagen; $B_1 + B_2 = 23.2\%$, $B_3 = 52.8\%$, $B_4 = 24\%$, for ichthyocol. Chromatography of standard amino alcohols such as glycinol, α-alaninol, serinol, and others is shown in Table I. It should be noted that glycinol and α-alaninol cochromatograph with peaks B_3 and B_4 of the basic fraction. Lysinol is retained on this column whereas serinol elutes at the position of peak B_1.

The neutral fraction contained most of the amino acids present in collagen with the exception of aspartic acid and hydroxyproline both of which were not usually eluted from Dowex 1 with 0.4 N acetic acid. Six distinct radioactive peaks were present as shown in Figure 2, with a low background of radioactivity in all the other amino acids. The relative distribution of radioactivity among these peaks was as follows: $N_1a = 3\%$, $N_1b = 4\%$, $N_1c = 4\%$, $N_1d = 4\%$, $N_2 = 16\%$, $N_3 = 69\%$, for ichthyocol; and $N_1a = 10\%$, $N_1b = 14\%$, $N_1c = 14\%$, $N_2 = 16\%$, and $N_3 = 45\%$, for calf skin collagen.

The total radioactivity of all the neutral peaks corresponded to 50–60% of the radioactivity of the neutral fraction. The remaining radioactivity was present as background counts. This corresponds on the average to 20% of the total radioactivity of the reduced protein.

Peak N_3, subsequently identified as α-aspartol, derived by reduction of α-aspartal in the protein, was almost certainly present as a lactone (β-aminobutyrolactone) in the acid hydrolysate of the reduced protein. The basicity of Dowex 1 in OH^- form was responsible for saponification of this lactone and of another lactone which apparently forms from N_2. The position of elution of an authentic sample of α-aspartol (β-amino-γ-hydroxybutyric acid) on an IR 120 column was identical with the position of elution of N_3. The authentic sample of α-aspartol in its lactone form (β-aminobutyrolactone) could be cochromatographed on the short column of IR 120 with the N_3 converted into its lactone form by treatment with HCl, eluting after ammonia as described in a previous publication (Blumenfeld and Gallop, 1962) and shown in Table I.

When an authentic sample of ε-hydroxynorleucine was chromatographed on a 54-cm column of IR 120, its position of elution was similar to peak N_1d. However, because of their small quantities, no positive identification of N_1 peaks has as yet been undertaken. If the component in N_1d proves to be ε-hydroxynorleucine its amount corresponds only at most to 5–10% of the total radioactivity present in the treated protein.

ε-Hydroxynorleucine, upon hydrolysis in 6 N HCl for 22 hr followed by evaporation *in vacuo*, was shown to be converted, to an extent of about 50%, into another compound[5] that emerges from the IR 120 column between leucine and tyrosine (see Table I) and follows peaks N_2 and N_3.

Isolation and Identification of Amino Alcohols in the Form of Their 2,4-Dinitrophenylamino Derivatives. Figure 3 shows a typical chromatographic separation on a silicic acid column of the dinitrophenylated compounds present in the gross basic fraction obtained in an experiment with calf skin tropocollagen. Ten well-resolved peaks, all showing absorption at 348 mμ, are present. The radioactivity of individual fractions was determined only for peaks III–V; the radioactivity of the other peaks was determined on pooled fractions. The first two peaks, present in relatively large amounts, exhibited radioactivity only slightly above background. From their absorption maxima and chromatographic behavior on thin layers of silicic acid, these were identified as dinitrophenyl ethyl ether and dinitroaniline, respectively.

Occasionally radioactive peaks with somewhat broader absorption maxima, suggesting *O*-DNP compounds, were found immediately following the dinitrophenyl ethyl ether and dinitroaniline peaks. One of these peaks was present in the experiment depicted in Figure 3 and corresponds to peak III. All other peaks were radioactive and had absorption characteristics of standard DNP-amino alcohols, *i.e.*, maximum at 348 mμ, shoulder at 405 mμ, and trough at 293 mμ.

Peak III was identified by mass spectrum and by comparison to the authentic compound as *O,N*-di-DNP-glycinol. In other experiments, *O,N*-di-DNP-alaninol was also found. These *O,N*-di-DNP compounds formed as a result of the relatively long time of reaction with FDNB, a condition found necessary to obtain good yields of *N*-DNP-amino alcohols.

The specific activity of peak III was found to be about 83% (see Table IV) of the specific activity obtained for a onefold-reduced compound. Actually, a compound in which the ratio of DNP groups to carbonyl or alcohol functions is equal to two should have a specific activity (as calculated by the convention adopted) equal to one-half of that found for a onefold-reduced compound, provided that the second DNP group also contributes to the maximum absorption of the compound in the 330–350-mμ range. In this convention of specific activity (disintegrations per minute per micromole), the concentration of the compound is computed from the absorption of its DNP group in this wavelength range. In *O,N*-di-DNP-glycinol, the *O*-DNP group contributes little to the light absorption in this range, and this is undoubtedly the reason why the

[5] This compound has been identified as ε-chloronorleucine which on base treatment or by treatment with Dowex 1 (OH^-) is converted in part to pipecolic acid. The extent to which ε-hydroxynorleucine during acid hydrolysis is converted, when in peptide bond, into such products cannot at this time be assessed. ε-Hydroxynorleucinol during acid hydrolysis behaves in a similar manner, yielding ε-chloronorleucinol which on base treatment or treatment with Dowex (OH^-) yields hydroxymethylpiperidine. The mass spectral properties of some of these compounds are included in the Appendix.

specific activity of this compound is close to that of a onefold-reduced compound.

By mass spectrometry and cochromatography with authentic dinitrophenylated compounds, peaks IV and V have been respectively identified as *N*-DNP-α-alaninol and *N*-DNP-glycinol. As shown in Table IV, these compounds were onefold reduced and therefore derived from α-alaninal and glycinal in the protein. As can be seen in Figure 3, the amounts of DNP-α-alaninol and DNP-glycinol are larger than those of any other compound, and the quantity of DNP-glycinol is about twice that of DNP-α-alaninol. This result was found generally in all the experiments performed. Whether this circumstance reflects a larger amount of glycinal in the protein or its greater susceptibility in bound form to reduction by $NaBH_4$-^3H remains to be established. One may note in Figure 3 that peaks III–V show considerable isotope effects. DNP derivatives of authentic radioactive *N*-DNP-glycinol and *N*-DNP-α-alaninol showed the same effects. As noted previously, a consideration of these isotope effects is of utmost importance in selection of tubes for pooling of fractions for rechromatography and for calculation of specific activity. On this basis one may account for the slightly higher than expected specific activities of some compounds shown in Table IV.

Even though peaks III–V appeared pure by specific activity measurements and rechromatography on thin layers or columns of silicic acid in benzene–ethyl acetate systems, subsequently these samples were found to contain other compounds which these systems did not resolve. This was particularly true for *N*,*N*′-di-DNP-lysinol. This compound was usually present in peaks containing DNP-α-alaninol and DNP-glycinol and could be separated from them by two-dimensional chromatography, as shown in Figure 4. As expected, the specific activity of *N*,*N*′-di-DNP-lysinol was one-half that of the specific activity of a model carbonyl compound containing a single dinitrophenylated amino group. Thus, lysinol most probably originated from lysinal in the protein. *N*,*N*′-Di-DNP-lysinol was isolated from rat skin collagen (Schneider *et al.*, 1967) and from ichthyocol (see Table IV). As shown in a previous publication (Schneider *et al.*, 1967), some of the radioactivity of lysine-^{14}C administered to rats can be recovered in *N*,*N*′-di-DNP-lysinol isolated from their skin collagen after $NaBH_4$ reduction, hydrolysis, and dinitrophenylation.

Other compounds may also be present in peaks containing DNP-α-alaninol and DNP-glycinol, and they can be resolved by virtue of their lack of mobility in the water system. This is shown in Figure 4; materials labeled 2 and 3 contained mainly *O*,*N*-di-DNP-glycinol and *O*,*N*-di-DNP-α-alaninol.

Peak VI was identified by mass spectrometry as DNP-enosoline, previously isolated in experiments with ichthyocol and characterized (Blumenfeld and Gallop, 1966). It is derived from enosaline in the protein; enosaline *per se* is probably an intermediate in formation of a proposed cross-linking compound, dienosaline.

The structures of compounds contained in peaks VII and VIII, eluted from the column with more polar solvents, have been identified as *N*-DNP-threoninol and *N*-DNP-serinol, respectively.

Experiments are now in progress on the isolation in larger amounts of the more polar radioactive *N*-DNP compounds in peaks IX and X. We are now using ethanol, pyridine, and formic acid for their elution. It appears that some of these compounds as their DNP derivatives have a low volatility, and work is in progress on the synthesis of more volatile derivatives suitable for use in the mass spectrometer. They appear to resemble adducts of the amino aldehydes with sugars.

One should note in Table IV that no compound has yet been isolated with a specific activity equal to that expected of a twofold-reduced compound. Clearly, under the conditions used here for reduction with sodium borohydride, no amino alcohols resulting from reduction of peptide or ester bonds were isolated. Thus, no significant side reactions occurred in the course of reduction. Moreover, no evidence was found for the presence of the *N*-DNP derivatives of prolinol, hydroxyprolinol, valinol, leucinol, isoleucinol, phenylalaninol, or tyrosinol, any of which would have been detected and identified readily by our techniques. The presence of other α-amino alcohols related to α-amino acids such as argininol and histidinol, possibly, but not likely, could have escaped detection.

In another series of experiments, components of the basic fraction resolved on the 10-cm columns of IR 120 (peak B_3 and B_4, and combined peaks B_1 and B_2) were treated separately with FDNB, and their derivatives were purified and characterized (see Table IV). Peaks B_3 and B_4 elute from 10-cm columns of IR 120 at the exact positions of elution of authentic samples of glycinol and alaninol (see Table I). The DNP derivatives of these peaks resolved well, both on columns and thin layers of silicic acid, and their specific activities corresponded to onefold-reduced compounds. The mobility of the DNP derivative of peak B_3 corresponds to that of DNP-glycinol and the DNP derivative of B_4 has the mobility of DNP-α-alaninol. Moreover, the respective structures of these compounds were definitely confirmed by mass spectrometry.

The DNP derivatives of the combined fractions B_1 and B_2 have a mobility slower than either DNP-α-alaninol or DNP-glycinol in the benzene–ethyl acetate system and were proved by mass spectrometry to be *N*-DNP-serinol and *N*-DNP threoninol, respectively.

From this series of experiments and the quantitative results obtained from chromatography on the 10-cm IR 120 columns, one may conclude that the basic fraction consists chiefly of glycinol and α-alaninol with smaller levels of lysinol, serinol, and threoninol.

The dinitrophenylated derivatives of peaks N_2 and N_3 obtained from the 54-cm IR 120 column were obtained in pure form after chromatography on silicic acid columns using benzene–pyridine–glacial acetic acid (80:20:2). The specific activities of these compounds indicated that they were onefold reduced (see Table IV), and therefore derived from aldehyde or keto compounds in the protein.

Pure "DNP-N_3," in lactone form, was identified as *N*-DNP-β-aminobutyrolactone by mass spectrome-

TABLE IV: Structures and Specific Activities of the Dinitrophenylated Derivatives of Amino Alcohols Obtained from Reduced Collagens (related to specific activities of model compounds used in calibration of tritiated sodium borohydride used in their preparation).

DNP Peak	Collagen Used	Established Structure of Compd (DNP)	Sp Act. of Isolated Compd (dpm/μmole of DNP group)	Model Compd Reduced	Sp Act. of Model Compd (dpm/μmole of DNP group)	Nature of the Parent Compd in Collagen
III	Calf skin preparation D	*O*,*N*-Di-DNP-glycinol	4.0×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Glycinal
IV	Calf skin preparation D	α-Alaninol	5.9×10^{6a}	γ-Keto-δ-aminovaleric acid	4.8×10^6	α-Alaninal
V	Calf skin preparation D	Glycinol	5.6×10^{6a}	γ-Keto-δ-aminovaleric acid	4.8×10^6	Glycinal
VI	Calf skin preparation D	Enosoline	3.6×10^{6b}	γ-Keto-δ-aminovaleric acid	4.8×10^6	Enosaline
VII	Calf skin preparation D	Threoninol	4.7×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Threoninal
VIII	Calf skin preparation D	Serinol	4.9×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Serinal
IX	Calf skin preparation D	Unknown	2.5×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Unknown
X	Calf skin preparation D	Unknown	0.3×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Unknown
	Rat skin	Glycinol	2.75×10^4	γ-Keto-δ-aminovaleric acid	3.6×10^{4c}	Glycinal
	Rat skin	Lysinol	1.6×10^4	γ-Keto-δ-aminovaleric acid	3.6×10^{4c}	Lysinal
N_2	Ichthyocol preparation C	A lactone; structure unknown	4.5×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Unknown
N_3	Ichthyocol preparation C	β-Aminobutyrolactone	4.6×10^6	γ-Keto-δ-aminovalaeric acid	4.8×10^6	α-Aspartal

a	Ichthyocol preparation C	α-Alaninol	5.4×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	α-Alaninal
b	Ichthyocol preparation C	Dienosoline	4.9×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Dienosaline
c	Ichthyocol preparation C	Glycinol	6.8×10^{6a}	γ-Keto-δ-aminovaleric acid	4.8×10^6	Glycinal
d	Ichthyocol preparation C	Enosoline	4.7×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Enosaline
1	Ichthyocol preparation E	Lysinol	1.94×10^6	DNP-aminobutyraldehyde	3.7×10^6	Lysinal
6	Ichthyocol preparation E	α-Alaninol	2.92×10^{6b}	DNP-aminobutyraldehyde	3.7×10^6	α-Alaninal
7	Ichthyocol preparation E	Glycinol	2.98×10^{6b}	DNP-aminobutyraldehyde	3.7×10^6	Glycinal
15	Ichthyocol preparation E	Enosoline	3.0×10^6	DNP-aminobutyraldehyde	3.7×10^6	Enosaline
22	Ichthyocol preparation E	Threoninol	3.3×10^6	DNP-aminobutyraldehyde	3.7×10^6	Threoninal
23	Ichthyocol preparation E	Serinol	3.3×10^6	DNP-aminobutyraldehyde	3.7×10^6	Serinal
Peaks from 10-cm column of IR 120						
B_{1+2}	Ichthyocol preparation C	Threoninol	4.6×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Threoninal
	Ichthyocol preparation C	Serinol	4.3×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Serinal
B_3	Ichthyocol preparation C	Glycinol	4.7×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	Glycinal
B_4	Ichthyocol preparation C	α-Alaninol	4.7×10^6	γ-Keto-δ-aminovaleric acid	4.8×10^6	α-Alaninal

[a] Slightly higher due to isotope effect in selection of fractions for analysis. [b] Lower because impurities still present; peaks not rechromatographed. [c] Tritiated sodium borohydride was diluted 1:100 since used in a double-labeled experiment with ^{14}C.

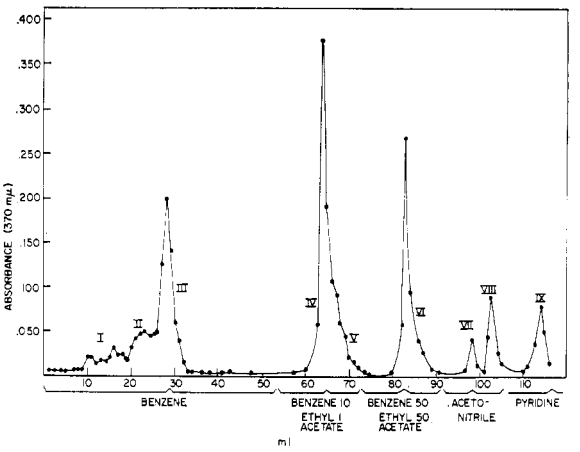


FIGURE 6: Elution pattern of precipitate I obtained from ichthyocol after treatment with dinitrophenylhydrazine (on a 1 × 21 cm silicic acid column).

try and by cochromatography with an authentic sample. This compound must have originated from dinitrophenylation of β-aminobutyrolactone formed by reduction of an α-aspartal component in the protein.

N_2, in the form of its DNP derivative, appeared to lactonize easily. Its structure has not yet been established.

Reaction of Various Tropocollagens with 2,4-Dinitrophenylhydrazine. Two types of products are obtained from various collagens treated for 30 min at 60° with dinitrophenylhydrazine in 2 N HCl. One type consists of DNP-hydrazones and DNP-osazones that are insoluble in aqueous acid; these appear as a dark red-brown precipitate. The second type are DNP-hydrazones and DNP-osazones that are soluble in aqueous acid. The insoluble osazones consist of products derived from α-amino aldehydes present in the protein, having been cleaved off in part by a conversion of the α-acylamino aldehydes into acylamides and to DNP-osazones (bisdinitrophenylhydrazones) by the Fischer reaction (Fischer, 1893). The second type include di nitrophenylhydrazine derivatives of aldehydes that contain an ω-amino group in addition to the α-amino group, and of DNP-hydrazones of the acylated α-amino aldehydes still covalently attached to the peptide chain. Extended reaction with dinitrophenylhydrazine causes more of these α-aminohydrazones to be converted to osazones, but the time needed for the conversion also allows significant amounts of the glucose and galactose, present in collagen in *O*-glycosidic linkage, to react with the reagent. This sequence of events was established by examination of precipitate II (see Methods). Only small amounts of the dinitrophenylhydrazine derivatives of these carbohydrates and their reaction products appear to be present in the precipitate obtained from the protein after mild treatment. (α-Methyl glucoside, galactose, or lactose yields no precipitate under the same conditions. An additional heating period of 10 min at 90° is needed to yield visible precipitates with these model carbohydrates.) Chromatography of the $DNPNHNH_2$ compounds obtained from ichthyocol after reaction for 30 min at 60° (precipitate I), on a silicic acid column, is represented in Figure 6. Several peaks absorbing at 370 mμ are present, emerging mostly with the solvent front. Peak III can be resolved further into two components on thin layers of silica gel. These compounds have mobilities and absorption spectral properties identical with the DNP-osazones of glyoxal and methylglyoxal and have been identified positively as such by their mass spectra (see Appendix). One may conclude that they derive from glycinal and α-alaninal which were bound to the protein as α-acylamino aldehydes (see Table V). A compound with the mobility of peak III is present in the reaction mixture of 4-amino-3-hydroxybutyric acid treated first with chloramine T and then with dinitrophenylhydrazine (see Table II). This compound has been identified positively as methylglyoxal DNP-osazone and arises from carboxymethylglyoxal DNP-osazone by an acid-catalyzed β decarboxylation as shown in Scheme I.

Under various conditions, comparison on thin-layer chromatography with a sample of authentic carboxymethylglyoxal DNP-osazone revealed that the osazone precipitates I and II obtained from collagen contained both the lactam (peak VI) and acid forms (peak VIII) of this derivative. The acid form could be dissolved in $NaHCO_3$. Strong acidification then caused some of the material to decarboxylate to methylglyoxal DNP-osazone and some to be converted into the lactam. Clearly, then, carboxymethylglyoxal DNP-osazone can be isolated from the protein treated with dinitrophenylhydrazine; it probably originates in the protein from α-aspartal, bound as β-acylaminosuccinic semialdehyde (Table V).

Reaction products of dinitrophenylhydrazine with *O*-methyl glucoside, galactose, or glyceraldehyde emerge at positions corresponding to peaks IV and VI obtained from the treated protein (see Figure 6). Part of peak IV obtained from the treated protein indeed may originate from hexoses bound to collagen in *O*-glycosidic linkage (Blumenfeld *et al.*, 1963). Peak VI, however, also contains a compound derived from α-aspartal. Peaks I and II, obtained from the treated protein, have not yet been identified and are also present in small amount in reaction products of certain carbohydrates with dinitrophenylhydrazine. Nevertheless, in the case of reactions with the protein in which relatively short reaction times are used, these peaks are considered to derive from aldehydes generically related to lysine and not from the sugars known to occur in collagen. The osazones which would be derived from serinal have not been positively identified yet, a difficulty being that they are similar to products obtained from the sugars.[6]

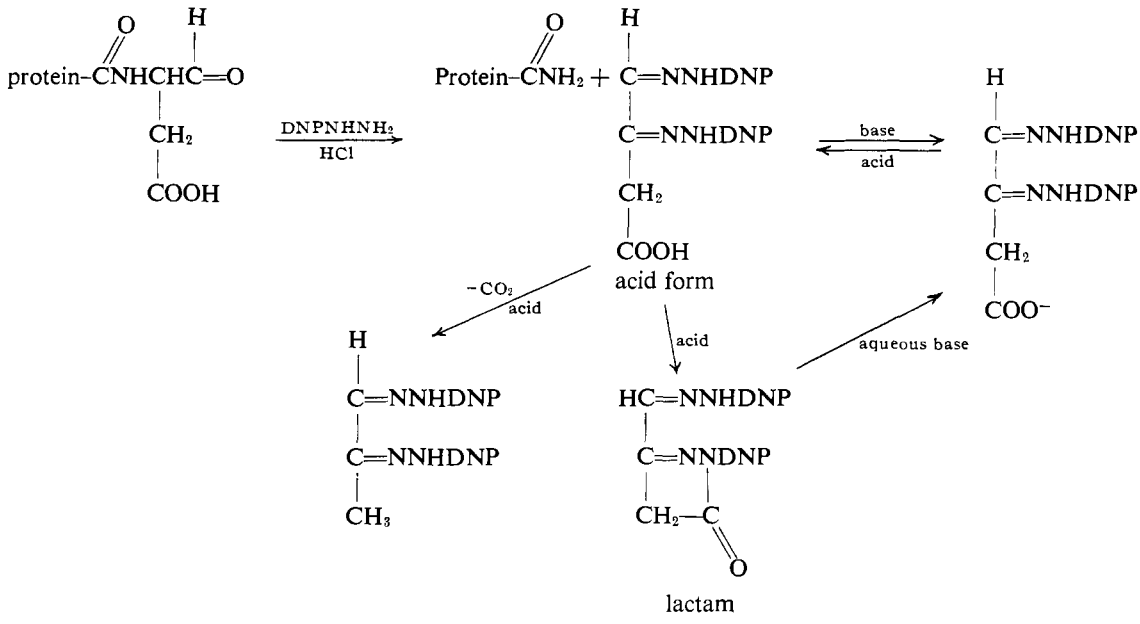
Peak IX obtained from the treated protein has a chromatographic behavior similar to the DNP-osazone which could be derived from lysinal and, in fact, is identical with the hydrochloride of δ-aminobutyl-DNP-osazone prepared by oxidation of 2-hydroxypiperidine

[6] Hydroxymethylglyoxal *N*-methylbenzothiazolone osazone has been isolated from ichthyocol treated with *N*-methylbenzothiazolone hydrazone, along with osazones of glyoxal and methylglyoxal. The former derives from serinal on the protein, and the latter two, from glycinal and alaninal, respectively.

TABLE V: Products of Reaction, Isolated on Silicic Acid Columns, of 2,4-Dinitrophenylhydrazine with Collagen.

Peak No.	Structure of the DNP Derivative Present in Peak	Nature of the Parent Compd in the Protein
I	Unknown	
II	Unknown	
III	HC=NNHDNP | HC=NNHDNP Glyoxal DNP-osazone	HC=O | CH_2NH_2 Glycinal
	HC=NNHDNP | CH_3—C=NNHDNP Methylglyoxal DNP-osazone	HC=O | H_2NCHCH_3 α-Alaninal
		HC=O | H_2NCHCH_2COOH α-Aspartal (after decarboxylation)
IV	A product of glucose and galactose with dinitrophenylhydrazine	Glucose and galactose
VI	A product of glucose and galactose with dinitrophenylhydrazine HC=NNHDNP | C=NNDNP | | CH_2-C || O Carboxymethylglyoxal DNP-osazone (in lactam form)	Glucose and galactose HC=O | $CHCH_2COOH$ | NH_2 α-Aspartal
VIII	HC=NNHDNP | C=NNHDNP | CH_2COOH Carboxymethylglyoxal DNP-osazone (in open form)	HC=O | $CHCH_2COOH$ | NH_2 α-Aspartal
IX	HC=NNHDNP | C=NNHDNP | CH_2 | CH_2 | CH_2 | CH_2 | NH_2 δ-Aminobutylglyoxal DNP-osazone	HC=O | $CHNH_2$ | CH_2 | CH_2 | CH_2 | CH_2 | NH_2 Lysinal

SCHEME I: For Rearrangement Reactions of Carboxymethylglyoxal Osazones Derived from α-Aspartal.



with chloramine T. The acid-soluble products obtained from reaction of dinitrophenylhydrazine with the protein contain a large amount of material with no mobility when chromatographed on thin layers with benzene–ethyl acetate (1:1). However, in 0.1 N HCl–ethanol (1:1) this material has a mobility identical with that of the 2,4-dinitrophenylosazone of δ-aminobutyl DNP-osazone hydrochloride. Again, these results suggest formation of the 2,4-dinitrophenylosazone derived from lysinal. Some of the derivative was insoluble in 2 N HCl and was recovered in peak IX; the bulk, however, was soluble in 2 N HCl and could be obtained from this solution after extraction of excess 2,4-dinitrophenylhydrazine. These results are summarized in Table V.

Oxidation with Periodate of Collagen First Reduced with Sodium Borohydride and of the Products of This Collagen after Hydrolysis with Acid. Dialysis of ichthyocol, first reduced with tritiated $NaBH_4$ and then oxidized with periodate, causes no significant loss of radioactivity. Thus, the protein-bound amino alcohols had not undergone oxidation. In contrast, free α-amino alcohols of fraction B, obtained by chromatography on Dowex 1, are, as expected, sensitive to periodate oxidation. Thus, after periodate treatment of the B fraction all but 10% of the radioactivity is no longer held to columns of IR 120 (see Figure 1), but counts are eluted with the hold-up volume of the column. Standard amino alcohols, such as glycinol, α-alaninol, and serinol, behave in an identical manner.

During the course of these experiments, about 10% of the total radioactivity of the basic fractions was observed to be resistant to periodate oxidation. This result may be explained by the presence of other aldehyde components not reduced to α-amino alcohols by $NaBH_4$, but instead reduced to certain types of ω-amino alcohols; such aldehydes may be exemplified by enosaline and dienosaline (Blumenfeld and Gallop, 1966).

The Mode of Attachment to the Protein of the α-Amino Aldehydes and of the Amino Alcohols Obtained by Their Reduction. Two observations permit the conclusion that the α-amino aldehydes of the protein and the amino alcohols obtained by their reduction are attached to the protein through their α-amino groups as acylamino compounds. These are: (a) the resistance to periodate oxidation of the intact, reduced protein, in which the alcohol function is free, in contrast to the susceptibility of the acid-hydrolyzed products to this oxidation; and (b) the facile formation of DNP-osazones of the α-amino aldehydes. As is well known, free α-amino aldehydes and the amino sugars do not form DNP-osazones as readily as *N*-acetylhexosamines and *N*-acetyl-α-amino aldehydes. Here protonation of the free amino groups somewhat retards the Fischer reaction in the formation of osazones. However, *N*-acetylglucosamine gives rise to the osazone of glucose and to acetamide. The acylated α-amino aldehyde compounds react readily, behaving like α-hydroxy aldehydes in this regard.

Discussion

The presence of the following α-amino aldehydes has been demonstrated in collagen: glycinal, α-alaninal, α-aspartal, serinal, threoninal, and lysinal. Two independent methods have been used. One involves a one-stage reduction with $NaBH_4$ of the aldehyde in the protein followed by isolation of the corresponding amino alcohols from the acid hydrolysate, and finally

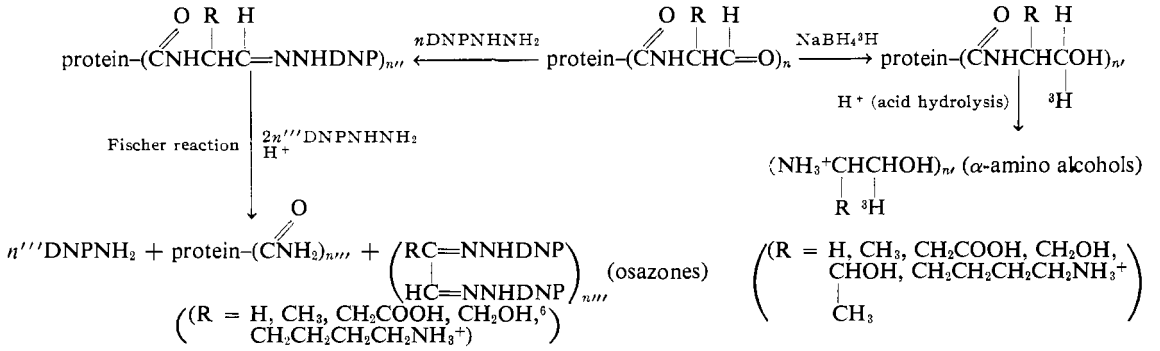


FIGURE 7: A schematic representation of the reactions of the protein-bound α-acylaminoaldehydes with tritiated sodium borohydride and 2,4-dinitrophenylhydrazine, depicting the relationships between the amino alcohols and osazone products. For simplicity the acylaminoaldehydes on the protein are depicted as free aldehydes although they are present in covalent attachments, the nature of which are still undefined; they are sensitive to and exposed by nucleophilic reagents and strong acid. If the molecular weight of the protein is taken as 300,000 then n would be at least 12; under the conditions indicated in the text, n' would be about 7–8 and n''' about 4; no estimate of n'' is as yet possible.

identification of the amino alcohols or their derivatives by chromatographic behavior, absorption spectral properties and mass spectrometry. The second method involves direct formation of 2,4-dinitrophenylhydrazine derivatives of the aldehydes in the protein, isolation of the dinitrophenylosazones, and finally their identification by chromatographic and optical spectral behavior and, in some instances, by mass spectrometry. Identification, in both methods, was confirmed by comparison with authentic samples of the presumed compounds, or with suitable model compounds. (This is summarized in a schematic diagram depicted in Figure 7.) Several other amino alcohols which occur in collagen were discussed in a previous publication (Blumenfeld and Gallop, 1966).

At the outset it would be useful to discuss what is known concerning selectivity of reductions by $NaBH_4$ in aqueous media. As is well known, $NaBH_4$ is a useful reducing agent for aldehydes and ketones, whereas in general, this reagent, used under mild aqueous conditions, does not reduce most esters, lactones, and amides (Seki *et al.*, 1965; Brown *et al.*, 1956; Kupchan and Johnson, 1956). However, several instances, particularly among carbohydrates, have been reported in which certain esters and lactones have been reduced to primary alcohols (Wolfrom and Anno, 1952; Schenker, 1961). Also, a recent paper (Seki *et al.*, 1965) illustrates the facile reduction of free α-amino acid esters to α-amino alcohols. In these "abnormal" ester reductions by aqueous $NaBH_4$, a functional group such as a hydroxyl, keto, or amino exists in a position neighboring the ester groups and undoubtedly facilitates the positioning of the borohydride prior to the ester reduction.

Little concrete evidence exists for the aqueous reduction of amide or peptide bonds by aqueous $NaBH_4$. However, a recent paper (Cerutti and Miller, 1967) documents the selective reduction by aqueous $NaBH_4$ of dihydrouridine in tRNA. In this reaction the heterocyclic portion of dihydrouracil (essentially a cyclic imide between a carboxylic and a carbamic acid) is reductively cleaved in a twofold reduction to 3-ureidopropanol. Our own experience has revealed the removal of an ε-phthaloyl group in the aqueous reduction of ε-phthaloyllysine ethyl ester by $NaBH_4$; the phthaloyl group was recovered as the lactone of *o*-hydroxymethylbenzoic acid. Although these are special cases with imides, one may conceive that special peptide bonds in proteins may be cleaved reductively by aqueous $NaBH_4$. A suggestion of this possibility was reported (Crestfield *et al.*, 1963) in studies in which aqueous $NaBH_4$ was used to promote reduction of disulfides in ribonuclease. However, no detectable cleavage of the A chain of insulin was found with aqueous $NaBH_4$, which suggested a measure of specificity in any side reactions with $NaBH_4$.

A selective method for reductive cleavage of peptide bonds involving the imino group of prolyl residues has been described (Ruttenberg *et al.*, 1964). In this method a much more powerful agent than $NaBH_4$ is used under anhydrous conditions. During the cleavage reactions the acyl function of the residue preceding proline is reduced in a one-stage reduction to the aldehyde level. A subsequent one-stage reduction with aqueous $NaBH_4$ is employed to convert the aldehyde into the alcohol. It is noteworthy that during this aqueous reduction step no side reactions involving peptide-bond cleavage have been reported.

A variety of investigations during which aqueous $NaBH_4$ has been used to reduce carbonyl groups of Schiff bases in the presence of proteins have been reported (Horecker *et al.*, 1961; Grazi *et al.*, 1963; Holmquist and Schroeder, 1966; Meloche and Wood, 1964). Little attention has been paid to possible side reactions; nevertheless, most of the studies suggest that few side reactions, if any, occurred during the treatment with $NaBH_4$. Our own investigation with α and β chains of hemoglobin, to be reported at a later date, indicate that no significant reduction of peptide bonds by $NaBH_4$ occurs.

However, since this communication shows that tropocollagen contains six α-amino aldehydes related to α-amino acids, special precautions were taken to show that the α-amino alcohols obtained after $NaBH_4$ treatment resulted from reduction of an aldehyde or keto carbonyl group to an alcohol group and not by reduction of carboxylic acid, peptide bond, or carboxylate

ester or amide to an alcohol. Accordingly, the isolation of the onefold-reduced amino alcohols, glycinol, α-alaninol, α-aspartol, lysinol, serinol, and threoninol, from the various $NaBH_4$-reduced collagens, clearly indicates the presence in collagen of the corresponding α-amino aldehydes. Since no twofold-reduced compounds have yet been found, the absence of significant side reactions is indicated.

To demonstrate the presence of some of abovementioned aldehydes in yet another way, various collagens upon treatment with 2,4-dinitrophenylhydrazine in 2 N HCl were found to yield a mixture of products from which the following 2,4-DNP-osazones could be isolated: glyoxal, methylglyoxal, carboxymethylglyoxal, and δ-aminobutylglyoxal. These are the products that would be expected with four of the α-amino aldehydes listed above (see Figure 7). Accordingly, the evidence from two independent lines of investigation is quite conclusive that these α-amino aldehydes are components of various collagens. The osazones resulting from serinol and threoninol, though likely present, are mixed with some of the sugar osazones and have not yet been isolated in pure form.[6]

The reactivity of the protein-bound α-amino aldehydes with 2,4-dinitrophenylhydrazine and the facile formation of DNP-osazones suggest that the α-amino aldehydes are linked to the protein through their α-amino groups, as acylamino compounds. The results with oxidation by periodate support this conclusion. Thus, the $NaBH_4$-reduced but unhydrolyzed protein retains its tritium in the protein-bound α-acylamino alcohols; in contrast, the free α amino alcohols obtained after acid hydrolysis are, as expected, readily attacked by periodate. It would seem likely that the α-amino aldehydes are located either at C-terminal positions in collagen subunits or are acylated to side-chain carboxyl groups of aspartic or glutamic acid residues and serve as branch points. More evidence in this regard is essential, and such investigations are in progress.

From their obvious relation to α-amino acids it would seem that the six α-amino aldehydes are derived biosynthetically from α-carboxyl reductions of corresponding C-terminal, or branching, α-amino acid residues. In the case of lysinal this is already apparent (Schneider *et al.*, 1967). Biochemical precedent suggests that carboxyl groups are not reduced directly to aldehydes but require an activation stage, usually as a phosphate mixed anhydride, prior to reduction. Such an α-carboxyl kinase and reductase system should be present in connective tissue unless more specialized systems are involved. On the other hand, if some of these α-amino aldehydes are at C-terminal positions, as suggested, conceivably a reduction step occurring at the conclusion of biosynthesis of subunits might serve to release them from their ester attachment to tRNA and lead to the formation of C-terminal aldehydes.

This paper has concentrated on the isolation and identification of α-amino aldehydes from three collagens. There are indications reported in a preliminary publication (Blumenfeld and Gallop, 1966) and also indicated here (see Table IV) that various ω-amino aldehydes are also present in collagens. Some of these appear to be related to lysine and, in one instance, seem to involve a modification of a lysyl residue near an N-terminal position (Piez *et al.*, 1966); in another case, the modification appears to involve either a C-terminal or branching lysine, which is reduced to lysinal as an intermediate in further reactions (Schneider *et al.*, 1967).

Brief mention should be made of the level of these amino aldehydes in tropocollagen. There is about 0.5–1.0 μmole of directly reactive (*i.e.*, rapidly reacting) aldehydes/100 mg of protein, and about 3–4 μmoles of indirectly reactive (covered or slowly reactive) aldehydes/100 mg of protein (Gallop, 1964), or at least 12/tropocollagen molecule. It would appear that the α-amino aldehydes are in the main the indirectly reactive aldehydes requiring either strong acid dinitrophenylhydrazine treatment, deesterification by hydrazine, or mild saponification to release them from their covalent attachment to the protein. $NaBH_4$ also facilitates this release and immediately reduces the free aldehydes thus formed. Of the total of 12 α-amino aldehydes, we roughly estimate that approximately 7–8 are reduced per tropocollagen molecule under the conditions used. The remaining α-amino aldehydes either require stronger conditions for reduction, are in portions of the molecule not accessible to the reagent, or are in covalent attachments not labile to aqueous, mildly alkaline $NaBH_4$.

The quantitation of total aldehydes as derivatives of 2,4-dinitrophenylhydrazine can be only approximate, since under the conditions used the reaction does not proceed to completion and, as indicated, several types of derivatives are obtained. The amount of insoluble 2,4-dinitrophenylosazones obtained corresponds roughly to four per mole of tropocollagen.

Because of difficulties in obtaining good recoveries, at the present time one may only estimate the relative amounts of the various α-amino aldehydes obtained. Thus the quantity of glycinal appears to be about twice that of either α-alaninal, α-aspartal, or lysinal; serinal and threoninal are recovered in somewhat lesser amounts. The quantities of ω-amino aldehydes and the yet unidentified aldehydes are smaller. It would appear that the ω-amino aldehydes, enosaline, dienosaline, and those derived from lysine near the N-terminal positions of chains, constitute most of the directly reactive aldehydes. This group includes both saturated and α,β-unsaturated aldehydes; the latter are enriched in the more cross-linked or more mature collagens (Blumenfeld and Gallop, 1966; Piez *et al.*, 1966).

The presence of at least nine different types of aldehydes in acid-soluble tropocollagen, of which several are present in quantities corresponding to fractions of residues per mole, only reflects the fact that some may be intermediates on the way to final cross-linking compounds.

The nature of the yet unidentified aldehydes is of great interest. Are they a separate group of compounds, or are they additional intermediates in the formation of ω-amino aldehydes or other cross-linking compounds of nature yet unknown? Soluble tropocollagen appears to contain a family of biochemical intermediates on the pathway of connective tissue maturation.

APPENDIX TABLE I: Mass Spectra of Selected 2,4-Dinitrophenylamino Alcohols and Lactones.

Base Ion *m/e* (%)	Structure of Base Ion	*N*-2,4-DNP Derivative of	Parent Ion (%)[a]	Other Important Ions (%)[a]
196	$DNPN^+H{=}CH_2$	Glycinol	227 (15)	
		β-Alaninol	241 (22)	206 (8.7)
		4-Amino-1-hydroxybutane	255 (13)	220 (8.0)
		5-Amino-1-hydroxybutane	269 (12.2)	234 (14.4)
		6-Amino-1-hydroxybutane	283 (9.3)	248 (7.4)
		δ-Aminovalero-γ-lactone	281 (22.5)	85 (76.1)
		4-Amino-3-hydroxybutyric acid	285 (9.6)	267 (2.9), 239 (11.5)
		Glycinol (*O*,*N*-di-DNP)	393 (14)	363 (12.7)
210	$DNPN^+H{=}CH(CH_3)$	α-Alaninol	241 (11.4)	
		DL-2-amino-3-hydroxybutane	255 (8.3)	
		α-Alaninol (*O*,*N*-di-DNP)	407 (4.4)	377 (6.5)
223	$DNPN^+H{=}\dot{C}CH_2CH_3$	2-Amino-2-ethyl-1-3-dihydroxypropane	285 (0)	254 (54)
224	$DNPN^+H{=}C(CH_3)_2$	2-Amino-2-methylpropanol	255 (8.3)	
	$DNPN^+H{=}C(H)CH_2CH_3$	2-Amino-1-hydroxybutanol	255 (12.3)	239 (0.6)
226	$DNPN^+H{=}CH(CH_2OH)$	Serinol	257 (13.7)	
		Threoninol	271 (22.2)	240 (27.8)
		Serinol (*O*,*N*-di-DNP)	423 (12.8)	392 (65.3)
250	$DNPN^+{=}CH(CH_2CH_2CH_2CH_2)$	ε-Hydroxynorleucine	313 (50)	268 (86), 240 (44), 234 (64), 222 (79)
		ε-Hydroxynorleucinol	299 (15.8)	268 (67.1), 266 (10.6), 222 (22.0), 216 (50.0)
		Lysinol (*N*,*N'*-di-DNP)	464 (0)	448 (1.8), 447 (1.6), 433 (22.2), 415(13.6), 280 (24.4), 266 (40), 222 (20.4), 196 (22.2)
		Hydroxymethylpiperidine	281 (3)	216 (20), 217 (25), 222 (37), 233 (6), 204 (11)
286 (100%) 288 (38)	$DNPN^+H{=}CH(CH_2CH_2CH_2CH_2Cl)$	ε-Chloronorleucinol	317 (7) 319 (2.5)	226 (4), 240 (3), 250 (4), 256 (2), 270 (3)

[a] Intensity relative to base ion (100%).

APPENDIX TABLE II: Mass Spectra of 2,4-Dinitrophenylosazones.[a]

Compound	Base Ion *m/e*	Parent Ion *m/e* (%)[b]	Other Important Ions *m/e* (%)[b]
Glyoxal	183	418 (28)	235 (33)
Methylglyoxal	183	432 (48)	415 (21), 250 (56), 249 (58), 219 (52)
(2-Methyl)propylglyoxal	183	474 (58)	457 (11), 439 (15), 380 (37), 292 (37)
Benzylglyoxal	508	508 (100)	183 (36), 442 (27), 491 (14), 326 (23), 295 (18)

[a] Compounds were volatilized at a temperature of over 250°. [b] Intensity relative to base ion.

At this stage of our information, the α-amino aldehydes established in this study as components of tropocollagen may in part be considered to participate in formation of subunit attachments and may somehow be related to the aspartyl ester-like bonds (Blumenfeld and Gallop, 1962). In this regard, the fact that these aldehydes are exposed by deesterification is of interest. The aldehyde modifications of lysine residues appear to be involved in formation of α-chain attachments and in intertropocollagen unit attachments. The α-amino aldehydes may also function in covalent attachments of the carbohydrate of ground substance to the collagen fiber during the maturation process. Clearly, much more information is now needed.

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