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Studies on the Reduction of Elastin. II. Evidence for the Presence of α-Aminoadipic Acid δ-Semialdehyde and Its Aldol Condensation Product*

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ABSTRACT: Studies on the reduction of elastin with sodium borotritide have shown: (1) Most of the tritiated compounds in an acid hydrolysate of elastin previously reduced with sodium borotritide arise from modified lysine residues in the backbone of the peptide chains. This was confirmed by studies on [^{14}C]lysine-labeled elastin obtained from chick embryo aortas grown in tissue culture. (2) Residues of α-aminoadipic acid δ-semialdehyde which are formed by the deamination of the ε-amino group of certain lysine residues in elastin, are reduced by sodium borohydride to ε-hydroxynorleucine residues. Hydrolysis of reduced elastin in 6 N HCl leads to substantial conversion of the ε-hydroxynorleucine into ε-chloronorleucine, which in turn is converted into pipecolic acid upon treatment with dilute alkali. Studies on pure ε-hydroxynorleucine confirmed these results. It was also found that ε-hydroxynorleucine was stable to treatment with 2 N NaOH at 110° for 22 hr. Hydrolysis of reduced tritiated elastin in 2 N NaOH revealed 2–3 residues of ε-hydroxynorleucine/1000 amino acid residues. (3) The properties of the most prominent radioactive compound in an alkaline hydrolysate of reduced elastin were consistent with the reduced derivative of an aldol condensation product of two residues of α-aminoadipic acid δ-semialdehyde. Mass spectrum of its ethyl ester derivative, oxidation with a mixture of periodate and permanganate, hydrogenation over palladium, and the calculated specific activity all agree with the proposed structure. In elastin the aldol condensation product may serve a dual role: first, as an independent cross-link, and second as a precursor for the desmosine cross-links. In bovine elastin, there are 4–5 residues of the aldol condensation product per 1000 amino acids.

It has been clearly established by Partridge *et al.* (1966) and Miller *et al.* (1964) that lysine is the precursor of the desmosine and isodesmosine cross-links in elastin. Recently, Franzblau *et al.* (1965) described another amino acid, lysinonorleucine, occurring in hydrolysates of elastin. The structure of this compound indicates that it too may serve as a cross-linking agent in elastin. We suggested that the precursor of lysinonorleucine also is lysine. Studies on the reduction of bovine elastin with sodium borotritide led to the proposal that synthesis of lysinonorleucine probably occurs *via* formation of the Schiff base, $\Delta^{6,7}$-dehydrolysinonorleucine (Lent and Franzblau, 1967). This could arise from the condensation of one residue of α-aminoadipic acid δ-semialdehyde and one residue of lysine. In addition to that incorporated into the lysinonorleucine fractions, significant amounts of tritium were found in several other fractions obtained from hydrolysates of elastin. In particular, in column amino acid analysis, one tritium-containing fraction appeared in the region of isoleucine and one eluted just before tyrosine. The desmosines are apparently reduced by $NaBT_4$ as well (Lent and Franzblau, 1967). Pretreatment of bovine elastin with 2,4-dinitrophenylhydrazine followed by reduction with $NaBT_4$ leads to the presence of tritium only in the reduced desmosine fractions. Conversely, reduction of elastin abolishes subsequent reaction with 2,4-dinitrophenylhydrazine (Lent and Franzblau, 1967).

Miller and Fullmer (1966) reported the presence of both saturated and unsaturated aldehydes in an elastin preparation, which was purified by enzymatic procedures. Recent studies by Miller *et al.* (1967) indicated that oxidation of [^{14}C]lysine-labeled elastin with performic acid results in the formation of [^{14}C]α-aminoadipic acid. According to these authors, certain of the radioactive peaks obtained from hydrolysates of non-oxidized elastin appear to be degradation products of α-aminoadipic acid δ-semialdehyde.

The present communication reports studies on elastin of chick embryo grown in tissue culture in the presence of [^{14}C]-lysine. Additional studies on the reduction products of bovine elastin are also reported here. Our results clearly indicate incorporation of [^{14}C]lysine into lysinonorleucine. If, as suggested by Miller *et al.* (1967), performic acid oxidation of elastin causes almost exclusively the formation of α-amino-

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adipic acid, then reduction should lead only to ε-hydroxynorleucine, since the residues being reduced or oxidized are α-aminoadipic acid δ-semialdehyde residues. It is known that ε-hydroxynorleucine itself is not stable to acid hydrolysis, appearing after this treatment as two major ninhydrin-positive peaks on the amino acid chromatogram. One of these appears just before glycine and the other before tyrosine. Evidence suggests that all the [^{14}C]lysine incorporated into elastin does not follow this pattern. Nevertheless, ε-hydroxynorleucine is a principal product of the reductive treatment. However, studies on alkaline hydrolysates of reduced elastin reveal still another major radioactive component. Data presented indicate that this new component is the reduced aldol condensation product of two residues of α-aminoadipic acid δ-semialdehyde.

Materials

Bovine *ligamentum nuchae* elastin was prepared according to the method of Partridge *et al.* (1955). $NaBT_4$ and uniformly labeled [^{14}C]lysine were obtained from New England Nuclear Corp. and L-pipecolic acid from Calbiochem Corp. ε-Hydroxynorleucine was synthesized according to the procedure of Gaudry (1948). Hanks Medium 199 was purchased from GIBCO, N. Y. White Leghorn chick embryos (12-days old) were obtained from Spafas Inc., Norwich, Conn.

Methods

Amino acid analyses were performed automatically on a Technicon amino acid analyzer. When required a stream-splitting device was employed.

Mass spectrum were obtained with the use of a Hitachi-Perkin-Elmer Model RMU-6E mass spectrometer.

Radioactivity was determined with a Packard Model 3310 liquid scintillation counter employing Bray's solution (15 ml) as the scintillation fluid.

Reduction of elastin was carried out as described previously (Lent and Franzblau, 1967). Briefly, 179.0 mg of either $NaBH_4$ or a standardized mixture of $NaBH_4$ and $NaBT_4$ (Blumenfeld and Gallop, 1966) was added to 1 g of bovine elastin or 1 g of dried defatted chick embryo aorta suspended in 15 ml of 0.001 M EDTA (pH 9.0). Reduction was allowed to proceed for 2 hr while keeping the pH constant by addition as required of small amounts of 0.01 N HCl. The reaction was then stopped and remaining sodium borohydride destroyed by adjusting the reaction mixture to pH 3.0 with 50% acetic acid. The reduced bovine elastin or chick embryo aortas were then thoroughly washed with water, alcohol, and ether, and finally dried *in vacuo*.

[^{14}C]Lysine-labeled elastin was prepared from 12-day-old chick embryo aortas in the following manner. From each embryo a segment of ascending aorta, approximately 3 mm, was placed in a tissue culture tube containing 3 ml of Hanks Medium 199, modified to contain per ml, 100 units each of penicillin and streptomycin and 0.5 μCi of uniformly labeled [^{14}C]lysine. After incubation of the aortas at 37° for 24 hr, the medium was replaced with fresh medium devoid only of radioactive lysine. The culture was then incubated for 8 days more, during which time the medium was replaced every other day. The number of aortas handled at any one time was 150. After 750 had been cultured, a procedure requiring 6 weeks, all were pooled and washed several times with 0.9% NaCl followed by H_2O. The aortas were then extracted with alcohol followed by ether. The total yield was 105 mg of defatted aortic tissue. In one preparation β-aminopropionitrile fumarate (15 μg/ml) was also added to the tissue culture medium.

Oxidation with a periodate–permanganate mixture was carried out by a modification of the procedure of Lemieux and von Rudloff (1955). To 0.35 ml of test solution, 0.25 ml of 0.1 M K_2CO_3 (pH 7.5) was added followed by 0.40 ml of 0.02 M $NaIO_4$ and 0.04 ml of 0.005 M $KMnO_4$. Oxidation was then allowed to proceed for varying periods of time. Determination of formaldehyde was carried out as follows. To 0.2 ml of reaction mixture, 0.02 ml of 1.0 M $NaAsO_2$ and 0.02 ml of 2 N H_2SO_4 were added. After standing 15 min, 0.3 ml of H_2O and 5.0 ml of 0.2% chromotropic acid solution (19.2 N H_2SO_4) were added successively and the entire mixture was placed in a boiling-water bath for 30 min, cooled, and the absorbance at 570 mμ was determined. Formaldehyde and erythritol were used as standards.

Reduction of Periodate–Permanganate Oxidation Mixture. After oxidation for 45 min as described above, 3.0 ml of H_2O was added. For each 0.5 μmole of formaldehyde present in the reaction mixture above, 14.0 mg of $NaBT_4$ was added after the pH was adjusted to 8.5 with 0.01 N NaOH. Reduction was allowed to proceed for 90 min at which time 50% acetic acid was added until the pH was 3.0. The entire mixture was evaporated to dryness and dissolved in 0.01 N HCl. Unlabeled pipecolic acid and proline were added and the mixture was desalted on a Dowex 50 column. The desalted mixture was redissolved in 0.01 N HCl and placed on the amino acid analyzer equipped with a stream-splitting device.

Experimental Procedures and Results

Studies with [^{14}C]Lysine-Labeled Elastin. The [^{14}C]lysine-labeled aortas were divided into equal portions. One portion (35 mg) serving as a control was treated with 2 ml of 0.1 N NaOH for 45 min at 95° according to the procedure of Lansing *et al.* (1952). The sample was then rinsed well with water and subjected to hydrolysis in 6 N HCl at 105° in a sealed vial. The hydrolysate was evaporated to dryness, redissolved in 0.01 N HCl, and a portion was placed on the amino acid analyzer equipped with a stream-splitting device. Collected fractions were assayed for radioactivity. The distribution of radioactivity is shown in Figure 1A. Major radioactive peaks appeared in the desmosine, lysinonorleucine, glycine, and leucine regions. Similar results have been obtained from elastin of rats fed uniformly labeled [^{14}C]lysine (Cleary *et al.*, 1966). Because of the poor separation of lysinonorleucine, merodesmosine, lysinoalanine, and lysine on the long column (130 cm), another aliquot was run on a 50-cm column at pH 4.5 (0.38 M sodium citrate). Good separation of all the components listed above was thereby achieved. The specific activities of the isolated lysine, desmosine, lysinonorleucine, and lysinoalanine are reported in Table I. Examination of aortas incubated in the presence of β-aminopropionitrile in a manner similar to Miller *et al.* (1965) showed no radioactivity in the lysinonorleucine region; in fact only the lysine contained radioactivity.

A second portion (35 mg) of the dried, pooled aortas was treated as above with one additional step. Before application of the alkaline-extracting procedure, the material was reduced with sodium borohydride. The distribution of radioactivity is given in Figure 1B. The specific activities of lysine, the des-

TABLE I: Specific Activities of Several Amino Acids Obtained from [^{14}C]Lysine-Labeled Elastin.

Amino Acid	cpm/μmole[a]	
	Not Reduced	Reduced
Lysine	34,000	31,000
Desmosine	27,000	44,000
Isodesmosine	31,000	
Lysinonorleucine	33,000	37,000
Merodesmosine		31,000
Lysinoalanine	22,000	18,000

[a] Based on lysine equivalents assuming: (a) desmosine and isodesmosine equal to 4 lysine equiv; (b) merodesmosine equal to 3 lysine equiv; and (c) lysinonorleucine equal to 2 lysine equiv.

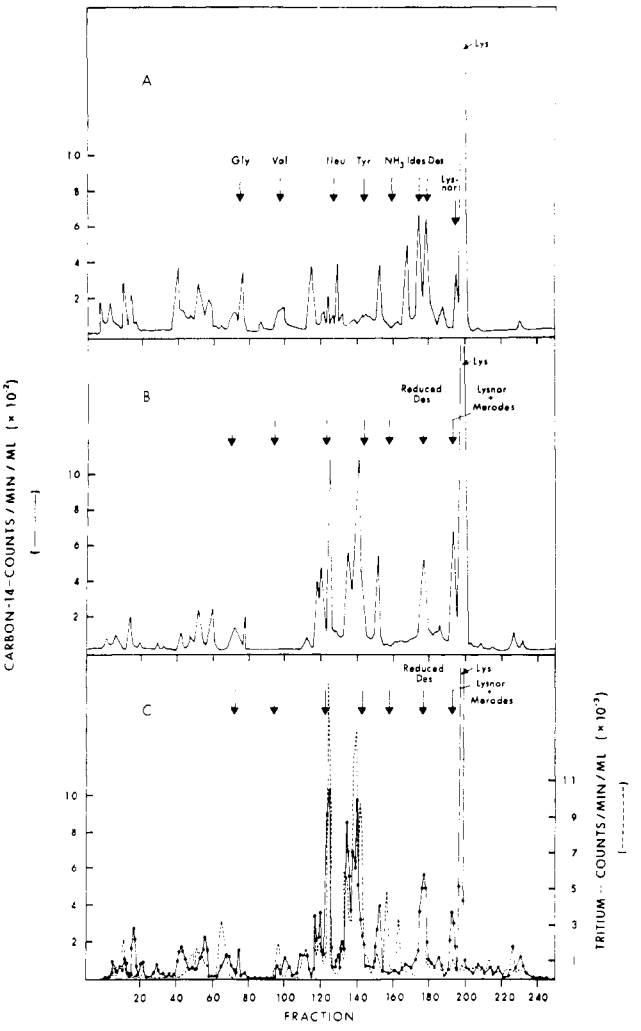


FIGURE 1: Radioactivity profile of [^{14}C]lysine-labeled elastin grown in tissue culture as described in text. Chromatography performed on amino acid analyzer equipped with split-stream device. (A) Elastin hydrolyzed in 6 N HCl; (B) elastin reduced with $NaBH_4$, then hydrolyzed in 6 N HCl; and (C) elastin reduced with $NaBT_4$, then hydrolyzed in 6 N HCl.

mosines, lysinonorleucine, and merodesmosine are reported in Table I. The isolated lysine had the same specific radioactivity as the lysine obtained in the control experiment. The desmosines in the two experiments also had almost identical specific activities; however, two discrete peaks were observed in the desmosine region before reduction, but only one broad peak was detected after reduction. The latter observation agrees with already reported experiments in which $NaBT_4$ was used (Lent and Franzblau, 1967). Of the radioactivity recovered, a significant portion occurs in two peaks eluting respectively near the isoleucine and tyrosine regions. These peaks were not present significantly before reduction, *i.e.*, in the control experiment. Small radioactive peaks in proximity to the valine and postphenylalanine regions, respectively, were observed in both control and reduced samples. Merodesmosine isolated in this manner also had a specific radioactivity approximately equal to that of the lysine provided that one ascribes to merodesmosine, in the ninhydrin assay, a color equivalent to three times the equivalent value for leucine. The latter results are compatible with the structure of merodesmosine proposed by Starcher *et al.* (1967).

Table I also includes data obtained for lysinoalanine, an artifact of alkaline hydrolysis of certain proteins (Bohak, 1964).

The third portion of dried aortas (35 mg) was treated with $NaBT_4$ as described in the methods. Alkaline extraction and analysis then revealed the distribution of both ^{14}C and ^{3}H radioactivities shown in Figure 1C. The ^{14}C-labeled peaks appearing in proximity to the isoleucine and tyrosine regions, observed after reduction with nonradioactive $NaBH_4$ in the previous experiment, now also contained tritium. The only significant peak containing ^{14}C and no tritium was that of lysine. However, there are at least three peaks containing tritium but no ^{14}C; two eluted in the region near ammonia, and one just before glycine.

The data in Figure 1C were corrected for low counting efficiency of ^{14}C due to the presence of tritium. As expected, the merodesmosine peak contained both tritium and ^{14}C.

Studies on the Hydrolysis of ε-Hydroxynorleucine. As mentioned in the introduction, ε-hydroxynorleucine, after treatment with 6 N HCl, divides into two major components observed as peaks on the amino acid analyzer, one eluting just before glycine and the other in the region of tyrosine. The possibility that this also occurred in acid-prepared hydrolysates of reduced elastin led us to reexamine the behavior of ε-hydroxynorleucine after treatment in 6 N HCl as compared with treatment in 2 N NaOH. A stock solution of synthetic ε-hydroxynorleucine was prepared. An aliquot placed on the amino acid analyzer yielded the pattern shown in Figure 2A. A second aliquot, heated for 20 hr at 105° in 6 N HCl and subsequently placed on the amino acid analyzer, gave the pattern shown in Figure 2B. A third aliquot, heated at 110° for 22 hr in 2 N NaOH and then neutralized with HCl, gave the analytical pattern shown in Figure 2D. A fourth aliquot, heated in 6 N HCl in the usual manner, evaporated to dryness, then treated with 1.0 ml 0.2 N NaOH for 2 hr at 95°, and finally neutralized with 6 N HCl, gave the chromatograph depicted in Figure 2C.

Results of these experiments are as follows. The chromatograph of ε-hydroxynorleucine after treatment with 6 N HCl

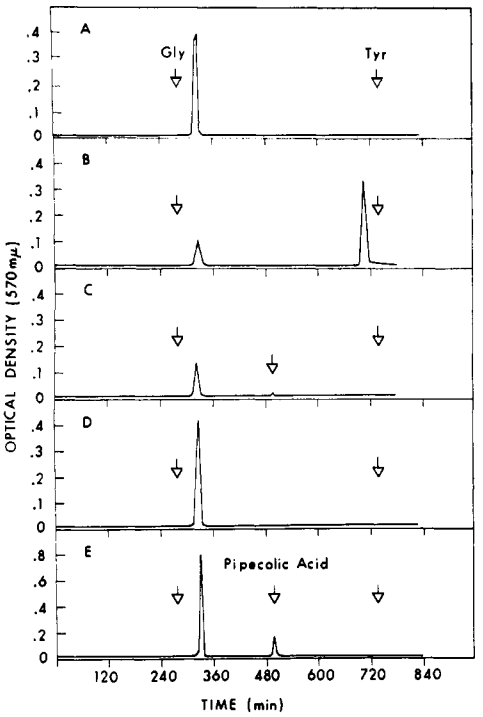


FIGURE 2: Behavior of ϵ-hydroxynorleucine on amino acid analyzer after several treatments. (A) Control sample, no treatment; (B) hydrolyzed in 6 N HCl; (C) hydrolyzed in 6 N HCl, followed by treatment with 0.2 N NaOH for 2 hr at 95°; (D) hydrolyzed in 2 N NaOH for 22 hr at 110°; (E) cochromatography of L-pipecolic acid and C.

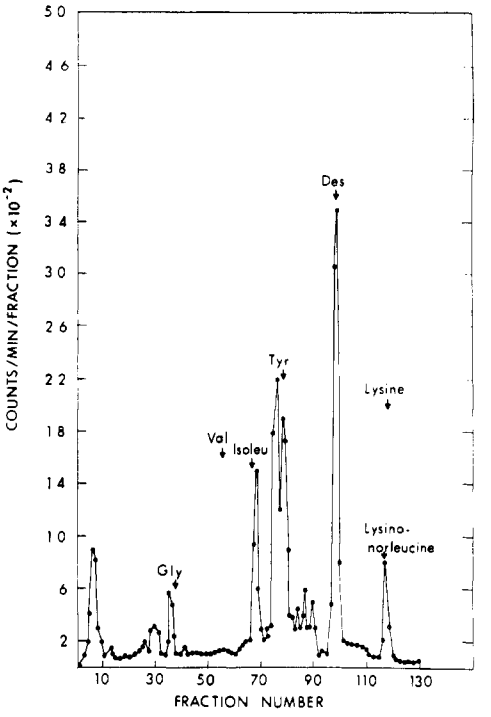


FIGURE 3: Distribution of tritium in acid hydrolysate (6 N HCl) of bovine elastin previously reduced with $NaBT_4$. Arrows indicate the peak positions of certain amino acids on the amino acid analyzer.

shows two major distinct peaks. Of the total ninhydrin-assayable material, 30% appeared near the position at which glycine would be eluted, and 70% near tyrosine.

In contrast, the ninhydrin-reactive material found after treatment of ϵ-hydroxynorleucine with 2 N NaOH, appears solely in the peak near the position characteristic of glycine. Treatment with 0.2 N NaOH of ϵ-hydroxynorleucine that had received prior treatment with 6 N HCl, indicated that the peak near the glycine region was not altered when compared with material given the direct acid treatment (Figure 2B), but the peak near the region for tyrosine disappeared. In addition, a small peak appeared near the region for valine.

Two possibilities were considered for the structure of the compound with elutes proximate to tyrosine: ϵ-chloronorleucine and $\Delta^{5,6}$-dehydronorleucine. The latter, synthesized according to the method of Albertson (1946), appeared in a chromatograph just before the position for isoleucine, and did not cochromatograph with the unknown compound as a single peak. In addition, $\Delta^{5,6}$-dehydronorleucine was stable to treatment with alkali. On the other hand, if the compound was ϵ-chloronorleucine, treatment with alkali would lead to formation of pipecolic acid. Cochromatography of authentic pipecolic acid and ϵ-hydroxynorleucine treated successively with 6 N HCl and 0.2 N NaOH (Figure 2C), yielded a single peak eluting near the position for valine (Figure 2E). The same characteristic spectral absorption ratio at 440 mμ/570 mμ was obtained after reaction with ninhydrin of both commercial pipecolic acid and the compound arising from ϵ-hydroxynorleucine after HCl–NaOH treatments. We were unable to synthesize ϵ-chloronorleucine, but did prepare the homologous 2-amino-6-chloroheptanoic acid according to the method of Aspen and Meister (1962). This compound eluted just after the position for phenylalanine, and was converted by treatment with dilute NaOH into a compound eluting near the position for glycine, and having a higher absorbance at 440 than at 570 mμ.

Products of the treatment of ϵ-hydroxynorleucine with 6 N HCl were separated on an amino acid analyzer equipped with a split-stream divider. The peak contents eluting near the position for tyrosine were pooled and desalted. The desalting procedure, which includes elution with 0.25 N NH_4OH, led to substantial conversion of the material into pipecolic acid. Thin-layer chromatography, using silica gel and a developing solvent system of butanol–acetic acid–water (4:1:1, v/v), was then used to separate pipecolic acid from its precursor. Both compounds were eluted from the thin-layer plates with 0.01 N HCl. The ethyl esters were prepared according to the method of Biemann *et al.* (1961). The mass spectrum of the precursor indicated intense peaks at *m/e* values of 84, 120, and 122. The peaks at 120 and 122 occurred in a ratio of 3:1, and accorded with the amine fragment expected to arise from ϵ-chloronorleucine. The expected ratio of 3:1 is due to the natural distribution of ^{35}Cl and ^{37}Cl, which is 3:1. The mass at 84 indicates cyclization of the amine fragment with elimination of HCl. Thus, the spectrum agrees fully with the structure of ϵ-chloro norleucine. The mass spectrum of the second component accorded with that of pipecolic acid.

Studies on Hydrolysates of Reduced Bovine Elastin. A. HYDROLYSIS WITH ACID. Reduced bovine elastin (50.0 mg) was refluxed for 22 hr in 6 N HCl. The HCl was evaporated, and the sample was washed thoroughly with water and dried by evaporation. A portion (2.2 mg) of the hydrolysate was placed on

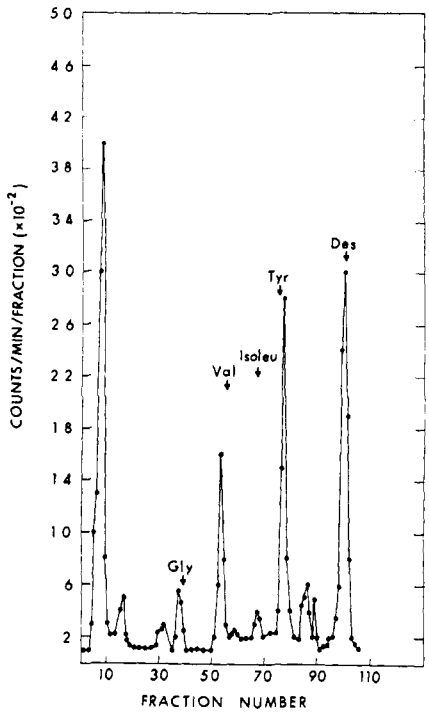


FIGURE 4: Distribution of tritium from reduced bovine elastin ($NaBT_4$) hydrolyzed in 6 N HCl followed by mild alkali treatment. Arrows indicate the peak positions of certain amino acids on the amino acid analyzer.

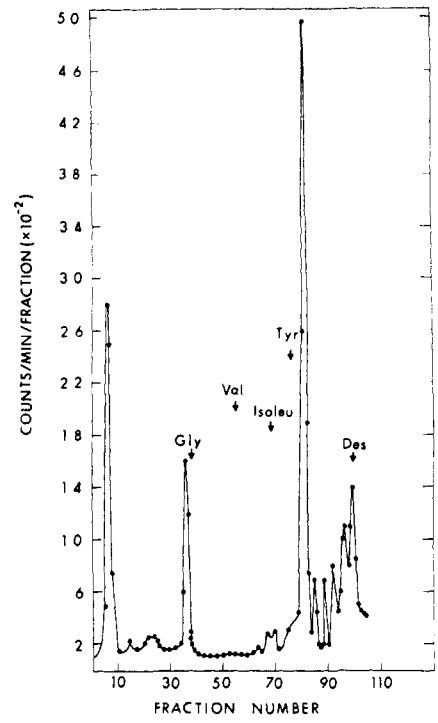


FIGURE 5: Distribution of tritium in 2 N NaOH hydrolysate of bovine elastin previously reduced with $NaBT_4$. Arrows indicate positions of certain amino acids on amino acid analyzer.

the amino acid analyzer (Type C-2 resin) equipped with a split-stream device. The total time for a complete amino acid separation using this system was 12 hr. The portion of eluent not analyzed was collected in fractions of 1.15 ml. An aliquot (0.5 ml) of each fraction was examined for radioactivity. The distribution of radioactivity is reproduced in Figure 3. Of the radioactivity placed on the column, 80% was recovered in the collected fractions, and the remainder in the subsequent wash with 0.2 N NaOH used for regeneration of the column. In Figure 3 one may note that only a small portion of total radioactive counts were recovered in the region near glycine. As already shown in the model experiments, this peak corresponds to that portion of ε-hydroxynorleucine remaining under these conditions of treatment with 6 N HCl. There was also a considerable number of counts in the region near tyrosine, as expected if ε-chloronorleucine were present. In addition, significant radioactivity occurred in the regions proximate to isoleucine and the desmosines.

B. TREATMENT WITH ALKALI OF ACID-HYDROLYZED ELASTIN. If an HCl hydrolysate of reduced elastin contains ε-chloronorleucine, then subsequent treatment with alkali should lead to formation of pipecolic acid. This could be detected in analysis by diminution of the material eluting in the peak near tyrosine. An aliquot (2.2 mg) of the acid hydrolysate described above was dried by evaporation and then treated with 0.5 ml of 0.2 N NaOH at 95° for 2 hr. The solution was acidified to pH 2.0 with concentrated HCl and diluted to 1.0 ml with water. The entire sample was placed on the amino acid analyzer. The chromatographic distribution of radioactivity is shown in Figure 4. A radioactive peak eluting in the region near valine is evident where no peak occurred in the control analysis. This new peak corresponds to the position at which authentic pipecolic acid elutes in this system.

In the alkali-treated sample, the total number of counts which have moved out to the peak proximate to the tyrosine region, observed in the control HCl-hydrolyzed sample, are almost completely recovered in the new peak. However, not all of the counts observed in the control in the region near tyrosine are removed by the subsequent treatment with alkali. The newly appearing radioactive peak which arises after treatment with alkali cochromatographed with added authentic pipecolic acid.

In a separate experiment, the radioactive fractions obtained from HCl-treated reduced elastin in the region near tyrosine were pooled, desalted, and treated with 0.2 N NaOH, and the solution was analyzed. Again, the counts proximate to the valine region cochromatographed with an authentic sample of pipecolic acid. It should be pointed out that even without treatment with alkali, a significant portion of the material is converted into pipecolic acid by the desalting procedure. Nevertheless, the desalted material was treated with alkali to ensure that all of the ε-chloronorleucine present in the sample was converted into pipecolic acid.

C. ALKALI HYDROLYSIS OF REDUCED ELASTIN. As already demonstrated, ε-hydroxynorleucine is apparently stable to treatment with 2 N NaOH. Because of this, we examined the radioactive elution profile of a sample of reduced elastin hydrolyzed under these alkaline conditions. Reduced tritiated elastin (50.0 mg) was hydrolyzed in 2.0 ml of 2 N NaOH at 110° for 22 hr in an alkali-resistant sealed vial. Previous studies have indicated that, under these conditions, a small but significant amount of valylproline is obtained (Cannon *et al.*, 1967). In addition, some destruction of the desmosines ap-

TABLE II: Distribution of Tritium from Reduced Elastin after Various Treatments.[a]

Fraction (region)	Treatments (total cpm/fraction)				
	Acid[b]	Acid, Then Base[c]	Base[d]	Base,[d] Then Acid	Base,[d] Then Acid, Followed by Base[c]
ε-Hydroxynorleucine	7,400	7,700	21,000	6,750	7,900
Pipecolic acid	0	14,600	0	0	15,100
"Isoleu"	17,600	4,900	950	19,250	4,500
"Tyr I"[e]	30,250	33,500	0	29,150	31,800
"Tyr II"[e]	26,400		0	27,500	
"Post-Phe" (aldol condensate)	0	0	55,000	0	0
Desmosines	42,500	40,100			

[a] The same quantity of protein was used for each treatment (2.2 mg). [b] All acid treatments consisted of 6 N HCl in a sealed vial at 110° for 22 hr. [c] Base treatment 0.2 N NaOH at 95° for 2 hr. [d] Base hydrolysis 2.0 N NaOH at 110° for 22 hr. [e] ε-Chloronorleucine occurs in these fractions.

parently occurs (Anwar and Oda, 1966). The alkaline hydrolysate was then neutralized with concentrated HCl and the solution was diluted to 30.0 ml with water. An aliquot of this solution corresponding to 2.2 mg of original protein was placed on the amino acid analyzer and assayed for radioactivity. The distribution of radioactivity is shown in Figure 5. A significant portion of the counts was found in the region near glycine corresponding to the position of ε-hydroxynorleucine. The counts obtained in the first fractions from the column are apparently due to tritiated water most likely arising from destruction of the desmosines. The major radioactive peak appears after phenylalanine and does not correspond to either of the peaks seen near the tyrosine region in samples obtained after hydrolysis in the HCl (see Figure 3). In the latter case, the radioactivity appeared in peaks near the isoleucine and tyrosine regions, and does not appear here (Figure 5).

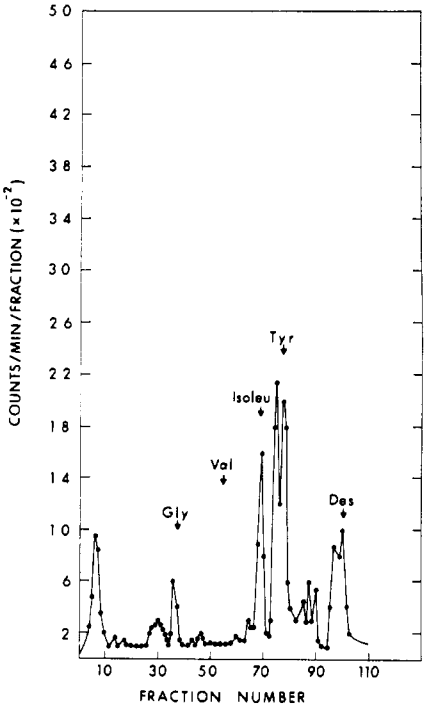


FIGURE 6: Distribution of tritium from reduced bovine elastin hydrolyzed in 2 N NaOH followed by hydrolysis in 6 N HCl. See text for details. Arrows indicate the peak positions of certain amino acids on the amino acid analyzer.

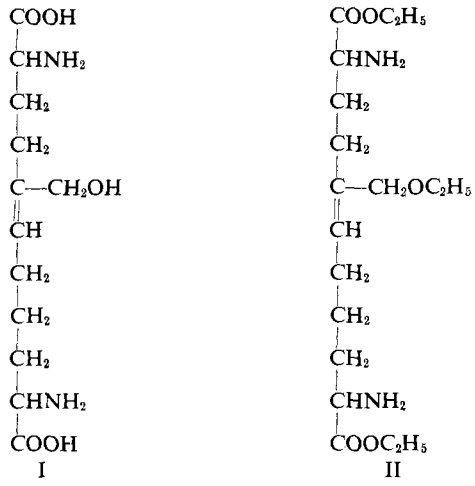
D. HCl HYDROLYSIS OF HYDROLYSATE OBTAINED BY TREATMENT WITH NaOH. A portion of the diluted alkaline hydrolysate from above, equivalent to 2.2 mg of original reduced elastin, was treated in 6 N HCl at 110° for 22 hr in a sealed vial. The solution was evaporated to dryness, the residue was washed thoroughly with water and then dissolved in 0.01 N HCl, and the solution was placed on the amino acid analyzer. The distribution of radioactivity found after these treatments is shown in Figure 6. As can be seen, the radioactivity in the peak near the glycine region was decreased to approximately 30% of the control, which in this case was the sample treated only to alkaline hydrolysis (Figure 5). Radioactivity appeared in the regions near isoleucine and tyrosine and concomitantly was markedly reduced near the phenylalanine region. The counts in the desmosine region are again relatively diminished indicating irreversible destruction due to the alkaline treatment. There were no counts in the pipecolic acid region. However, reduced tritiated elastin, treated successively with 2 N NaOH, 6 N HCl, and then again with 0.2 N NaOH for 2 hr at 95°, showed a significant number of counts in the pipecolic acid region, in fact, twice as many counts were found in this region as compared with the region proximate to the position of glycine. This would be expected if the ratio of ε-hydroxynorleucine to ε-chloronorleucine had been 30:70 after treatment with 6 N HCl.

The total counts recovered in the several pertinent fractions described in the above experiments are given in Table II. Using the procedure of Blumenfeld and Gallop (1966) one can estimate the concentration of these various reduced substances if one assumes that only one tritium atom per molecule is incorporated during the reduction procedure. On

this basis, an alkaline hydrolysate of reduced elastin contained 2–3 residues of ε-hydroxynorleucine/100 total amino acid residues and 4–5 residues of the compound eluting after phenylalanine.

Isolation and Partial Characterization of Individual Radioactive Components from Reduced Tritiated Elastin. A. POSTPHENYLALANINE PEAK AFTER ALKALINE HYDROLYSIS. To isolate and detect the material in the postphenylalanine peak using the ninhydrin reaction, we employed a buffer gradient (Burns *et al.*, 1965) which tends to spread out the amino acid chromatogram between isoleucine and ammonia. The elution pattern of the hydrolysate of elastin made with 2 N NaOH and then resolved on the amino acid analyzer (type A resin) employing this gradient is shown in Figure 7A. In contrast, the pattern of elastin previously reduced with $NaBT_4$, and then hydrolyzed in 2 N NaOH, shows a ninhydrin-positive peak between leucine and tyrosine (Figure 7B). This peak had significant radioactivity and, in all systems tested appeared to represent the material of the postphenylalanine peak described above. As expected, an acid hydrolysate of the base-hydrolyzed reduced elastin did not show this unique peak. The material in this peak was isolated in large quantities, desalted, and characterized as follows.

Mass Spectrum. The ethyl ester was prepared using anhydrous ethanolic HCl according to the procedure of Biemann *et al.* (1961). The mass spectrum is consistent with structure (I). It is the reduced derivative of the product of an aldol condensation of two residues of α-aminoadipic acid δ-semialdehyde.



The mass spectrum of the ethyl ester derivative (Figure 8) shows a parent molecular ion (M^+) with a mass of 358. From the structure depicted (I), one should have expected a molecular ion of 330. However, under the conditions of esterification, the α,β-unsaturated alcohol group apparently reacts with 1 mole of ethanol to form the corresponding ether (II). The fragmentation pattern is entirely consistent with this structure as the parent molecular ion (M^+). In addition, the parent molecular ion may actually exist also in a cyclized form, *i.e.*, either of the α-amino groups is capable of adding across the double bond. Evidence for both open form and cyclic forms appears in the mass spectrum. The parent ion in either form is capable of further cyclization, by elimination of C_2H_5OH, to yield a significant peak at *m/e* of 312. There are significant

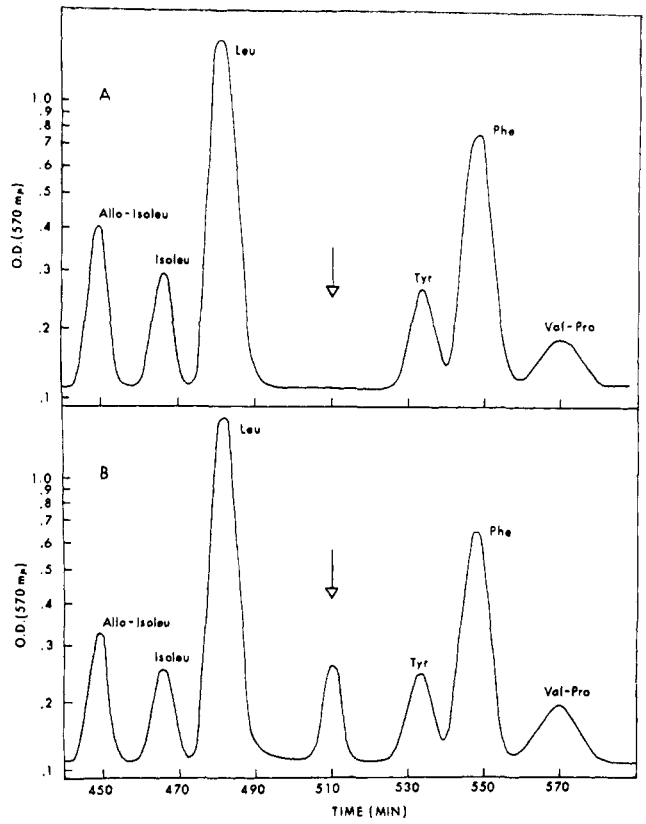


FIGURE 7: Amino acid chromatogram of bovine elastin (A) and elastin reduced with $NaBT_4$ (B). Both samples were hydrolyzed in 2 N NaOH for 22 hr at 110°. The gradient employed is that of Burns *et al.* (1965). The ninhydrin reaction is measured at 570 mμ.

peaks for losses corresponding to $M^+ - 45$ (loss of C_2H_5O), $M^+ - 59$ (loss of $CH_2OC_2H_5$), $M^+ - 73$ (loss of $COOC_2H_5$), $M^+ - 119$ (loss of $C_2H_5OH + COOC_2H_5$), $M^+ - 136$ (loss of $C_2H_5OH + COOC_2H_5 + NH_3$), and $M^+ - 192$ (loss of $C_2H_5OH + 2COOC_2H_5$). The last peak occurs at *m/e* of 83, the most abundant fragment. A fragment also occurs at 83.5, corresponding to a loss of $C_2H_5O + 2COOC_2H_5$. The fragments at 83 and 83.5 correspond to different diamine fragments (*i.e.*, the loss of two $COOC_2H_5$ groups) with *m/e* values of 166/2 and 167/2, respectively.

Specific Activity. Based on the standardized $NaBT_4$ used and ninhydrin reactivity, the specific activity of the isolated purified amino acid contains 1 tritium atom/2.1 leucine equiv. This result agrees with the proposed structure (I) which shows two α-amino acid functional groups and one alcohol (or tritium) group.

Hydrogenation. Hydrogenation over palladium at 2 atm of pressure yielded another compound which eluted in the amino acid analyzer just before ammonia.

Oxidation. The purified compound was oxidized with a periodate–permanganate mixture as described in the methods. A stock solution of the unknown compound was prepared containing 1.0 μM leucine equiv/ml as measured by the ninhydrin reaction (each mole of reduced aldol condensation product yields 2 leucine equiv). Aliquots of this solution were oxidized for varying periods of time. After 15 min, 0.90 mole of formaldehyde per mole of aldol condensation could be measured by reaction with chromtropic acid. An allylic alcohol,

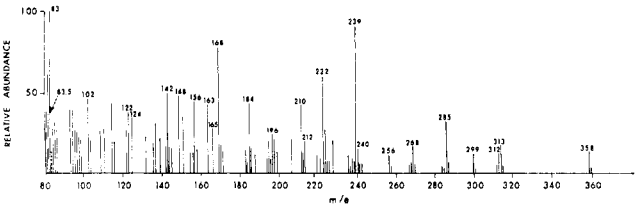


FIGURE 8: Mass spectrum of ethyl ester derivative of reduced aldol condensation product of two residues of α-aminoadipic acid δ-semialdehyde (see text for details).

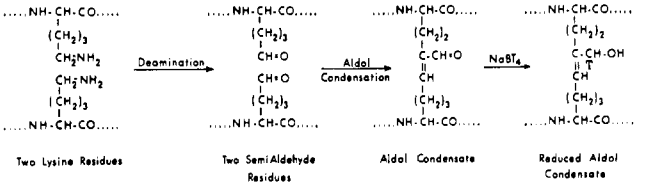


FIGURE 9: Formation of aldol condensate from two residues of lysine. Included is formation of the reduced aldol condensate after treatment with $NaBT_4$.

when oxidized in this manner, should yield 1 equiv of formaldehyde. When the formaldehyde was distilled approximately 60% of the radioactivity (tritium) was collected. Recovery of formaldehyde in the distillate also corresponded to 60%.

Amino acid analysis showed that for each mole of leucine equivalent oxidized, 0.3 mole of aspartic acid, 0.1 mole of glutamic acid, and 0.03 mole of α-aminoadipic acid appeared. If the reaction mixture after oxidation is reduced with the standardized $NaBT_4$, desalted, and placed on the amino acid analyzer equipped with a split-stream divider, significant radioactivity appears in the regions of pipecolic acid and proline. Nonradioactive aspartic acid, glutamic acid, and α-aminoadipic acid also appear. This suggests that oxidation is not complete and, specifically, α-aminoadipic acid δ-semialdehyde and glutamic acid δ-semialdehyde are still present. Each of these compounds could cyclize with formation of an internal Schiff base; then, on reduction, α-aminoadipic acid δ-semialdehyde would yield pipecolic acid and glutamic acid γ-semialdehyde would yield proline. Based on the specific activity of the $NaBT_4$ used, there appear approximately 0.2 mole of pipecolic acid and 0.1 mole of proline per 0.5 mole of aldol condensate (1 leucine equiv) oxidized and then subsequently reduced.

Acid Hydrolysis. A portion of the radioactive aldol condensation product containing 100,000 cpm was treated with 6 N HCl in the usual manner. Approximately 45% of the counts was lost during subsequent evaporation of the 6 N HCl and of applied water washes. The residue was dissolved and placed on the amino acid analyzer with the split-stream divider. Although only 60% of applied radioactivity was recovered, over 90% of recovered counts appeared in the region near isoleucine.

It should be noted that a 6 N HCl hydrolysate of reduced elastin shows a similar radioactive peak in the region near isoleucine, while the reduced aldol region is devoid of any significant radioactivity. Thus, the material in the peak near isoleucine obtained after treatment of reduced elastin with 6 N HCl apparently is a degradation product of the reduced aldol condensate. This conversion is not reversible since alkaline treatment of the previously acid-hydrolyzed elastin does not result in appearance of the reduced aldol.

B. DESMOSINE REGION. The radioactive fractions in the desmosine region resulting from an acid hydrolysate were pooled and desalted. Treatment of this material with 2 N NaOH at 110° for 22 hr followed by chromatography indicated a major portion of the radioactive counts came through the amino acid analyzer column with the void volume. Evaporation of the alkaline-treated material before placing it on the analyzer caused this latter peak to disappear, suggesting that it was in fact tritiated water as considered above. To establish that the aldol condensation product described above did not result from the alkaline hydrolysis of reduced desmosine in peptide linkage, elastin, which had been treated with 2,4-dinitrophenylhydrazine and subsequently reduced with $NaBT_4$, was subected to alkaline hydrolysis in 2 N NaOH. No radioactivity and no ninhydrin-positive peak was found in the region between leucine and tyrosine, but again significant radioactivity came right through the column.

Discussion

Data presented indicate that during hydrolysis in HCl, 70% of the ε-hydroxynorleucine residues present in reduced elastin are converted into ε-chlorononorleucine. The latter compound, when treated with dilute alkali, is converted into pipecolic acid. However, a very significant finding was that ε-hydroxynorleucine is stable to treatment with 2 N NaOH at 110° for 22 hr. Unfortunately, the concentrations of glycine and alanine in the hydrolysate of elastin used in these experiments were so overwhelming that the ninhydrin color due to ε-hydroxynorleucine could not be measured on the amino acid analyzer. However, one can estimate the quantity of ε-hydroxynorleucine present by determining the counts in the radioactive peak and relating this value to the specific activity of the standardized $NaBH_4$–$NaBT_4$ mixture which was employed in the reduction. By this procedure 2–3 residues of ε-hydroxynorleucine per 1000 amino acid residues were calculated to be present in the protein. In the untreated elastin this would correspond to two to three residues of α-aminoadipic acid δ-semialdehyde. That this radioactive peak was in fact ε-hydroxynorleucine was proved by further studies on the base-hydrolyzed elastin. Acid treatment of the base-hydrolyzed protein produced a decrease in the counts in the region of ε-hydroxynorleucine and an increase in the counts near the tyrosine region. As shown in Table II, the counts are distributed as one would predict based on studies of ε-hydroxynorleucine alone. When the acid-treated base hydrolysate was treated again with dilute alkali, pipecolic acid was formed, once more in the exact quantity one would have expected from the original concentration of ε-hydroxynorleucine present. These data together with the mass spectrum demonstrate that a significant quantity of α-aminoadipic acid δ-semialdehyde is present in elastin.

In alkaline hydrolysates of reduced elastin, the reduced derivative of the aldol condensation product of two residues of α-aminoadipic acid δ-semialdehyde was the most prominent radioactive peak, greater even than the desmosines. Because the usual procedure for hydrolysis of proteins in 6 N HCl re-

sult in its destruction, this compound had hitherto escaped detection. Use of a modified buffer gradient in our amino acid analyzer allowed its separation from all other amino acids in an alkaline digest of elastin. The concentration of this compound, both by the ninhydrin reaction and by radioactivity, was then estimated to be between 4 and 5 residues per 1000 amino acid residues in reduced mature bovine elastin. This amount is at least as great as the contents of desmosine, isodesmosine, and lysinonorleucine combined. Unpublished work by Salcedo and Franzblau, indicate that approximately 5–7 residues of the compound/1000 total residues exist in reduced elastin obtained from chick embryo aorta.

The oxidation studies suggest the possibility that the double bond may exist in either of two positions in the molecule. If the double bond exists as shown in structure I, then oxidation should lead to α-aminoadipic acid and glutamic acid or, if oxidation is not complete, to their respective ω-semialdehydes. If the double bond migrates to the alternate position α,β to the alcohol group, then oxidation would lead to aspartic acid and α-aminopimelic acid. Alternatively, aspartic acid could have occurred by an enolization mechanization of structure I during the oxidation procedure. It is interesting to note that Starcher *et al.* (1967) refrain from assigning the position of the double bond in merodesmosine as well.

The aldol condensate could be an independent cross-link in the protein and/or could serve as a precursor for the desmosine cross-link. Thus the aldol could combine with a dehydrolysinonorleucine residue to form a desmosine cross-link or it could join with a lysine residue to form an intermediate dehydromerodesmosine. The latter could then react with a residue of α-aminoadipic acid δ-semialdehyde to form a desmosine cross-link. Finally, the aldol could add to another α-aminoadipic acid δ-semialdehyde residue to form a "di-aldol." In the latter case, three semialdehyde residues condense leaving a single carbonyl function; in turn this adduct could then react with a lysine residue to form the carbon and nitrogen skeleton of desmosine.

In any case the aldol is most likely synthesized from two lysine residues as shown in Figure 9. Reduction with sodium borohydride adds stability to the natural cross-link so that it may be isolated after alkaline hydrolysis.

A recent paper by Smith *et al.* (1968) has described the isolation of a soluble "proelastin" from aortas of copper-deficient pigs. This soluble protein has an amino acid composition similar to that of insoluble elastin but does not contain desmosine or isodesmosine. The lysine content was approximately 42–45 residues/1000 amino acid residues. If this value is meaningful, it should be possible to account for these lysine residues in the mature elastin fiber. If one can overlook species and tissue differences in the elastin, a reasonable accounting can be obtained. Assuming that one desmosine or isodesmosine is derived from four lysine residues and one aldol condensate or one lysinonorleucine from two lysine residues one can account for 22–24 lysine residues/1000 amino acids. Added to this, the 2–3 residues of α-aminoadipic acid δ-semialdehyde and 4 residues of lysine present in elastin account for 28–31 lysine residues/1000 amino acid residues. Possibly one additional aldehyde component is present which derives from 3 lysine equiv.

Finally, the same aldol compound has been proposed as a cross-link between α chains in collagen (Bornstein *et al.*, 1966; Rojkind *et al.*, 1968). Preliminary experiments have indicated that it is present in small quantities in calf skin collagen (Paz *et al.*, 1969).

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