STUDIES ON THE REDUCTION OF BOVINE ELASTIN: EVIDENCE FOR THE PRESENCE OF $\Delta^{6,7}$-DEHYDROLYSINONORLEUCINE*

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A crosslinking amino acid in elastin, designated lysinonorleucine, has been described by Franzblau et al. (1965a, 1965b). Its structure suggested that it may be fashioned biosynthetically from two residues of lysine pre-existing in polypeptide chains of elastin or, indeed, of a pre-elastin. The proposed scheme of biosynthesis included: (a) oxidative deamination of the ε-amino group of one residue of lysine to yield a residue of α-amino adipic semialdehyde; (b) condensation of the aldehyde residue with the ε-amino group of a second residue of lysine to form a Schiff base (i.e., a residue of $\Delta^{6,7}$-dehydrolysinonorleucine) resulting in crosslinking of two elastin chains or two segments of a single chain; and (c) reduction of the aldimine function of the Schiff base to produce the final crosslinking residue of lysinonorleucine. The postulate that residues of lysine are converted to those of α-amino adipic semialdehyde is also fundamental to the scheme proposed by Partridge et al. (1964) and Miller et al. (1964) for biosynthesis of other crosslinking amino acids of elastin, desmosine and isodesmosine, and evidence for the occurrence of the aldehyde has been forthcoming (Partridge, 1966; Miller and Fullmer, 1966). In the present communication

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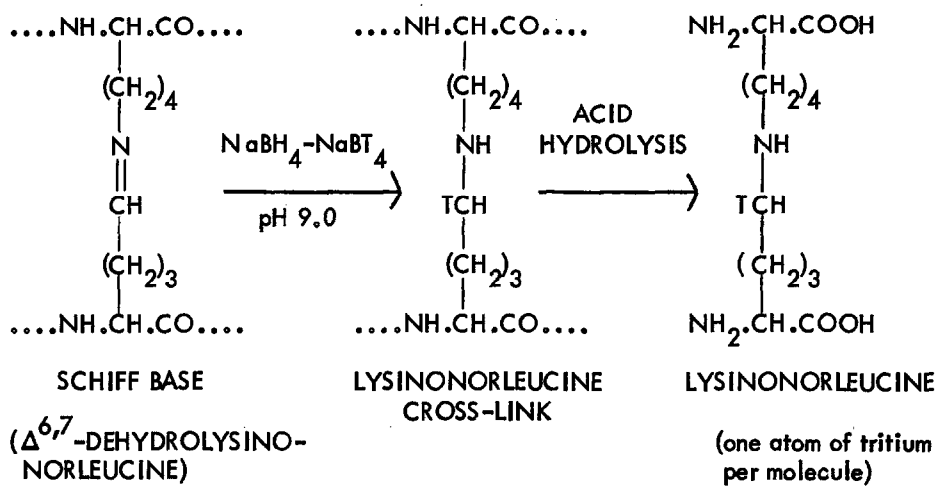
we give evidence that the proposed Schiff base intermediate is present in elastin and can be reduced chemically to lysinonorleucine according to reactions shown in Figure 1. Thus, treatment of elastin with tritiated sodium borohydride ($NaBT_4$) resulted in the introduction of tritium into chromatographically separable fractions containing lysinonorleucine and indeed in an increase of the content of lysinonorleucine. Incidental to these findings, tritium was found incorporated into the desmosine fractions and into other compounds yet unidentified, suggesting that the desmosines and certain other components of elastin also become reduced under the conditions used.

FIGURE 1



METHODS AND RESULTS

Reduction and analysis of bovine elastin. The elastin used in these studies was prepared from bovine ligamentum nuchae by the autoclaving method of Partridge et al. (1955). For reduction reactions, a mixture of $NaBH_4$ and $NaBT_4$ was prepared and standardized for radioactivity as described by Blumenfeld and Gallop (1966). Standardization includes reaction of the borohydride with 2-acetamido-3-butanone, a model compound that undergoes a one-step reduction with incorporation of one atom of hydrogen or tritium. The standardized $NaBH_4$-$NaBT_4$ (179 mg) was then added to elastin (1 gm) suspended in 50 ml of 0.001M EDTA previously adjusted to pH 9.0 with

NaOH. The reaction was allowed to proceed for two hours at pH 9, this being held constant by addition of 0.01N HCl. After this time excess borohydride was destroyed by acidification to pH 3.0 with 50% acetic acid. The reduced elastin was filtered, washed with water until the washings were neutral, and dried with alcohol followed by ether.

Reduced elastin (400 mg) was refluxed for 22 hours in 6N HCl. The HCl was evaporated and the sample washed thoroughly with water and dried by evaporation. An aliquot of hydrolysate was placed on the column of a Technicon Amino Acid Analyzer equipped with a split-stream device. Elution was carried out in a manner similar to that described by Hamilton et al. (1963), the portion of the eluant not analyzed being collected in fractions of 2.8 ml.

An aliquot (0.1 ml) of each fraction was examined for radioactivity in an Ansitron liquid scintillation counter. The observed distribution of radioactivity is depicted in

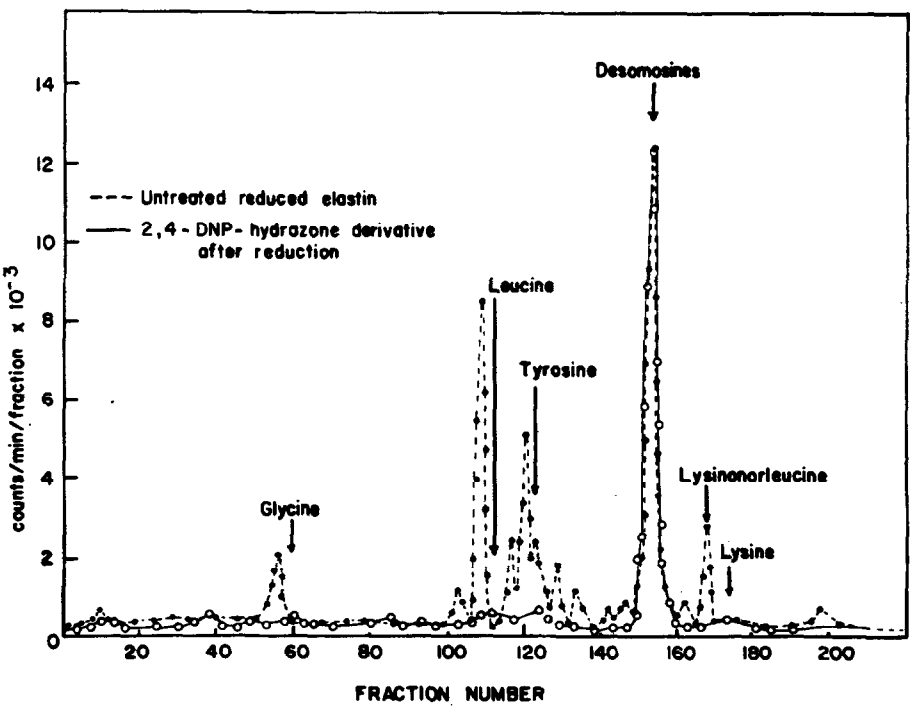


Figure 2 Distribution of tritium in reduced elastin hydrolysates. Arrows indicate the peak position of certain amino acids.

Figure 2, and total counts recovered in the desmosine and lysinonorleucine fractions are shown in Table I. The table also shows the contents of lysinonorleucine before and after reduction.

The results show that 80% of the radioactivity incorporated into the elastin was recovered in all of the collected fractions; of this about 22 and 5% were found in the desmosine and lysinonorleucine fractions respectively. The specific activity of the lysinonorleucine isolated after reduction was 1.03×10^6 dpm per micromole. On the basis of the knowledge that in a one-step reduction of 2-acetamido-3-butanone, the model compound of Blumenfeld and Gallop (1966), the borohydride reagent would yield a specific activity of 4.6×10^6 dpm per micromole, one may then calculate from dilution of counts that for each 3 lysinonorleucine molecules occurring before reduction an additional one was formed by reduction. The actual measured increase in lysinonorleucine occurring with reduction, as shown in the table, is approximately 25% (from 0.85 to 1.15 residues per 1000 total residues).

Incorporation of tritium in the desmosine fractions is accompanied by a skewing of the chromatographic peaks, so that discrete desmosine and isodesmosine peaks are no longer observed; and absorption of ultraviolet light at 275 mμ decreases significantly compared with that shown by the desmosines before borohydride treatment. These results suggest that the desmosines also become reduced, and indeed reduction of pyridinium compounds by borohydride has been amply documented (Anderson et al., 1965; Lyle et al., 1962). Figure 2 also shows additional prominent peaks containing radioactivity after borohydride treatment of elastin. These are in the regions where glycine, leucine and tyrosine normally appear.

Preparation of 2,4-dinitrophenylhydrazone (DNP-hydrazone) of elastin before and after reduction: 1.0 gm of elastin was suspended in 20 ml of 0.8% 2,4-dinitrophenylhydrazine (2,4-DNPH) in 2N HCl and stirred for one hour. The protein was washed successively with 0.01N HCl, water, alcohol and ether. A similar DNP-

hydrazone derivative of aortic elastin was prepared by Miller and Fullmer (1966). A portion was solubilized by treatment with elastase so that chemical determinations could be made on homogeneous aliquots. The number of DNP-hydrazone groups was determined by the spectrophotometric method of Rojkind et al. (1964) and, as seen in Table I, was 10.2 per 1000 total residues.

TABLE I

ANALYSES OF BOVINE ELASTIN AND ITS 2,4-DINITROPHENYLHYDRAZONE DERIVATIVE BEFORE AND AFTER REDUCTION[a]

	Residues/1000 residues		Total Counts Recovered		
Treatment[d]	Lysino-norleucine[b]	2,4-DPNH[c]	Complete analysis	Desmosine fractions	Lysinonorleucine fractions
None	0.85	---	---	---	---
Reduction with $NaBH_4$-$NaBT_4$	1.15	---	160,000	35,000	7,700
Reaction with 2,4-DNPH	0.80	10.2	---	---	---
Reduction with non-tritiated $NaBH_4$ followed by 2,4-DNPH	1.10	0.15	---	---	---
2,4-DNPH followed by reduction with $NaBH_4$-$NaBT_4$	0.85	10.2	52,000	40,600	1,050

[a] All reductions were carried out with the same batch of standardized $NaBH_4$-$NaBT_4$ mixture.
[b] The ninhydrin color equivalent of lysinonorleucine is twice that of leucine.
[c] Based on Kjeldahl nitrogen assuming an average residue weight of 100.
[d] Experimental procedures described in the text.

Elastin previously reduced with non-tritiated sodium borohydride was also treated in the same manner. The DNP-hydrazone content of this preparation was 0.15 equivalents per 1000 total residues (Table I).

Reduction of the DNP-hydrazone derivative of bovine elastin: The DNP-hydrazone derivative of elastin was reduced with standardized $NaBH_4$-$NaBT_4$ mixture in the same manner as described for elastin. The reduced protein resulting from this treatment was hydrolyzed in 6N HCl and radioactivity determined in the fractions obtained from the split stream analysis. The observed distribution of tritium is shown in Figure 2 and the counts in specific fractions in Table I. In this experiment 80% of all recovered radioactivity was found in the desmosine-isodesmosine fractions.

Chemical synthesis of tritiated lysinonorleucine by a model reduction: The chemical synthesis of lysinonorleucine was accomplished by modification of a method previously reported from this laboratory (Franzblau et al., 1965b). The intermediate Schiff base was reduced using standardized $NaBH_4$-$NaBT_4$. The reduction of similar Schiff bases with $NaBH_4$ has been described (Rosen et al., 1964; Schellenberg, 1963). The results when compared with a model one-step reduction of 2-acetamido-3-butanone indicated that reduction of the Schiff base also was a one-step reduction.

DISCUSSION

Our results indicated that reduction of elastin caused an increase in lysinonorleucine from approximately 0.85 to 1.15 residues per 1000 total residues. This increase, approximately 25%, is highly significant because the radioactivity incorporated into the lysinonorleucine fractions also indicated an approximate 25% increase in lysinonorleucine content.

Reaction of 2,4-DNPH with elastin before reduction should produce DNP-hydrazones of carbonyl and Schiff base components present in the protein, assuming they are accessible to the reagent. Treatment of the DNP-hydrazone derivative with sodium borohydride, in contrast to elastin per se, would not result in reductive formation of alcohols and secondary amines since the precursor aldehydes and Schiff bases respectively would be present as DNP-derivatives.

In point of fact, treatment of elastin with 2,4-DNPH before reduction prevented

any significant incorporation of tritium except as exhibited in the region of the desmosines, where 80% of all incorporated counts were found. Reduction of elastin before treatment with 2,4-DNPH only allowed a slight amount of DNP-hydrazone to be formed subsequently. These results support the conclusion that carbonyl and Schiff base components are present in the bovine elastin preparation utilized.

From the measured incorporation of radioactivity into elastin obtained by use of a standardized $NaBH_4$-$NaBT_4$ reagent, one can calculate that the equivalent of 14 single-step reductions occurred per 1000 amino acid residues. Assuming 20% of the incorporated tritium is in the desmosine fractions, one can calculate that 11 single-step reductions ascribable to aldehyde and Schiff base components took place. This number compares favorably with the figure of 10.2 dinitrophenylhydrazone groups found after treatment of elastin with 2,4-DNPH.

In summary, bovine elastin contains, per 1000 amino acid residues, 10 or 11 groups that can form dinitrophenylhydrazones. Of this number, approximately 0.25 equivalents per 1000 residues is in the form of a Schiff base which on reduction is transformed into 0.25 equivalents of lysinonorleucine. Thus, the number of residues of lysinonorleucine is found to increase after reduction, and it would appear that for every 3 residues of lysinonorleucine occurring in untreated elastin there is one residue of the presumed precursor Schiff base, $\Delta^{6,7}$-dehydrolysinonorleucine. Reduction of elastin with borohydride affects the desmosine components, perhaps by reduction of the pyridinium rings, causing a change in their spectral and chromatographic properties. Other carbonyl components in elastin become transformed by reduction and appear as distinct peaks in chromatograms. The nature of the responsible components and their possible significance as precursors of the desmosines is now being investigated.

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