ALDEHYDES IN NATIVE AND DENATURED CALF SKIN TROPOCOLLAGEN[+]

M.A. Paz, R.W. Lent, B. Faris, C. Franzblau[1], O.O. Blumenfeld[2], and P.M. Gallop[3]

Departments of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, N.Y., and Boston University School of Medicine, Boston, Mass.

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Some of the aldehydes which have recently been found in collagen and elastin, have been postulated to play a major role in the cross-linking and maturation of these two fibrous proteins. The aldehydes in elastin are derived from lysine residues in the protein backbone. By a presumed oxidation of the ε-amino group, lysine is converted to α-amino adipic acid semialdehyde (Lent and Franzblau, 1968), an intermediate, which can either form a Schiff base with another lysine yielding dehydrolysinonorleucine or condense with another residue of the semialdehyde to yield the aldol product, α-amino adipic acid semialdehyde aldol (formula below). The presence of these compounds was recently demonstrated in elastin by Lent and Franzblau (Lent and Franzblau, 1968; Franzblau et al. 1965). Dehydrolysinonorleucine and α-amino adipic acid semialdehyde aldol may either serve as intermediates in the biosynthesis of desmosines or other as yet unknown cross-linking compounds, or may themselves serve the ultimate cross-linking role.

In collagen, in addition to lysine, several other amino acids serve as aldehyde precursors. Since α-amino aldehydes derived from glycine, alanine,

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aspartic and glutamic acids*, serine, threonine and lysine have recently been demonstrated in collagen (Gallop et al. 1968), some type of biosynthetic transformation must occur, in which the α-carboxyl groups of these amino acids are ultimately reduced to aldehydes. Lysinal, shown to be derived from lysine (Schneider et al. 1967) can apparently undergo a further deamination at its α-amino group giving rise to enosaline and subsequently dienosaline (Blumenfeld and Gallop, 1966). Alternatively, as in elastin, lysine can undergo a presumed oxidative deamination to yield α-amino adipic acid semialdehyde which was recently shown to be present in collagen (Gallop et al. 1968), as well as in peptides derived from collagen (Bornstein and Piez, 1966). The presence in collagen of α-amino adipic acid semialdehyde aldol has been proposed and its role as a cross-link postulated (Bornstein and Piez, 1966; Rojkind et al. 1968). In this communication we describe its isolation as a reduced derivative, from native and denatured collagens. Moreover, we demonstrate that α-amino aldehydes are exposed in collagen upon denaturation suggesting that the chain separation which occurs upon denaturation may involve the rupture of special sensitive covalent bonds.

Acid soluble calf skin tropocollagen prepared by the method of Gallop and Seifter (1963) was used in this study. Denaturation was achieved by heating a suspension of collagen in water for 10 minutes at 60° C. Reduction was carried out with tritiated sodium borohydride (New England Nuclear Corporation, Boston, Mass.), calibrated by reduction of δ-amino levulinic acid, as described previously (Gallop et al. 1968).

A 2% denatured collagen solution (20 ml) was reduced with tritiated sodium borohydride (35 mg) in the presence of 0.001 M EDTA at pH 9 - 9.5, at toom temperature for 1.5 hours. The reaction was terminated by adding 0.5 N HCl dropwise until the pH of the solution was 4.

Native collagen was reduced both in solution and suspension. In the former

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*The α-amino aldehyde derived from glutamic acid was identified after reduction as α-glutamol (4-amino, 5-hydroxypentanoic acid). The presence of this aldehyde was not previously reported and will be discussed in another publication.

case 140 mg of collagen was dissolved in 65 ml of 0.05 N acetic acid by stirring overnight at 4° C. The pH was then carefully adjusted to 7.5 and 18 mg of tritiated sodium borohydride added. Reduction was carried out at 4° C. and the pH maintained between 9 and 9.5. After 1.5 hours, an aliquot equal to half of the solution was adjusted to pH 4 to terminate the reaction. The remaining solution was reduced for an additional 1.5 hours. Reduction of native collagen in suspension was carried out on 170 mg of finely cut collagen in 7.5 ml of 0.001 M EDTA at 4° C., for 1.5 hours at pH 9 - 9.5. Reaction was terminated as indicated above and the reaction mixture heated to 37° C. to effect denaturation and facilitate subsequent handling. All solutions were then lyophilized to constant specific activity.

Knowing the specific activity of tritiated sodium borohydride for a one-fold reduced compound and the uptake of radioactivity, the concentration of tritium per 100 mg of protein (determined by a Kjeldahl nitrogen determination) was computed and is shown in Table I.

TABLE I

Reduction of calf skin collagen with $NaBH_4$.

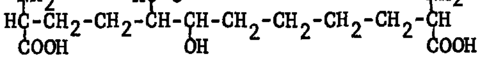
STATE OF COLLAGEN	DURATION OF REDUCTION	$\frac{\mu \text{ Moles TRITIUM}^*}{100 \text{ mg PROTEIN}}$
a. Native in suspension	1.5 h	1.1
b. Native in solution	1.5 h	1.0
c. Native in solution	3.0 h	1.3
d. Denatured	1.5 h	9.0
e. Native in suspension reduced and denatured	1.5 h	7.0

*This value reflects only an approximate number of carbonyl groups reduced since some exchangeable tritium is present in the lyophilized preparations which appears as T_2O after acid or alkaline hydrolysis.

It is clear that the uptake of radioactivity is much less in the native than in the denatured protein. The value obtained for the latter is higher than previously reported (Gallop et al. 1968) due to the substitution of lyophilization for dialysis steps. Upon dialysis, some small molecular weight components with high specific activity are lost.

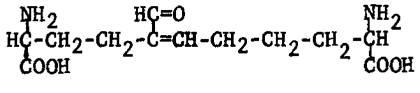
To isolate the reduction products of the aldehydes, namely the tritiated alcohols, 20-30 mg of the reduced proteins were hydrolyzed in 4 ml of 2 N NaOH for 20 hours at 105° C. Lent and Franzblau (1968) have previously established that the reduction products of α-amino adipic acid semialdehyde and its aldol condensate are stable to alkaline hydrolysis, whereas, they are destroyed by acid hydrolysis; most α-amino alcohols are unaltered by either acid or alkaline hydrolysis (Blumenfeld and Gallop; unpublished data); however, with α-aspartol and α-glutamol, lactone formation is promoted under conditions of acid hydrolysis.

The tritiated alcohols were isolated by use of the Technicon Amino Acid Analyzer equipped with a split stream device. Figure 1a shows the chromatogram obtained from a hydrolysate of native collagen reduced in suspension. It can be seen that the only radioactive peaks present are in fractions corresponding to tritiated water (fractions 5-16), ε-hydroxynorleucine (fractions 78-81), and a compound present in fractions 107-110. Native collagen reduced in solution gave a similar pattern. The position of emergence of ε-hydroxynorleucine was established by chromatography of the authentic compound (Lent and Franzblau, 1968; Gallop et al. 1968); the nature of the compound in fractions 107-110 was deduced by comparison with a compound present in an alkaline hydrolysate of reduced elastin which exhibits identical chromatographic behavior under a variety of experimental conditions, as shown below. The structure of this compound in elastin has been established by Lent and Franzblau (1968) to be the reduced dehydrated aldol condensation product of two residues of α-amino adipic acid semialdehyde.



$$\underset{\mathrm{COOH}}{\overset{\mathrm{NH_2}}{\mathrm{HC}}}\mathrm{-CH_2-CH_2-}\overset{\mathrm{HC=O}}{\mathrm{CH}}\mathrm{-}\underset{\mathrm{OH}}{\mathrm{CH}}\mathrm{-CH_2-CH_2-CH_2-CH_2-}\underset{\mathrm{COOH}}{\overset{\mathrm{NH_2}}{\mathrm{CH}}}$$

aldol product of α-amino adipic acid semialdehyde



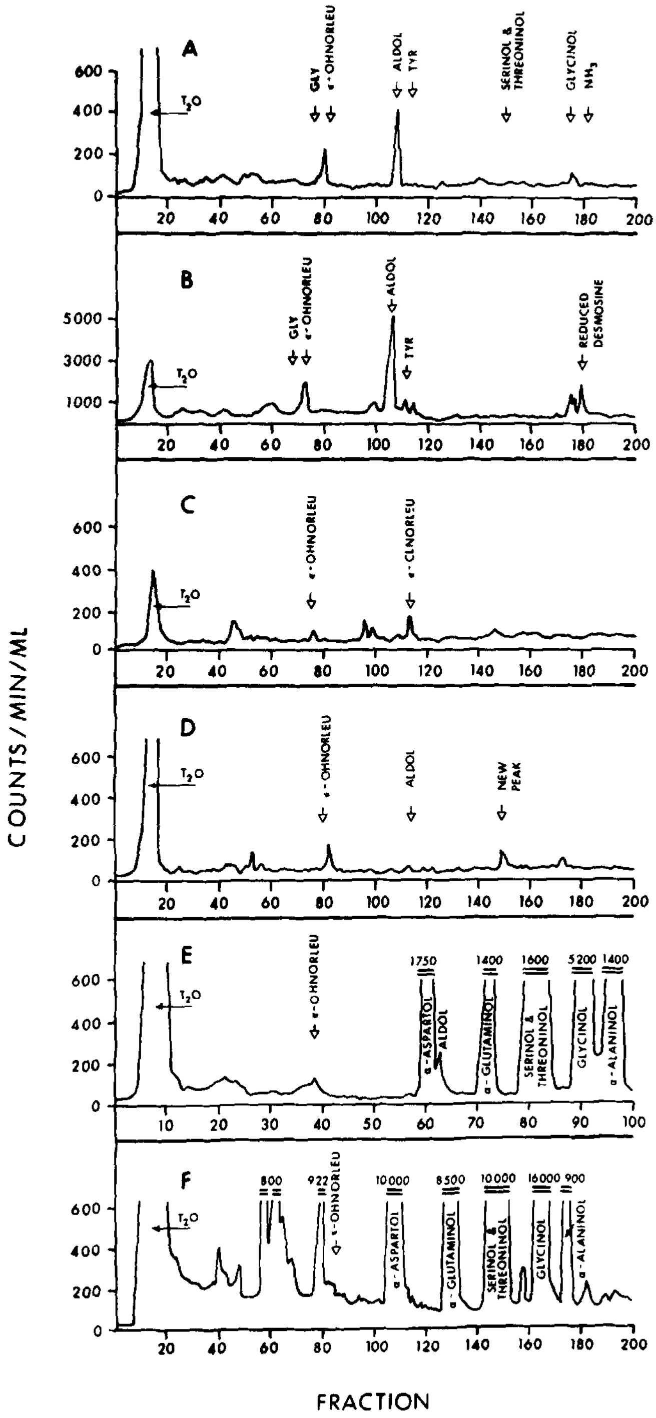
$$\underset{\mathrm{COOH}}{\overset{\mathrm{NH_2}}{\mathrm{HC}}}\mathrm{-CH_2-CH_2-}\overset{\mathrm{HC=O}}{\mathrm{C}}\mathrm{=CH-CH_2-CH_2-CH_2-}\underset{\mathrm{COOH}}{\overset{\mathrm{NH_2}}{\mathrm{CH}}}$$

dehydrated aldol product of α-amino adipic acid semialdehyde

In Figure 1b, it can be seen that the radioactive compound isolated from an alkaline hydrolysate of reduced elastin (reduced with tritium labeled sodium borohydrode) emerges at the same position as the radioactive compound in an alkaline hydrolysate of reduced native suspension of collagen. As seen in Figure 1c, this compound is not stable to acid hydrolysis (6 N HCl, 22 h., 110° C.); this is also found in elastin. The small amount of radioactivity in fractions 110-113 is due to ε-chloronorleucine derived from ε-hydroxynorleucine during acid hydrolysis (Lent and Franzblau, 1968; Gallop et al. 1968). The radioactivity in fractions 95-100 is most likely due to a compound called N_2, which has not as yet been identified (Gallop et al. 1968). Hydrogenation overnight, in the presence of palladium black under two atmospheres of hydrogen, revealed a new radioactive peak appearing in fractions 146-155 (see Fig. 1d). The eluting volume is identical to that of the hydrogenated compound in reduced elastin. From these experiments we conclude that the reduced radioactive compound is identical to that found in elastin and is derived by reduction from an aldol condensation compound of α-amino adipic acid semialdehyde.

Chromatography of an alkaline hydrolysate of reduced denatured collagen is shown in Figure 1e. One should note the presence of six α-amino alcohol peaks (their chromatographic positions have been previously established (Gallop et al. 1968)), in addition to the peaks of ε-hydroxynorleucine and the reduced aldol condensate. Upon hydrogenation of the alkaline hydrolysate, 80% of radioactivity corresponding to the reduced aldol compound is retarded and emerges in the region of the hydrogenated unsaturated aldol compound described above. The aldol condensation compound of α-amino adipic acid semialdehyde is therefore also present in denatured collagen.

As can be noted in Figures 1a-1d, no α-amino alcohols are formed from reduced native collagen. This is true whether the collagen is reduced in suspension or solution. Also as seen in Table I, the uptake of total radioactivity is significantly less in the native than denatured collagen. When the reduced native suspension is denatured and reduced again with tritiated sodium borohydride, the α-



Distribution of radioactivity from hydrolysates of collagen and elastin reduced with tritiated sodium borohydride. Technicon Amino Acid Analyzer was employed using the gradient system of Burns et al. (1965) which spreads the elution region between leucine and ammonia. An 0.6 x 130 cm column was employed (type A resin)in A,B,C,D,F and in E an 0.6 x 60 cm column (type C-2 resin). In all cases a split stream device was used so that one half of the eluate was collected in fractions at 5 minute intervals; the other half was analyzed automatically by ninhydrin in the usual manner. Radioactivity was determined in each fraction by using Bray solution in a Packard Liquid Scintillation Counter. The radioactivity indicated corresponds to: A) 4.2 mg reduced native collagen in suspension-alkaline hydrolysate; B) 2.5 mg reduced chick-embryo elastin-alkaline hydrolysate; C) 4.0 mg reduced native collagen in suspension-acid hydrolysate; D) 4.0 mg reduced hydrogenated native collagen in suspension-alkaline hydrolysate; E) 1.25 mg reduced denatured collagen-alkaline hydrolysate; F) 4.0 mg reduced native collagen in suspension, denatured and reduced again-alkaline hydrolysate. The position of elution of certain amino acids and amino alcohols are indicated by arrows. Aldol refers to the dehydrated aldol condensation product of two residues of α-amino adipic acid semialdehyde, as indicated by the formulas in the text. The peaks marked α-glutaminol should be labeled α-glutamol, to be consistent with the trivial name that we have used in the text for 4-amino, 5-hydroxypentanoic acid.

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amino alcohols are formed and the specific activity of the reduced protein increases significantly. This is seen in Table I and Figure 1f. We can conclude that α-amino aldehydes in collagen become exposed and available to reduction upon conversion of collagen to gelatin.

Table II shows the distribution of total radioactivity in reduced denatured collagen. In denatured collagen over 95% of the radioactivity is distributed among the seven α-amino alcohols in similar ratios to those found previously (Gallop et al. 1968). Only about 1% of total radioactivity is present in either ε-hydroxynorleucine or the reduced α-amino adipic acid semialdehyde aldol condensation compound.

TABLE II

Distribution of total radioactivity in tritiated alcohols in an alkaline hydrolysate of reduced denatured collagen.

TRITIATED ALCOHOL	% TOTAL RADIOACTIVITY*	TRITIATED ALCOHOL	% TOTAL RADIOACTIVITY*
ε-hydroxynorleucine	0.9	serinol plus threoninol	14.9
α-aspartol	10.0	glycinol	39.2
α-amino adipic acid semialdehyde aldol	0.8	α-alaninol	13.8
α-glutamol	8.5	lysinol**	10.8

*Average of 4 determinations (total amino alcohols 6-9 residues/1000 but does not indicate complete reduction).

**Eluted from column with 0.2 N NaOH.

In native collagen only 3 and 4% of total radioactivity applied to the colu emerges in the ε-hydroxynorleucine and reduced aldol peaks, respectively. About 20 of the radioactivity is present in a basic fraction eluted from the column with 0.2 N NaOH. The nature of this compound is now under study; the remaining radioact ty is present as tritiated water.

Knowing the effective specific activity of tritiated sodium borohydride, it possible to calculate the quantity of ε-hydroxynorleucine and reduced aldol compoun in the reduced protein. This calculation shows that in both the native and the denatured collagens these reduced compounds amount each to approximately 0.05 residue 1000 residues or 0.15 residues/tropocollagen molecule.

Bornstein and Piez (1966) isolated peptides containing α-amino adipic acid semialdehyde from α-chains of collagen subjected to cyanogen bromide cleavage; from β-chains similar peptides were postulated to contain the aldol condensation compoun The significance of the low non-stoichiometric quantities of these compounds found in this study is not clear and remains to be elucidated. Possibly they are intermediates for other cross-linking compounds, or they may not be accessible to reduction, or perhaps destroyed during isolation. When one compares the aldehydic components in collagen and elastin it is of great interest that both α-amino adipic aci semialdehyde and its aldol condensation product are present in hydrolysates of both proteins. In acid-soluble calf skin collagen, however, they are apparently present in trace, non-stoichiometric amounts. On the other hand, no α-amino aldehydes have yet been found in elastin whether reduced in suspension, or as a soluble elastase digest (Franzblau; unpublished data). Furthermore, neither dehydrolysinonorleucine (Franzblau et al. 1965) nor desmosines, present in elastin (Thomas et al. 1963),are apparently present in collagen. Although there are similarities, significant differences in biosyhthesis of cross-links and subunit attachment sites exist in these two major fibrous proteins.

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