THE PRESENCE OF LYSINAL (2,6-DIAMINOHEXANAL) IN TROPOCOLLAGEN+

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Small amounts of certain aminoaldehydes have been demonstrated in tropocollagen (Blumenfeld and Gallop, 1966). One of these compounds, termed "enosaline", was tentatively identified as a 6-aminohexenal on the basis of the mass spectra obtained with a derivative formed by reduction of the aldehyde with $NaBH_4$ and dinitrophenylation of the amino group. The structure of the parent compound suggested that it could arise biosynthetically from lysine. Accordingly, experiments have been performed to test this hypothesis. The present communication describes the isolation and proof of structure of C^{14}-lysinol (2,6-diaminohexanol) obtained after $NaBH_4$ reduction of skin tropocollagen prepared from growing rats injected with uniformly labeled lysine C^{14}. These results suggest strongly that tropocollagen contains lysinal (2,6-diaminohexanal), and show the origin of this aldehyde from lysine. The possible relationship of lysinal to enosaline is considered.

METHODS AND RESULTS

Preparation of Lysine-C^{14}-Collagen

A solution of uniformly labeled lysine-C^{14} (New England Nuclear Corp.) was adjusted to pH 7 and diluted to 30 ml; each ml. of this solution con-

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tained 0.0166 mc. Each of ten rats, weighing approximately 140 grams, received a daily intraperitoneal injection, for three days, of 1 ml of this solution. Four days after the last injection, the rats were sacrificed. Acid soluble tropocollagen was prepared from the skins by the procedure of Gallop and Seifter, (1963), and was determined to have a specific activity of 2.0×10^5 dpm/100 mg. of protein.

Isolation of the C^{14} Labeled Reduced, Dinitrophenylated Derivative ($X\text{-}C^{14}$).

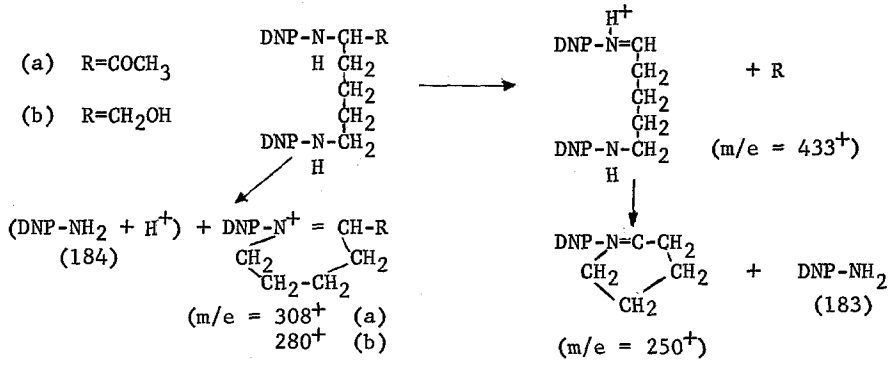
Heat denatured tropocollagen (880 mg.) was reduced with $NaBH_4$ and then hydrolyzed for 22 hours in 6N HCl (Blumenfeld and Gallop, 1966). Passage of the hydrolysate through a 70 cm x 1.5 cm. column of Dowex-1 X 8 (OH^-) with water as eluant yielded a ninhydrin-positive fraction (basic fraction B) that contained 0.3% of the total radioactivity of the hydrolysate. Analysis of the pooled effluent (B), which was the fraction obtained by elution with 40 ml of water, showed the presence of basic amino alcohols, ammonia and non-radioactive arginine (Blumenfeld and Gallop, 1966). However, the fraction did not contain lysine, hydroxylysine or histidine. Subsequent elution of the column with 0.4 N acetic acid yielded a fraction ("neutral fraction", N) that did contain lysine, hydroxylysine and histidine together with most of the neutral and acidic acids. Aspartic acid and hydroxyproline were still retained on the column. The specific activity of lysine obtained after a subsequent separation on the short column of the Beckman-Spinco amino acid analyzer was 3750 dpm/μmole. Hydroxylysine was found to have a specific activity of 3100 dpm/μmole.

Dinitrophenylation of fraction B produced DNP-amino alcohols that could be separated on a silicic acid column (Blumenfeld and Gallop, 1966). Several DNP-compounds were eluted from silicic acid with 10:1 benzene-ethyl acetate; with 9:2 benzene-ethyl acetate others could be eluted, at least two of which were radioactive. The second of these, $X\text{-}C^{14}$, was purified, by rechromatography to a constant specific activity of 1030 dpm/μmole of DNP group. The extinction coefficient for the DNP group at 348 mμ, used

in the calculation of specific activity, was assumed to be 16 x 10^3. Since, as shown subsequently, X-C^{14} has two DNP-amino groups per molecule and an extinction coefficient of 34.4 x 10^3, its specific activity was actually 2215 dpm/μmole. This indicates that lysine-C^{14} (3,750 dpm/μmole) was probably the precursor of X-C^{14}.

Identification of the radioactive dinitrophenylated amino alcohol (X-C^{14}) as di-DNP lysinol.

X-C^{14} has an R_f of 0.45 in 1:1 benzene:ethyl acetate, on thin layers of silicic acid. It does not cochromatograph with any of previously isolated compounds such as DNP-glycinol or DNP-alaninol (Blumenfeld and Gallop, 1966). X-C^{14} was examined in a MS-9 AEI mass spectrometer. At 300°C, a component volatilized that gave a spectrum with strong positive ions at 196, 222, 250, 266, 280, 433; the peak at 250 was the base peak. Di-DNP-lysine methyl ester was reported previously by Penders et al, (1966), to give strong peaks at m/e, 196, 222, 250, 266, 308, 433, with 250 as the base peak. It appears that X-C^{14} and di-DNP-lysine methyl ester have in common at least five carbons (indicated by the 433^+ peak and its fragmentation). Furthermore, the 308^+ peak in the lysine methyl ester (a) and the 280^+ peak in the unknown (b) are considered analogous:



Accordingly, the mass spectral interpretation permits identification of X-C^{14} as di-DNP-lysinol-C^{14} or X as di-DNP-lysinol. Additional smaller peaks (434, M-30; 448, M-16; 447, M-17) support this interpretation. The mass spectrumwas identical to the authentic compound which is shown below.

Synthesis of di-DNP-lysinol.

Dibenzoyl-l-lysine was esterified in ethanol and dry HCl. The ester was reduced in dry tetrahydrofuran, at room temperature, with a slight excess of two equivalents of $LiAlH_4$. The reaction was terminated by addition of alcohol and dilute aqueous HCl; the reaction mixture was evaporated *in vacuo* and dibenzoyl lysinol extracted with ethyl acetate. It was recrystallized from ethyl acetate: pet. ether; mp., 133-135°.

Dibenzoyl lysinol was hydrolyzed in 6N HCl, under reflux overnight, and the HCl removed by evaporation *in vacuo*. Benzoic acid was removed with ethyl acetate, and lysinol treated with excess FDNB at pH 9.0 for three hours. The product was extracted into ether, contaminating dinitrophenol

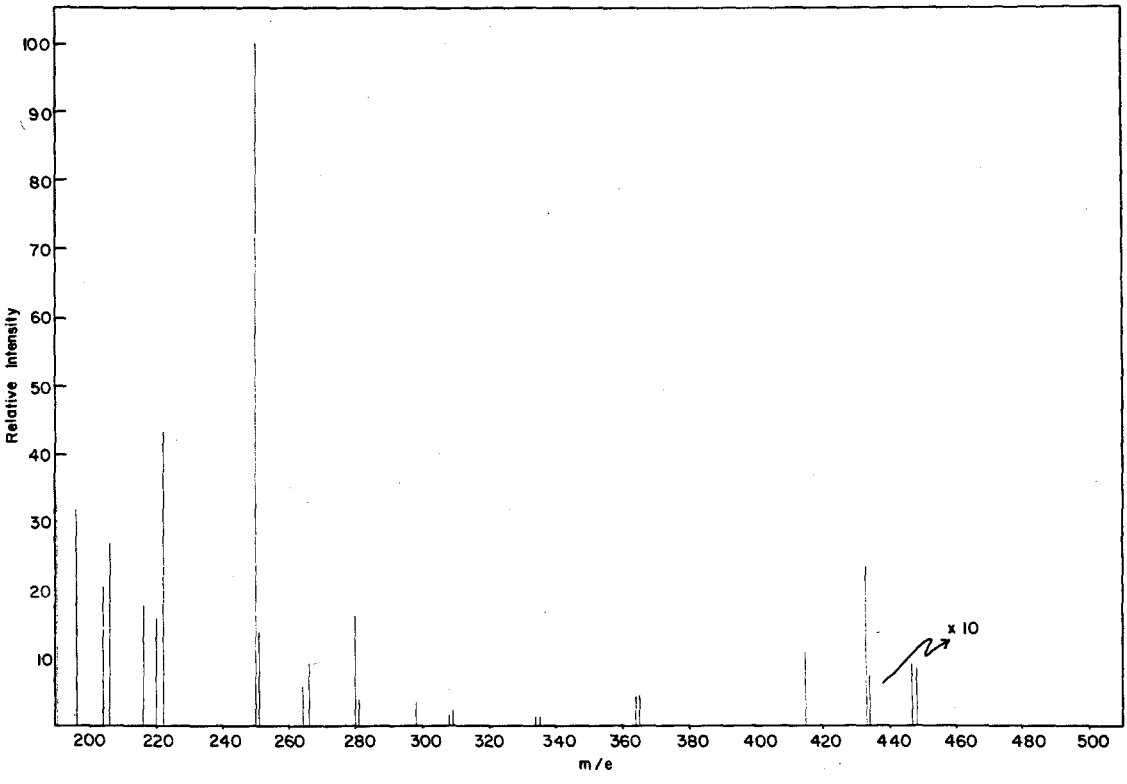


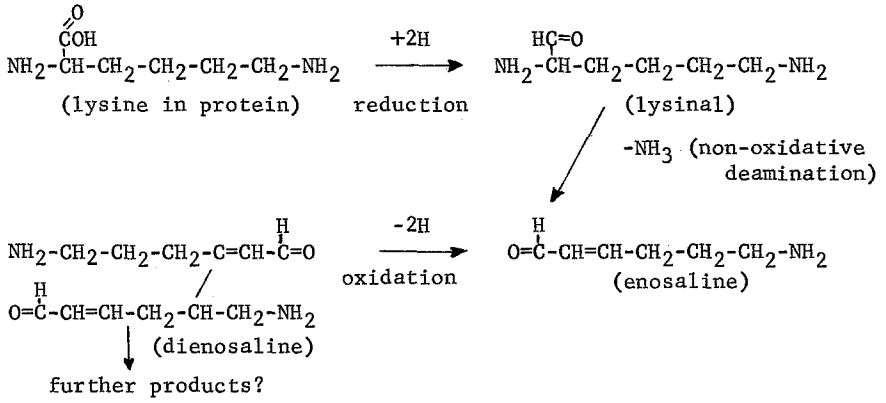
Figure 1. The mass spectrum of di-DNP-lysinal obtained on an AEI MS9 Mass Spectrometer, volatilized in the vicinity of 300°C in the direct sample insertion technique.

recovered by washing with $NaHCO_3$ and di-DNP-lysinol isolated on a silicic acid column with benzene:ethyl acetate (1:1) as eluant. It solidified on removal of solvent and was crystallized by treatment with petroleum ether. It melted at 58°-60° and had a molecular extinction coefficient of 34.4 x 10^3 at 348 mμ. Anal. Calc. for C_{18} H_{20} N_6 O_9 (464.4): C, 46.55%; H, 4.34%; N, 18.10%. Found: C, 46.44%; H, 4.52%; N, 17.85%.

The mass spectrum on a AEI (MS 9) mass spectrometer is shown below in figure 1. Co-chromatography of this material and X-C^{14} on thin layers of silicic acid in several solvent systems also indicated their identity.

DISCUSSION

Lysinal-C^{14} is present in biosynthetically labeled acid soluble rat skin tropocollagen at approximately the same level of radioactivity as lysine-C^{14}, suggesting that lysine is a precursor of lysinal. Lysinal in turn might then be the precursor of enosaline (6-aminohexenal) a compound surmised to have a role in the crosslinking of collagen (Blumenfeld and Gallop, 1966). Apparently, certain lysine residues at C-terminal positions on subunits of α chains or branches of tropocollagen, in a series of reactions within the rat, undergo reduction of their carboxyl groups to produce lysinal residues. A lysinal residue might subsequently undergo a non-oxidative deamination at the α-amino group to form enosaline (6-aminohexenal), now bound in the protein through the amino group that previously was the ϵ -amino group of the precursor lysyl residue(s). The type of non-oxidative deamination postulated here, involving the formation of an α-β double bond, is well known in biochemistry. For example, aspartase, histidase, and arginino-succinidase participate in similar non-oxidative deamination reactions. This series of reactions is pictured schematically as follows:



The occurrence of lysinal in tropocollagen reported here, and the presence of α-alaninal (β-aminopropionaldehyde) and α-aspartal (β-aminosuccinic semialdehyde) reported earlier (Blumenfeld and Gallop, 1966), indicate that collagen contains a family of α-amino aldehydes. Recent investigations, to be reported separately, indicate that glycinal (aminoethanal) also occurs in collagen. This suggests that collagen-forming tissues may contain an enzyme system with a capacity to reduce C-terminal amino acid residues of tropocollagen subunits, transforming them to C-terminal residues of α-amino aldehydes. In other experiments to be reported elsewhere, we have isolated the 2,4-dinitrophenylosazones of the same group of α-aminoaldehydes. Lysinal, one of this family appears capable of further biosynthetic modifications to form a crosslink or subunit attachment associated with maturation of tissue collagen.

REFERENCES

1. Blumenfeld, O.O. and Gallop, P.M. Proc.Nat'l.Acad.Sci., 56, 1260-67, 1966.
2. Gallop, P.M. and Seifter, S. in Methods in Enzymology, Vol. VI, pg. 635, ed. Colowick and Kaplan, Acad.Press, 1963.
3. Penders, Th.J., Copier, H., Heerma, W., Dijkstra, G. and Arens, J.F. Recueil des Trav. Chim., 85, 216, 1966.