Lysinonorleucine. A New Amino Acid from Hydrolysates of Elastin*

C. Franzblau,† B. Faris, and R. Papaioannou

ABSTRACT: This paper describes the characterization and synthesis of a new amino acid isolated from hydrolysates of elastin. Results from dinitrophenylation studies, nuclear magnetic resonance spectra, infrared spectra, and mass spectra suggest that the structure is N^{ϵ}-(5-amino-5-carboxypentyl)lysine. To this compound we have assigned the trivial name lysinonorleucine. Synthesis can be achieved *via* two different procedures: (1) N^{α}-acetyllysine is oxidized by *N*-bromosuccinimide to N^{α}-acetylaminoadipic acid δ-semialdehyde, which is then condensed with N^{α}-acetyllysine to a Schiff base. The Schiff base is reduced with sodium borohydride and the resulting product is hydrolyzed by acid to lysinonorleucine (5–10% yield); (2) 1 equiv of 5-(δ-bromobutyl)hydantoin is condensed with 1 equiv of N^{α}-benzoyllysine ethyl ester by refluxing in tetrahydrofuran. The condensate is then treated with alkali at elevated temperature to yield lysinonorleucine (57% yield).

Treatment of the naturally occurring amino acid with snake venom L-amino acid oxidase indicated that both α-carbons are in the L configuration.

Peptide fractions from elastin which were enriched in desmosine and isodesmosine, were shown also to contain another ninhydrin-positive substance, hitherto undescribed (Franzblau *et al.*, 1965a). This compound was isolated by ion-exchange chromatography and demonstrated to be an amino acid. Its electrophoretic mobility, aqueous titration, behavior on dinitrophenylation, and various chemical analyses suggested that the compound was N^{ϵ}-(5-amino-5-carboxypentyl)lysine. To this, the trivial name lysinonorleucine was assigned (Franzblau *et al.*, 1965b). Tissue culture studies performed in a similar manner to those of Miller *et al.* (1964) indicated that lysine was the precursor of this new amino acid. In the present communication, conclusive evidence is given that the structure of the compound is N^{ϵ}-(5-amino-5-carboxypentyl)lysine.

Materials

Elastin of bovine *liqamentum nuchae* was prepared according to the procedure of Partridge *et al.* (1955). N^{α}-Methyllysine and N^{ϵ}-methyllysine were obtained from Cyclo Chem. Corp.; 1-fluoro-2,4-dinitrobenzene and 5-(δ-bromobutyl)hydantoin were obtained from Eastman Kodak Co.; N^{α}-benzoyllysine was obtained from Calbiochem. Corp., and L-amino acid oxidase from Worthington Biochemical Corp.

The new amino acid, designated lysinonorleucine above, was obtained from bovine elastin in relatively large amount by slight modification of the procedure described previously (Franzblau *et al.*, 1965a). Elastin (185 g) was refluxed in 1200 ml of 6.0 N HCl for 28 hr. The resulting hydrolysate was placed on a 4.5 × 102 cm column of Dowex 50-X8 (200–400 mesh) and eluted with various concentrations of HCl as previously described.

Experimental Procedures and Results

Dinitrophenylation Studies. To 3.0 mg of the purified amino acid dissolved in water, cupric carbonate was added in slight excess over the amount needed to make the copper derivative. The mixture was boiled for 5 min, cooled, and then centrifuged to remove excess copper carbonate. To the supernatant, 0.5 ml of 10% $NaHCO_3$ was added; the total volume was then adjusted to 1.0 ml with water. An equal volume of 2.5% 1-fluoro-2,4-dinitrobenzene in ethanol was added and the reaction mixture was stirred for approximately 30 min. After this time, excess 1-fluoro-2,4-dintrobenzene was removed by extraction with ether. The yellow-green residual solid was removed by centrifugation, washed with water, and then dissolved in a small amount of 0.1 N HCl. Hydrogen sulfide was bubbled through the solution, CuS was removed by filtration, and the filtrate was evaporated to dryness and redissolved in 0.1 N HCl. The absorption spectrum of this product is shown in Figure 1. The absorption at 385 mμ is characteristic of dinitrophenylated N-substituted α-amino acids such as sarcosine, pipecolic acid, and proline.

Dinitrophenylation of the copper complexes of N^{α}-methyllysine and N^{ϵ}-methyllysine was carried out in a similar manner. The absorption spectrum of the dinitrophenylated derivatives is shown in Figure 2. The N^{α}-DNP-derivative of N^{ϵ}-methyllysine has a maximum absorption of 385 mμ and the N^{ϵ}-DNP derivative of N^{α}-methyllysine at 355 mμ, the latter being the same as that of N^{ϵ}-DNP-lysine.

An aliquot of dinitrophenylated amino acid (lysinonorleucine) was placed on a Technicon amino acid analyzer employing a 15-cm column similar to that of Spackman *et al.* (1958), and a chromatogram was obtained as shown in Figure 3. As expected, the product was still reactive to ninhydrin, indicating the presence of free amino groups. Chromatographically, it behaved quite similar to N^{ϵ}-DNP-lysine (see Figure 3).

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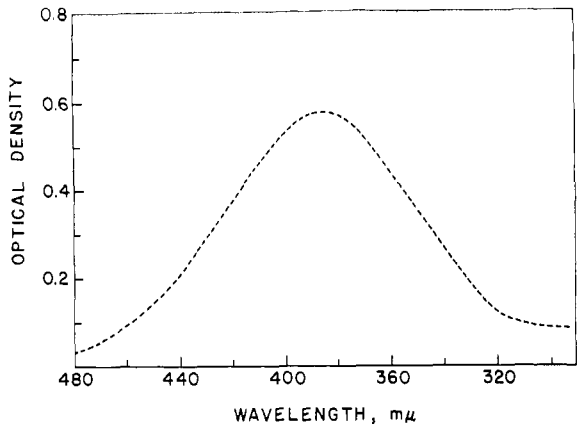


FIGURE 1: The absorption spectrum of dinitrophenylated lysinonorleucine. The dinitrophenylation was carried out on the copper complex of lysinonorleucine and the copper was subsequently removed (see text for details).

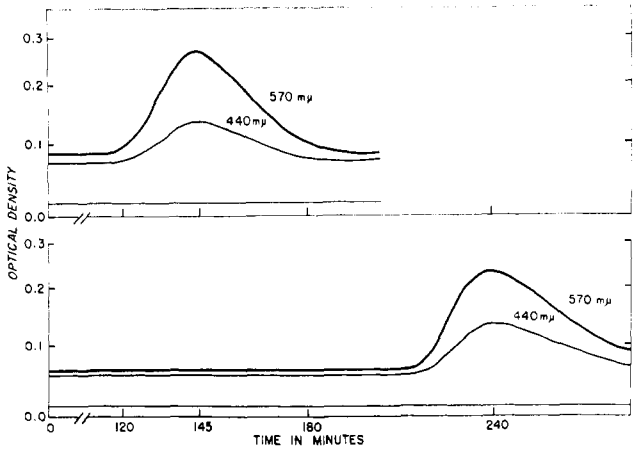


FIGURE 3: Chromatography of dinitrophenylated lysinonorleucine (upper curve) and N^{ϵ}-DNP-lysine (lower curve) on a Technicon amino acid analyzer (type A resin). The eluting buffer was 0.38 M sodium citrate (pH 5.28) and the flow rate was 30.0 ml/hr.

Peaks obtained with both compounds are skewed and absorption at 440 mμ is relatively large, a consequence, in part, of the dinitrophenyl group. From the micromoles of ninhydrin-reacting leucine equivalents calculated from amino acid analysis, and from the extent of absorption at 385 mμ, a molar extinction coefficient of approximately 9×10^3 was calculated; this is about one-half the value reported in the literature for DNP-imino acids including sarcosine and proline (Fraenkel-Conrat *et al.*, 1955). The molar extinction coefficient for N^{ϵ}-DNP-lysine calculated in this manner was approximately 17×10^3. Therefore, the putative amino acid appeared to have two free α-amino groups and one secondary amino group. The two primary α-amino groups could be complexed with copper, so that subsequent dinitrophenylation yielded a derivative with a single DNP group with the spectral characteristics of an amino acid containing a DNP secondary amino group such as N^{ϵ}-DNP-N^{ϵ}-methyllysine. After dinitrophenylation, the copper could be removed to reveal the two nondinitrophenylated α-amino groups.

The dinitrophenylated material, free of copper, was then redinitrophenylated. The resulting compound was now ninhydrin negative and gave the spectrum shown in Figure 4.

Nuclear Magnetic Resonance Spectrum of Lysinonorleucine. The nuclear magnetic resonance spectrum of the naturally occurring amino acid (lysinonorleucine) in trifluoroacetic acid was examined, employing a Varian A 60, 60 Mcps spectrometer. Approximately 20 mg of sample was dissolved in 0.4 ml of trifluoroacetic acid containing the internal reference, tetramethylsilane (1% v/v). Results obtained in trifluoroacetic acid are especially instructive because the τ values are based on an internal standard and because RNH_3^+ or $RNH_2{}^+R$ are observable only in strong acid (Bovey and Tiers, 1959). The spectra of lysinonorleucine and of lysine given in Figure 5 are identical except for the fact that a peak occurs in lysine at 3.1 and in lysinonorleucine at 2.8. The peak at 3.1 is due to the ϵ-NH_3^+ and that at 2.8 is most probably due to $CH_2NH_2{}^+CH_2$ present in the lysinonorleucine.

Manometric Analyses. Values obtained previously (Franzblau *et al.*, 1965b) for total nitrogen, total carbon, nitrous acid–nitrogen, and ninhydrin–CO_2 are consistent with the proposed structure as elucidated from the studies above.

Treatment with L-*Amino Oxidase.* Approximately 1.7 mg of lysinonorleucine isolated from elastin was dissolved in 1 ml of 0.01 N HCl. Two aliquots, 0.25 ml each, of this solution were evaporated to dryness. To one tube 0.50 mg of L-amino acid

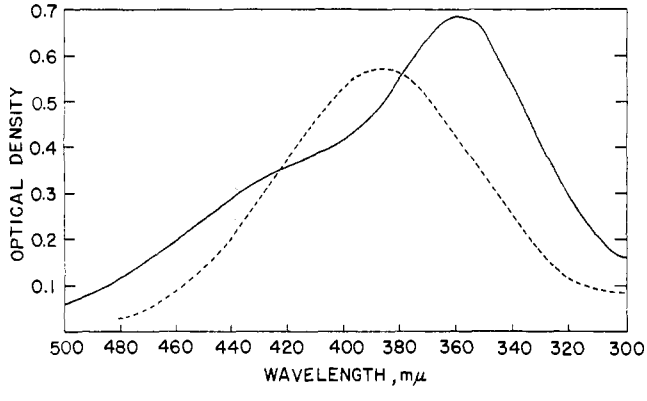


FIGURE 2: The absorption spectrum of N^{ϵ}-DNP, N^{α}-methyllysine (———), and N^{ϵ}-DNP-N^{ϵ}-methyllysine (———).

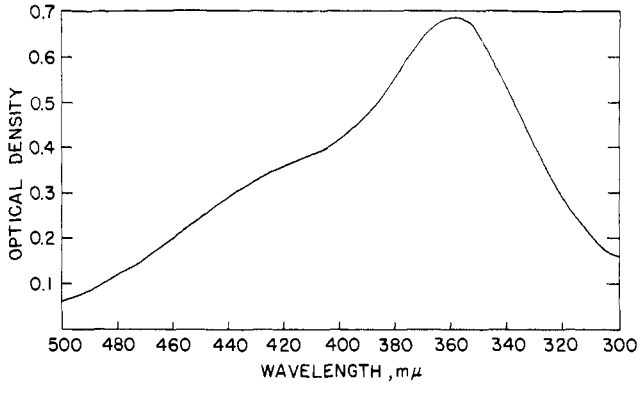


FIGURE 4: The absorption spectrum of completely dinitrophenylated lysinonorleucine (see text for details).

TABLE I: Effect of L-Amino Acid Oxidase on Lysinonorleucine.

	μmoles of Lysinonorleucine[a]	μmoles of NH_3
Lysinonorleucine (no treatment)	0.404	0.046[b]
First treatment with L-amino acid oxidase	0.084	0.270
Second treatment with L-amino acid oxidase	0.006	0.470

[a] Expressed in leucine equivalents. [b] Small amount of ammonia present in the amino acid chromatogram.

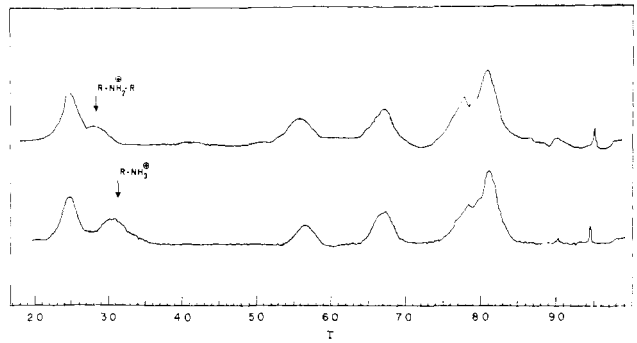


FIGURE 5: The nuclear magnetic resonance spectrum of lysinonorleucine (upper curve) and lysine (lower curve) in trifluoroacetic acid.

oxidase dissolved in 1.0 ml of 0.1 M Tris buffer (pH 7.70) was added. To the other tube 1 ml of the same buffer containing no enzyme was added. Both tubes were incubated at 37° for 26 hr, an aliquot was removed from each tube, and amino acid analysis was performed. The tube containing enzyme received a second portion of enzyme corresponding to 0.57 mg of protein. Buffer was added to the control. The tubes were reincubated and analyzed for amino acids. The results of these experiments are given in Table I. The data for lysinonorleucine are expressed as micromoles of leucine equivalents in the ninhydrin assay; 1 μmole of lysinonorleucine in this assay yields 2 leucine equiv in the automatic procedure of Spackman *et al.* (1958). The results indicated that 98.5% of the lysinonorleucine reacted with L-amino acid oxidase, reducing the peak due to lysinonorleucine and yielding an almost equivalent amount of ammonia. Thus both α-amino groups of the natural lysinonorleucine must be in the L configuration.

Synthesis of N^{ϵ}-(5-Amino-5-carboxypentyl)lysine (Lysinonorleucine). A. OXIDATIVE PROCEDURE. N^{α}-Acetyllysine (1 mmole; 188 mg), prepared according to the method of Neuberger and Sanger (1943), was dissolved in 7 ml of water and 89 mg (0.5 mmole) of *N*-bromosuccinimide was added. The mixture was maintained at pH 3.0 by addition of 0.2 N NaOH during the 30 min required for completion of the reaction. The reaction mixture showed the presence of aldehyde in tests with 2,4-dinitrophenylhydrazine. The mixture was then taken to dryness and the residue was suspended in 5.0 ml of ethanol containing a few drops of acetic acid. The solution was reduced over palladium black by hydrogenation at 2 atm of pressure and room temperature overnight. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. Deacetylation of the amino groups was accomplished by hydrolysis of the residue in 6.0 N HCl at 110° in a sealed vial for 1 hr.

An aliquot of the hydrolysate was placed on a Technicon amino acid analyzer equipped with a 50-cm column similar to that of Spackman *et al.* (1958). The chromatogram showed unreacted lysine and a peak which emerged in exactly the same position as did a sample of lysinonorleucine isolated from hydrolysates of elastin. Another aliquot was cochromatographed with a known quantity of authentic lysinonorleucine from elastin and the two materials emerged as a single peak.

Synthetic lysinonorleucine was isolated by large batch separation employing the 50-cm column described above, followed by desalting on a Dowex 50 column as previously described for the isolation of lysinonorleucine from elastin (Franzblau *et al.*, 1965a). The over-all yield in the synthesis varied from 5 to 10%. This material gave the same characteristic dinitrophenylated derivatives described for the naturally occurring substance.

B. NONOXIDATIVE SYNTHETIC PROCEDURE. The method employed was similar to that described by Speck *et al.* (1963) for synthesis of N^{ϵ}-glyceryllysine. N^{α}-Benzoyllysine (50.0 mg) was dissolved in 5 ml of dry ethanol containing 1.6 mmoles of HCl. The solution was refluxed for 21 hr and then evaporated to dryness *in vacuo*. A thick oil of N^{α}-benzoyllysine ethyl ester remained. The ester then was dissolved in 5.0 ml of tetrahydrofuran, and this solution received 43.6 mg of 5-(δ-bromobutyl)hydantoin and 0.05 ml of triethylamine. The reaction mixture was refluxed for 21 hr, cooled, and filtered. The filtrate was concentrated to a thick syrup, 2 ml of 2.0 N NaOH was added, and the solution was placed in a sealed vial and heated at 110° in an oven for 17 hr. The hydrolysate was filtered and the pH was adjusted to approximately 2.0. Water was added to bring the volume to 10.0 ml and a small aliquot (0.02 ml) of this solution was then placed on the amino acid analyzer employing a 50-cm column as described previously. A peak of lysinonorleucine was identified; it corresponded to 57% of the theoretical yield. Cochromatography of this material with lysinonorleucine isolated from elastin yielded a single homogeneous peak. In subsequent preparations the peak corresponding to lysinonorleucine was collected and desalted on a Dowex 50 column. The desalted material was evaporated to dryness, washed with alcohol, and dried with ether to yield finally a solid white material. However, all attempts at crystallization failed. *Anal.* Calcd: C, 52.0; H, 9.0; N, 15.0. Found: C, 51.9; H, 9.05; N, 13.70. Of interest is the fact that the product was extremely hygroscopic, a property described also for lysinoalanine by Bohak (1964). The infrared spectrum of the synthetic material was identical with that of natural lysinonorleucine.

Mass Spectrum of Lysinonorleucine Ethyl Ester. Final proof that the structure of synthetic or natural lysinonorleucine in fact is N^{ϵ}-(5-amino-5-carboxypentyl)lysine was achieved by mass spectrometry of suitable derivatives. Beginning with 0.4 mg of synthetic lysinonorleucine, the ethyl ester was prepared according to the method of Biemann *et al.* (1961). The mass spectrum of this compound was obtained using an AEI MS-9

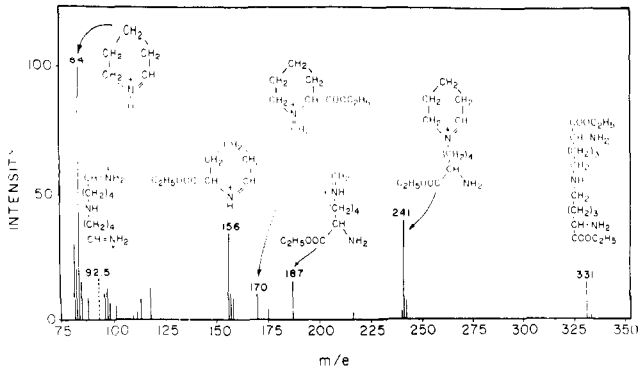
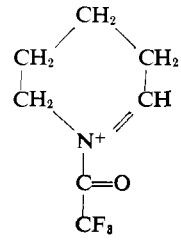


FIGURE 6: The mass spectrum of the ethyl ester derivative of lysinonorleucine.

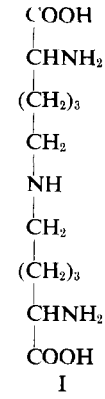
mass spectrometer with the kind help of Dr. Gerald O. Dudak of Harvard University. The results are shown in Figure 6. As expected, a *m/e* peak of 331 corresponding to the parent molecular ion (M^+) is present. Several other strong peaks occur at *m/e* of 241, 187, 170, 156, and 84, respectively. A significant peak at 92.5 is also present. The peak at 241 can be explained by formation of the amine fragment ($M^+ - 73$) with the additional loss of 17 due to cyclization by elimination of NH_3. The proposed structure of the fragment is included in Figure 6. The loss of 73 from each of the ester groups in the molecule, leaving a doubly charged amine fragment of mass 185 would appear on the mass spectrum at 92.5. Fragmentation of the secondary amine leads to a peak of 187, the structure of which is also included in the figure. This fragment cyclizes by loss of NH_3 to give a peak at 170. Additional evidence for this conversion is a metastable peak at 130.1. This same peak at 187 also loses 31 mass units to provide a peak at 156, a conversion substantiated by a metastable peak at 154.5. The most abundant peak occurs at *m/e* of 84, also, it should be pointed out, the most abundant in the spectra for the ethyl esters of lysine (Biemann *et al.*, 1961), N^{ϵ}-methyllysine (Murray, 1964), and lysopine (Biemann *et al.*, 1960), respectively. As suggested by Biemann *et al.* (1961), the most probable structure for this fragment is that indicated in Figure 6. The mass spectrum confirms that the molecular structure of lysinonorleucine is as depicted. The mass spectrum of the naturally occurring lysinonorleucine yielded identical results.

Mass Spectrum of Trifluoroacetyl Butyl Ester of Lysinonorleucine. The trifluoroacetyl butyl ester of the synthetic lysinonorleucine was prepared according to the method of Hartman and Wotiz (1964). The mass spectrum showed the expected molecular ion of 675. The most abundant peak occurred at 180. The trifluoroacetyl butyl ester of lysine was also examined. Again the most abundant peak was at 180. The most probable structure for this fragment is



Discussion

The structure of lysinonorleucine (I) suggests that, like the



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desmosines, it serves as a cross-linking amino acid in elastin. Evidence presented elsewhere (Lent and Franzblau, 1967) suggests that it is synthesized *via* an intermediate Schiff base, formed by the condensation of one residue of α-aminoadipic acid δ-semialdehyde and one residue of lysine. In elastin obtained from bovine *Liqamentum nuchae*, approximately 1 residue of lysinonorleucine is present per 1000 amino acid residues.

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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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† Established Investigator of the American Heart Association. To whom inquiries should be made.