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Isolation and Partial Characterization of a New Amino Acid from Reduced Elastin*

B. C. Starcher,† S. M. Partridge, and D. F. Elsden

ABSTRACT: This paper describes the isolation of an amino acid, $C_{18}H_{34}N_4O_6$, which appears in the hydrolysis products of elastin from young animals when the protein has been treated with alkali followed by borohydride reduction. Like desmosine and isodesmosine the new amino acid incorporates the label from [^{14}C]lysine in tissue culture experiments. Evidence from the mass spectrum of the tetra-*N*-trifluoroacetyl tri-*n*-butyl ester (mass 954) indicates that the compound can be regarded as derived from three molecules of lysine with the loss of two nitrogen atoms. From its empirical formula and relationship to lysine a structure is proposed for the new amino acid and preliminary chemical and spectral evidence is given in support. To mark its structural relationship to the isomeric desmosines we propose to name the new compound merodesmosine.

Feeding experiments with labeled amino acids carried out by a number of authors have shown that the turnover rate of elastin becomes extremely slow as the animal reaches maturity and growth ceases. Walford *et al.* (1964) extended their observations with rats up to 930 days after the injection of [^{14}C]lysine and found that after an initial decline due to the growth of the rat during the first 120 days the radioactivity remained constant throughout life. These experiments suggest that apart from the repair of lesions, the elastic structures of the large blood vessels are retained unchanged once growth has ceased. However, it is equally clear that during the early life of mammals there is both growth and remodeling of elastin in structures such as the arterial walls (Gillman and Hathorn, 1958), and that in young animals the elastic tissue is in a state of active metabolism.

The problem of the biogenesis of elastin is complicated by the circumstance that in the mature protein the peptide chains are cross-linked by covalent bonds to form a continuous network (Partridge, 1962). It has become clear that elastin fibres are formed from a soluble precursor protein by a process of cross-linking which is dependent upon the oxidation of certain lysine side chains in the precursor molecule. The cross-links so formed are stable structures which retain their configuration after enzymic or acid hydrolysis of purified elastin (Partridge *et al.*, 1963). Two unusual amino acids, containing cyclized structures forming the link, have been

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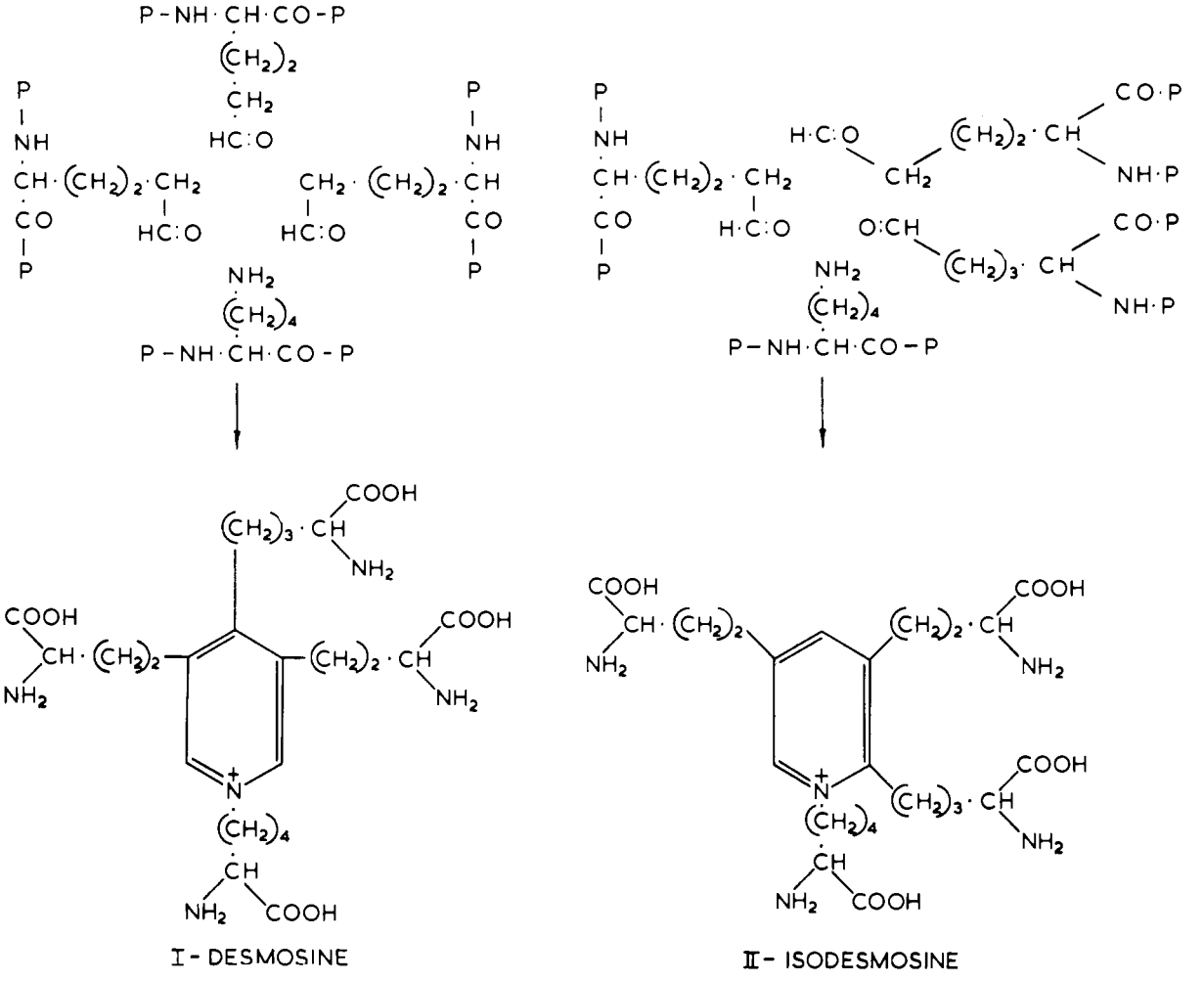

FIGURE 1: The scheme illustrates the over-all reaction resulting in the formation of desmosine cross-linkages from the lysine side chains of a soluble protein precursor of elastin. P represents the peptide chains of the elastin precursor. The reaction requires the oxidative deamination of three lysine ϵ-amino groups to give an intermediate which is represented formally as the semialdehyde of α-aminoadipic acid.

isolated from the hydrolysis products of elastin from bovine *Ligamentum nuchae*, and have been shown to have the structures I and II in Figure 1 (Thomas *et al.* 1963; Partridge *et al.*, 1966).

It should be noted that the empirical formula of the desmosine isomers, $C_{24}H_{40}N_5O_8$, is equivalent to four lysine residues less N_3H_{16} and it has been proposed that the first step in the biosynthesis of the cross-link is the oxidative deamination of the side chains of three of the four lysine residues involved in the link. The over-all process has been expressed as shown in Figure 1 (Partridge, 1965).

The incorporation of four lysine residues into the structure of the desmosines has now been confirmed by several workers (Partridge *et al.*, 1964; Miller *et al.*, 1964; Partridge *et al.*, 1966; Anwar and Oda, 1966) but details of the synthetic route have so far remained obscure. It is known, however, that at least one of the steps in the synthesis is a slow process, since both in tissue culture and in animal experiments full incorporation of [^{14}C]lysine given as a pulse dose occupies 10–15 days from the time of injection. This, and other evidence (Partridge, 1966), suggests that the process of cyclization to form the quaternary pyridinium ring of the desmosine isomers occurs, not as a single event, but as a series of linked reactions involving unstable intermediates.

Inspection of the structures given in Figure 1 suggests that Schiff bases and aldols might be implicated as intermediates in the over-all process. This view has been strengthened by the isolation of lysinonorleucine from the hydrolysis products of elastin by Franzblau and his colleagues (1965). Lysinonorleucine, N^{ϵ}-(5-amino-5-carboxypentanyl)lysine, may be regarded as the product of reduction of the Schiff base formed between lysine and α-aminoadipic semialdehyde produced by the oxidative deamination of the ϵ-amino group of a second molecule of lysine. It should be observed that the biosynthesis of lysinonorleucine, apparently involving the reduction of a Schiff base as a normal function of the metabolism of elastic tissue, has resulted in the transformation of an

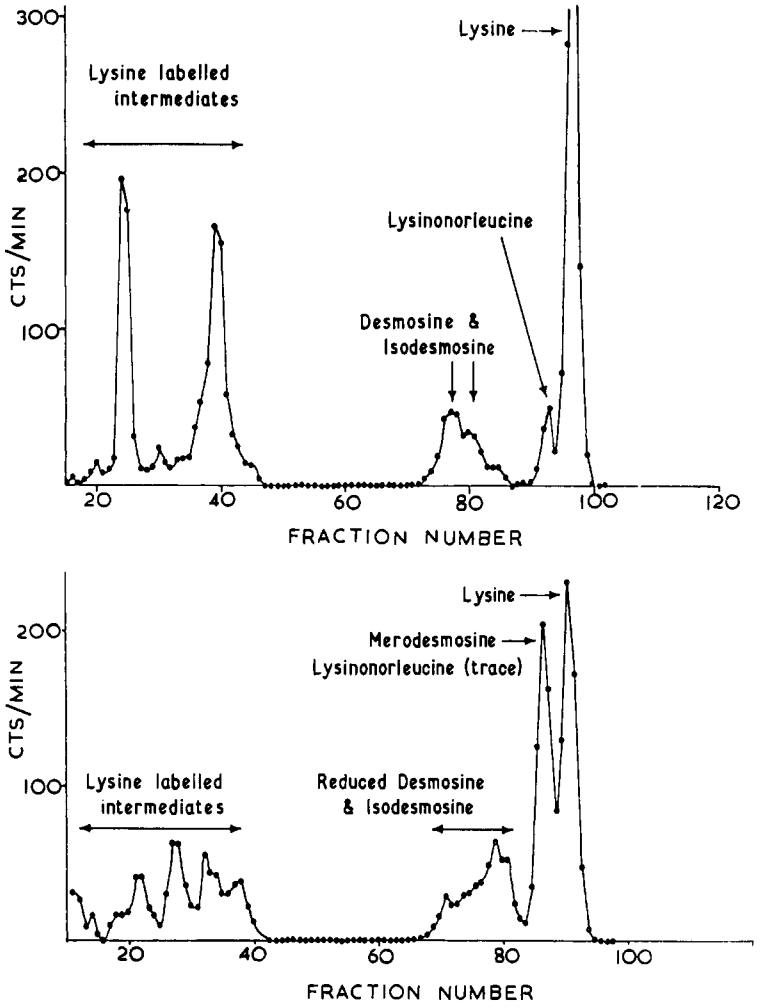

FIGURE 2: Chromatography of the hydrolysis products of elastin from duck aorta pulse labeled with [^{14}C]lysine in tissue culture. (a) Control (upper): elastin was isolated using hot 0.1 N NaOH and was hydrolyzed without further treatment. (b) Reduced elastin (lower): elastin was isolated by hot alkali treatment and reduced with sodium borohydride at pH 7.2. The peak due to merodesmosine is much increased in chromatogram b and this increase is accompanied by a reduction in the peaks due to lysine and unidentified lysine-labeled intermediates.

unstable intermediate product to a compound stable enough to be isolated after the acid hydrolysis of the protein. A similar process of reduction could be deliberately employed to stabilize other of the possible Schiff bases in order to study details of the train of intermediates in desmosine biosynthesis. The object of the experiments presently described was to attempt this procedure by borohydride reduction of elastin isolated from growing elastic tissue.

Experimental Section

Incorporation of Labeled Lysine in Tissue Culture Experiments. Aorta preparations of young ducklings 1–3-days old were grown in organ culture under the conditions described by O'Dell *et al.* (1966). The aortae from 36 ducklings were pulse labeled in medium containing [^{14}C]lysine (1 μc/ml) for 24 hr and then retained in unlabeled normal medium for a further 48 hr. The aortae were harvested and divided into three equal groups. Two groups were combined and elastin (40 mg) was isolated from the aortae by treatment with 0.1 N NaOH at 98° for 40 min. One-half of the elastin preparation (20 mg) was suspended in 0.5 M Tris-HCl buffer (pH 7.2, 5 ml) and sodium borohydride (20 mg) was added in small quantities over a period of 1 hr. The mixture was allowed to stand with occasional stirring for an additional 12 hr at room temperature to complete the reaction. The reduced elastin was recovered by filtration and washed with water. Reduced and unreduced specimens were both hydrolyzed under nitrogen in sealed tubes with 6 N HCl for 24 hr at 110°. The hydrolysates, after removal of HCl by vacuum evaporation, were separated on columns (41 × 0.6 cm) packed with Amberlite CG 120-X8 (400 mesh/in.) using a system of volatile buffers essentially as described by Schroeder *et al.* (1962). For development

of the buffer gradient the mixing chamber contained 70 ml of pyridine–acetic acid buffer (0.2 M with respect to pyridine, pH 3.1) and the reservoir contained 1.0 M pyridine acetate buffer (pH 5.0). The rate of flow was 2.5 ml/hr, temperature 20°. The eluates from both experiments were collected in 0.6-ml fractions.

Radioactivity was measured by drying 0.1 ml from each fraction onto 1-in. stainless-steel planchets and the counts were determined using the Tracerlab BLB-570 low-background counting system. Figure 2a,b shows the counts obtained from the hydrolysate of reduced and unreduced elastin. One drop from each fraction was placed on the base line of a paper chromatogram developed with butanol–acetic acid–water (4:1:1) in order to observe the position of the individual amino acids. Comparison of Figure 2b (reduced elastin) with Figure 2a (unreduced) reveals that, as a result of borohydride reduction, the peaks due to unidentified lysine-labeled intermediates (fractions 10–40) and that due to lysine itself (fractions 95–99) were much diminished. The peaks due to desmosine and isodesmosine were replaced by two slightly slower moving peaks apparently due to the corresponding reduced products. This shift in position, due to reduction, was not unexpected since although reduction of pyridine derivatives with metal hydrides is known to be difficult, quaternary pyridinium salts are reported to be easily reduced to tetrahydropyridine derivatives by means of sodium borohydride (Schenker, 1961). The most striking feature of the chromatogram of reduced elastin, however, is that a new peak (fractions 90–94) with a high count, due to an unknown ninhydrin-reacting compound, has appeared in a position immediately preceding lysine. Later in this paper the isolation and characterization of this compound are described, and to avoid circumlocution it is proposed to refer to it hereafter as merodesmosine.

To confirm that merodesmosine carries the lysine label, the three center fractions from the merodesmosine-lysinonorleucine peak were mixed and an aliquot was subjected to high-voltage paper electrophoresis following the procedure detailed later in this paper. The paper strip was sprayed with ninhydrin. The bands due to lysinonorleucine and merodesmosine from reduced and unreduced elastin were then cut out and the radioactivity was measured in a liquid scintillation counter. Unreduced elastin showed a faint ninhydrin band due to merodesmosine which gave 19 cpm while the band due to lysinonorleucine gave 15 cpm. Reduced elastin showed a strong ninhydrin band for merodesmosine which gave 256 cpm while the band due to lysinonorleucine was not much changed at 30 cpm.

In order to demonstrate the effect of the alkali treatment used during the isolation of purified elastin from aorta tissue, the 12 aortae which were not alkali treated were combined and homogenized in 0.5 N Tris buffer (pH 7.2) and sodium borohydride (40 mg) was added slowly with stirring. After completion of the reaction, elastin was isolated from the reduced aorta tissue by alkaline treatment. The elastin was then hydrolyzed and separated by column chromatography, and the fractions were counted as described above. The radioactivity of the merodesmosine peak from the reduced aorta tissue represented 8.6% of the total counts compared with 3.4% for unreduced elastin. This increase was small when compared with the increase (to 25.5% of the total counts) observed when elastin was treated with hot alkali before reduction. Since most of the desmosine and isodesmosine appeared in the reduced form it was apparent that the reduction of the aorta tissue had been successful and it was concluded that treatment with hot sodium hydroxide favors the formation of an unsaturated precursor of merodesmosine.

Isolation of Merodesmosine. Elastin (about 1.5 kg) was prepared from the *L. nuchae* of a group of heifers and steers about 18 months old by treatment with 0.1 N sodium hydroxide at 98° for 45 min following the procedure of Lansing *et al.* (1952). The fiber preparation (1.0 kg) was suspended in 0.2 N Tris buffer (pH 7.2) and 5.0 g of $NaBH_4$ was slowly added. The reaction mixture was stirred at room temperature for 24 hr. The reduced product was recovered by filtration and washing and was hydrolyzed under nitrogen by refluxing with constant-boiling HCl for 48 hr. Most of the hydrochloric acid was removed by repeated evaporation with added water. The residue was taken up in sufficient water to reduce the HCl concentration to 0.1 N and absorbed on a 7.6-cm diameter column packed with Zeo Karb 225-X8 (100–200 mesh). The column was washed with 0.4 M pyridine acetate buffer (pH 3.5) which removed the acidic and most of the neutral amino acids. The basic amino acids were eluted with pH 5 pyridine acetate buffer, and the effluent was decolorized by filtering through charcoal. This fraction was further separated by elution with a volatile buffer gradient system using a column of Zeo Karb 225-X8 (138 × 2.9 cm). The mixing chamber (1 l.) was filled with the pH 3.1 volatile pyridine–acetic acid buffer of Schroeder *et al.* (1962) and pyridine–acetic acid buffer (pH 5.0) was supplied from the reservoir. Fractions of 10 ml were collected and a drop from each was chromatographed on paper using butanol–acetic acid–water (4:1:1) as the solvent. Fractions enriched with the new amino acid (R_F 0.005) but containing reduced desmosine and isodesmosine (R_F 0), lysinonorleucine, and lysine were concentrated, streaked on sheets of Whatman No. 3 paper, and developed for 48 hr in butanol–pyridine–9.0 M aqueous ammonia (1:1:1). The band due to the new amino acid (R_F 0.05) was located under an ultraviolet lamp by observing the quenching of background fluorescence. (The following R_F's were observed in butanol–pyridine–ammonia: lysine, 0.21; lysinonorleucine, 0.10; merodesmosine, 0.05; and desmosine, 0.02.) Strips containing the amino acids were cut out and eluted with water. The product, after refractionation by the same method, contained no other amino acid and was further purified and desalted by application to a narrow bore Dowex 50 column and displacement with 0.2 N NaOH. The purified amino acid (yield, 110 mg) was recovered as microcrystals by dropwise addition of ethanol to the concentrated aqueous solution. It chromatographed as a single peak on the Technicon amino acid analyzer. With this instrument, using standard buffer solutions, the peak due to the new amino acid

appeared just before, and tended to overlap, that due to lysinonorleucine.

In order to confirm the identity of the purified amino acid (obtained in these experiments from bovine *L. nuchae* elastin) with merodesmosine isolated as a ^{14}C-labeled amino acid from aortic elastin, the two preparations were mixed and chromatographed together using the solvent mixtures described above. A more sensitive method for separating the various lysine derivatives found in elastin appears to be by use of high-voltage electrophoresis as described later in this paper. No separation of the radioactivity from the ninhydrin color could be obtained by any of these methods. The crystalline amino acid was ash free. *Anal.* Calcd for $C_{18}H_{34}N_4O_6 \cdot H_2O$ (420): C, 51.3; H, 8.6; N, 13.2; O, 26.7. Found (combustion): C, 50.25; H, 9.34; N, 13.5; O, 26.91. Found (Kjeldahl): N, 13.1. Using the Mecrolab vapor pressure osmometer the molecular weight found was 436.

The ninhydrin color yield was 2.50 leucine equiv/mole, calculated for mol wt 420. Compared under the same conditions the color yield of desmosine was 3.6 leucine equiv/mole (mol wt 543).

Proton magnetic resonance spectra in deuterium oxide showed absorption as broad-banded envelopes in the spectral regions expected for $CH(ND_3^+)CO_2^-$, CH_2 and CH_3 attached to N^+, and aliphatic CH_3 and CH_4. There was no absorption in the aromatic region, suggesting the absence of heterocyclic rings.

The infrared spectrum (solid phase, 1.5-mg sample in 200 mg of KCl) was typical of an α-amino acid in zwitterion form (NH_3^+ stretching, 3040 cm^{-1}; NH_3^+ deformation, 1630 and 1519 cm^{-1}; COO^-, 1588 cm^{-1}). The un-ionized carboxyl group was absent. There was no evidence of an aromatic structure. A broad band at 3400 cm^{-1} was present and could be due to either hydroxyl, water of hydration, or NH.

Absorption in the Ulraviolet Region. The compounds showed no absorption peak or inflexion in the range 230–350 mμ, suggesting the absence of an aromatic structure.

High-Voltage Paper Electrophoresis. Merodesmosine, together with lysine and other lysine derivatives, lysinonorleucine and desmosine, were pipetted onto the base line of a Whatman No. 1 paper strip. Small spots of glucose were also applied at both sides of the base line in order to correct for movement of buffer due to endosmosis. The buffer system was ammonium carbonate (1%, w/v) (pH 8.9); the paper strip was cooled by immersion in white spirit. A potential of 43 v/cm was applied for 70 min. The amino acid spots were developed with ninhydrin and side strips carrying glucose were cut off and developed with aniline phthalate. In Figure 3 the mobilities of the components (corrected for endosmotic flow) are plotted against the gross charge ratio for each component. The points lie on a smooth curve, from which it can be deduced that the gross ratio of positive to negative charges associated with merodesmosine is near 1.33. This is consistent with a molecule containing four positively and three negatively charged groups.

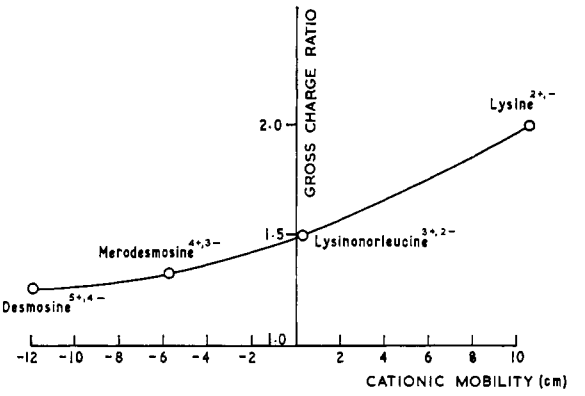

FIGURE 3: Paper electrophoresis of α-amino acid derivatives of lysine. Buffer solution, ammonium carbonate (1%, w/v), pH 8.9. Field strength, 43 v/cm. The mobilities are corrected for endosmotic flow by use of a glucose reference. The mobilities of lysine, lysinonorleucine, and desmosine fall on a smooth curve when plotted against their gross charge ratios. From its corrected mobility merodesmosine contains four potential positive and three potential negative charges.

Mass Spectrum of the N-Trifluoroacetyl n-Butyl Ester. Gehrke *et al.* (1965) have given a general procedure for the preparation of the *N*-trifluoroacetyl *n*-butyl esters of the amino acids for the purpose of preparing volatile amino acid derivatives for analysis by gas chromatography. This procedure was adopted, without modification, for the preparation of the merodesmosine derivative (10 mg).

The mass spectrum of the derivative is shown in Figure 4. The highest recorded ion occurs at *m/e* 954 which corresponds to the expected molecular ion. Exact mass measurement gave the value 954.3628 ± 0.009. These measurements were made with an A.E.I. MS.9 mass spectrometer. The sample was introduced by direct insertion into the ion source at 100°. Perfluorokerosene was used as the mass standard.

If it is assumed that the molecule contains 12 fluorine and 4 nitrogen atoms, then only three formulae give exact masses within the experimental error of 10 ppm. Of these, two, $C_{52}H_{46}F_{12}N_4$ and $C_{45}H_{50}F_{12}N_4O_5$, are inconsistent with other evidence about the molecule, while the third, $C_{38}H_{54}F_{12}N_4O_{10}$ (2-ppm error), fits the elemental analysis and other evidence, and is therefore the only reasonable result.

This confirms that this derivative of merodesmosine is the expected tetra-*N*-trifluoroacetyl tri-*n*-butyl ester ($C_{38}H_{54}F_{12}N_4O_{10}$, mol wt 954). The formation of this derivative confirms that the four nitrogen atoms in merodesmosine are present as primary or secondary amino residues, and locates the six oxygen atoms as three carboxyl residues. The evidence from the mass spectral analysis of the derivative, combined with evidence of the derivation of merodesmosine from lysine, leads to the proposal that merodesmosine has the structure V (Figure 5). The position of the double bond has not been

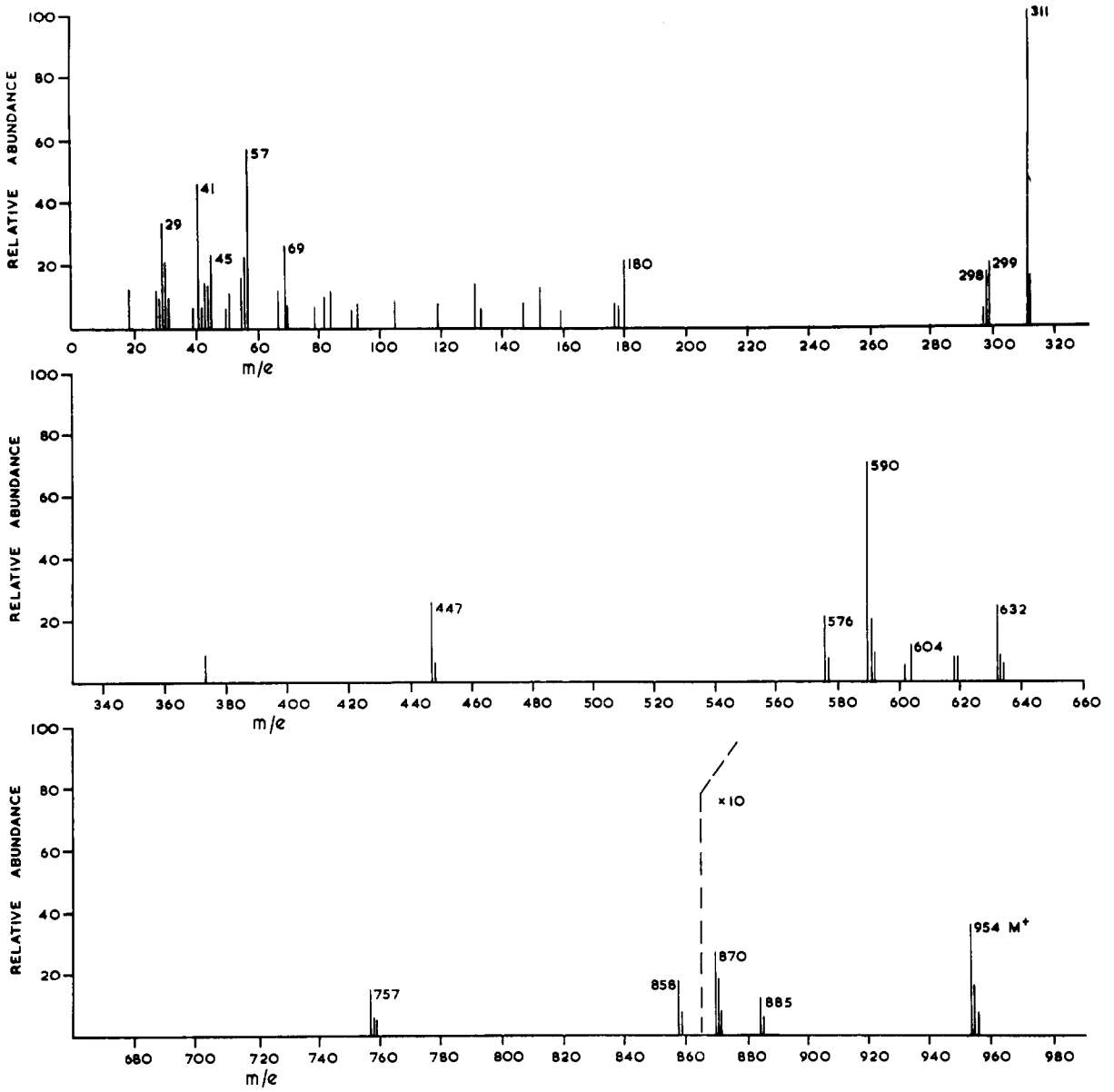

FIGURE 4: Mass spectrum of the tetra-*N*-trifluoroacetyl tri-*n*-butyl ester of merodesmosine. The sample was introduced by direct insertion into the ion source at 100°. The highest recorded ion is at *m/e* 954 which corresponds to the expected molecular ion. Exact mass measurement gave the value 954.3628 ± 0.009 corresponding to $C_{38}H_{54}F_{12}N_4O_{10}$ (2-ppm error).

determined but the two most probable positions are indicated in the figure.

Discussion

The isolation of the new amino acid in labeled form from lysine-labeled aorta tissue shows that the compound, like the demosines and lysinonorleucine, incorporates the lysine skeleton in its structure. None of the usual amino acid constituents of proteins incorporate ^{14}C from lysine under these conditions (O'Dell *et al.*, 1966; Anwar and Oda, 1966). Merodesmosine is found only in trace quantities in the hydrolysis products of elastin from young or old animals and appears as a substantial peak only in chromatograms of the hydrolysis products of young elastin that has been treated with alkali and subsequently reduced with borohydride. The trace quantities which appear in normal alkali-treated elastin may have arisen as a result of incipient reduction by sugar breakdown products in the presence of alkali. It seems clear that the immediate precursor of merodesmosine is a labile substance, probably present in small amount, which is converted by reduction to the acid-stable compound isolated in these experiments.

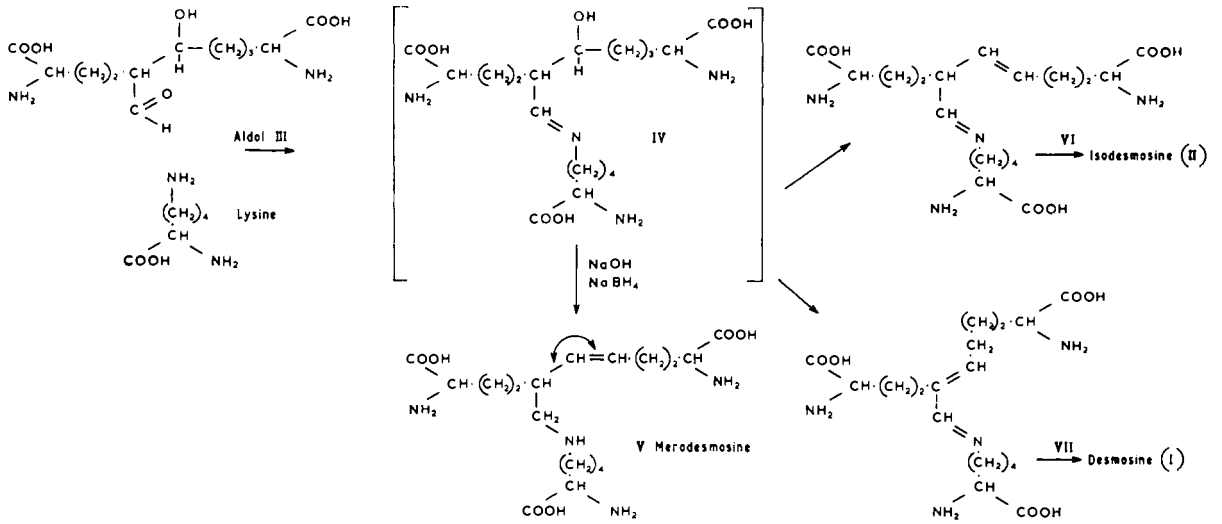

FIGURE 5: The scheme represents part of the biosynthetic route to desmosine and isodesmosine. Structure IV is inferred from the isolation of merodesmosine (V) from young growing elastin after treatment with hot alkali followed by borohydride reduction. Dehydration of IV would be expected to yield a mixture of the isomers VI and VII which could form isodesmosine (II, Figure 1) and desmosine (I, Figure 1), respectively, by cyclization with α-aminoadipic semialdehyde.

The isolation of merodesmosine from the products of borohydride reduction of purified elastin completes a range of three lysine-derived compounds and their isomers (lysinonorleucine, merodesmosine, and desmosine) which incorporate, respectively, two, three, and four residues of lysine. It appears probable that the synthetic route for each compound follows a common path initiated by the oxidative deamination of lysine to α-aminoadipic semialdehyde followed by condensation with the ϵ-amino group of a single unchanged lysine residue.

A scheme to represent these reactions is proposed in some detail in Figure 5. In this reaction sequence compound IV, containing aldol and Schiff base structures, is regarded as the common precursor of merodesmosine (V) and of both desmosine (I) and isodesmosine (II). In each case the reaction involves dehydration about the carbinol group to give a double bond which may occupy one or other of the positions in a three-carbon prototropic system. It is suggested that the distribution of the isomers so formed may determine the desmosine:isodesmosine ratio found in mature elastin. In elastin which has not been treated with alkali the amount of merodesmosine formed by reduction is small, and this suggests that the action of alkali is to accelerate the formation of IV and its dehydration to an unsaturated Schiff base. This product is then removed from the equilibrium system by borohydride reduction to merodesmosine at neutral pH.

Lysinonorleucine, like the desmosines, appears to occur naturally in elastin but it is not yet known whether it represents a cross-link reinforcing the desmosine system or if the synthesis of a fixed amount of lysinonorleucine is the reflection of a mechanism by which the random and continuous development of cross-links is blocked in order to produce fenestrations or to delineate fiber size.

The proposal that the first stage in the cross-linking reaction is represented by a dynamic equilibrium system of aldols and Schiff bases, the balance of which can be influenced by hydrogen ion concentration or reduction, provides an explanation for a number of puzzling features of the biosynthetic process. Thus, although the lysine content of elastin from young animals steadily decreases with age, it has been observed in this laboratory that the sum moles of lysine + (moles of lysinonorleucine/2) + (moles of desmosine/4) does not remain constant as the elastin ages. It is suggested that the observed discrepancy may be due to the presence, in significant amount, of a train of unstable intermediate products which do not appear in the gross amino acid analysis of elastin after acid hydrolysis. Miller and Fullmer (1966) found that the amino acid composition of lathyritic elastin differed from normal elastin only with respect to a higher content of lysine. The total amount of lysine and quarter-desmosine plus isodesmosine in the lathyritic elastin equalled 15.8 residues/1000 total residues and the corresponding value in control elastin was 12.7. This result suggests that in control elastin at least 3 lysyl residues/1000 total amino acid residues exist in a form which is undetected after acid hydroysis of the protein. This apparent loss of lysine residues in control elastin was associated with an increase in aldehyde content, providing evidence that lysine is converted to an aldehyde-containing intermediate during the biosynthesis of desmosine and isodesmosine. Comparison of the absorption spectra of elastin after reaction with *N*-methylbenzothiazolone hydrazone hydrochloride (MBTH) suggested that at least two types of aldehydes, saturated

and α,β unsaturated, were associated with normal elastin from young growing animals. Recently Lent and Franzblau (1967) have reduced elastin with tritiated sodium borohydride and have shown that the tritium label is taken up, not only by desmosine and isodesmosine, but also by a group of unidentified amino acids appearing near leucine and tyrosine in ion-exchange chromatograms. If the elastin is first treated with 2,4-dinitrophenylhydrazine in HCl, to cover carbonyl and Schiff base radicals present in the protein, the tritium label is found only in the desmosines. The presence of aldehyde and other derivatives of lysine has also been confirmed by Miller *et al.* (1967) in experiments with chick aorta labeled with [^{14}C]lysine. One of these derivatives was α-aminoadipic semialdehyde since this substance was recovered as α-aminoadipic acid after oxidation with performic acid followed by acid hydrolysis.

In the present report evidence is given for the derivation of merodesmosine from lysine and for its empirical formula both by elementary analysis and from the mass spectrum of a volatile derivative. The four nitrogen atoms have also been accounted for as amino or imino groups and the six oxygen atoms as three carboxyl residues. No formal chemical proof of other details of the structural configuration is as yet available but the structure V is proposed as the most reasonable hypothesis in keeping with present evidence. Since the compound contains a double bond the position of which has not been determined, it remains possible that merodesmosine is a mixture of geometric and structural isomers.

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