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Isolation, Purification, and Cross-linking Profiles of Elastin from Lung and Aorta†

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ABSTRACT: Elastin from anatomically defined regions of young calf lung and dog aorta was isolated and purified by a procedure which sequentially removed lipids, collagen, structural glycoproteins, and the microfibrillar proteins without apparent damage to the cross-linking residues, which have been shown to be sensitive to autoclaving and hot alkali treatment. One of the methods described was effective in obtaining pure elastin from lung parenchyma. Visceral pleura was found to be the richest source (25% dry weight) of elastin in the lung tissues examined. The amino acid compositions of the elastins purified by different methods were compared for purity and for the detection of possible damage to cross-linking compounds. Cross-linking profiles were obtained by column chromatography either after reduction with ^{3}[H]$NaBH_4$ or after reaction with ^{14}[C]NaCN and NH_3. The ^{3}[H]$NaBH_4$ method, under carefully controlled conditions, proved not to be quantitatively reproducible. The reaction of elastin with ^{14}[C]NaCN and NH_3 appeared preferable due to its reproducibility; this procedure required one type of hydrolysis for the analysis of all the cross-linking compounds. Examination of the cross-linking profiles of the elastins from various tissue regions revealed differences in the type, distribution, and quality of cross-links.

Elastin is an insoluble, extensively cross-linked protein found in lung, ligamentum nuchae, aorta, skin, and elastic cartilage. It may be isolated from tissues after other more soluble connective tissue elements have been removed, most often by the use of harsh methods involving autoclaving and exposure to hot alkali (Lansing et al., 1952; Partridge et al., 1955). Such procedures can remove collagen and other components, leaving behind "intact" but undoubtedly "degraded" elastin. In these elastin preparations, a number of N-terminal amino acid residues have been found (Gotte et al., 1963; Serafini-Fracassini and Smith, 1974), demonstrating the probable hydrolysis of peptide bonds in elastin. Richmond (1974) also noted that elastin preparations obtained after hot alkali treatment are altered, as evidenced by a decrease in the level of the desmosine types of cross-links, when compared with milder, albeit not yet entirely satisfactory methods of extraction.

Elastin is a vital constituent of lung and aorta. In lung it participates with surfactant in the maintenance of elastic recoil and in the stabilization of the alveolar volume (Johanson and Pierce, 1973; Wright, 1961; Snider et al., 1974). In addition, the continuity of elastin fibers, as demonstrated histologically (Oderr, 1964; Pierce and Ebert, 1965; Pump, 1974), helps to maintain the patency of small airways and blood vessels during all phases of the respiratory cycle. A loss of elastin integrity, as may occur in emphysema, will result in an increase in the size of the air spaces distal to the terminal bronchioles due to dilatation and destruction of the alveolar walls. As examples, individuals with homozygous α_1-antitrypsin deficiency show a high incidence of emphysema, a probable result of the diminution of plasma antielastase activity (Erikson, 1964; Pierce et al., 1969). Johanson and Pierce (1972) induced emphysema in laboratory animals by the introduction of papain or elastase

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into the lower airways of the lung; they were able to produce an increased compliance in excised rat lungs which was not due to alterations in surface forces. On histological evidence, altered elastic recoil correlated well with damaged elastin fibers. Bacterial collagenase produced a marked susceptibility to pleural rupture, but did not alter the elastic behavior. These investigators concluded that elastin is a major connective tissue determinant of pulmonary structure and elastic behavior.

In aorta, elastin plays a vital role in withstanding the intermittant forces that are exerted on the tissue. In Marfan's syndrome, where there are extensive defects in connective tissue, a most critical weakness of the tunica media of the aorta and pulmonary artery results in either diffuse dilatation or dissecting aneurysm. The histological appearance of such aortas suggests defects in elastin. Similar lesions are seen in lathyritic or copper deficient animals where lysyl oxidase, the key enzyme involved in the maturative cross-linking of collagen and elastin, is inhibited.

The study reported here set out to isolate and purify elastin from histologically defined regions of bovine lung and other tissues and to measure the nature and distribution of cross-links. Much of the reported chemical work on lung collagen and elastin has involved "whole lung" sections or structures which have been poorly defined anatomically. One of the long-term objectives of the present study is the development of methods which will attempt to relate the characteristics and properties of the elastin isolated to the etiology of specific human pathological states. Accordingly, it seemed pertinent to separate tissues as far as technically possible into anatomically defined regions. Furthermore, procedures for elastin isolation should be as free as possible from degradative action which might obscure or alter significant features of the protein.

Experimental Procedures

Fresh calf lungs from animals less than 3 months old were obtained from a nearby slaughter house. Adult dog aortas were obtained from animals sacrificed by other investigators at this institution. Lungs were stored frozen or dissected immediately on ice, into the following regions.

Trachea: Dissection was performed from below the larynx to the carina.

Bronchial Tree: From the carina, the bronchial tree was dissected peripherally and cleaned of parenchyma and blood vessels. Bronchioles with a diameter of about 2 mm could be dissected free of surrounding tissue.

Pleura: Visceral pleura could be stripped off the parenchyma. This was more easily accomplished when the lung was distended with air.

Parenchyma: Parenchyma was more difficult to isolate as a defined entity. Peripheral lung, 3–4 mm thick, freed of pleura, major airways, and major blood vessels was used.

Thoracic dog aortas were dissected free from loose connective tissue adherent to the adventitia.

Representative samples were taken for histological examination to confirm that the tissues were normal and that adequate isolation of the defined areas had occurred. A modified Verhoeff's iron haematoxylin stain (Verhoeff, 1908) was used for this purpose and, in addition, a fluorescent technique for the identification of elastin through detection of elastin aldehydic cross-link intermediates was developed (to be reported elsewhere).

Elastin Extraction and Purification (Procedure A_2). The anatomically defined regions were minced finely in 50% aqueous pyridine at 4 °C. The suspension was centrifuged and the residue washed thoroughly by centrifugation with water until free of pyridine and dried under vacuum. This material was finely powdered in a nitrogen mill for 3 min and then extracted for two 24-h periods with 2 M $CaCl_2$ at 4 °C followed by four water washes. The remaining tissue was further defatted and dehydrated with ethanol for 20 min, filtered through a sintered glass filter, treated with chloroform–methanol (2:1 by volume) for 20 min, washed with ether, and lyophilized. The defatted dry weight was obtained. This material was then extracted with 5% trichloroacetic acid at 90 °C for 30 min to denature and remove much of the collagen.[1] After centrifugation, the residue, washed with water three times to remove the excess trichloroacetic acid, was incubated with purified collagenase (ratio of substrate to enzyme approximately 50:1) at pH 7.5 in the presence of 0.5 mM $CaCl_2$ at 37 °C overnight to remove the remaining collagen. The supernatant was separated by centrifugation, and the residue was again digested with collagenase. The collagenase-insoluble residue was washed with water and was further extracted with 5 M guanidine, 0.05 M dithioerythritol, 0.1% EDTA[2] in 0.1 M Tris buffer at pH 8.5 at 37 °C overnight under a N_2 atmosphere in a stoppered bottle to remove microfibrillar and other remaining proteins. The residue obtained after centrifugation was twice washed with H_2O and extracted with 6 M urea, 1% sodium dodecyl sulfate, 0.05 M dithioerythritol in 0.05 M Tris buffer at pH 7.7 at room temperature under N_2 in a stoppered bottle. This last extraction was repeated twice. After centrifugation, the residue was dialyzed vs. distilled H_2O until sodium dodecyl sulfate was no longer detectable and was finally lyophilized. Elastin was converted to a fine powder in a Spex freezer mill. This was designated procedure A_2 and differs from Richmond's procedure (Richmond, 1974) in the initial steps. We added treatment with 50% pyridine and 2 M $CaCl_2$ to remove cell membranes and other tissue components, and 5% Cl_3CCOOH to remove some of the collagen and denature the remaining collagen. This treatment made the subsequent collagenase digestions more efficient. Procedure A_2 was applied to lung parenchyma to obtain pure elastin and proved to be very effective in removing other tissue components. Procedure A_1 was employed with elastin from calf visceral pleura and dog aorta as these tissues were more easily purified. Procedure A_1 is similar to A_2 but omits the treatments with 50% pyridine and 2 M $CaCl_2$. Calf ligamentum nuchae elastin was purified by the method of Partridge et al. (1955) using hot alkali extraction (procedure B).

Determination of Elastin Cross-linking Profiles. Reaction of Elastin with $^3[H]NaBH_4$. Samples of calf visceral pleura, ligamentum nuchae, and dog thoracic aorta elastin were reduced with calibrated tritiated sodium borohydride. The procedure was standardized as follows: 10 mg of finely powdered elastin was suspended in 10 ml of 0.1 M phosphate buffer pH 7.8. To the suspension was added 2.5 mg of calibrated ^{3}[H]sodium borohydride (specific activity 5.05×10^6 dpm/μM) and kept for 30 min at room temperature with

[1] It is possible that hot trichloroacetic acid could break aldimine linkages, but at this stage of development of suitable methods it was desirable to include this step. The fact that dehydrolysinonorleucine was present on analysis indicates that treatment with hot trichloroacetic acid does not entirely remove this cross-link. Further experiments will be carried out to examine this point in more detail.

[2] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HNL, ϵ-hydroxynorleucine; LNL, lysinonorleucine; CLNL, carboxylysinonorleucine; DAP, α,ϵ-diaminopimelic acid; OHAP, ϵ-hydroxy-α-aminopimelic acid; CAA, carboxyaminoaldol.

TABLE I: Elastin Content of Pulmonary Tissues and Aorta.

Reference	Tissue	Procedure	Elastin % Dry Wt
	Lung Parenchyma		
Pierce and Hocott (1960)	Human	B	1.3–11.2[b,c]
Pierce and Ebert (1965)	Human	B	26.5[b]
John and Thomas (1971)	Ox	B	12.5[b]
John and Thomas (1972)	Human	B	25.0[b]
Hoffman et al. (1972)[a]	Rat	B	3.7–4.4[b,c]
Richmond (1974)	Guinea pig	A	20[d]
Present study[a]	Calf	A_2	14
		A_1	22[b]
	Visceral Pleura		
Pierce and Ebert (1965)	Human	B	4–14[b,c]
John and Thomas (1971)	Ox	B	28[b]
John and Thomas (1972)	Human	B	8–16[b,c]
Present study[a]	Calf	A_1	25[b]
	Dog	A_1	25[b]
	Thoracic Aorta		
Present study[a]	Dog	A_1	70[b]
Grant (1967)[a]	Human	B	33[b]
	Sheep	B	39[b]
	Goat	B	45[b]
	Pig	B	50[b]

[a] These studies paid particular attention to the anatomical dissection of the tissues. [b] Elastin content expressed as percent dry defatted weight. [c] The range reported by the authors. [d] Expressed as percent of the partially purified residue.

TABLE II: Amino Acid Composition[a] of Calf Parenchymal Elastins Prepared by Various Methods.

	Procedure of Purification				
	B	A_2	A_2 less Pyridine	A_2 less $CaCl_2$	A_1
Hyp	15.0	8.4	14.8	9.9	9
Asp	10.5	10.1	10.1	29.9	19
Thr	7.0	10.1	9.0	21.0	17
Ser	8.0	10.3	12.6	22.2	23
Glu	20.9	24.2	23.3	55.1	71
Pro	110	108	114	96.6	75
Gly	313	302	293	248	243
Ala	220	213	205	177	192
Val	135	148	134	122	166
Met		1.4	2.0	14.6	5
Ile	25.9	17.1	37.7	30.4	22
Leu	63.3	66.3	62.2	70.6	84
Tyr	8.5	7.1	5.2	10.5	T[b]
Phe	37.1	37.7	25.6	37.4	26
Isodesmosine[c]	1.4	3.2	2.7	2.6	1.1
Desmosine[c]	3.9	7.6	5.8	6.2	1.5
Lys	8.8	13.0	14.7	25.1	23
His	3.5	2.9	7.2	7.0	4
Arg	7.8	10.1	9.3	13.4	18

[a] Expressed as residues per 1000 residues. [b] T, traces. [c] Expressed as lysine equivalents.

continuous stirring. The procedure was continued as previously described (Paz et al., 1971), except that washing by centrifugation was replaced by a 36-h dialysis against several changes of H_2O to minimize the loss of material. The lyophilized, reduced elastin was divided into two aliquots: one was hydrolyzed with 3 N HCl in a sealed evacuated tube for 44 h at 105 °C, the other was hydrolyzed with 2 N KOH at 105 °C for 22 h. After evaporation of the HCl from the acid hydrolysate under vacuum in a rotary evaporator, the residue was dissolved in 2 ml of H_2O. To the alkaline hydrolysates, perchloric acid was added dropwise to pH 3.0 at 4 °C and the precipitate of potassium perchlorate was separated by centrifugation. The cross-linking profiles were obtained by chromatography of the hydrolysates on a single column amino acid analyzer using a gradient of sodium citrate buffers from pH 2.8 to 7.0.

Reaction of Elastin with ^{14}[C]NaCN and NH_3. Ten milligrams of finely powdered elastin was suspended in 2 ml of H_2O. To the suspension were added 2.0 mg of ^{14}[C]NaCN (Pereyra et al., 1973, 1974; specific activity 1.1 mCi/mM) and 2 ml of 30% NH_4OH. The procedure followed is as previously described (Pereyra et al., 1973). The hydrolysate was chromatographed on a single column amino acid analyzer using the gradient described above.

Results and Discussion

Elastin Content and Amino Acid Composition of Pulmonary Tissues. The elastin content of various tissues of the dog and calf have been estimated (Table I) and compared with other published results. Visceral pleura was the richest source of elastin from the lung tissues examined and our results were comparable to previous reports. However, values obtained by some investigators using other procedures for human material could be the result of either species differences or differences in the methods employed. A similar comment could be made about parenchyma, where a wide variety of values have been reported. In the present study, a lower value for the elastin content was obtained with procedure A_2 which, however, gave a parenchymal elastin preparation which appeared to be quite pure.

The value obtained for the elastin content of the dog thoracic aorta is considerably higher than that found by other investigators for the same tissue in different species and may be due to our use of milder procedure. In Table II, the amino acid compositions of elastin from calf parenchyma which has been prepared by several different methods are compared. In the case of lung parenchyma elastin, procedure A_1 gives rise to a relatively impure elastin (column 5). The addition of the $CaCl_2$ extraction step (column 3) partially improved the quality of the elastin. Less satisfactory results were obtained with the use of 50% pyridine alone, omitting the $CaCl_2$ extraction (column 4). The amino acid composition of the elastin prepared by addition of both the $CaCl_2$ and the pyridine steps to the extraction (column 2, procedure A_2) is comparable to that obtained using procedure B (column 1). That the values for desmosine and isodesmosine are higher indicates a better preservation of the cross-linkages. This milder procedure may also retain some elastin which might have been removed in the hot alkali procedure by virtue of its somewhat higher solubility. A larger lysine content as is present in elastin purified by procedure A_2 would be expected. By the amino acid composition criteria procedure A_2 renders a pure elastin from calf lung parenchyma without the use of "degradative" conditions.

Table III compares the amino acid analysis of calf liga-

TABLE III: Amino Acid Composition of Elastins from Bovine Pleura and Dog Aorta.[a]

Tissue:	Calf L.N.	Calf Pleura	Calf Pleura	Calf Pleura	Dog Aorta
Procedure of Purification:	B	A_1	B	A_2 (less TCA and DTE)	A_1
Hyp	7.9	10.4	8.8	9.5	13.5
Asp	6.7	7.6	4.9	13.7	5.9
Thr	9.7	9.6	6.4	12.5	20.1
Ser	11.3	9.4	6.5	13.5	14.6
Glu	18.0	19.7	14.7	23.5	23.8
Pro	120	101	119	115	103
Gly	316	316	324	299	339
Ala	219	230	232	219	239
Half-cystine		2.5		5.6	
Val	145	141	137	126	99.2
Met	T	0.5		2.1	T
Ile	27.3	24.1	24.7	26.2	25.9
Leu	62.0	55.7	61.3	61.9	43.5
Tyr	3.6	7.8	8.2	11.1	24.4
Phe	32.3	28.4	32.4	40.9	24.4
Isodesmosine[b]	4.3	5.4	3.4	3.8	3.2
Desmosine[b]	9.3	12.8	6.8	6.0	7.1
Lys	5.6	9.2	5.0	3.1	3.8
His	2.8	2.6	1.9	10.3	2.4
Arg	6.0	6.7	4.2	8.6	7.1
LNL	1.9	1.9		1.9	1.9

[a] Abbreviations: T, traces; L.N., ligamentum nuchae; TCA, trichloroacetic acid; DTE, dithioerythritol. [b] Expressed as lysine equivalents.

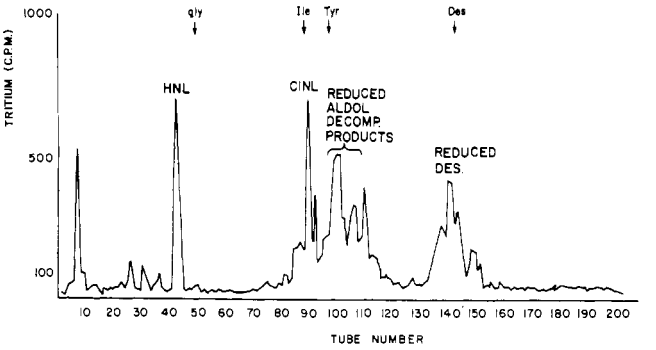

FIGURE 1: Cross-linking profile of ^{3}[H]$NaBH_4$ reduced elastin hydrolyzed with 3 N HCl. Elastin from calf pleura purified by procedure A_1.

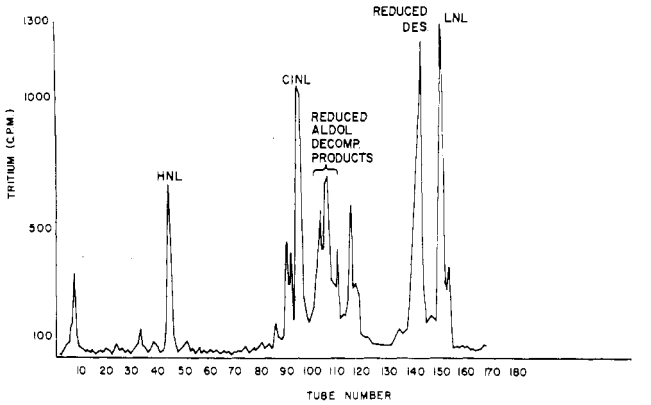

FIGURE 2: Cross-linking profile of ^{3}[H]$NaBH_4$ reduced elastin from calf pleura purified by procedure B and hydrolyzed with 3 N HCl.

mentum nuchae prepared by procedure B (column 1) and calf pleura prepared by the various procedures, as well as a preparation of dog aorta elastin. Elastin preparations which are quite "pure" were obtained when procedure A_1 was applied to calf visceral pleura (column 2) and dog aorta (column 5). Comparing the amino acid composition of the calf pleura elastins prepared by procedure A_1 (column 2) and hot alkali extraction (procedure B, column 3), it is clear that the latter method results in the destruction of some of the cross-linkages particularly the isodesmosine and desmosine. The omission of trichloroacetic acid and dithioerythritol from the extraction procedure resulted in a somewhat "impure" calf pleura (column 4) with a lower content of isodesmosine and desmosine than the calf pleura elastin prepared by procedure A_1 (column 2).

Figure 1 shows the cross-linking profile[3] of ^{3}[H]$NaBH_4$ reduced elastin from calf pleura (procedure A_1). Upon reduction, allysine residues in elastin are reduced to ϵ-hydroxynorleucine (HNL) residues. Hydrolysis with 6 N HCl converts about two-thirds of the ϵ-hydroxynorleucine into chlorononorleucine (Franzblau and Lent, 1969); the extent of this conversion is much less with 3 N HCl hydrolysis (Tanzer et al., 1970). The reduced aldol compound which results from the chemical reduction of the aldol condensation product of two allysine residues is also unstable to acid hydrolysis. After acid hydrolysis it appears as two or more radioactive peaks in the tyrosine region of the chromatogram. The pyridinium ring compounds, desmosine and isodesmosine, upon reduction are converted to tetrahydrodesmosines and after hydrolysis and chromatography appear as a broad heterogenous peak; this indicates the presence in that area of several related, probably isomeric, compounds with similar chromatographic properties. The cross-linkages, lysinonorleucine and dehydrolysinonorleucine formed by the reactions between allysine and lysine, are present in elastin as both the naturally reduced compound lysinonorleucine (75%) and as dehydrolysinonorleucine (25%); the latter compound will incorporate tritium upon chemical reduction with ^{3}[H]$NaBH_4$ to form labeled lysinonorleucine. The lysinonorleucine peak appears in the chromatogram of reduced elastin after the reduced desmosine peak; in the elastin examined (Figure 1), this radioactive peak is relatively small. A small radioactive peak would be expected if most of the cross-link was in naturally reduced form, or if the hot trichloroacetic acid treatment used in the purification procedure partially destroyed this perhaps labile aldimine bond.

The cross-linking profiles of the reduced elastins from calf pleura (purified by procedure A_1), calf ligamentum nuchae (procedure B), and dog aorta (procedure A_1) are qualitatively quite similar. With the alkali treatment of ligamentum nuchae elastin, less heterogeneity is apparent in the area of the reduced desmosines as compared with the other mildly treated elastins. This difference might have resulted from a destructive effect of the sodium hydroxide on some of the "desmosine" isomers. In this regard when calf pleura elastin is purified by procedure B and the cross-linking profile determined after ^{3}[H]$NaBH_4$ reduction and acid hydrolysis, less heterogeneity is also observed in the reduced desmosines region (Figure 2). Furthermore, a large and significant peak appears in the lysinonorleucine area. These results indicate a destructive effect of NaOH on some of the "desmosine" isomers, with the formation of dehydrolysinonorleucine as a decomposition product.

The cross-linking profiles were also examined after alkali hydrolysis since the reduced aldol condensation product and

[3] See paragraph at end of paper regarding supplementary material.

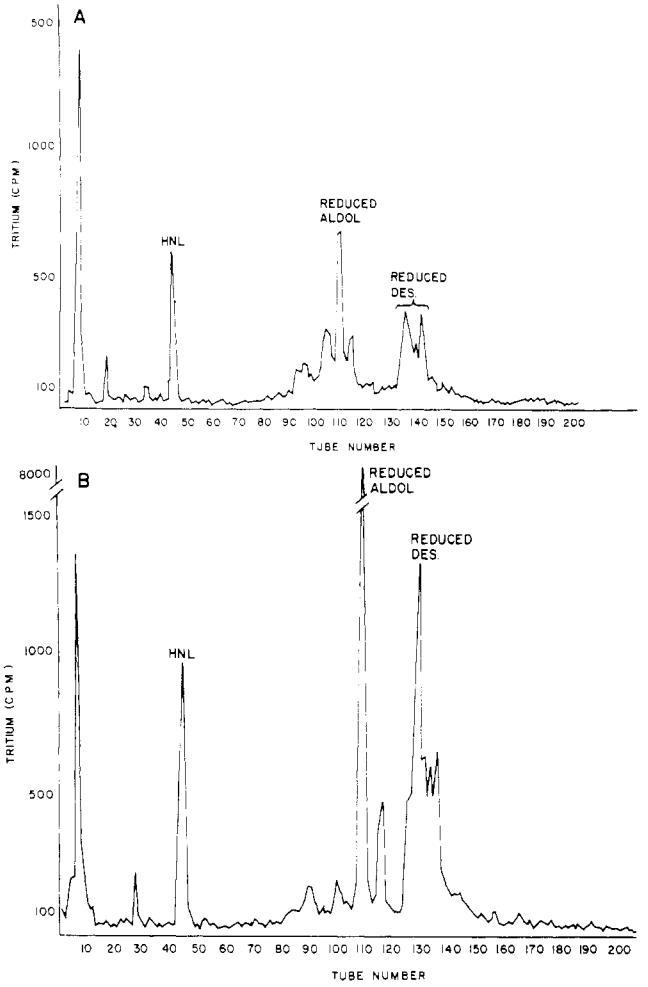

FIGURE 3: Cross-linking profiles of ^{3}[H]$NaBH_4$ reduced elastins hydrolyzed with 2 N KOH. (A) Elastin from calf pleura purified by procedure A_1. (B) Elastin from calf ligamentum nuchae purified by procedure B.

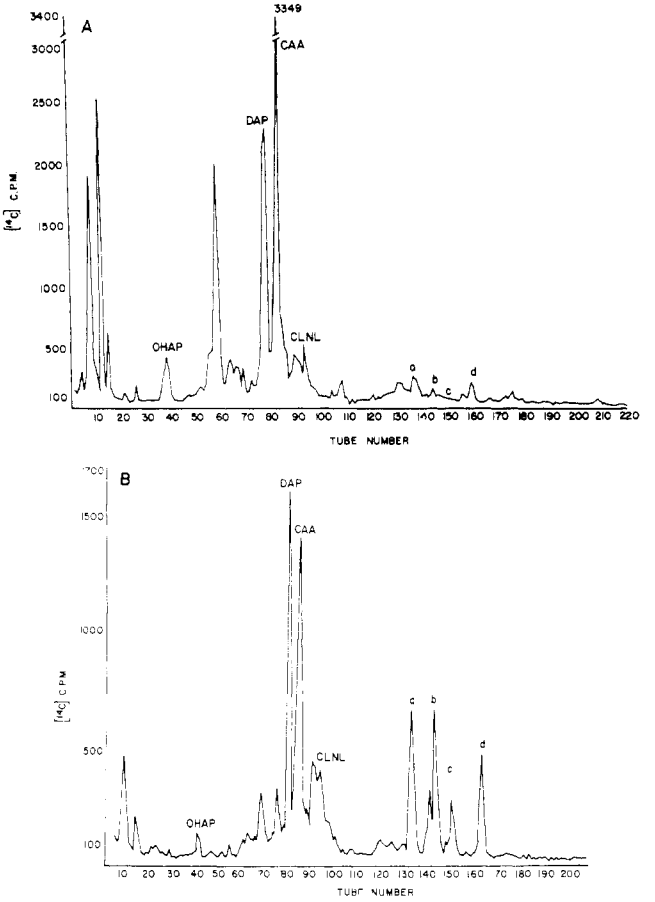

FIGURE 4: Cross-linking profiles of acid hydrolysates of elastins treated with ^{14}[C]NaCN and NH_3. (A) Elastin from calf parenchyma purified by procedure A_2. (B) Elastin from dog aorta purified by procedure A_1.

the hydroxynorleucine are more stable to alkaline hydrolysis. However, reduced desmosines are extensively destroyed by alkali. A distinct reduced aldol peak is present in reduced elastin from calf pleura (procedure A_1) and ligamentum nuchae (procedure B), after reduction and alkaline hydrolysis (Figures 3A and 3B). The cross-linking profiles of these two elastins are similar and, in the alkali processed ligamentum nuchae (Figure 3B), the aldol peak is particularly prominent. Apparently, the alkaline extraction has no destructive effect on the reduced aldol or hydroxynorleucine peaks.

The cross-linking profiles of elastins derived from calf lung parenchyma (procedure A_2) and dog aorta (procedure A_1) obtained by the ^{14}[C]NaCN procedure are shown in Figures 4A and 4B. The reaction of elastin with ^{14}[C]NaCN and NH_3 leads to stabilization and labeling of certain cross-links and their intermediates. These modified compounds can be isolated after acid hydrolysis as new ^{14}C-labeled amino acids. Four such amino acids have been isolated and their structures previously established (Pereyra et al., 1974). They are: α,ϵ-diaminopimelic acid (DAP), and ϵ-hydroxy-α-aminopimelic acid (OHAP), derived from α-aminoadipic acid δ-semialdehyde; 1,9-diamino-1,9-dicarboxyglycino-non-4-ene [carboxyaminoaldol, (CAA)] derived from the aldol condensate of two residues of α-aminoadipic acid δ-semialdehyde; and 2-amino-6-lysinopimelic acid [carboxylysinonorleucine, (CLNL)] derived from dehydrolysinonorleucine. Some qualitative as well as quantitative differences are apparent. Quantitation of the identified peaks from these chromatograms as well as those from calf pleura (procedure A_1) and calf ligamentum nuchae (procedure B) is shown in Table IV. Desmosine, isodesmosine, and lysinonorleucine were calculated from the ninhydrin reaction in the amino acid analyzer, and OHAP, DAP, CLNL, and CAA were calculated from radioactivity. The results are expressed as lysine equivalents. In this table, the sum of OHAP and DAP would measure the allysine content of the elastin. The values obtained for the ligamentum nuchae elastin are close to the values previously reported with the exception of CAA which is lower (Pereyra et al., 1973). The values obtained for the other elastins calculated from radioactivity are lower than the corresponding values for ligamentum nuchae. In particular, in the calf parenchymal elastin the dehydrolysinorleucine, the lysinonorleucine, and the aldol compounds are present in low amounts. Moreover, in the same preparation the presence of some soluble immature elastin is suggested by a high value of lysine which brings the balance of the total lysines accounted for to the same level observed for the other elastins (Table IV). Several other radioactive peaks, of as yet unknown composition, are present in the chromatograms. Peaks marked a, b, c, and d are particularly prominent in dog aorta elastin and the total radioactivities found in these peaks as well as in the peaks present between OHAP and DAP are shown in Table IV and one lysine equivalent is assumed for each peak. If these peaks represent di-, tri-, or tetrafunctional cross-links, the amount present particularly in aorta becomes quite significant.

TABLE IV: Analysis of Elastin Cross-links.[a]

Source of Elastin Procedure	Des[b,h]	Iso-des[b,h]	LNL[b]	OHAP and DAP[c] Measures Allys[e]	CAA[c] Measures "Aldol"	CLNL[c] Measures de-LNL[f]	Pre-DAP[c] (between OHAP and DAP)	Others[c] (a, b, c, d)	Lys Derived Cross-links & Intermediates	Lys Residues	Total[d] Lys Residues Accounted for
Calf pleura (A_1)	8.0	3.1	1.9	0.44	0.95	0.65	0.15	0.39	15.58	9.2	24.78
Calf L.N.[g] (B)	9.3	4.3	1.9	1.02	2.16	0.95	0.73	0.14	20.50	5.6	26.10
Dog aorta (A_1)	7.1	3.1	1.9	0.59	1.78	1.22	0.51	0.96	17.16	3.8	20.96
Calf paren.[g] (A_2)	7.6	3.2	0.5	0.43	0.57	0.17	0.54	0.09	13.10	13.0	26.10

[a] Average residue molecular weight of elastin is 83.8; number of μM/83.8 mg elastin is equivalent to the number of residues per 1000 residues; all the data are expressed as lysine equivalents per 1000 residues. [b] Calculated from absorbance at 570 nm (ninhydrin) using the color value of lysine. [c] Calculated from radioactivity. [d] Pig aortic tropoelastin contains about 46 residues of lysine, assuming the same value for tropoelastin of other species, about one-half are accounted for by lysine remaining and lysine derived cross-links and intermediates. There are other unidentified radioactive peaks not considered in this calculation. [e] Allys, allysine. [f] de-LNL, dehydrolysinonorleucine. [g] L.N., ligamentum nuchae; paren., parenchyma. [h] Des, desmosine; Isodes, isodesmosine.

Considerable effort was devoted to the preparation and purification of lung elastin. After careful dissection, calf visceral pleura was found to be quite rich in elastin and, using denaturing and reducing agents as well as collagenase under mild conditions, we were able to obtain a relatively pure elastin preparation. Lung parenchymal proved to be more difficult to dissect and the complexity of the tissue required the use of longer additional extractions in order to obtain "pure" elastin. The addition of the $CaCl_2$ and pyridine extraction steps greatly improved the quality of the parenchymal elastin preparation. The amino acid composition of this elastin showed the absence of significant contamination with collagen or other structural glycoproteins. As parenchymal elastin isolated from calf by procedure A_2 showed an increase in the lysine content as compared with elastin purified by procedure B, it is possible that some of the more soluble elastin derived from tropoelastin was present. This more soluble, less cross-linked elastin would be removed in preparations obtained with the use of the hot alkali extraction. The use of other procedures which might help remove small blood vessels prior to the chemical purification might also reduce the possibility of contamination and yield a superior elastin preparation from lung parenchyma. The use of a collagenase purified by affinity chromatography on a column of alkali-treated elastin (Serafini-Fracassini et al., 1975) might also be helpful and may remove other contaminating proteases with elastolytic activities (Spina et al., 1975) from the collagenase.

Purified elastins from calf pleura and ligamentum nuchae and dog aorta were used to compare the cross-linking profiles obtained by reduction and those obtained with ^{14}[C]NaCN and NH_3. The first procedure applied to calf pleura and ligamentum nuchae elastins and dog aorta elastin gave similar qualitative results if analogous procedures were used for purification of the elastins. Some of the cross-linking compounds present in elastin are sensitive to acid and to base and, in consequence, mild procedures should be used for the purification of the elastin. The so-called desmosine and isodesmosine residues, which are pyridinium compounds, also contain a complex mixture of isomers including the dihydrodesmosines. On acid hydrolysis, these dihydrodesmosine isomers probably undergo some oxidation to pyridinium compounds and decomposition to other products which include LNL.

In this study, although the reduction method was used under standardized conditions with careful control of the concentration of reactants, pH, temperature, and time of reaction, the method was found neither to be quantitative nor entirely reproducible. All elastin samples were similarly ground to a very fine powder as the accessibility of the cross-links to $NaBH_4$ reduction was clearly dependent on the physical state of the elastin. In spite of this precaution, the incorporation of tritium upon reduction was quite variable and was not considered to be quantitative. We have preferred the ^{14}[C]NaCN-NH_3 procedure which proved to be reproducible in duplicate analyses and requires only one type of hydrolysis. However, it still remains to be seen if this procedure is entirely quantitative with regard to the detection of the various types of cross-links and their intermediates.

With the cyanide procedure, significant differences in the type and distribution of cross-links were apparent upon examination of the profiles of the different elastins. Quantitative differences were determined for the identified cross-linking compounds using total dpm in each peak and the specific activity of ^{14}[C]NaCN. We are attempting to identify some of the other compounds which show significant quantitative differences in elastin from different species and tissues. The reaction conditions are also being investigated to obtain maximum incorporation of ^{14}[C]cyanide. It would appear that the ^{14}[C]cyanide procedure as employed here is sufficiently reproducible, so that a responsible comparison of normal and pathological tissue elastins can be made. This might show whether or not defective maturation of elastin has some role in the etiology of pulmonary and other disorders.

Supplementary Material Available

Five figures containing additional cross-linking profiles as noted in footnote 3 (5 pages). Ordering information is given on any current masthead page.

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Resonance Raman Spectra of Iron(III)–, Copper(II)–, Cobalt(III)–, and Manganese(III)–Transferrins and of Bis(2,4,6-trichlorophenolato)diimidazolecopper(II) Monohydrate, a Possible Model for Copper(II) Binding to Transferrins†

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ABSTRACT: Fe(III), Cu(II), Co(III), and Mn(III) complexes of ovo- and human serum transferrins show resonance enhanced Raman bands near 1600, 1500, 1270, and 1170 cm^{-1} upon excitation with laser frequencies which fall within the visible absorption bands of those metalloproteins. Comparison of the visible absorption and resonance Raman spectra of the Cu(II)–transferrin complexes with those for the Cu(II) model compound, bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate, indicates that the resonance Raman bands are due to enhancement of phenolic vibrational modes. For the model Cu(II) compound, a normal coordinate analysis was used to aid our assignment of the observed resonance bands at 1562, 1463, 1311, and 1122 cm^{-1} to A_1 vibrational modes of the 2,4,6-trichlorophenolato moiety. These assignments are consistent with those made for Cu(II)–transferrins. The latter assignments were based upon calculated A_1 frequencies for *p*-methylphenol (Cummings, D. L., and Wood, J. L. (1974), *J. Mol. Struct. 20,* 1). The wavelength shifts in the resonance bands for the model compound from those for Cu(II)–transferrins are due to the influence of the chloro substituents on the planar vibrations of phenol. These results clearly identify tyrosine as a ligand in copper binding to transferrins.

Unlike that for heme proteins, the physical structure of the two metal binding sites of transferrins has not been resolved. From studies of proton displacement upon introduction of the metal, Warner and Weber (1953) suggested that the phenolic group of tyrosine residues acts as ligands in Fe(III) and Cu(II) binding to ovotransferrin. This suggestion was supported by difference spectra (Wishnia et al., 1961; Tan and Woodworth, 1969; Lehrer, 1969), NMR[1] (Woodworth et al., 1970) and chemical modification (Komatsu and Feeney, 1967) studies. Fluorescence measurements (Luk, 1971) also pointed to tyrosine as ligand in the binding of trivalent lanthanide ions to transferrins. ESR measurements, which showed extra hyperfine splitting of the $g_\perp$ peak (Windle et al., 1963; Aasa et al., 1963; Aasa and Aisen, 1968), provided evidence for participation of nitrogen-containing ligands in Cu(II) binding to transferrins. The imidazole group of histidine residue has been proposed as the nitrogen-containing ligand (Aasa et al., 1963), although other nitrogen-containing groups, such as amide (Feeney and Komatsu, 1966) and guanidyl (Windle et al., 1963), have also been suggested. Chemical modification studies indicate the possible participation of histidyl groups (Line et al., 1967; Bezkorovainy and Grohlich, 1971) but not amino

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[1] Abbreviations used: NMR, nuclear magnetic resonance; TCPI, bis(2,4,6-trichlorophenolato)diimidazolecopper(II) monohydrate; ESR, electron spin resonance.