The Biosynthesis of Elastin Cross-links

THE EFFECT OF COPPER DEFICIENCY AND A LATHYROGEN

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Recently, two new amino acids have been isolated from hydrolysates of bovine ligamentum nuchae elastin by Partridge, Elsden, and Thomas (1). Further studies from the same laboratory (2) have shown the compounds to be structural isomers composed of a pyridium ring with four side chains located at positions 1, 3, 4, and 5 (desmosine), as depicted in Fig. 1, or at positions 1, 2, 3, and 5 (isodesmosine). Each side chain has a carboxyl-terminal and an α-amino-terminal group, and since these amino acids were found in peptides containing multiple amino- and carboxyl-terminal groups (3), it has been proposed (2, 4) that either isomer could serve as a cross-link between as many as four different polypeptide chains.

Consideration of the structure of the desmosines¹ and the empirical formula \( (C_{40}H_{20}N_5O_{22}H_2O) \) suggested that these compounds might arise from the condensation of the side chains of 4 lysine molecules with the loss of the ε-amino group from 3 of them. Direct evidence that lysine serves as a precursor has been obtained from studies on the incorporation of radioactivity into the desmosines from lysine-¹⁴C (4, 5). Furthermore, we have found that the amino acid composition of chick aorta elastin remains constant with age except that there is an increase in the content of the desmosines accompanied by an equivalent decrease in lysine content (5). These results (4, 5) indicate that the conversion of lysine to the desmosines occurs after the incorporation of lysine into elastin or an elastin precursor.

In this report, we examine in further detail the biosynthesis of the desmosines. Since copper deficiency is known to produce defects in aortic elastin (6-8) and since lathyrism has been shown to cause both an interference in the cross-linking of collagen and copper-deficient chicks as well as those from tissue culture were pooled within experimental groups and washed several times with cold (5°) 0.9% NaCl solution. The aortas were then defatted by successive 24-hour periods of extraction with acetone and ether at 5°. After drying at room temperature, the specimens were minced into fine particles and extracted with 0.1 N HCl per mg of protein at 108° for 72 hours. This period of hydrolysis was necessary to effect complete release of desmosine, isodesmosine, and valine.

Amino Acid Analyses of the Elastin Hydrolysates—Portions of the residue remaining after alkali extraction were hydrolyzed in a nitrogen atmosphere in a sealed tube with 1 ml of 6 N HCl per mg of protein at 108° for 72 hours. This period of hydrolysis was necessary to effect complete release of desmosine, isodesmosine, and valine.

Amino acid analyses of the elastin hydrolysates, equivalent to about 1 mg of protein, were performed on an automatic amino acid analyzer (16). In this system, the desmosines chromatograph as two incompletely resolved peaks between phenylalanine...
The structure of desmosine as given by Thomas, Elsden, and Partridge (2). The side chain at carbon 4 is at position 2 in isodesmosine. The number of methylene groups in the side chains at positions 3, 4, and 5 is based on the assumption that the molecule is derived from 4 lysine residues.

FIG. 1. The incorporation of isotope into alkali-insoluble elastin. Embryonic chick aortas were incubated for the first day in medium containing approximately 0.5 μC of lysine-14C per ml and then transferred to the same medium without isotope. O, lysine; ●, quarter-desmosine plus isodesmosine; ▲, average specific activity.

and hydroxylysine. As both isomers exhibit identical color yields (3.6 times the leucine color yield) with the ninhydrin reagent, the total amount in any given hydrolysate could be easily calculated. (We are indebted to Dr. S. M. Partridge for samples of desmosine and isodesmosine which were used to standardize the analyzer.)

The results were expressed as quarter residues, in a manner analogous to that of half-cystine, since each molecule is presumed to occupy four positions in the polypeptide. Correction factors employed for those amino acids which were found to be partially destroyed during the course of hydrolysis were as follows: threonine, +6%; serine, +22%; tyrosine, +4.5%; phenylalanine, +3.1%. Each sample analyzed contained at least 0.035 μmole of lysine and 0.07 μmole of the desmosines (quarter residues).

Radioactivity determinations were made on the amino acids in the elastin hydrolysates by continuous scintillation counting of the effluent from the automatic amino acid analyzer (17). In all of the cases, sufficient counts were obtained to give a standard counting error of less than 8%. In some experiments, the average specific activity of lysine and quarter-desmosine plus isodesmosine was calculated as follows: counts per min of (quarter-desmosine + isodesmosine)/micromoles of lysine + micromoles of (quarter-desmosine + isodesmosine).

RESULTS

Tissue Culture—The specific activities of lysine and the desmosines in elastin were measured at various intervals after an initial 1-day exposure of embryonic chick aortas to lysine-14C. The lysine-14C was removed by rinsing the aortas once with nonradioactive medium and then by placing them in either a complete or a lysine-free medium.

In the complete medium, the specific activity of the lysine decreased and the specific activity of the desmosines increased with time (Fig. 2). The decrease in lysine specific activity was considerably greater than the increase observed in the specific activity of the desmosines, causing the average specific activity to decrease from the 1st to the 9th day. Thus, it seemed likely that sufficient amounts of elastin were being synthesized to dilute the labeled pool.

In the absence of lysine (Fig. 3), the decrease in lysine specific activity was less rapid, and it was accompanied by an equivalent increase in the specific activity of the desmosines, the average specific activity remaining constant. These results presumably reflect the absence of elastin synthesis and the continuation of cross-linking in the previously synthesized radioactive elastin after the initial 1-day incubation in the presence of lysine-14C.

Copper Deficiency—The alkali-insoluble elastin content of aortas from chicks fed the copper-supplemented diet for 1 week was approximately 40% of the dry, fat free weight, and it increased to approximately 60% after an additional 2 weeks. On the copper-deficient diet, the elastin content remained at about 40%. However, this may not reflect a depressed elastin synthesis since the decrease in content of the desmosines (see below) may render the elastin more soluble in alkali.

The number of residues of lysine and desmosines in elastin isolated at weekly intervals from copper-supplemented chicks (dietary copper at 5 ppm) is shown in Fig. 4. There was an increase of 5 residues of desmosine plus isodesmosine over the
period studied, and this was accompanied by the loss of 1 residue of lysine. After 3 weeks of dietary copper supplementation, the total amount of lysine and desmosine plus isodesmosine (the latter two expressed as quarter residues) appeared to remain constant at approximately 13.5 residues/1000 total residues. In further experiments, copper was added to the control diet at a level of 25 ppm and elastin was isolated at weekly intervals for 3 weeks. The higher level of dietary copper supplementation appeared not to alter the relationship between lysine and the desmosines during this period.

Fig. 4 also contains comparable data obtained from the aortas of chicks raised on the same diet without copper supplementation. In this case, although the rate of formation of new cross-links, expressed as content of the desmosines, was equal to that observed in control animals for the 1st week, the process appears to have ceased after this time. Accompanying the apparent cessation of cross-link formation was a 2- to 3-fold increase in lysine content which reached its maximum at 3 weeks. An increased lysine content in elastin from chicks fed a copper-deficient diet for 2 weeks has also been reported by Starcher, Hill, and Matrone (18). As was the case with elastin isolated from chicks fed the copper-supplemented diet, the total amount of lysine and desmosine plus isodesmosine remained constant after 3 weeks, but the sum was higher, viz. 19.5 against 13.5 residues/1000 total residues.

As may be seen in Columns 2 and 3 of Table I, the amino acid composition of elastin isolated from chicks fed copper-supplemented and copper-deficient diets was identical except for the differences in lysine and cross-linking residues already noted. As a point of reference, the amino acid composition of the elastin from 1-day-old chicks is also given in Table I. It will be noted that the sum of lysine and cross-linking residues in these chicks was 10.4 residues/1000 which increased to 13.2 residues/1000 after 3 weeks on the copper-supplemented synthetic diet. Our previous results (5) indicated that the total amount remained constant at 10 or 11 residues/1000. The latter result was again observed during the course of the present experiments when the chicks were maintained on a diet of Purina Chow rather than the synthetic diet. Therefore, there seem to be other nutritional factors involved.

**TABLE I**

| Amino acid composition of elastin from aortas of normal and copper-deficient chicks |
|---------------------------------|----------------|
|                                 | Copper supplemented | Copper deficient |
|                                 | 1-day-old | 3-week-old |
| Hydroxyproline                  | 23        | 24        | 23  |
| Aspartic acid                   | 1.9       | 1.6       | 1.5 |
| Threonine                       | 4.9       | 4.0       | 3.9 |
| Serine                          | 5.0       | 4.7       | 4.5 |
| Glutamic acid                   | 12        | 11        | 11  |
| Proline                         | 124       | 122       | 123 |
| Glycine                         | 350       | 350       | 350 |
| Alanine                         | 176       | 175       | 174 |
| Half-cystine                    | <0.5      | <0.5      | <0.5|
| Valine                          | 180       | 180       | 178 |
| Isoleucine                      | 19        | 19        | 19  |
| Leucine                         | 60        | 59        | 58  |
| Tyrosine                        | 8.5       | 10        | 9.3 |
| Phenylalanine                   | 21        | 23        | 21  |
| Quarter-desmosine*              | 5.9       | 9.2       | 7.7 |
| Lysine                          | 4.5       | 4.0       | 12  |
| Histidine                       | <0.2      | <0.2      | <0.2|
| Arginine                        | 3.6       | 4.5       | 4.1 |

* Includes isodesmosine.

![Fig. 4](image-url) The effect of dietary copper supplementation (left) and copper deficiency (right) on the content of lysine and the desmosines in alkali-insoluble elastin from chick aortas. O, lysine; ●, quarter-desmosine plus isodesmosine; ▲, sum of lysine and quarter-desmosine plus isodesmosine.

![Fig. 5](image-url) The effect of penicillamine on the incorporation of isotope into alkali-insoluble elastin. Chick embryo aortas were incubated in medium containing 0.1 μC of lysine-14C per ml for 8 days with varying levels of penicillamine hydrochloride. O, lysine; ●, quarter-desmosine plus isodesmosine.

Effect of Penicillamine-HCl and β-Aminopropionitrile in Tissue Culture—Copper analyses (19) of the media used for tissue culture indicated that it contained 12 ppb of copper. This was apparently above the minimal level necessary since the rate of isotope incorporation into the desmosines by embryonic aortas was not altered if the copper level was increased to 1 or 2 ppm. For this reason penicillamine, a chelator of copper (20), was added to the medium in an attempt to create a copper deficiency.
the possibility that penicillamine causes a general inhibition of protein synthesis was investigated by incubating aortas with lysine-14C per ml for 8 days with varying levels of B-aminopropionitrile fumarate. O, lysine; •, quarter-desmosine plus isodesmosine.

As shown in Fig. 5, penicillamine was found to inhibit the formation of the desmosines. The addition of 1 mg per ml to the culture medium completely inhibited the incorporation of radioactivity into these compounds. In addition, this level of penicillamine reduced the specific activity of lysine by 50%. At 0.5 mg per ml, the specific activity of lysine was unchanged while the specific activity of the desmosines was reduced to about 40% of the control value.

The possibility that penicillamine causes a general inhibition of protein synthesis was investigated by incubating aortas with lysine-14C for 8 days and then measuring the incorporation of radioactivity into the triethylacetic acid-precipitable material. As shown in Table II, protein synthesis was inhibited less than 20% in the presence of 2 mg per ml of penicillamine. This finding suggests that the decrease in specific activity of lysine was due at least in part to the partial loss of the more recently synthesized, radioactivity-containing elastin during the extraction procedure owing to the decreased amount of cross-linking.

As illustrated in Fig. 6, B-aminopropionitrile (BAPN) on the incorporation of isotope into alkali-insoluble elastin. Chick embryo aortas were incubated in medium containing 0.1 μC of lysine-14C per ml for 8 days with varying levels of B-aminopropionitrile fumarate. O, lysine; •, quarter-desmosine plus isodesmosine.

In this report, we have presented additional evidence that lysine in peptide linkage serves as a precursor of the desmosines. After a 1 day incubation of aortas with lysine-14C, the specific activity of lysine in alkali-insoluble elastin was found to be 4 to 5 times greater than the specific activity of the desmosines. Upon subsequent incubation in a non-radioactive medium, the specific activity of lysine declined and that of the desmosines increased. Under conditions limiting the further synthesis of elastin (incubation in a lysine-free medium), the amount of radioactivity lost from lysine was equal to that gained in the desmosines. Under these conditions, it is likely that the decrease in lysine specific activity is due to the preferential cross-linking of the recently formed, radioactive elastin. Previously formed elastin must also undergo further cross-linking since elastin obtained from older chickemb has a greater content of the desmosines and less lysine (5).

It is reasonable to presume that the formation of desmosine and isodesmosine from lysine proceeds through one or more as yet unidentified intermediates. This assumption is supported by the finding that elastin prepared from the aortas of copper-deficient chicks contains 8 more residues of lysine than can be accounted for by the decrease in content of the desmosines. It appears that in a state of copper deficiency, lysine normally destined for conversion to the intermediate remains as lysine. That is, the extra residues represent the normal content of intermediate.

The common consequence of copper deficiency observed in chicks (6) and in swine (22), rupture of the aorta, can be attributed to the inability to synthesize elastin cross-links resulting in a decrease in the rate of accretion of new fibers and subsequent general weakening of the aortic wall.

The attempt to create a copper deficiency in tissue culture by adding penicillamine did result in an inhibition of cross-link formation. However, the very high levels, relative to copper, necessary, and the observation that the lysine specific activity did not rise above the control value, as might be expected from the increased lysine content of the elastin from aortas of copper-deficient chicks, may mean that penicillamine is acting in a manner other than by chelating copper. It is known, for example, that it combines with pyridoxal (23).

The finding that a lathyrogen (B-aminopropionitrile) also inhibits the synthesis of the desmosines suggests a similarity in the mechanism of cross-link formation for elastin and collagen. Collagen does not contain desmosine or isodesmosine, but chemically related intermediates could be involved in both cases. In this regard, aldehydes have been suggested as participating in collagen cross-linking (24), and Partridge et al. (4) have suggested that the lysine in elastin which is destined for formation of the desmosines may be converted to aldehyde intermediates prior to condensation.

So far, desmosine and isodesmosine are the only compounds identified as possible cross-links in elastin. However, since elastin, which is rich in lysine and deficient in the desmosines, is accumulated in copper deficiency, and since an appreciable quantity of radioactive lysine is incorporated into the alkali-insoluble protein under conditions where the formation of des-
mosine and isodesmosine are completely blocked, it is possible that other types of cross-links are present.

**SUMMARY**

Factors influencing the formation of aortic elastin cross-links (desmosine and isodesmosine) from lysine were studied in chicks in vivo and in isolated embryonic chick aortas in tissue culture. Alkali-insoluble elastin from copper-deficient chicks showed a marked increase in lysine content which was not accounted for by the observed decrease in content of the desmosines, suggesting the presence of an unidentified intermediate in normal elastin whose synthesis is copper-dependent. In tissue culture, the incorporation of lysine-14C into desmosine and isodesmosine was inhibited by a lathyrogen (β-aminopropionitrile) and by penicillamine. These agents may prevent the formation or the condensation of the intermediates which normally produce the cross-links. Although penicillamine chelates copper, it is not clear that its effect in culture is based on this property.

**REFERENCES**

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