Tropoelastin production and tropoelastin messenger RNA activity

Relationship to copper and elastin cross-linking in chick aorta

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The elastin content of the chick thoracic aorta increases 2–3-fold during the first 3 weeks post-hatching. The deposition of elastin requires the covalent cross-linking of tropoelastin by means of lysine-derived cross-links. This process is sensitive to dietary copper intake, since copper serves as cofactor for lysyl oxidase, the enzyme that catalyses the oxidative deamination of the lysine residues involved in cross-link formation. Disruption of cross-linking alters tissue concentrations of both elastin and tropoelastin and results in a net decrease in aortic elastin content. Autoregulation of tropoelastin synthesis by changes in the pool sizes of elastin or tropoelastin has been suggested as a possible mechanism for the diminished aortic elastin content. Consequently, dietary copper deficiency was induced to study the effect of impaired elastin cross-link formation on tropoelastin synthesis. Elastin in aortae from copper-deficient chicks was only two-thirds to one-half the amount measured in copper-supplemented chicks, whereas copper-deficient concentrations of tropoelastin in aorta were at least 5-fold higher than normal. In spite of these changes, however, increased amounts of tropoelastin, copper deficiency and decreased amounts of elastin did not influence the amounts of functional elastin mRNA in aorta. Likewise, the production of tropoelastin in aorta explants was the same whether the explants were taken from copper-sufficient or -deficient birds. The lower accumulation of elastin in aorta from copper-deficient chicks appeared to be due to extracellular proteolysis, rather than to a decrease in the rate of synthesis. Electrophoresis of aorta extracts, followed by immunological detection of tropoelastin-derived products, indicated degradation products in aortae from copper-deficient birds. In extracts of aortae from copper-sufficient chicks, tropoelastin was not degraded and appeared to be incorporated into elastin without further proteolytic processing.

INTRODUCTION

Elastin is important to the elasticity of blood vessels, ligaments, skin and lung (Sandberg *et al.*, 1981; Foster *et al.*, 1983; Rucker & Dubick, 1984). Despite the biological importance of elastin, knowledge of the factors responsible for regulation of elastin expression is incomplete.

It has been observed that the elastin content is decreased in tissues where tropoelastin and elastin concentrations have been altered by inhibiting cross-linking reactions (Smith *et al.*, 1977; Buckingham *et al.*, 1981; Rucker, 1982; Keeley & Johnson, 1983). However, previous investigations have not conclusively verified that changes in elastin concentrations influence elastin expression. In studies by Frisch *et al.* (1985) involving the use of monensin to block secretion of tropoelastin, the precursor of elastin, it was reported that tropoelastin synthesis is repressed at a pretranslational level, and that intracellular concentrations of tropoelastin may regulate tropoelastin synthesis. By analogy with collagen regulatory processes (Paglia *et al.*, 1979), it has also been suggested that peptides derived from tropoelastin might influence rates of elastin synthesis. For example, it has been postulated that receptor-mediated endocytosis may be a mechanism whereby tropoelastin or elastin-derived peptides are recycled back into cells (Mecham *et al.*, 1984; Griffin *et al.*, 1983). Studies by Mecham *et al.* (1981) have

also indicated that properties of the extracellular matrix can influence the rates of elastin synthesis. Ligament cells grown on dead ligament tissue are stimulated to produce more elastin than do cells grown on plastic culture dishes.

The concept that inhibition of cross-link formation results in inhibited tropoelastin synthesis comes from observations of repressed tropoelastin production in aortic tissues cultured in the presence of the cross-linking amino acid inhibitor BAPN (Keeley & Johnson, 1983). However, Schein *et al.* (1977) and Mecham *et al.* (1981) did not detect changes in elastin synthesis when tropoelastin accumulation was induced by inclusion of 3,4-dehydropyrroline or BAPN respectively. In clear contrast, a report by Hill & Davidson (1986) suggests that aorta minces from copper-deficient pigs are capable of increased tropoelastin synthesis rates. Consequently, we examined tropoelastin synthesis in the copper-deficient and -sufficient chick aorta to try to resolve the extent to which increased tissue contents of tropoelastin or a decrease in elastin influences tropoelastin synthesis. If intact elastic fibres are required for maximal rates of elastin synthesis, then submaximal rates of elastin synthesis might be expected in copper-deficient tissues, where the elastic fibres are often sparse, fragmented and disarrayed (O'Dell *et al.*, 1961). When animals are rendered copper-deficient, covalent cross-linking of elastin decreases (Kagan & Sullivan, 1982), resulting in increased tissue concentrations of tropoelastin, and

decreased quantities of elastin. As an alternative hypothesis, elevated tissue concentrations of tropoelastin might be a signal for inhibition of further tropoelastin synthesis.

In the growing chick, elastin synthesis and accumulation occur primarily during the first 2–3 weeks post-hatching (Keeley & Johnson, 1983; Rucker *et al.*, 1977). The effects of copper deficiency on aortic elastin metabolism are most profound during this period. Clearly, the content of mature elastin in copper-deficient aorta is less than normal (O'Dell *et al.*, 1961).

Herein, it is demonstrated that, despite a greater than 5–10-fold increase in aortic tropoelastin (copper-deficient versus -supplemented chicks), synthesis of elastin by poly(A)⁺ mRNA fractions in a cell-free system is the same whether the RNA is isolated from aortae of normal or copper-deficient chicks. It also is demonstrated that the production of tropoelastin by aorta explants from copper-sufficient and -deficient chicks is the same.

EXPERIMENTAL

Materials

Groups of 1-day-old White Leghorn cockerel chicks were given one of four diets based on spray-dried skim milk (O'Dell *et al.*, 1961; Buckingham *et al.*, 1981). The diets were: (1) copper-sufficient (25 µg of copper/g, added as CuSO₄); (2) copper-deficient (< 1 µg of copper/g of diet); (3) copper-sufficient, but restricted to the food intake of the copper-deficient group; or (4) copper-sufficient, but with 0.2% BAPN added. The diets were assayed for copper by flame atomic-absorption spectrophotometry (Clegg *et al.*, 1981).

Chemical reagents were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.), Pierce Chemical Co. (Rockford, IL, U.S.A.), Bethesda Research Laboratories (Bethesda, MD, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[6-³H]Valine (15 Ci/mmol) and L-[³H]proline (55 Ci/mmol) were products from ICN Radiochemicals (Irvine, CA, U.S.A.). Tissue-culture supplies were obtained from Grand Island Biochemical Co. (Grand Island, NY, U.S.A.). *Staphylococcus aureus* was purchased from Meloy Laboratories (Springfield, VA, U.S.A.).

Determinations of soluble and insoluble elastin

Soluble elastin in the guanidine thiocyanate extracts of aorta (see 'RNA isolation' below) was measured by competitive enzyme-linked immunosorbent assays (Mecham & Lange, 1982; Voller *et al.*, 1980). Highly purified chick tropoelastin (Rucker, 1982) was used to coat the enzyme-linked immunosorbent assay plates and establish standard curves. Guanidine thiocyanate/CsCl was added to the standard solutions in amounts equivalent to those in tissue extracts to compensate for any effects of these agents on the binding capacity of the anti-(chick elastin) antibodies. The characterization of the antibody preparation has been described (Buckingham *et al.*, 1981).

For insoluble elastin, the residue remaining after extraction with guanidine thiocyanate was extracted further with 95% (v/v) ethanol overnight. The residue was then freeze-dried, and insoluble elastin isolated and measured as described previously (O'Dell *et al.*, 1961; Buckingham *et al.*, 1981).

RNA isolation

The procedures were adapted from Chirgwin *et al.* (1979) and Foster *et al.* (1982). For each isolation, 20 aortae from 13- or 21-day-old chicks from each of the treatment groups were used. Ten aortae from 6-month-old birds were also used. The aortae were excised, cleansed of blood and adhering tissue and pulverized in liquid N₂. The tissue was next suspended in 4 ml of guanidine thiocyanate reagent (5 M-guanidine thiocyanate, 0.5% N-lauryl sarcosine, 10 mM-EDTA, 0.7% 2-mercaptoethanol, 0.1 ml of antifoam A and 10 mM-Tris buffer, pH 7.5) and further homogenized (1–2 min) at medium speed with a Tekmar (Cincinnati, OH, U.S.A.) homogenizer.

After extraction procedures similar to those described by Foster *et al.* (1982), the RNA was then precipitated (Chirgwin *et al.*, 1979). Total RNA was estimated by assuming 25 A₂₆₀ units/mg of RNA. As noted above, samples of the extracts and the resulting insoluble residues were utilized for tropoelastin and elastin assays.

Poly(A)⁺ RNA isolation

To 1 mg samples of RNA (after solubilization by heating to 60 °C) in 4 ml of 25 mM-Tris, pH 7.5, containing 12.5 mM-EDTA were added 1 ml of 5% (v/v) SDS and then 5 ml of 1 M-LiCl, pH 7.5. These samples were applied to oligo(dT)-cellulose columns (2 ml packed volume) equilibrated previously with a binding buffer [10 mM-Tris (pH 7.5)/5 mM-EDTA/0.5 M-LiCl/0.5% SDS]. Each column was washed with 10 column vol. of the binding buffer. The poly(A)⁺ RNA was eluted from the column with distilled water and precipitated as described by Foster *et al.* (1982).

Cell-free translation of poly(A)⁺ RNA

Each assay used at least 1 µg of poly(A)⁺ RNA per 61 µl of rabbit reticulocyte-lysate cocktail, with translation reaction conditions and reagents similar to those described by Foster *et al.* (1982). L-[³H]Proline (10 µCi) was used to label translation products. The reaction mixtures were terminated by adding 6 µl of ribonuclease A. For product analysis, 5 µl samples were spotted on Whatman GF/C filters previously soaked in 1% L-proline/0.02% NaN₃. Protein was precipitated (with 10% trichloroacetic acid, containing 0.3% H₂O₂ to decolorize the sample), and measured as radioactivity by scintillation counting.

Immunoprecipitation and characterization of cell-free translation products

The immunoprecipitation procedure was adapted from that of Kessler (1975). A 20 µl portion from each translation mixture was transferred to 280 µl of immunoprecipitation buffer (0.5% Nonidet P-40, a non-ionic detergent, 150 mM-NaCl, 5 mM-EDTA, 40 mM-Tris, 0.02% NaN₃, 1 mg of bovine serum albumin/ml, 2 mM-L-proline, 40 mM-ε-aminohexanoic acid, 1 mM-phenylmethanesulphonyl fluoride, pH 7.4) containing 2 µl of anti-(chick α-elastin) antiserum. After incubation (8–10 h) at 4 °C, 150 µl of a 10% (v/v) suspension of formalin-fixed *S. aureus* (Cowan strain) in the immunoprecipitation buffer was added and the samples were incubated further (30 min). The immunocomplexes bound to *S. aureus* were then pelleted by centrifugation (1000 g for 30 min) through 500 µl of immunoprecipita-

tion buffer containing 1 M-sucrose, washed (twice) with the immunoprecipitation buffer and re-pelleted. Labelled proteins were then extracted from the pellets as described by Kessler (1975). Samples of eluted products were assessed by polyacrylamide-gel electrophoresis (Laemmli, 1970) and fluorographic detection (Chamberlain, 1979).

Characterization of guanidine thiocyanate-extractable proteins

The guanidine thiocyanate in aortic extracts was first exchanged for urea by passage through a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated with 4 M-urea. The eluates were electrophoresed, and the proteins blotted on to nitrocellulose and immunostained by using a goat anti-rabbit horseradish peroxidase complex (1:2000) and 4-chloro-1-naphthol as substrate (Bowen *et al.*, 1980).

DNA determinations

DNA was precipitated from 1 ml portions of the guanidine thiocyanate/CsCl aorta extracts by adding 0.1 vol. of 3 M-sodium acetate, pH 5, and 2 vol. of 95% ethanol. The precipitates were digested with proteinase K in the presence of urea, as described by Barth & Willershausen (1978). DNA was then precipitated and assayed by the methods of Thomas & Farquhar (1978) and Hinegardner (1971).

Organ culture

Two aortae (minced into 1 mm³ pieces) were used per culture (Heng-Khoo *et al.*, 1979). The culture medium was valine-free Eagle's basal medium with Earle's salts. L-[³H]Valine (10 μ Ci/ml) was the only source of L-valine. The ratio of this medium to tissue was 4:1 (v/w). To some of the incubations, D-penicillamine (1 mg/ml) was added to inhibit elastin cross-linking (see the Discussion section). The medium was flushed continually with CO₂/O₂ (19:1) and the aortae were incubated at 40 °C. After 1.5 h of incubation, the L-[³H]valine-labelled medium was decanted and a sample of tissue (about 20 mg from each of the cultures) was taken. Culture medium containing non-radioactive L-valine (5 mM) was then added, and the incubations were continued for a further 4 h. Tissue samples were taken during this 'chase' period as described by Heng-Khoo *et al.* (1979). Radioactivity incorporated into soluble proteins was measured and the proteins were partially characterized by polyacrylamide-gel electrophoresis (Laemmli, 1970) and detection by fluorography (Chamberlain, 1979).

RESULTS

Tropoelastin and elastin

In Fig. 1 are values indicating the changes in tropoelastin and elastin concentrations in relationship to dietary copper. For copper-supplemented birds, the concentration of tropoelastin in aortae was increased 3–4-fold during the period of most rapid aortic accumulation of insoluble elastin. From day 5 throughout the course of the observations, aortae from copper-deficient chicks contained from 5 to as much as 15 times the tropoelastin concentration as in aortae from corresponding copper-supplemented chicks. This increase in tropoelastin concentration occurred concomitantly with a decrease in the cross-linked elastin content. By day 21, the insoluble elastin concentration in copper-deficient

Fig. 1. Dietary copper and the concentration of tropoelastin and insoluble elastin in developing chick aorta

The continuous line connects values obtained from copper-supplemented chicks fed *ad libitum*. The broken line connects values from copper-deficient chicks. At day 13 the concentration of tropoelastin in aorta from birds given 0.2% BAPN from hatching was $445 \pm 55 \mu\text{g}/100 \text{ mg}$ of aorta. Restricting the food intake of copper-supplemented birds to that consumed by copper-deficient birds also resulted in a slight, but significant, increase by day 21, to $90 \pm 43 \mu\text{g}$ of tropoelastin/100 mg of fresh aorta, compared with less than $50 \mu\text{g}$ of tropoelastin/100 mg in copper-supplemented chicks fed *ad libitum*. Each value is the mean $\pm$ S.E.M. for three to ten determinations: ** $P < 0.01$.

chick aorta was about one-half that normally found in aortae from copper-supplemented chicks. Aorta elastin concentrations in birds given BAPN for 13 days were nearly identical with those for 13-day-old copper-deficient chicks.

Nucleic acid content of aortae

Quantities of nucleic acids extracted from aortic tissues are given in Table 1. The yield of RNA per g of aortae from 21-day-old copper-sufficient chicks was twice that

Table 1. Total DNA, RNA and poly(A)⁺ RNA extracted from chick aortae

The total DNA, RNA and poly(A)⁺ RNA fractions were initially extracted from chick aortae with guanidine thiocyanate. Values for total RNA are based on the A_{260} of RNA purified from the guanidine thiocyanate extracts. Values for poly(A)⁺ RNA are based on the A_{260} of the RNA fraction eluted from oligo(dT) columns. Values represent means $\pm$ s.e.m. for three to nine determinations per group: *statistically significant at $P < 0.05$ with respect to the copper-supplemented group fed *ad libitum*.

Diet	Nucleic acid fractions ($\mu\text{g/g}$)							
	13-day-old chicks				21-day-old chicks			
	DNA	Total RNA	RNA/DNA	Poly(A) ⁺ RNA	DNA	Total RNA	RNA/DNA	Poly(A) ⁺ RNA
Copper-supplemented, <i>ad lib.</i>	631 $\pm$ 49	828 $\pm$ 45	1.3 $\pm$ 0.2	22 $\pm$ 1	573 $\pm$ 32	958 $\pm$ 51	1.7 $\pm$ 0.1	23 $\pm$ 4
Copper-deficient	709 $\pm$ 7	1084 $\pm$ 150	1.7 $\pm$ 0.2	26 $\pm$ 4	783 $\pm$ 20*	1212 $\pm$ 71*	1.6 $\pm$ 0.1	28 $\pm$ 3
BAPN	792 $\pm$ 54	1079 $\pm$ 84	1.4 $\pm$ 0.2	24 $\pm$ 7	—	—	—	—
Food restricted	—	—	—	—	596 $\pm$ 59	1099 $\pm$ 95	1.9 $\pm$ 0.2	26 $\pm$ 3

Table 2. Total protein and tropoelastin synthesis in organ culture and by cell-free translation of poly(A)⁺ RNA extracted from chick aortae

The net radioactivity incorporated into total protein and tropoelastin is given. For cell-free translations, 1 μg of the poly(A)⁺ RNA extracted from 21-day-old chicks was used per assay. Two aortae from 28-day-old chicks were used for each organ culture. Nearly identical results were obtained by using poly(A)⁺ RNA isolated from 13-day-old copper-deficient or -sufficient chicks or chicks fed with BAPN. Translations were for 90 min and results are expressed per assay. Values represent means $\pm$ s.e.m. for five to eight samples for each treatment: N.D., not determined. D-Penicillamine was added at 1 mg/ml in the culture medium.

(a) Organ culture				
Treatment of animals	Penicillamine	$10^{-4} \times$ Total protein (d.p.m./mg)	$10^{-4} \times$ Tropoelastin (d.p.m./mg)	$100 \times$ Tropoelastin/total protein (%)
Copper-supplemented, <i>ad lib.</i>	—	1.0 $\pm$ 0.2	N.D.	N.D.
	+	0.8 $\pm$ 0.06	0.26 $\pm$ 0.03	33 $\pm$ 6
Copper-deficient	—	N.D.	N.D.	N.D.
	+	1.0 $\pm$ 0.2	0.3 $\pm$ 0.08	29 $\pm$ 4
(b) Cell-free translation of poly(A) ⁺ RNA				
Treatment of animals		$10^{-5} \times$ Total protein synthesis (d.p.m./assay)	$10^{-5} \times$ Tropoelastin synthesis (d.p.m./assay)	$100 \times$ Tropoelastin/total protein (%)
Copper-supplemented, <i>ad lib.</i>		5.5 $\pm$ 0.5	1.4 $\pm$ 0.3	25 $\pm$ 4
Copper-deficient		7.2 $\pm$ 0.6	1.7 $\pm$ 0.2	24 $\pm$ 3
Food restricted		4.4 $\pm$ 2.1	1.1 $\pm$ 0.2	25 $\pm$ 4

reported from embryonic-chick aorta tissues (Barrineau *et al.*, 1981), perhaps reflecting greater cellular hypertrophy during this developmental period. Consistently, lower amounts of both RNA and DNA were extracted from aorta of copper-supplemented birds as compared with corresponding aortae from copper-deficient or BAPN-treated birds. The ratios of RNA to DNA were similar for all groups (1.3–1.7 for aortae from 13-day-old chicks and 1.6–1.9 for aortae from 21-day-old chicks).

Cell-free translation of poly(A)⁺ RNA

The results from cell-free translations are summarized in Table 2. Tropoelastin accounted for about 25% of the total L-[³H]proline incorporated into translated protein,

regardless of prior dietary history. The data were similar to results reported previously for translation of poly(A)⁺ RNA from embryonic-chick aortae (Barrineau *et al.*, 1981; Burnett *et al.*, 1980). In contrast, in cell-free translations of aortic mRNA from adult birds (approx. 6 months old), the amount of tropoelastin relative to the total protein was less than 1% (results not shown).

Differences among groups were not significant, as judged by one-way analysis of variance of the arcsin-transformed percentages. These results were taken to indicate that copper deficiency, BAPN poisoning or energy restriction does not alter the amounts of functional tropoelastin-specific mRNA. Fluorograms of the translation products from the various treatments were virtually indistinguishable (Fig. 2).

Fig. 2. Fluorograms of L-[^3H]proline-labelled proteins obtained when poly(A) $^+$ RNA extracted from copper-supplemented or -deficient chick aortae was translated in a cell-free rabbit reticulocyte system

Lane (a), immunoprecipitated tropoelastin from control-chick aorta poly(A) $^+$ RNA; lane (b), immunoprecipitated tropoelastin from copper-deficient-chick aorta poly(A) $^+$ RNA; lane (c), total L-[^3H]proline-labelled proteins from control-chick aorta poly(A) $^+$ RNA; lane (d), total L-[^3H]proline-labelled proteins from copper-deficient-chick aorta poly(A) $^+$ RNA; lane (e), L-[^3H]proline-labelled proteins after translation without added poly(A) $^+$ RNA.

Synthesis of tropoelastin and insoluble protein by aorta explants from copper-sufficient and -deficient chicks

To determine if copper deficiency altered the efficiency of tropoelastin synthesis or increased degradation of elastin intermediates, chick aortae explants were pulsed with L-[^3H]valine and then chased in radioisotope-free medium with or without the addition of penicillamine. At the end of the 90 min incubation, approx. 10000 d.p.m. of L-[^3H]valine was incorporated per mg of aorta, independent of dietary treatment or the presence or absence of penicillamine in the culture medium (Table 2). This is also shown in fluorograms (Figs. 3a and 3b). The decrease in the intensity of bands corresponding to tropoelastin correlated with the increased incorporation of radioactivity into insoluble protein (Fig. 3a). These observations were comparable with those by Kao *et al.* (1982).

The rapid incorporation of tropoelastin into the insoluble protein fraction was effectively inhibited by penicillamine (Fig. 3b). Moreover, there was no indication that the newly synthesized tropoelastin was degraded significantly in cultures when cross-linking was blocked to increase the probability of detecting tropoelastin degradation.

Fig. 3. Distribution of radioactivity between soluble and insoluble proteins in aortae cultured with or without added D-penicillamine

The data represent the 'chase' phase which followed a 1.5 h incubation of the aorta in medium containing L-[^3H]valine. In (a), TE designates tropoelastin. Radiochemically labelled soluble and insoluble protein fractions are designated $\bullet$ — $\bullet$ or $\bullet$ — $\bullet$ respectively for data from copper-supplemented chicks. The distribution of radiochemically labelled proteins when D-penicillamine was present in the medium at 1 mg/ml is shown in (b). At this concentration of penicillamine the incorporation of soluble protein into insoluble protein is blocked. The fluorograms (insets) indicate that there is no evidence that substantial tropoelastin degradation occurs during the chase period ($\blacksquare$ — $\blacksquare$, soluble protein from copper-supplemented chick aorta; $\square$ — $\square$, soluble protein from copper-deficient chick aorta; $\blacksquare$ — $\blacksquare$, insoluble protein from copper-supplemented chick aorta; $\square$ — $\square$, insoluble protein from copper-deficient chick aorta).

Characterization of guanidine thiocyanate-extractable proteins

Fig. 4 depicts immunostained protein bands in the guanidine thiocyanate extracts from aortae of copper-deficient, BAPN-treated and copper-supplemented chicks. Sample concentrations were adjusted so that approximately equal amounts of tropoelastin (as determined by enzyme-linked immunoadsorbent assay) were applied to each well. This required concentrating the aortic extracts from copper-sufficient chicks about 20-fold. Therefore, all non-elastin proteins were present at unusually high concentrations in the extracts from copper-sufficient chicks.

Numerous bands were detected in extracts of aorta from copper-deficient or BAPN-fed chicks. Since the specificity of the antisera was established (Buckingham *et al.*, 1981; Romero *et al.*, 1986), it is concluded that bands were derived from the proteolytic cleavage of tropoelastin or partially cross-linked elastin. The higher- M_r bands appeared to be derived primarily from the association of degraded tropoelastin with other extracted proteins. For example, they were not observed when samples were treated with CNBr (Kao *et al.*, 1982) before electrophoresis, although bands of M_r less than 70 000 persisted. Moreover, since there was little evidence of tropoelastin degradation in the aortic extracts from copper-sufficient chicks, it was unlikely that the proteolysis was an artifact that occurred *post mortem*. Similarly

there was no evidence of labelled tropoelastin degradation during the 4 h 'chase' in aorta explant culture (Fig. 3b), indicating that intrinsic elastolytic activity must be relatively low in chick aortae.

DISCUSSION

Despite all the potential mechanisms whereby tropoelastin or elastin might modulate its own expression, there was no indication that tropoelastin synthesis was changed by varying the amount of elastin, tropoelastin or elastin-derived products. No significant differences were detected in the relative amounts of elastin-specific mRNA when aortae from copper-deficient or -supplemented birds were compared.

Previous investigations have shown that elastin synthesis is directly proportional to the cellular content of elastin-specific mRNA (Shibahara *et al.*, 1981; Foster *et al.*, 1982; Davidson *et al.*, 1982, 1985). Failure to detect significant differences in the amounts of aorta elastin-specific mRNA from copper-deficient or copper-sufficient chicks, as well as BAPN-poisoned or energy-restricted chicks, suggests that there were no differences in the potential for elastin synthesis among these groups. Indeed, elastin synthesis was the same despite tissue contents of tropoelastin that were at least 5–10 times higher than normal. Moreover, tropoelastin synthesis by aortic explants was the same in all of the experimental groups evaluated. This finding corroborates the mRNA data and, in addition, indicates that the efficiency of translational processes was not impaired by copper deficiency, increased amounts of tropoelastin, or decreased amounts of elastin.

Rather than decreased synthesis, the basis for decreased elastin accumulation appeared to be extracellular proteolysis of tropoelastin. Immunodetection of elastin in nitrocellulose blots indicated significant tropoelastin degradation in extracts of aorta from copper-deficient and BAPN-treated chicks, but not in extracts of copper-sufficient aortae. Potential sources for the elastolytic activity include proteinase(s) secreted by elastin-synthesizing cells, proteinases from phagocytic cells, or plasma-derived proteinases that penetrate the vessel wall. In organ culture, pulse-chase experiments were used to test for significant amounts of intrinsic aortic proteinase activity. In normal aorta, labelled tropoelastin is almost quantitatively transferred from the soluble to the insoluble elastin fraction, indicating that during normal processing tropoelastin sustains negligible losses caused by proteolysis. Moreover, even when cross-linking was blocked by addition of D-penicillamine to cultures, data from tissue-culture experiments suggested very little, if any, degradation of tropoelastin. Failure to demonstrate proteinase activity in the cultured explants suggested that phagocytic cells or serum might be possible sources of the proteinase activity. Of these two possibilities, serum is the most probable source (cf. Romero *et al.*, 1986).

In summary, we suggest that the loss of aorta elastin from copper-deficient or lathyritic animals results from proteolysis of tropoelastin and partially cross-linked elastin, possibly caused by exposure to plasma proteinases. The expression of tropoelastin by chick aortae does not appear to be influenced by the presence of elevated tissue concentrations of tropoelastin or the presence of elastin peptides. Disruption of elastic fibres in the aorta, at least in the chick, does not appear to affect elastin synthesis.

Fig. 4. Tropoelastin and elastin degradation products in aortic extracts from chicks fed diets sufficient (Cu^+) or deficient (Cu^-) in dietary copper or diets containing 0.2% BAPN fumarate

The soluble elastin content of each aortic extract (13-day-old chicks) was estimated by enzyme-linked immunosorbent assay. Before polyacrylamide-gel electrophoresis, sample volumes were adjusted until concentrations of soluble elastin in each sample were approximately equal. Elastin-derived peptides were detected by immunostaining the aortic proteins after their transfer from the gels on to nitrocellulose. Similar results were obtained when aorta extracts from 7- or 21-day-old copper-deficient and -supplemented chicks were compared.

This work was supported by Grant HL 15965 and Training Grant HL 07013 awarded by the National Heart, Blood and Lung Institute, N.I.H.

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Received 13 September 1985/30 December 1985, accepted 24 February 1986