Composition of Proteoglycans in the Aortas of Copper-Deficient Rats[1] (42835)

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Abstract. **Copper deficiency adversely affects the extracellular matrix of the arterial wall, leading to cardiovascular lesions. To study the lesions resulting from copper deficiency, the composition of proteoglycans from aortas of copper-deficient rats was compared with proteoglycans of aortas from copper-supplemented rats. Copper deficiency in rats was verified by copper levels in adrenal glands (mean ± SE, 0.37 ± 0.07 vs 1.03 ± 0.17 μg/g wet wt in supplemented rats). The proteoglycans were isolated from the aorta by extraction with 4 *M* guanidine-HCl and by digestion of the tissue with elastase. The proteoglycans were purified by CsCl isopycnic centrifugation and fractionated by gel filtration. The fractions were characterized for molecular size and glycosaminoglycan composition. Total uronate in the aortas from copper-deficient rats was 25% greater than in aortas from copper-supplemented rats, and the proteoglycans from copper-deficient rat aortas were of greater molecular size. Among the glycosaminoglycans the concentration (μg/mg tissue) of isomeric chondroitin sulfates, particularly dermatan sulfate, was greater in copper-deficient animals than in copper-supplemented animals. These observations are similar to earlier findings in experimental atherosclerosis and to a response of cardiovascular connective tissue to injury.** [P.S.E.B.M. 1989, Vol 190]

The first cardiovascular damage attributed to copper deficiency was found by Bennetts *et al.* (1). The gross pathology of copper deficiency and the pathologic similarities between animals deficient in copper and people with ischemic heart disease have been reviewed (2–5). Ventricular aneurysm, calcification, edema, fibrosis, hypertrophy, rupture, and thrombosis are accompanied by cellular infiltration of the myocardium and necrosis of myocardial cells. Arteries undergo elastic degeneration, fibrosis, intramural hemorrhage, and glycosaminoglycan (GAG) increase. Foamy histocytes are seen (6). Coronary arteries are hyalinized and necrotic. Smooth muscle degeneration, sudanophilia, and thrombosis occur.

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Animals deficient in copper are glucose intolerant, hypercholesterolemic, hyperuricemic and hypertensive, and have abnormal electrocardiograms. These phenomena (with the exception of hypertension) and evidence that humans react similarly to copper depletion have been reviewed (5). The data on blood pressure (7–9) were too few for inclusion.

The adverse effects of copper deficiency on all three classes of connective tissue (collagen, elastin, and ground substance) in several species of animals have been reviewed (4). Some of these effects are the result of impaired activity of lysyl oxidase, a copper metalloenzyme (10). Because a close relationship exists between the fibrous proteins and proteoglycans in the aorta, it was of interest to investigate the effect of copper deficiency on arterial wall proteoglycans.

Materials and Methods

Materials. Ultrapure guanidine (Gdn)-HCl was purchased from the United States Biochemical Corporation and preparative grade cesium chloride (CsCl) from Gallard-Schlesinger. ϵ-Aminocaproic acid, benzamidine-HCl, EDTA-disodium salt, phenylmethylsul-

fonyl fluoride, *N*-ethylmaleimide, iodoacetamide, 5,5′-dithiobis[2-nitrobenzoic acid], Streptomyces hyalurolyticus hyaluronidase, and *Escherichia coli* were obtained from Sigma Chemical Company. High purity elastase (115 units/mg, porcine pancreatic) was from Elastin Products Company, Inc. (Pacific, MO), and papain 2× crystallized (29 units/mg) was from ICN Biochemicals. Glucuronolactone was from Corn Products Refinery Company (New York, NY). Chondroitinases came from Miles Laboratory. Glycosaminoglycan standards were generous gifts from Drs. J. A. Cifonelli and M. B. Mathews, University of Chicago. All other reagents were of analytical grade.

Animals and Tissues. Weanling male rats (Harlan Sprague-Dawley, Madison, WI) were housed under conditions similar to those described (11). They were divided into two groups of 10, matched by mean weight to differences of less than 0.1 g, and were fed a purified diet (12), based on 62% sucrose, 20% egg white solids, and 10% corn oil (by weight), containing all of the nutrients known to be essential for rats including 2.0 mg of biotin/kg diet (13). As this diet is deficient in copper and zinc, one group (supplemented) received a drinking solution containing 2 μg of copper and 10 μg of zinc/ml (as sulfate and acetate, respectively) in demineralized water (Super Q System; Millipore Corp., Bedford, MA). The other group (deficient) received a similar solution containing only zinc.

Forty-one days later, after the unexpected death of two deficient rats over a weekend, the remaining rats were anesthetized with pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL), and heparinized blood was collected by cardiac puncture. Aortas and adrenal glands were removed quickly, stripped of all extraneous matter, and frozen with liquid nitrogen. Aortas were kept at −80°C until shipped with dry ice from Grand Forks to New Orleans, where they were stored at −60°C until used for proteoglycan extraction.

Isolation of Proteoglycans. The frozen aortas were thawed at 4°C, blotted to remove excess water, and weighed. They were then pooled according to groups, copper-deficient (total wet weight of eight aortas, 201 mg) and copper-supplemented (total wet weight of nine aortas, 324 mg). The aortas were finely minced and proteoglycans were extracted from the minced tissue by procedures described (14, 15). Briefly, the tissue was extracted by 4 *M* Gdn-HCl containing nonspecific protease inhibitors (0.1 *M* ϵ-aminocaproic acid, 0.005 *M* benzamidine-HCl, 0.01 *M* EDTA, 0.005 *M* *N*-ethylmaleimide, 0.001 *M* phenylmethylsulfonyl fluoride, and 0.001 *M* iodoacetamide) for 48 hr at 4°C. The extract was separated from the residue by centrifugation at 6000*g*. The supernatant, after clarification by filtration through a pad of Hyflo Supercell (Fisher Scientific Co.), was adjusted to a density of 1.33 g/ml by the addition of solid CsCl and centrifuged at 100,000*g* for 40 hr at 8°C in a Beckman L2-75 ultracentrifuge in a Ti 50 rotor. Following centrifugation 2.0-ml fractions were collected through a 2.0-ml syringe with a long needle starting from the bottom of the tube (14). The densities of the fractions were determined by weighing 0.1 ml. The bottom three fractions containing more than 95% of the total uronate of the extract were pooled, and the density of the pooled solution was adjusted to 1.46 g/ml by the addition of solid CsCl and centrifuged again for 40 hr. Six 2-ml fractions were collected from each tube and their densities determined. The fractions were then exhaustively dialyzed against distilled water and lyophilized. The lyophilized materials were reconstituted in a small volume of distilled water and analyzed.

Elastase Hydrolysis of Tissue. The tissue remaining after Gdn-HCl extraction was exhaustively dialyzed against 0.2 *M* Tris-HCl buffer (pH 8.8) containing all protease inhibitors at 4°C (15 volumes, 6 changes over 4 days) to remove Gdn-HCl that was bound to the tissue. It was then hydrolyzed twice by elastase (each time 25 units/100 mg original tissue) in the Tris buffer containing protease inhibitors at 37°C for 24 hr each time. The digest was separated from undigested tissue by centrifugation at 6000*g* and the clear supernatant was subjected to CsCl isopycnic centrifugation at a density of 1.65 g/ml (16, 17). (This density allows good resolution of elastase-solubilized proteoglycan.) After centrifugation six 2-ml fractions were collected from each centrifuge tube starting from the bottom. The densities of the fractions were determined, and these were then exhaustively dialyzed against distilled water, lyophilized, and analyzed.

Undigested Residue. The undigested residue from elastase hydrolysis of the tissue was digested by 0.5 *M* NaOH (15 ml/g tissue) at 4°C for 16 hr. The digest was adjusted to pH 6.4 with 0.5 *N* phosphoric acid and hydrolyzed by papain (10 mg in 6.0 ml of EDTA buffer, pH 6.4). The samples were deproteinized by passing through a column of Dowex 50W-X2 H^+. The effluent was exhaustively dialyzed against distilled water, lyophilized, and analyzed for GAG.

Gel Filtration. Gel filtration of different proteoglycan fractions was performed on a Sepharose CL-4B column (0.9 × 60 cm) equilibrated with 6 *M* urea, at a flow rate of 0.5 ml/min at 24°C collecting 1.0 ml in each fraction. The void volume of the column was determined with *E. coli* by measuring turbidity at 600 nm and total volume with 5,5′-dithiobis[2-nitrobenzoic acid] by measuring absorption at 280 nm. The column was eluted by 6 *M* urea and effluent fractions of the proteoglycans from the column were analyzed for uronic acid.

β-Elimination of Proteoglycans. β-Elimination of proteoglycans was carried out in 0.05 *M* NaOH and 1.0 *M* $NaBH_4$ for 18 hr at 45°C (18, 19). Following

neutralization with acetic acid the samples were deproteinized through a Dowex 50W-X2 H^+ column. The effluent from the column was neutralized with Na_2CO_3, dialyzed against distilled water, and lyophilized.

Analytical Methods. Cholesterol in plasma was measured by fluorescence (20); copper and zinc in adrenal glands were measured by atomic absorption spectroscopy after destruction of organic matter with nitric and sulfuric acids and hydrogen peroxide (21). Means were compared by Student's *t* test (22).

Because the amounts of proteoglycans from the aorta tissues were limited, uronic acid content in the proteoglycans and GAG was determined by a modified procedure of Bitter and Muir (23). To 0.1 ml of sample containing 1–10 μg of uronic acid was added 0.02 ml of 0.1% carbazole reagent in 95% ethanol and 1.0 ml of borate-H_2SO_4 reagent. The sample and the reagents were well mixed, heated in a boiling water bath for 20 min, cooled to room temperature, and the absorbance read at 530 nm in a Beckman DU spectrophotometer. A linearity in absorbance was noted between 1 and 10 μg of glucuronolactone in this procedure. Protein content in the proteoglycan fractions was measured according to the procedure of Hartree (24).

Analysis of Glycosaminoglycans. GAG mixtures containing heparan sulfate, hyaluronic acid, and isomeric chondroitin sulfates were analyzed by a sequential chemical and enzymatic procedure. The sample was first subjected to oxidative deamination by nitrous acid for heparan sulfate degradation using the procedure of Lindahl et al. (25). The oligosaccharides of heparan sulfate that were formed during degradation were separated from the remaining GAG by gel filtration on a Sephadex G-50 column (0.3 × 20 cm), eluting the column with 0.1 *M* ammonium formate (pH 6.0). The void volume fraction that contained hyaluronic acid and isomeric chondroitin sulfates was sequentially digested by Streptomyces hyaluronidase, chondroitinase AC, and chondroitinase ABC. After each digestion the undigested GAG were separated from the enzyme, and oligosaccharides formed during the digestion, to avoid their interference in the carbazole reaction of uronic acid in the subsequent step. The enzyme was first removed by passing the digestion mixture through a Dowex 50W-2X H^+ column (0.3 × 10 cm) and washing the column with 10 ml of distilled water. The effluent and washings containing the GAG and oligosaccharides were combined, neutralized by NH_4OH, lyophilized, and reconstituted in a small volume of water. The oligosaccharides were then separated from the GAG by gel filtration on a Sephadex G-50 column (0.3 × 20 cm) with distilled water as eluent. The amount of uronate eluted in the void volume of the column was determined by the modified procedure of Bitter and Muir (23) in a small portion of the sample and the remainder was used for the next enzyme digestion. After digestion of the sample by chondroitinase ABC, uronate was not detected in the void volume fraction of the gel filtration column.

Prior to analysis of GAG from proteoglycans, we analyzed a synthetic mixture of GAG containing 10 μg (uronic acid) each of heparan sulfate, hyaluronic acid, chondroitin 6-sulfate, and dermatan sulfate. Replicate analyses (five times) of the GAG mixtures showed good reproducibility (coefficent of variation of 3.8% for heparan sulfate, 2.4% for hyaluronic acid, 5.1% for chondroitin 6-sulfate, and 3.4% for dermatan sulfate). The mean observed values of individual GAG were between 92 and 105% of the theoretical values.

Results

Animals. Before the termination of the experiment, one of the control rats died from unknown reasons. Of the two deficient rats that died, one had ventricular aneurysm. In the remaining deficient rats, cholesterol in plasma increased (mean ± SE, 134 ± 18.0 mg/dl vs 86 ± 2.9 mg/dl; $P < 0.04$) and copper in adrenal glands decreased (mean ± SE, 0.37 ± 0.07 vs 1.03 ± 0.17 μg/wet g; $P < 0.01$). Zinc in adrenal glands was unchanged (12.8 μg/wet g, both groups).

Aortas from copper-supplemented rats weighed more than the aortas from copper-deficient rats (mean ± SE, 0.036 ± 0.002 g vs 0.025 ± 0.009 g). Because the amount of tissue from each animal was limited, proteoglycans were isolated from pooled tissue.

Proteoglycans. For complete extraction of proteoglycans and hyaluronic acid from the aortic tissue, we used a sequential procedure we described (16). The first isopycnic centrifugation for the Gdn-HCl extract was essential in this procedure to remove contaminating tissue proteins from proteoglycans. The aortas from copper-deficient rats had about 25% greater concentration of uronate than the aortas from copper-supplemented rats (Table I). The relative distribution of uronate in different extracts varied little between the groups, except for papain-solubilized GAG in which copper-supplemented rats had a greater amount of GAG than copper-deficient rats.

Figure 1 illustrates dissociative CsCl isopycnic centrifugation profiles of proteoglycans in the extracts. Although there is no difference in the profiles between the groups of rats, fractions 1 and 2 from the copper-deficient group had higher uronate values than those from the copper-supplemented group. Based on the uronate profiles, fractions were pooled as shown in Figure 1. Fractions from Gdn-HCl extracts were pooled into three fractions, I–III, and the elastase-solubilized extracts into two fractions, I and II. Because there was a large amount of proteinaceous material in fraction III of Gdn-HCl extracts, we did not study this fraction further. (High absorbance at 530 nm of this fraction in the carbazole-H_2SO_4 reaction was caused by spurious color because of the presence of a large amount of protein.)

Table I. Uronate Distribution in Sequential Extracts of Proteoglycans from Aortas of Rats fed Copper-Deficient and Copper-Supplemented Diets[a]

Diet	Total UA[b] (μg/mg tissue)	UA Distribution in different extraction steps		
		Gdn-HCl Extract	Elastase solubilized	Papain solubilized
		(% of Total UA)		
Copper deficient	1.66	49	36	15
Copper Supplemented	1.34	46	32	22

[a] The aortas were sequentially extracted by 4 *M* Gdn-HCl and digested by elastase and by papain.
[b] UA, uronic acid.

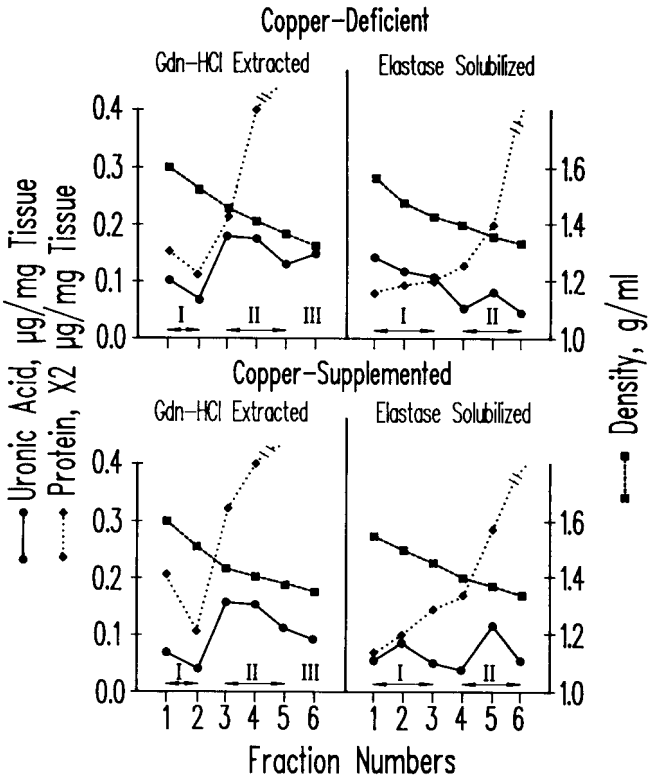

Figure 1. Ultracentrifuge profiles of proteoglycans from aortas of rats on copper-deficient and copper-supplemented diets. Proteoglycans were extracted sequentially by Gdn-HCl and by elastase digestion of the tissue. Centrifugation was carried out under dissociative conditions (4 *M* Gdn-HCl) in CsCl at a density of 1.46 g/ml for Gdn-HCl extracts and 1.65 g/ml for elastase-solubilized extracts. Fractions were pooled, as shown, based on uronate profiles. For details please refer to the text.

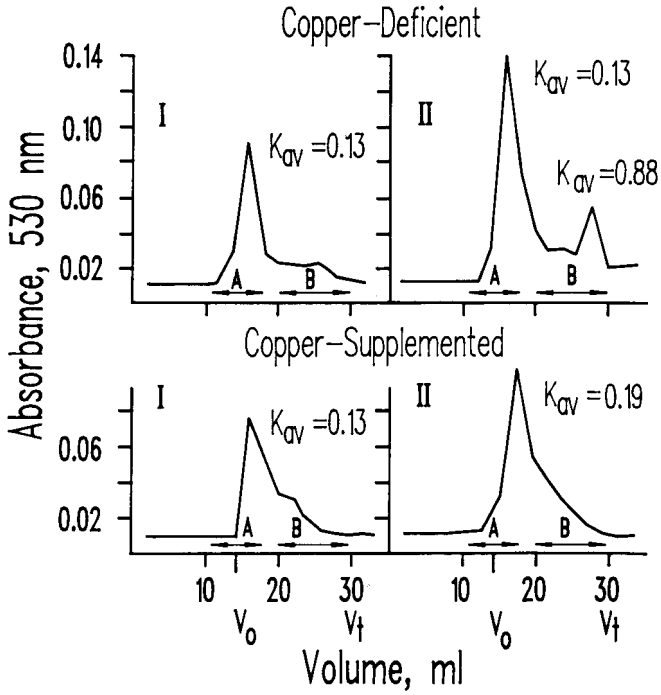

Figure 2. Sepharose CL-4B gel filtration profiles of Gdn-HCl-extracted proteoglycans fractions. The column was eluted with 6.0 *M* urea and monitored by uronic acid. Fractions were pooled as shown by arrows. The K_{av} values were calculated using the formula: $K_{av} = V_e - V_o/V_t - V_o$, where V_o = void volume and V_t = total volume of the column, and V_e = elution volume of the peak.

Gel filtration profiles of proteoglycan fractions are illustrated in Figures 2 and 3. All of the fractions demonstrated heterogeneity with a major peak eluting near the void volume. Each fraction was arbitrarily divided into two subfractions, A and B. Although fraction IA from Gdn-HCl extracts of copper-deficient and copper-supplemented rat aortas had similar K_{av} values, fraction IIA from the copper-deficient group had a lower K_{av} value than that from the copper-supplemented group. The elastase-solubilized proteoglycans had greater K_{av} values than those of Gdn-HCl-extracted proteoglycans from both groups of rats. The K_{av} values of peaks of elastase-solubilized proteoglycans from copper-deficient rat aortas were somewhat lower than those from copper-supplemented rat aortas (Fig. 3). The peaks of fractions II had higher K_{av} values and were broader than peaks of fractions I.

Composition of Glycosaminoglycans. The GAG composition of proteoglycan fractions from gel filtration column is reported in Table II. With few exceptions the fractions from the copper-deficient group had greater concentrations of total uronate than the fractions from the copper-supplemented group. The predominant GAG in the Gdn-HCl-extracted fractions were chondroitin sulfates and dermatan sulfate, with lower density fractions IIA and IIB containing considerable quantities of heparan sulfate and hyaluronic acid. Among Gdn-HCl-extracted fractions, greater concen-

trations of chondroitin sulfates and dermatan sulfate and lower concentrations of hyaluronic acid were noted in the copper-deficient group than in the copper-supplemented group. No difference in heparan sulfate between the groups in Gdn-HCl-extracted fractions was observed. Greater amounts of heparan sulfate were noted in elastase-solubilized fractions than in Gdn-HCl-extracted fractions. In contrast to Gdn-HCl-extracted fractions, elastase-solubilized fractions contained greater concentrations of heparan sulfate and hyaluronic acid in the copper-deficient group than in the copper-supplemented group. Like Gdn-HCl-extracted fractions, elastase-solubilized fractions had greater concentrations of chondroitin sulfates and dermatan sulfate in the copper-deficient group than in the copper-supplemented group. Among the papain-solubilized GAG, the concentrations of heparan sulfate and hyaluronic acid were greater in the copper-supplemented group than in the copper-deficient group. In overall composition greater concentrations of isomeric chondroitin sulfates, particularly dermatan sulfate, and lower concentrations of hyaluronic acid were noted in the copper-deficient group, while there was no difference in heparan sulfate between the groups.

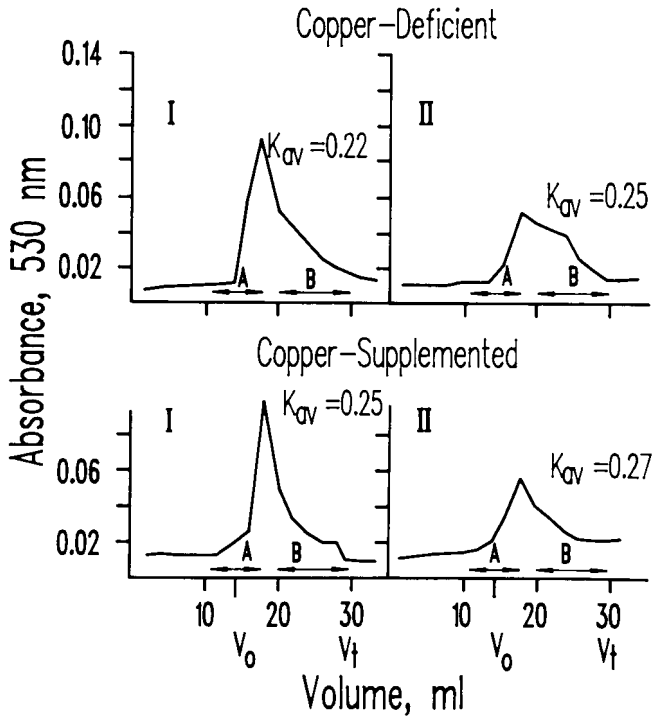

Figure 3. Sepharose CL-4B gel filtration profiles of elastase-solubilized proteoglycan fractions.

Discussion

The diet used in this experiment has been used for more than a decade; it rapidly produces copper deficiency by numerous criteria. Copper deficiency was verified by an aneurysm, hypercholesterolemia, and decreased adrenal copper.

The results obtained in this study indicate that changes in the composition of aorta proteoglycans occur in copper-deficient rats. Because the availability of tissue from each rat was limited, these observations were made on pooled samples. Although multiple recovery steps are involved in this isolation procedure of proteoglycans, good reproducibility in quantitation of proteoglycans from tissues was noted in earlier studies (26, 27). The observed difference of 25% in the total uronate content in the aortas between experimental and control animals is significant. In earlier studies, Linker *et al.* (28) also observed increased concentrations of GAG in the aortas from copper-deficient swine. In contrast, in other connective tissue disorders resulting from nutrient deprivation, vitamin C or vitamin A deficiency, or lathyrism, the levels of GAG are often

Table II. GAG Composition of Proteoglycan Fractions of Rat Aortas

Fraction	Copper-deficient group (μg/100 mg tissue)					Copper-supplemented group (μg/100 mg tissue)				
	Total UA[a]	HS[b]	HA	CS	DS	Total UA	HS	HA	CS	DS
Gdn-HCl-extracted PG										
IA	16.4	0	0	14.9	1.5	11.0	1.0	0	9.4	0.6
IB	7.5	1.1	0	2.5	3.9	5.0	1.0	0	2.5	1.5
IIA	30.8	2.2	4.6	19.4	4.6	23.4	2.3	5.1	15.0	1.0
IIB	11.9	2.4	1.0	2.1	6.4	11.4	1.4	4.6	3.6	1.8
Elastase-solubilized PG										
IA	27.8	8.3	0	12.0	7.5	15.4	5.9	0	6.5	3.0
IB	9.0	5.5	0	1.0	2.5	9.6	5.9	0	0.6	3.1
IIA	11.9	4.9	3.4	1.9	1.7	9.9	3.2	0	5.5	1.2
IIB	10	5.1	1.6	1.1	2.2	8.0	3.7	1.2	0.6	2.5
Papain-solubilized GAG[c]	25	5.0	1.5	10.5	8.0	30	9.6	4.2	7.8	8.4

[a] UA, hexuronic acid; PG, proteoglycan; HA, hyaluronic acid; CS, chondroitin 4- and 6-sulfates; DS, dermatan sulfate; HS, heparan sulfate.
[b] Individual GAG were analyzed by a combination of chemical and enzymatic procedures and not corrected for differences in color response in carbazole reaction of individual GAG.
[c] The residual tissue after elastase hydrolysis was digested by papain and GAG were isolated.

decreased (29–31). In our (32) recent studies of GAG from lungs of copper-deficient chicks, we did not observe any change in the levels of total GAG but we noted an alteration in the relative distribution of individual GAG, particularly chondroitin sulfates. It is likely that the changes occurring in the proteoglycan composition in copper deficiency vary with the tissue. Aorta is prone to develop atherosclerotic lesions and copper deficiency in experimental animals results in cardiovascular lesions (2, 6). We did not examine the intimal surface involvement of the aortas with lesions in this study, but we (33) previously observed significant histologic cardiovascular abnormalities in this experimental model. The aortas showed large areas of distorted and depleted elastic fibers which may be important in the initiation of atherosclerosis (34). We were primarily interested in the biochemical changes in aorta in this study.

The finding of increased concentrations of isomeric chondroitin sulfates, particularly dermatan sulfate, in the aortas of copper-deficient rats is similar to the findings in aortas with atherosclerotic lesions. In human atherosclerosis as well as in experimental atherosclerosis in several species of animals, higher concentrations of proteoglycans were observed, primarily due to increased levels of isomeric chondroitin sulfates (35). The proteoglycans in the arterial wall are synthesized by smooth muscle cells and endothelial cells (36). Proliferation of smooth muscle cells is one of the early events of atherogenesis (37), and the proliferating cells elaborate connective tissue components, including proteoglycans, particularly chondroitin sulfates and dermatan sulfate (38). Hill and Davidson (39) observed increased cellularity in both thoracic and abdominal aortas from copper-deficient pigs. It is likely that the higher concentrations of these proteoglycans in this study are primarily due to proliferation of smooth muscle cells. Even if there is a defect in proteoglycan metabolism (reduced rate of epimerization of glucuronic acid to iduronic acid) in the copper-deficient state, as previously suggested (32), it could be minor when compared with the synthesis by actively proliferating smooth muscle cells.

Gel filtration profiles of proteoglycans suggest higher molecular weights for proteoglycans from copper-deficient rat aortas than proteoglycans from aortas of a copper-supplemented group. Although this observation is in contrast to that of the lung proteoglycans in copper deficiency, proteoglycans isolated from atherosclerotic lesions have been shown to have higher molecular weights than proteoglycans of the normal tissue (27, 40). It is not clearly known whether the higher molecular weight proteoglycans from atherosclerotic tissue or injury response result because of abnormal synthesis of core protein with several GAG chains or because of formation of higher molecular weight complexes with other arterial wall proteins.

It may be pertinent to mention that there are species differences in affecting vascular changes by copper deficiency; for example, sheep (41) show little changes, whole swine (42), chicks (43), rabbits (44), and rats (33) do show vascular defects in copper deficiency.

Copper deficiency alters the concentration, size, and amount of certain proteoglycans in the aorta. The changes are somewhat similar to that which occurs in aortas with atherosclerotic lesions.

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