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Composition of Glycosaminoglycans in the Lungs of Copper-Deficient Chicks[1] (42194)

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Abstract. Copper deficiency results in defective elastin and collagen maturation in most tissues. A close relationship also exists between these components and proteoglycans in connective tissue. In an effort to obtain information on the nature of proteoglycans in copper deficiency, the composition of glycosaminoglycans in lungs from copper-deficient (1 μg/g of diet) or -supplemented (25 μg/g diet) chicks was studied. The total glycosaminoglycan concentration in copper-deficient chick lungs did not differ from that in control chick lungs. However, variations in individual glycosaminoglycan concentrations between lungs from copper-deficient and -supplemented chicks were observed. Heparan sulfate and dermatan sulfate concentrations were lower in copper-deficient chick lungs than in controls. The glycosaminoglycans from lungs of copper-deficient chicks also had lower molecular weights than glycosaminoglycans from lungs of control birds.

Dietary copper is essential for growth and development because of its role in numerous protein and enzyme systems. Several laboratory animal models have been used to demonstrate the essential role of copper (1–3). Some of the earliest signs of copper deficiency are anemia (4), cardiac fibrosis (5), hyperlipidemia (6), and abnormal arterial elastin formation (7). Thinning and apparent collapse of the air–blood capillary network of lungs were noted in copper-deficient chicks by Buckingham *et al.* (8) and Lefevre *et al.* (9). These signs also occur in some copper-related genetic disorders, such as Menke's disease (10) or various rodent mutants, e.g., mottled or brindled mice (11).

In connective tissue, copper deficiency affects the degree of crosslinking of collagen and elastin, because copper is essential for lysyloxidase activity (12). Since a close relationship exists between these fibrous proteins and proteoglycans in connective tissue it was of interest to investigate the effect of copper deficiency on connective tissue glycosaminoglycans (GAG). In particular, the GAG in the lung were of interest, since they serve structural roles as well as nonstructural roles as part of the mucociliary system. Reported herein are studies on the composition and molecular size characteristics of GAG from copper-deficient and -supplemented chick lungs.

Materials and Methods. *Animals and tissues.* Day-old white leghorn cockerels were fed semipurified diets based on spray-dried skim milk containing no added copper or copper added as $CuSO_4$ at 25 μg/g of diet. The diets were fed for 14 days. The composition of the basal diet was identical to that previously reported (8). The chicks were housed in stainless-steel brooders with free access to feed and deionized water. At the end of the dietary period, the birds were necropised and the lungs dissected, trimmed of extra parenchymal, tracheobronchial and vascular tissue, and stored at −20°.

Isolation of GAG. GAG were isolated from minced and acetone dry-defatted lung tissues by procedures previously described (13, 14). Briefly, the tissue was digested with papain at 65°C for 48 hr. An equal volume of 12% trichloroacetic acid (TCA) was added and the protein material precipitated. The latter was separated from GAG by centrifugation at 14,000*g* for 30 min. The supernatant fraction containing the GAG was exhaustively dialyzed against distilled water to remove TCA and then lyophilized. This material was further

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hydrolyzed by Pronase in 0.1 *M* phosphate buffer, pH 7.6, for 48 hr and GAG were isolated as previously described (13).

Fractionation of GAG. GAG were fractionated on Dowex-1 Cl^- columns (15). The GAG from the column were eluted using a NaCl–$MgCl_2$ gradient (0.5–3.0 *M*). The fractions were analyzed by orcinol–H_2SO_4 reaction in a Technicon sugar analyzer. Fractions corresponding to peaks were collected using a stream splitter in the manifold. Material corresponding to each peak was pooled, dialyzed against distilled water, and analyzed. Relative concentrations of hyaluronic acid, heparan sulfate, and chondroitin sulfates plus heparin were calculated from areas of peaks in the chromatograms. Isomeric chondroitin sulfates in mixtures were determined by the procedure of Saito *et al.* (16). Heparin was estimated by determining the glucosamine content. The automated chromatographic procedure for GAG analyses was reproducible and the results were comparable to those obtained by the manual chromatography followed by analysis of individual fractions for characterization (15). Replicate analyses showed a mean deviation of 2% from the average for hyaluronic acid, 4% for heparan sulfate, 7% chondroitin 6-sulfate, 10% for chondroitin 4-sulfate, 5% for dermatan sulfate, and 7% for heparin.

Gel filtration. Gel filtration of GAG fractions was performed on a Sepharose CL-6B column as previously described (17). GAG from the column were eluted with 0.5 *M* sodium acetate and the fractions were analyzed by orcinol–H_2SO_4 reaction in a Technicon sugar analyzer. The column was calibrated with *Escherichia coli* and standard samples of GAG as described earlier (17).

Electrophoresis. Electrophoresis of GAG was performed on cellulose acetate strips in pyridine/formate buffer, pH 3.6, and in 0.2 *M* cadmium acetate (18). GAG were localized by Alcian blue stain.

Analyses. Hexuronic acid was determined by the Dische method (19) and hexosamine by the method of Boas (20) after the sample had been hydrolyzed in 4 *N* HCl for 14 hr at 100°C. Differential determinations of glucuronic acid and iduronic acid and glucosamine and galactosamine were done by gas liquid chromatographic procedures (21, 22). Total sulfate was estimated by the procedure of Terho and Hartiala (23). Protein was determined by the procedure of Lowry *et al.* (24).

Results. Body and lung weights of birds were similar to those reported previously (8, 9). For example, body weights for 14-day-old copper-supplemented chicks ranged from 105 to 145 g (average 125 g) and body weights for copper-

TABLE I. GLYCOSAMINOGLYCANS (GAG) FROM LUNGS OF COPPER-DEFICIENT CHICKS AND CONTROLS

	Total UA[a] (μg/mg tissue)	Percentage of total GAG					
		HA	HS	C4-S	C6-S	DS	Hep
Controls	1.20	15.9	31.6	10.6	25.4	13.2	3.3
	1.15	14.8	29.8	9.8	28.1	12.4	5.1
	1.56	14.0	30.2	10.3	28.8	11.3	5.4
	0.80	18.1	33.4	10.9	20.7	11.1	5.8
	0.99	22.7	28.5	11.1	21.6	13.2	2.9
	1.30	17.8	30.4	13.2	24.6	11.0	3.0
Mean ± SE	1.17 ± 0.11	17.2 ± 1.28	30.7 ± 0.68	11.0 ± 0.48	24.9 ± 1.35	12.1 ± 0.41	4.30 ± 0.54
Copper deficient	1.00	14.2	26.7	16.3	31.2	9.4	2.2
	0.87	11.9	21.8	12.4	38.9	8.6	6.4
	1.42	12.7	23.5	15.8	34.7	10.5	2.8
	0.92	16.1	20.9	14.2	33.4	12.8	2.6
	1.08	15.2	18.5	14.7	40.6	8.6	2.4
	1.18	14.1	19.8	15.0	40.2	9.1	1.8
Mean ± SE	1.08 ± 0.08	14.0 ± 0.63	21.9 ± 1.62	14.7 ± 0.56	36.5 ± 1.60	9.80 ± 0.66	3.0 ± 0.69
ρ[b]	NS	NS	<0.001	<0.001	<0.001	<0.01	NS

[a] Abbreviations: UA, uronic acid; HA, hyaluronic acid; HS, heparan sulfate; C4-S chondroitin 4-sulfate; C6-S, chondroitin 6-sulfate; DS, dermatan sulfate; Hep, heparin; NS, not significant.

[b] ρ, Test of significance by student *t* test between copper-supplemented and -deficient birds.

deficient chicks ranged from 85 to 110 g (average 95 g). Fresh lung weights were 0.61 ± 0.23 g, copper-supplemented chicks or 0.48 ± .16 g, copper-deficient chicks.

The total uronic acid concentration and relative distribution of individual GAG in lungs from copper-deficient and -supplemented chicks are reported in Table I. The initial papain digestion of lung tissue was insufficient to remove excess protein. Therefore, GAG preparations were also subsequently hydrolyzed by Pronase. This resulted in GAG devoid of detectable protein by the Lowry *et al.* method (24). The mean total uronic acid concentration in lungs from copper-deficient birds was not significantly different from the mean value of the controls, although there were differences in the distribution of individual GAG fractions.

Figure 1 illustrates Dowex-1 Cl^- column chromatographic profiles of GAG from the lungs of copper-deficient and control chicks.

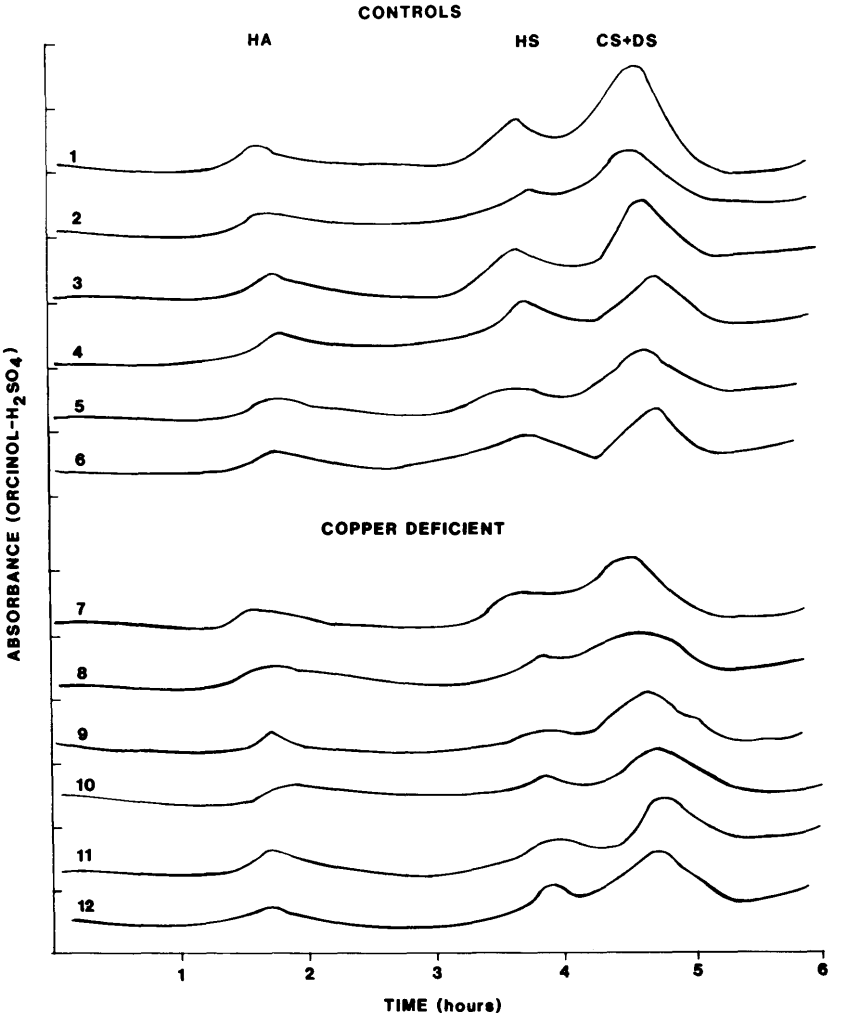

FIG. 1. A composite illustration of Dowex-1 Cl^- chromatography profiles of glycosaminoglycans from lung tissues of control (Nos. 1–6) and copper-deficient (Nos. 7–12) chicks. One hundred fifty microgram uronate material was used for each chromatogram. Absorbance units are not indicated in the figure because all chromatograms are shown together. All chromatographys were carried out under identical conditions using orcinol–H_2SO_4 reaction for continuous flow monitoring of column effluent. Abbreviations: HA, hyaluronic acid; HS, heparan sulfate; CS, chondroitin sulfates; DS, dermatan sulfate.

In all samples, GAG resolved into three peaks. Cellulose acetate electrophoresis of each peak indicated that peak 1 corresponded to hyaluronic acid, peak 2 to heparan sulfate, and peak 3 to a mixture of chondroitin sulfates and heparin. Relative proportions of individual GAG in each sample calculated from areas of the peaks and estimated by enzymatic and chemical analyses are reported in Table I. The predominant GAG in copper-deficient and control chick lungs were heparan sulfate and chondroitin 6-sulfate; each constituted between 20 and 35% of the total GAG, respectively.

Mean concentrations of chondroitin 4- and 6-sulfates were significantly greater in copper-deficient chick lung than in controls. Heparan sulfate and dermatan sulfate concentrations were significantly lower in copper-deficient chick lungs than in controls. Concentrations of hyaluronic acid and heparin plus keratan sulfate were not different between the groups.

Figure 2 illustrates gel filtration profiles of GAG fractions from Dowex-1 column chromatography from pooled samples. The hyaluronic acid fraction and chondroitin sulfate fractions each resolved into three peaks, while heparan sulfate gave a broad single peak. Analyses of GAG under each of the gel filtration peaks are reported in Table II. Peaks 2 and 3 in both GAG from copper-deficient birds were of lower molecular weights than similar samples from control birds. The relative proportions of areas of peaks 2 and 3 to

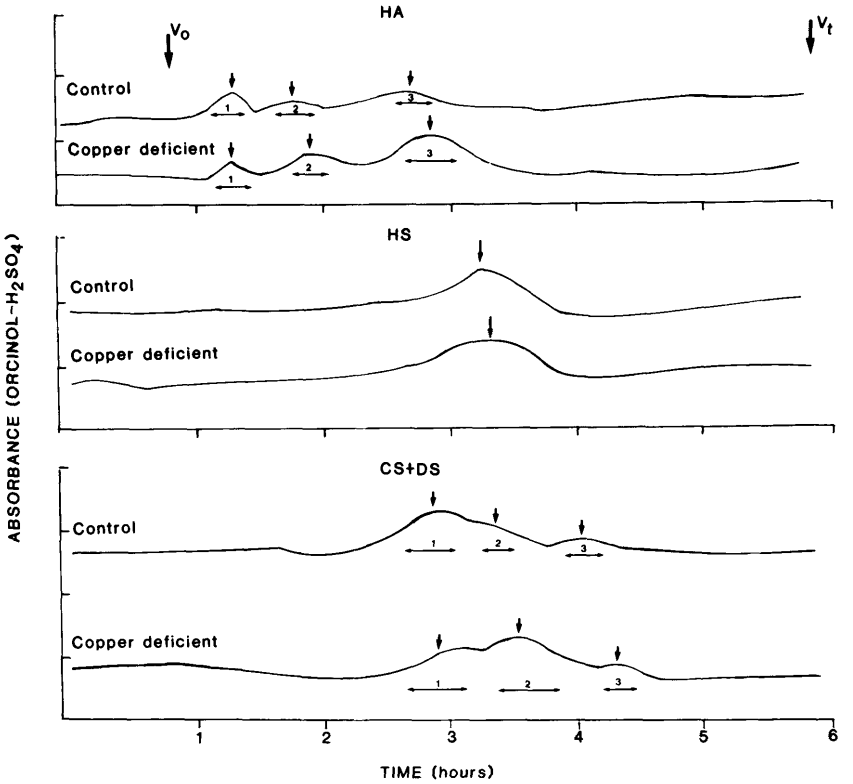

FIG. 2. Sepharose CL-6B gel filtration profiles of GAG fractions from lungs of control and copper-deficient chicks. GAG fractions corresponded to HA, HS, and CS and DS from each group were individually pooled for gel filtration. The column was continuously monitored by orcinol-H_2SO_4 reaction. The numbers underneath the peaks refer to peak numbers. The arrows over the peaks refer to the elution volumes used in calculating K_{av} and molecular weights. The column was caliberated with *Escherichia coli* for void volume (V_0), glucose for (V_t), and standard samples of GAG. Absorbance units are not indicated in the figure because all chromatograms are shown together.

TABLE II. ANALYSES OF GEL FILTRATION FRACTIONS OF GAG FROM CONTROL AND COPPER-DEFICIENT CHICK LUNGS

Fraction	Molecular[a] weight	UA[b]	Sulfate	$GLcNH_2$:$GalNH_2$	GlcUA:IdUA	Electrophoresis
		(mole/mole Hex HN_2)				
Hyaluronic acid						
Control						
Peak 1	210,000	0.97	0	100:0	100:0	HA
Peak 2	118,000	0.95	0	100:0	100:0	HA
Peak 3	61,700	1.01	0[c]	100:0[d]	100:0	HA
(Relative proportions of peaks: 1:1.47:2.67)[e]						
Copper deficient						
Peak 1	210,000	1.0	0	100:0	100:0	HA
Peak 2	103,000	0.93	0	100:0	100:0	HA
Peak 3	57,600	0.97	0	100:0	100:0	HA
(Relative proportions of peaks: 1:2.08:6.07)[e]						
Heparan sulfate						
Control	44,700	1.12	1.13	95:5	81:19	HS, CS[f]
Copper deficient	44,700	1.08	1.06	100:0	77:23	HS
Chondroitin sulfates + dermatan sulfate:						
Control						
Peak 1	57,600	0.89	1.02	0:100	86:14	CS, HS[f]
Peak 2	43,700	0.74	0.95	5:95	20:80	CS
Peak 3	26,900	—[g]	—	—	—	Hep
(Relative proportions of peaks: 1:0.78:0.24)[e]						
Copper deficient						
Peak 1	57,600	0.91	0.93	0:100	82:18	CS
Peak 2	37,200	0.72	0.89	7:93	15:85	CS
Peak 3	25,100	—	—	—	—	Hep, CS[f]
(Relative proportions of peaks: 1:1.41:2.93)[e]						

[a] Estimated from K_{av} values from gel filtration on Sepharose CL-6B.
[b] Abbreviations: UA, uronic acid; Hex-NH_2, hexosamine; $GlcNH_2$, glucosamine; $GalNH_2$, galactosamine; GlcUA, glucuronic acid; IdUA, iduronic acid. Other abbreviations are same as in Table I.
[c] Trace amount, <0.1% of sulfate was noted.
[d] Trace amount, <0.5% galactosamine.
[e] Relative proportions of areas of peaks were calculated with area of peak 1 in each of the fractions taken as 1.
[f] Faintly stained spot (Alcian blue).
[g] Not determined due to limitation of material.

peak 1 in both GAG fractions were also greater in lung samples from copper-deficient chicks than in control lung samples. However, there were no striking differences in chemical analyses of peaks of hyaluronic acid or chondroitin sulfate for samples from copper-deficient and control birds. In each group of birds the ratio of uronic acid to hexosamine was near 1.0 in all GAG fractions. Likewise, the ratio of sulfate to hexosamine was near 1.0 in heparan sulfate and chondroitin sulfate fractions. For the samples, results from electrophoresis confirmed chemical analyses with respect to identity of specific GAG.

Discussion. The results indicate that composition of specific GAG in chick lung is altered in copper deficiency without affecting the concentration of total GAG. In contrast, Linker *et al.* (25) in earlier studies, observed an increase in GAG in aortas from copper-deficient swine. In other connective tissue disorders resulting from nutrient deprivation, vitamin C or vitamin A deficiency or lathyrism, the levels of GAG are often found to be de-

creased (26–28). The increased concentrations of chondroitin sulfates, at the expense of heparan sulfate and dermatan sulfate in lungs from copper-deficient chicks compared to normal chicks, suggest that copper plays a role in the metabolism of specific GAG in birds.

With respect to possible sites where copper might act, both dermatan sulfate and heparan sulfate contain iduronic acid as one of their constituent monosaccharides. It is known that iduronic acid in these GAG is formed by epimerization of oligosaccharide bound glucuronic acid (29). The exact biochemical mechanism of this epimerization is not clearly understood. It is likely that in a copper-deficient state the epimerization takes place at a reduced rate, thus giving rise to lower levels of dermatan sulfate and heparan sulfate. The only other GAG that contains iduronic acid is heparin. Heparin was present in small amounts (about 5% of the total GAG). Since we used whole lung tissues in the study, it is possible that the heparin fraction could contain a small amount of keratan sulfate of bronchiolar origin. We did not attempt to identify and quantify this GAG. In contrast to these observations, Karlinsky (30) in emphysematous and fibrotic hamster lungs and we (31) in emphysematous rabbit lungs observed increased amounts of dermatan sulfate and hyaluronic acid. It is considered that these GAG in the lung exist electrostatically bound to collagen, and in this form they are less susceptible to degradation by glycohydrolases. These enzymes have increased activities in emphysematous lungs.

Both heparan sulfate and dermatan sulfate are important GAG in connective tissue and several biologic functions have been suggested for these GAG. They are anticoagulants and alter the ability of platelets to aggregate (32–34). Heparan sulfate is an ubiquitous component of animal cell surfaces (35) and is involved in a number of cell surface-related processes, such as cell adhesion (36). Moreover, heparan sulfate interacts with fibronectin (37) and with basement membranes (38). Dermatan sulfate generally occurs in skin and noncartilaginous tissues and may aid in collagen fiber formation (39); consequently, reduced levels of heparan sulfate and dermatan sulfate, as well as reduced activity of lysyloxidase, should contribute to the impaired maturation or accumulation of lung extracellular matrix components as previously reported (8, 9).

Gel filtration profiles also indicate a greater proportion of lower molecular weight species of GAG in lungs from copper-deficient compared to copper-supplemented birds. Copper deficiency results, in some respects, in emphysematous-like changes in developing rodent lungs (40). In a recent study of Pronase-induced experimental emphysema in rabbits, we (31) observed that GAG isolated from emphysematous lungs had lower molecular weights than the GAG from control animals. In the copper-deficient chicks, there also appears to be an abnormal dilation of the parabronchial airway (8, 9). The extent to which alteration in the size distribution of selected GAG contributes to this process deserves further attention.

Lastly, it should be noted that the distribution of GAG in 14-day-old chick lung appears to differ from that for the same age rabbit lung. It was reported previously (13) that in rabbit lung chondroitin 4-sulfate was the major GAG, accounting for 30% of the total GAG, while chondroitin 6-sulfate accounted for 16%. In avian lung chondroitin 4-sulfate accounts for 13% of the total GAG, while chondroitin 6-sulfate accounts for 24%. However, the greatest difference is in the percentage of heparan sulfate, 30% in chick lung compared to 14% in rabbit lung. But in both chick and rabbit lungs, hyaluronic acid constitutes 16% of the total GAG. In contrast, in dog lung (14), hyaluronic acid accounts for only 4% while heparan sulfate for 40%. The observations suggest that variations occur in the distributions of GAG in different animal species.

In summary, copper deficiency alters the size distribution and amounts of certain GAG in avian lungs, although the mechanism by which the changes in GAG occur is not clear. The changes were large enough to suggest that abnormal GAG metabolism contributes to the abnormal lung morphology observed in copper-deficient animals (9).

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