THE EFFECTS OF COPPER ON COLLAGEN CROSS-LINKING[1]

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Summary

Evidence in this report indicates that copper is directly involved in the process of collagen cross-linking. The bones from chicks fed copper-deficient diets became brittle and fragile. The collagen in copper-deficient bone was more easily extracted and contained less total aldehyde than corresponding controls. After chromatography on CM-cellulose columns and acrylamide gel electrophoresis, monomer or α-collagen and the cross-linked dimer, β-collagens, could be separated and quantitated. The α to β ratio of collagen from copper-deficient bone was approximately twice that of collagen from control bone, indicating a decrease in the cross-linking.

Evidence that aldehydic functional groups are involved in the intramolecular cross-linking of collagen has been provided by the observations of Bornstein et al. (2) and Bornstein and Piez (3) after characterization of peptides from monomer (α) and dimer (β) collagen. The extractability of tissue collagens appear to be inversely related to the degree of intramolecular cross-linking (14).

Recently, it has been shown that copper deficiency in chicks increases the amount of collagen which can be extracted from aortic tissue and tendon (5,12). In bone, copper deprivation also results in leg weakness and fragility resulting from defects in the organic matrix (4, 19). The purpose of the present investigation is to show that such anomalies may be related to the cross-linking of bone collagen.

METHODS

In three separate experiments, two groups of 25 chicks each were fed

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copper-deficient (< 1 ppm) or control (25 ppm) skim-milk diets[2] for 3 weeks from the day of hatching. At this time chicks were killed and the femurs and tibias removed. Bones were cleaned of all adhering and cartilaginous tissue. Soluble collagen was then extracted from the resulting tubes of diaphyseal bone (30-40 bones/experiment) with 0.5 M acetic acid (9) and purified in the following manner. The extracts were first centrifuged at 30,000 x g for 2 hours, and the collagen in the supernate was precipitated by dialysis against large volumes of 0.02 M Na_2HPO_4. After centrifugation, the collagen precipitate was redissolved in 0.5 M acetic acid and further purified by precipitation with NaCl (5-8% w/v). The precipitate (10 to 20 mg) was washed twice with deionized water and suspended in 0.06 $\Gamma/2$ sodium acetate buffer, pH 4.8, in preparation for carboxymethylcellulose (CM) chromatography. All procedures for isolation and purification were performed at 4°. The samples were chromatographed as described by Miller et al. (9) and Piez et al. (15). The column (1.5 x 15 cm) was maintained at 42°. The column flow rate was 150 ml/hour for most runs.

Disc electrophoresis of acetic acid extract was performed according to the procedure of Nagai, Gross and Piez (10). The collagen subunits separated by electrophoresis were quantitated by scanning with a densitometer adapted for acrylamide gels. The α/β ratio of collagens from copper-deficient and control bone was obtained from calculation of the area under the peaks after chromatography and gel electrophoresis.

Aldehyde content of acetic acid soluble collagens was determined by the method of Paz et al. (13). Acetaldehyde was used as the standard. The collagen content of the extracts was calculated after hydroxyproline determination (20). It was assumed that 130 ug of hydroxyproline was equivalent to 1 mg collagen.

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[2]General Biochemical, Cat. No. 170210, Chagrin Falls, Ohio.

RESULTS AND DISCUSSION

The collagen from copper-deficient bone was more soluble than collagen obtained from control bone (Table 1). When expressed as the percentage of total collagen (24% of the dry weight of diaphyseal bone), almost 4% of the

Table I The Effect of Copper Deficiency on the Solubility, Aldehyde Content and α/β Ratio of Acetic Acid Extracted Collagens.[1]

Treatment	Extracted Collagen (%)[2]	Aldehyde Content[3]	$\alpha_1 + \alpha_2/\beta_{11} + \beta_{12}$[4]	$\alpha_1 + \alpha_2/\beta_{12}$[5]
Deficient	0.92 ± 0.05	1.5 ± 0.1	6.7 ± 2.0	8.9 ± 2.0
Control	0.50 ± 0.14	2.0 ± 0.2	3.1 ± 0.5	4.3 ± 0.6

[1] Mean ± s.e. from 3 experiments.
[2] Expressed as the mg hydroxyproline/mg bone tube x 7.69 x 100 after acetic acid extraction.
[3] μM aldehyde/100 mg collagen.
[4] Calculated after quantitation of the collagens separated by acrylamide gel electrophoresis.
[5] Calculated from estimates of peak areas after carboxymethyl cellulose chromatography.

collagen could be extracted from deficient bones compared with approximately 2% from control bones. Soluble collagen from copper-deficient chicks also contained less aldehyde.

From chick bone, four subunits of native collagen can be resolved by CM-chromatography (9, 14), the two monomer subunits, designated as α_1 and α_2, and two dimer subunits, designated β_{11} and β_{12} (Figure 1). β_{11}-collagen (two condensed α_1-collagens) was present only in small amounts in acetic acid extracts and could not be quantitated. Assigning the value of one to represent the area for β_{12}-collagen, the relative values for α_1-plus α_2-collagens were calculated (Table 1). This ratio was approximately twice as great for soluble collagen from copper-deficient chicks as compared with controls.

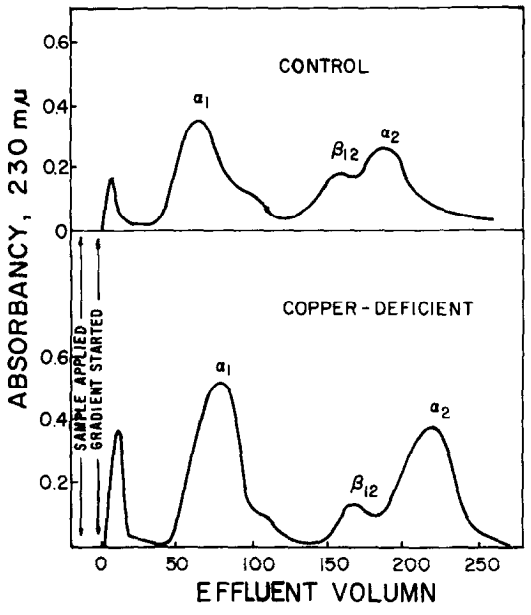

Figure 1. Chromatography on CM-cellulose (column, 1.5 x 15 cm, 45°) of collagen extracted from copper-deficient and control chick bone with acetic acid. Eluded with a linear gradient of ionic strength from 0.06 to 0.16 at pH 4.8 (NaCl in acetate buffer).

Acrylamide gel separation of collagens from control and copper-deficient chicks is depicted in Figure 2. The presence of β-collagens was visibly less in most cases in extracts of bones from copper-deficient chicks as compared with controls. Calculation of the $\alpha_1+\alpha_2/\beta_{11}+\beta_{12}$ ratio again revealed an approximate 2-fold increase in copper-deficient chicks as compared with controls (Table 1). These data, and the results from CM-chromatograms, indicate that copper deprivation results in a decrease in dimer collagen and thus a decrease in intramolecular cross-linking.

The mechanism of collagen cross-linking is presumed to involve the condensation of aldehyde groups which are derived from the ϵ-amino groups of collagen lysines (2, 17). The conversion of lysine to such an aldehyde (α-amino adipic-δ-semialdehyde) could be mediated by an amine oxidase. Copper containing amine oxidases have been shown to be present in vascular tissue (1, 6) and, more recently, in bone and tendon (16, 18). It is

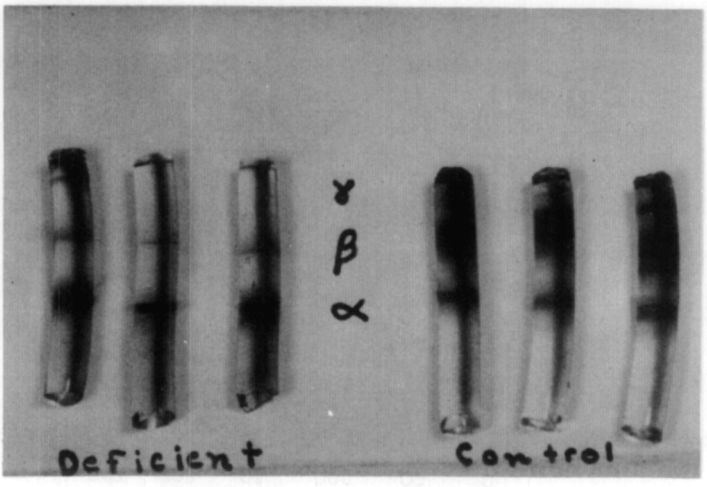

Figure 2. Acrylamide gels after disc electorphoresis of collagens extracted with 0.5 M acetic acid from normal and copper-deficient chick bone.

proposed that the decrease in bone collagen cross-linking is directly related to pathological defects in the organic matrix of bone, which have been shown to result from copper deficiency (4). Furthermore, the previous observations that bone amine oxidase is directly related to dietary copper levels (18) also indicate a relationship between the level of this enzyme and the degree of bone collagen cross-linking.

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