Cardiovascular Studies on Copper-deficient Swine

IV. CONTENT AND SOLUBILITY OF THE AORTIC ELASTIN, COLLAGEN, AND HEXOSAMINE*

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When pigs are reared from birth on a milk diet deficient in copper they develop characteristic cardiovascular defects that usually terminate in rupture of the heart or major vessels at 3 to 4 months of age (1). These events are preceded by demonstrable reduction of tensile strength of the aortas (2). The aortic elastic tissue shows histological defects (1), and alterations of its mechanical properties have been demonstrated (3). A reduction in insoluble elastin residue and an increase in a sodium chloride-extractable macromolecular component of these aortas has been reported (4). This report concerns the relation of the extractable material to the major intercellular components of the aorta, viz. collagen, elastin, and ground substance.

EXPERIMENTAL PROCEDURE

A detailed account has been published describing the nutritional aspects and pathological observations of this experiment (1). In summary, six control animals were on the copper-deficient diet supplemented with copper and iron; six were on the copper-deficient diet supplemented with vitamins and minerals (except copper); six were on the copper-deficient diet supplemented with iron alone. Each copper-deficient animal was autopsied as soon after death as possible, the aorta was measured, weighed, and sectioned into rings. Thoracic rings were cleaned, weighed, and frozen until subjected to fractionation. Two types of ring were fractionated in these experiments. Proximal rings which were cut from the ascending and descending aorta, near the arch, were large enough to be fractionated individually. Smaller rings, cut from the distal end of the descending thoracic aorta above the diaphragm, were too small to be fractionated separately. These were pooled into groups for fractionation. Each group weighed approximately as much as the larger individual rings and contained about four rings per group.

A simple fractionation procedure was devised to permit measurement of the viscosity of the buffer extract (5), determination of fat-free dry weight, and analyses for the desired major components (6). Fig. 1 shows the fractionation scheme employed. All operations with tissues were conducted in a cold room at 2°.

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1 This work has appeared in a preliminary communication (3) and will appear in a forthcoming publication as Paper III of this series in the Journal of Experimental and Molecular Pathology.

The tissues were minced finely with dissecting scissors and shaken with 6 times their weight of 1 M sodium chloride buffer. Centrifuging was done in a Spinco model L preparative centrifuge using the No. 40 head. Relative viscosity was measured in Ostwald viscometers with a flow time of 60 seconds at 20°. Protein hydrolysis was carried out in sealed Pyrex tubes for 3 hours at 138°. The tubes were cooled, opened, diluted to the desired volumes, and filtered through sintered glass filters to remove small amounts of humin. Mucopolysaccharides were hydrolyzed in 2 N HCl for 4 hours and analyzed for amino sugar according to Boas (7). Glucosamine served as the reference standard.

Fat-free Residue Weights—After the buffer extraction, the tissues were extracted in a Soxhlet apparatus successively with acetone, ethanol, and ether. They were dried in a vacuum at 70° and weighed.

Kjeldahl nitrogen was determined after digestion with a mercury catalyst (8), with the use of a Markham distillation apparatus (9). Amino acid analyses were done for hydroxyproline (10), proline (11), and glycine (12).

RESULTS

Sodium Chloride Buffer Extract—The small volumes of extract available from each ring limited the number of procedures to those listed in Table I. It is evident that more material was extracted from the copper-deficient aortas. The high relative viscosity of the copper-deficient extracts indicates that the material in solution was macromolecular. The proline and hydroxyproline analyses were done in an effort to see whether connective tissue protein components were affected by copper deficiency. The ratio of proline to hydroxyproline in pig Achilles tendon collagen was reported to be 1.06:1, whereas in elastin from pig aorta it was 10.3:1 (14, 15). The 10 extracts from proximal copper-deficient rings were close to the ratio reported for elastin from pig aorta. The ratio from the distal samples was slightly lower. This may be explained by the fact that these smaller rings came from a part of the aorta which has a decreased content of elastic tissue, with a larger proportion of collagen to elastin and an increased amount of muscular tissue (16). The ratios for both sets of control tissues are questionable because of the extremely small amounts of hydroxyproline actually determined in the assays. The hydroxyproline determined was of the order of 1 to 2 μg, below the level of reliability of the method used. The hexosamine results will be considered below in conjunction with a discussion of the autoclaved fraction.

Fat-free Residue Weights—Table II shows that the residue...
remaining from the copper-deficient tissue after buffer and fat extraction was significantly lower than that from the control tissue. This was true of the samples from both sites. It may be noted that the buffer extracts of the copper-deficient tissues contained more nitrogen and higher amounts of proline and hydroxyproline than the control tissues (Table I). The material extracted by the fat solvents was not studied.

**Autoclaved Extract ("Collagen")**—We anticipated that the fractionation scheme employed (Fig. 1) would yield an autoclaved extract containing collagen plus ground substance constituents. The low nitrogen values of Table III confirmed the prediction, since pure collagen would have had a nitrogen content of 18%. The per cent collagen was obtained by multiplying the hydroxyproline values by the factor 7.46 (8). There was twice as much hexosamine in the copper-deficient autoclaved extract as there was in the controls. The same ratio of hexosamine content prevailed in the sodium chloride buffer extracts (Table I).

There were no significant differences in the proline or glycine contents of the samples from control and copper-deficient animals. The hydroxyproline content of the proximal rings was also the same for control and deficient animals. The distal rings, from a segment of the aorta in which collagen is increased in abundance, showed a slightly higher hydroxyproline content. The small number of distal samples in the deficient group and the large standard deviation prevent further speculation on these results.

The one strikingly significant difference was in the amount of "noncollagen" material extracted by autoclaving. About 30% more was removed from the copper-deficient tissue than from controls.
TABLE III
Analyses of autoclaved extracts of aortic tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>Autoclaved extract (% fat-free dry wt.)</th>
<th>Nitrogen (% fat-free dry wt.)</th>
<th>Collagen* (% fat-free dry wt.)</th>
<th>Proline (% collagen)</th>
<th>Hexosamine (% fat-free dry wt.)</th>
<th>Glycine (% collagen)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal rings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (10)</td>
<td>28.7 ± 3.7</td>
<td>13.1 ± 1.5</td>
<td>17.3 ± 1.9</td>
<td>19.0 ± 2.0</td>
<td>n.d.</td>
<td>26.7</td>
</tr>
<tr>
<td>Copper-deficient (10)</td>
<td>36.4 ± 4.9</td>
<td>12.1 ± 1.4</td>
<td>18.0 ± 1.9</td>
<td>20.6 ± 3.0</td>
<td>n.d.</td>
<td>27.1</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.1</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Distal rings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (16)</td>
<td>30.0 ± 2.0</td>
<td>15.3 ± 0.62</td>
<td>19.8 ± 1.9</td>
<td>17.9 ± 0.83</td>
<td>0.18</td>
<td>27.1</td>
</tr>
<tr>
<td>Copper-deficient (15)</td>
<td>41.3 ± 5.2</td>
<td>13.9 ± 0.73</td>
<td>25.5 ± 5.2</td>
<td>17.8 ± 0.97</td>
<td>0.33</td>
<td>26.1</td>
</tr>
</tbody>
</table>

* Per cent hydroxyproline × 7.46 (see “Results”).

TABLE IV
Analyses of elastin residue of aortic tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>Per cent fat-free dry wt.</th>
<th>N (%)</th>
<th>Hydroxyproline (%)</th>
<th>Proline (%)</th>
<th>Glycine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal rings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (11)</td>
<td>68.8 ± 3.5</td>
<td>15.6 ± 0.5</td>
<td>1.23 ± 0.10</td>
<td>17.3 ± 0.6</td>
<td>23.9 ± 1.8</td>
</tr>
<tr>
<td>Copper-deficient (11)</td>
<td>60.8 ± 4.0</td>
<td>15.6 ± 0.2</td>
<td>1.19 ± 0.08</td>
<td>15.9 ± 0.7</td>
<td>28.1 ± 1.7</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.1</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&lt;0.1</td>
<td>&gt;5</td>
</tr>
<tr>
<td><strong>Distal rings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (16)</td>
<td>68.3 ± 1.6</td>
<td>15.8 ± 0.4</td>
<td>1.02 ± 0.06</td>
<td>16.9 ± 0</td>
<td>25.1</td>
</tr>
<tr>
<td>Copper-deficient (15)</td>
<td>57.4 ± 5.4</td>
<td>15.9 ± 0.9</td>
<td>1.12 ± 0.16</td>
<td>15.2 ± 0.8</td>
<td>24.2</td>
</tr>
</tbody>
</table>

TABLE V
Distribution of proline in various fractions

<table>
<thead>
<tr>
<th></th>
<th>Copper-deficient (pig 73-1)</th>
<th>Control (pig 73-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer extract</td>
<td>4.8</td>
<td>1.6</td>
</tr>
<tr>
<td>“Collagen” extract</td>
<td>9.5</td>
<td>7.4</td>
</tr>
<tr>
<td>Elastin residue</td>
<td>23.0</td>
<td>30.3</td>
</tr>
<tr>
<td>Total</td>
<td>37.3</td>
<td>39.3</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Neuman and Logan reported a collagen content of 16% in pig aorta samples taken near the arch (6). The collagen content reported here is in good agreement with theirs. However, their average elastin value of 55% for five samples was calculated from an assumed hydroxyproline content of only 1.5%. Since some investigators regard hydroxyproline as a contaminant in elastin (17), we chose to use the weight of residue remaining after “collagen” extraction to calculate elastin content.

The elastin residue of copper-deficient aortas was lower than that of controls. As shown in Table I, the deficient tissues yielded a buffer extract which contained 3 times as much proline as the controls. Table V gives the proline distribution for two typical aortic rings. Copper deficiency has resulted in a shift of proline out of the “elastin residue” compartment to the buffer extractable compartment. We have not determined the nature of the component which contains the excess proline in the buffer extract. Further work will be necessary to find out whether the proline is incorporated in a proelastin similar to the procollagen found in lathyrism (18). The total hexosamine value of 0.27% reported here for control aortas agrees with the values reported by Buddecke (19) for pig aorta and by Grant, Hathorn, and Gillman (20) for normal rat aorta. The doubled content of hexosamine found in our copper-deficient aortas resembles the value reported for lathyritic rat aortas (20).

Although we have not examined the lipids of the aortas, there is a possibility that the significantly lower solid content found after fat extraction (Table II) may be in part due to altered lipid content. Measurements made on aortas, subjected only to aqueous buffer extraction, yielded nearly equal residue weights for control and copper-deficient tissues.†

SUMMARY

1. The elastin, collagen, and hexosamine contents of aortas taken from pigs dying of copper deficiency have been determined. Suitable control aortas were also analyzed.

2. As compared with the aortas of nondeficient animals, copper deficiency resulted in (a) a lowered elastin content; (b) no observable change in collagen; (c) a doubled hexosamine content; (d) a statistically significant lowering of the proline content in the isolated elastin residue of the aorta.

3. Sodium chloride buffer extracts of copper-deficient aortas contained an increased amount of proline. The nature of the proline-containing component has not been determined.

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REFERENCES
