INTRACELLULAR DISTRIBUTION OF HEPATIC COPPER IN NORMAL AND COPPER-LOADED SHEEP

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SUMMARY
This investigation was conducted to determine the intracellular distribution of copper in the liver of normal sheep, and the changes associated with exposure to increased dietary copper. Normal rat liver (3.8 ± 1.0 µg Cu/g liver, wet tissue basis) and normal sheep liver (102.0 ± 37.2 µg Cu/g liver) was processed to give the four common subcellular fractions. When the intracellular distribution of copper in the two species was compared, the cytosol fraction of sheep liver contained a much lower (P<.001) percentage of copper than normal rat liver. Although the debris fraction of sheep contained a higher (P<.001) percentage than the rat, this copper did not appear to be associated with any specific component of this fraction.

Feeding a high level of dietary copper to sheep elevated hepatic copper concentration (238.5 ± 84.2 µg Cu/g liver) significantly (P<.05). Comparison with the normal group showed that with the higher liver copper concentration, proportionally less (P<.05) of the copper was found in the large granule fraction while proportionally more (P<.05) was present in the cytosol. These changes in the intracellular copper distribution of copper-stressed sheep directly oppose what is known for copper-stressed rats.

(Key Words: Hepatic Copper Metabolism, Intracellular Distribution, Copper-loaded Sheep.)

INTRODUCTION
Copper is an essential trace element which is also toxic if consumed in excess amounts (Underwood, 1977). Chronic copper toxicity can occur in all animals studied, but the level required to produce a toxic effect varies widely among species. Sheep are the most sensitive to copper poisoning of all domestic animals and reports of fatalities are not uncommon (Boughton and Hardy, 1934).

Rats are relatively insensitive to copper toxicity. This is reflected by their ability to maintain a fairly constant hepatic copper concentration as dietary copper levels are increased up to 200 ppm (Milne and Weswig, 1968). Sheep, on the other hand, show very little homeostatic control over hepatic copper concentration since there is a close relationship between dietary and liver copper (Dick, 1954).

The liver is the focal point of most studies on copper metabolism since it is the key organ in the metabolism, storage, and excretion of this element (Underwood, 1977). Subcellular fractionation of rat liver has shown characteristic patterns of the intracellular distribution of copper which reflect the copper status of the animal and are related to known cellular processes of copper metabolism and homeostasis (Gregoriadis and Sourkes, 1967; Verity et al., 1967; Evans et al., 1970; Alfaro and Heaton, 1974). Comparable information about the intracellular distribution of copper in sheep liver is unavailable for both the normal and copper-stressed states.

The objectives of this work were to elucidate the intracellular distribution of copper in the liver of normal or representative sheep and also to observe if this distribution is altered in response to a subacute dietary excess of this metal.

MATERIALS AND METHODS
Male Long-Evans hooded rats, weighing 300 to 400 g and fed a commercial diet (Ralston Purina Co., St. Louis, MO; 15.2 µg copper/g), were used as a source of normal rat liver.
Eight crossbred yearling wethers, average body weight 42.5 kg, were randomly assigned to either the control or experimental group. The animals were housed in individual metabolism crates with fresh tap water supplied ad libitum. Each animal received 227 g of chopped alfalfa hay (IRN 1-00-104) (6.15 μg copper/g) and 908 g of supplement (table 1). The control supplement contained 2.2 μg copper/g. The high-copper supplement fed to the experimental animals was prepared by adding CuSO₄·5H₂O to the control supplement to increase the copper content to 98.8 ppm. This level of copper was selected with the goal of producing elevated hepatic copper content without producing acute copper toxicity. This goal was accomplished since sheep in both groups had a slight loss of body weight during the experimental period. At the end of the 23-day feeding period, the animals were slaughtered and tissue samples collected.

Pieces of tissue taken from all lobes of the liver were minced with scissors, suspended in cold .25 M sucrose, and a 1:6 (w:v) homogenate prepared using a Potter-Elvehjem homogenizer with a Teflon pestle. Fractionation was carried out following the method of Porter et al. (1961), using a Sorvall Model RC2-B refrigerated centrifuge (SS-34 head) and a Beckman Model L2-65B ultracentrifuge (SW-27 rotor). The fractions collected by centrifugation were: 600 × G for 10 min to separate the debris pellet, 8,500 × G for 10 min to separate the large granule pellet, and 105,000 × G for 60 min to separate the microsomal pellet from the cytosol fraction.

The validity of the fractionation procedure was verified by assay of the following enzymes: lactic dehydrogenase by the method of Berman et al. (1965) as marker for the cytosol fraction, glucose-6-phosphatase by the method of Harper (1965) for the microsomal fraction, and acid phosphatase by the method of Bowers et al. (1967) for the large granule fraction. Protein was measured by the method of Lowry et al. (1951).

A preparation of pure nuclei was isolated from sheep liver by slight modification of the method of Blobel and Potter (1966). Homogenates were prepared as previously described and centrifuged for 10 min at 600 × G. The resulting pellet was taken up in .25 M sucrose, mixed with two volumes of 2.3 M sucrose (final sucrose concentration 1.62 M), layered over 2.2 M sucrose, and spun at 60,000 × G for 60 min in the ultracentrifuge (SW-27 rotor). Purity of the isolated nuclei was checked by measurement of the RNA:DNA ratio of the material. RNA was measured by the method of Fleck and Munro (1962), and DNA by the method of Burton (1956).

To minimize contamination of samples with exogenous copper, all glassware was rinsed with nitric acid and deionized-distilled water, and all solutions were prepared with deionized-distilled water.

Tissue and cellular fractions were digested with nitric, sulfuric, and perchloric acids and copper was analyzed by atomic absorption spectrophotometry. Statistical differences between means were determined by the method of Lapin (1975). Least squares regression analyses were performed by the methods of Neter and Wasserman (1974).

**RESULTS**

The results of the analyses of copper content of whole liver and cellular fractions of normal rats, control (normal) and copper-supplemented sheep are presented in table 2. The mean and range of tissue concentration for the two species agree well with previous investigators (Dick, 1954; Gregoriadis and Sourkes, 1967; Evans et al., 1970; Weiner et al., 1974). Furthermore, the percent distribution in cellular fractions of the rats agrees with the work of Alfaro and Heaton (1974), Milne and Weswig (1968) and Gregoriadis and Sourkes (1967). For control sheep, the mean liver copper concentration was much greater (P<.001) than for the rat. In addition, the range in values was far greater for the sheep. Difference between species is also seen in the distribution pattern of

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Internatl Ref No.</th>
<th>%a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground shelled corn</td>
<td>4-02-931</td>
<td>66.45</td>
</tr>
<tr>
<td>Oak sawdust</td>
<td></td>
<td>31.27</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>5-04-604</td>
<td>.90</td>
</tr>
<tr>
<td>Limestone</td>
<td>6-02-632</td>
<td>.69</td>
</tr>
<tr>
<td>Salt</td>
<td></td>
<td>.67</td>
</tr>
<tr>
<td>Vitamin-Antibiotic premixb</td>
<td></td>
<td>.02</td>
</tr>
</tbody>
</table>

aExpressed on a dry matter basis.

bSupplied 1250 IU vitamin A, 150 IU vitamin D, and 20 mg chlortetracycline per kilogram supplement.
TABLE 2. LIVER COPPER CONCENTRATION AND PERCENT DISTRIBUTION AMONG LIVER FRACTIONS OF NORMAL RATS, CONTROL AND COPPER-SUPPLEMENTED SHEEP

<table>
<thead>
<tr>
<th>Species</th>
<th>Liver copper concentration</th>
<th>Debris (%)</th>
<th>Large granule (%)</th>
<th>Microsome (%)</th>
<th>Cytosol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (8)</td>
<td>$3.8 \pm 1.0_\mathrm{d}$</td>
<td>12.8_\mathrm{d}</td>
<td>20.7_\mathrm{d}</td>
<td>12.7</td>
<td>53.9_\mathrm{d}</td>
</tr>
<tr>
<td>Control sheep (4)</td>
<td>$102.0 \pm 37.2_\mathrm{e,f}$</td>
<td>37.0_\mathrm{e}</td>
<td>37.7_\mathrm{e,f}</td>
<td>9.9</td>
<td>15.4_\mathrm{e,f}</td>
</tr>
<tr>
<td>Copper-supplemented sheep (4)</td>
<td>$238.5 \pm 84.2_\mathrm{g}$</td>
<td>34.5</td>
<td>24.9_\mathrm{g}</td>
<td>11.3</td>
<td>29.3_\mathrm{g}</td>
</tr>
</tbody>
</table>

*aMean ± SE, wet tissue basis.
*bPercent of total hepatic copper.
*cNumber in parenthesis indicates number of animals.
*d,eDifferent superscripts indicate differences ($P<.001$) between rats and control sheep.
*f,gDifferent superscripts indicate differences ($P<.05$) between control and copper-supplemented sheep.

Liver copper. In rat liver, the cytosol fraction contained over half of the cellular copper, whereas in sheep this fraction contained only 15%. Another major difference in the distribution is the proportion found in the debris fraction. This fraction in rat liver contained approximately 13% of total liver copper, while in sheep it accounted for 37% of the liver copper. The absolute amounts of copper in all four sheep liver fractions were greater than that found in comparable fractions from rat liver.

The high-copper supplement increased ($P<.05$) liver copper concentration. In sheep livers with elevated copper content, the proportion of copper found in the cytosol fraction increased while the proportion found in the large granule fraction decreased. This is in contrast to the results obtained with copper-loaded rats where the opposite effect is observed, i.e., an increase in the proportion in the large granule fraction concomitant with a decrease in the proportion present in the cytosol fraction (Gregoriadis and Sourkes, 1967; Verity et al., 1967; Evans et al., 1970; Lal and Sourkes, 1971). However, Milne and Weswig (1968) reported a study in which no change in the distribution pattern was observed with increased hepatic copper.

The cellular debris fraction, as noted above, accounted for the largest proportion of the copper found in sheep liver. This pellet consisted largely of nuclei, but also contains some intact cells, connective tissue, plasma membranes and red blood cells. To determine if the copper was specifically associated with the nucleus, a preparation of purified nuclei was isolated from the livers of five normal sheep. The RNA:DNA ratio ($0.16 \pm 0.02$) of the preparations confirms that they contained purified nuclei according to Tata (1974) and Maggio et al. (1963). Although the debris contained 41% of the total copper, only 31% of this (debris copper) was associated with purified nuclei. In preliminary studies, isolated plasma membranes have been found to contain only 34% of the debris fraction copper. The low recovery of copper in purified nuclei and isolated plasma membranes indicates that a major portion of the copper found in the debris fraction is not specifically associated with either organelle.

Discussion

In most mammals with normal copper status (including cattle), about half of the copper in the liver cell is found in the cytosol fraction (Gregoriadis and Sourkes, 1967; Porter et al., 1961; Verity et al., 1967). Although preferential, the binding in this fraction is limited. Thus, with increasing cellular copper content, the percentage bound in this fraction decreases with a concomitant increase in the debris and especially the large granule fractions. Verity et al. (1967) showed that in mice exposed to a high level of copper, the number and enzymatic activity of lysosomes increased and that these organelles accounted for most of the increase of copper in the large granule fraction.

The results of this investigation show that sheep liver presents a different pattern of
intracellular copper distribution. It could be argued that the results obtained in these experiments are misleading because of the great difference in hepatic copper content of the two species used in these investigations. In a study of copper loading of rat liver, Lal and Sourkes (1971) concluded that the partition of copper between the large granule and nuclear fractions was a function of actual hepatic concentration. Between 30 and 100 μg Cu/g tissue, a larger proportion was found in the mitochondrial (large granule) fraction. At 100 μg Cu/g tissue, the amount of copper in the two fractions was equal. Further increases in hepatic copper resulted in a plateauing of large granule copper while the debris fraction continued to accumulate the element.

To determine if the above observation could be applied to our data with sheep liver, data from this study, combined with data obtained from sheep slaughtered at The Pennsylvania State University Meats Laboratory, were compared with data for individual copper-loaded rats published by Gregoriadis and Sourkes (1967), Evans et al. (1970) and Lal and Sourkes (1971). A summary of these data is presented in table 3. When the data are expressed in this manner, it is clear that the sheep accumulate more (P<.05) copper in the cytosol fraction and less (P<.05) in the large granule fraction than do rats at comparable levels of liver copper. Furthermore, if the concentration of copper in the large granule fraction is plotted as a function of total hepatic copper (figure 1), the large granule fraction of sheep liver is saturated at a lower (P<.01) copper concentration than that of the rat.

At the present time, there is no clear explanation for the decrease in large granule copper and increase in cytosol copper associated with elevated liver copper concentration in copper-fed sheep. Harris and Dean (1973) reported finding an extra copper-binding protein in the cytosol of copper-toxic sheep. These investigators did not identify this extra protein, but speculated that either the protein was a specific copper-chelating mechanism induced by high, nonphysiological concentrations of copper or that the new protein was a derivative of the copper-binding proteins already present in liver.

Bremner and Marshall (1974) have provided the only other definitive information about the cytosol proteins found in sheep liver. Over half the cytosol copper was found in the metallothionein (12,000 M.W.) fraction. The second major component (32%) was a protein greater than 75,000 M.W. Although placing sheep on a zinc-deficient diet did not alter the copper content of either the liver or cytosol fraction, there was a shift in copper distribution among the proteins, with the higher molecular weight protein containing the majority of the cytosol copper.

### Table 3. Comparison of Hepatic Copper Distribution in Sheep and Rats at Comparable Tissue Copper Content

<table>
<thead>
<tr>
<th>Species</th>
<th>Hepatic copper</th>
<th>Debris</th>
<th>Large granule</th>
<th>Microsome</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/g</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Low hepatic copper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat (8)</td>
<td>55.5</td>
<td>24</td>
<td>60&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sheep (7)</td>
<td>54.5</td>
<td>30</td>
<td>39&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>22&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>High hepatic copper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat (4)</td>
<td>181.0</td>
<td>41</td>
<td>42&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sheep (8)</td>
<td>168.5</td>
<td>38</td>
<td>28&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>25&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number in parenthesis indicates number of animals.

<sup>b</sup>Data are derived from the individual values reported by Gregoriadis and Sourkes, 1967; Evans et al., 1970; and Lal and Gregoriadis, 1971.

<sup>c</sup>Data are derived from the individual values of the sheep reported in table 2 and other sheep obtained from The Pennsylvania State University Meats Laboratory.

<sup>d</sup><sup>e</sup>Differences (P<.05) between species within hepatic copper content are indicated by different superscripts.
copper. The significance of these observations in terms of the data presented in this paper is unclear, since data on sheep comparable to those used in these studies provide no evidence for the presence of zinc deficiency.

The inability of sheep to maintain copper homeostasis at relatively low levels of dietary copper appears to be related to differences in the metabolism of the metal by the liver of this species. The large granule (lysosome-containing) fraction of sheep liver does not respond to large doses of copper as it does in other species. Evans (1973) reports several studies elucidating the importance of the lysosomes in the excretion of copper into the bile. Therefore, the defect in copper homeostasis in sheep may be the inability of the lysosomes to sequester and excrete copper from the liver. Whether this is due to some basic difference in lysosomal properties or lysosomal numbers is unknown. This apparent reduced ability of sheep to utilize lysosomes as a secretory pathway merits further investigation.

LITERATURE CITED


Underwood, E. J. 1977. Trace Elements in Human and
