CHOLESTEROL METABOLISM IN COPPER DEFICIENT RATS

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SUMMARY

The influence of copper deficiency on the appearance of newly synthesized cholesterol in the plasma lipids of rats was examined following \( ^3\text{H} \) mevalonate injection. At 181 days copper deficient rats exhibited a highly significant increase in plasma cholesterol concentration. Copper deficiency was associated with a greatly enhanced appearance of \( ^3\text{H} \) in newly synthesized cholesterol and cholesteryl esters in the plasma lipids. A concomitant decrease in \( ^3\text{H} \) incorporation into liver lipids was also observed. The results suggest that copper deficiency markedly influences the clearance of hepatic cholesterol to the plasma pool, and a highly significant correlation was observed between plasma copper concentrations and \( ^3\text{H} \) incorporation into plasma cholesterol. The results are discussed in terms of a possible role for copper in lipoprotein metabolism, bile acid metabolism, and the uptake of cholesterol by extrahepatic tissues.

Copper has been recognized as an essential dietary component for nearly 50 years (1). Recent evidence suggests a role for copper in the metabolism of cholesterol, in particular the cholesterol concentration of plasma. Dietary copper deficiency has produced elevations in plasma cholesterol in rats (2-4), and it has been hypothesized that an absolute or relative deficiency of copper is a major factor in the etiology of coronary heart disease because of an association with many apparently dissimilar observations on the epidemiology of coronary heart disease and cholesterol metabolism (5). The influence of copper nutrition on plasma cholesterol concentrations assumes

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a particular significance since an elevation of plasma cholesterol concentrations is one of the principal indications of risk of coronary heart disease (6), the leading cause of death in the United States (7). Moreover, recent evidence suggests that many diets commonly consumed in the United States provide considerably less copper (8-11) than the 2 mg considered to be the daily requirement of adults (12,13).

Previous reports of copper deficiency induced increases in plasma cholesterol provide little information on the appearance of newly synthesized cholesterol in the plasma, since they describe only the alterations in plasma cholesterol concentrations (2-4). This paper describes the initial results of \(^{3}H\) incorporation into the plasma lipids of copper deficient rats following \(^{3}H\)-mevalonate injection.

**MATERIALS AND METHODS**

Weanling Sprague Dawley male rats approximately 43 g each, were assigned to two groups of seven animals each, matched by mean weight to within 0.4 g. Each member of the experimental group was paired by weight with a member of the control group, and the mean absolute difference in weight between members of the pair was 0.9 g. Rats were fed a cholesterol free basal diet comprising the following components: sucrose 51.3%, egg white 20%, cottonseed oil 9.5%, coconut oil 9.5%, fibrous cellulose powder 3.0%, choline chloride 0.15%, plus all necessary vitamins, essential fatty acids and minerals with the exception of copper (4). The diet is based on the recommendation of the National Academy of Science, Committee on Animal Nutrition (14). Experimental animals (copper-deficient) were fed the basal diet ad libitum which contained 0.57 \(\mu\)g Cu/g diet. Control animals were fed basal diet supplemented to 5.0 \(\mu\)g Cu/g diet by the addition of finely ground CuSO\(_4\)-5H\(_2\)O during diet preparation. On each day each rat in the control group was given an amount of food (to the nearest 0.1 g) equal to that consumed by the member of the pair in the experimental group (pair fed). All animals were provided with distilled demineralized water (Super Q Systems, Millipore Corp.) \(^{1}\) ad libitum. All animal cages, diet preparation and handling equipment were washed with Radiac metal scrubbing soap solution (Atomic Products Corp.). Animals were housed under conditions described previously (15), except that extreme filtration of the air was considered unnecessary in a non-industrial area.

Routine analysis of both experimental and control diets was accomplished by wet ashing using concentrated nitric and sulfuric acids. Complete digestion was accomplished using 50% hydrogen peroxide. Copper concentrations were determined by flame atomic absorption spectrophotometry (Perkin-Elmer, Model 503). Analysis of diets by this procedure has shown a 98% recovery for copper. Dietary analysis showed copper to be within 5% of the desired concentration in both experimental and control diets (4). Plasma copper concentrations were also determined by flame atomic absorption spectrophotometry, using samples diluted with distilled demineralized water. Appropriate blanks were run in all cases.

Preliminary studies indicated that the appearance of \(^{3}H\)-cholesterol in rat plasma lipids, following subcutaneous injection, was best measured four

\(^{1}\text{Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.}\)
hours after $^3$H mevalonate administration. By four hours the incorporation of $^3$H into plasma lipids was still increasing and had not peaked.

At 181 days the seven pairs of rats were fasted for 24 hours and injected subcutaneously with freshly prepared $^3$H-mevalonate (DL-mevalonate-5-$^3$H, 5.0 Ci/m mole, New England Nuclear) in neutral 0.2 M bicarbonate buffer at a dose of 3.0 $\mu$Ci/100 g body weight. Exactly four hours after mevalonate injection 6-10 ml blood was obtained from each animal by heart puncture (with ether anesthesia), centrifuged in heparinized tubes, and the plasma promptly removed and placed in metal free polypropylene tubes. Plasma cholesterol concentrations were determined by fluorimetry (16). Liver cholesterol concentrations were determined in a similar manner following extraction with chloroform methanol (2:1). The lipids in 1.0 ml of plasma and 1.0 g samples of liver, were extracted with 30.0 ml of chloroform/methanol (2:1) and filtered. Filtered extracts were shaken with 1/5 the extract volume of 0.1 M NaCl. The phases were allowed to separate at room temperature for 3 hours, and the chloroform phase evaporated to dryness using a rotary evaporator. The lipids were immediately redissolved in chloroform to a final volume of exactly 2.0 ml. Lipid extracts were stored at $4^{\circ}$C. A 0.5 ml sample of lipid extract from each animal was pipetted into liquid scintillation vials, and the chloroform purged with nitrogen. Lipids were counted in 10.0 ml of Aquasol-2 (New England Nuclear) using a liquid scintillation counter (Nuclear Chicago).

In addition 500 $\mu$l samples of lipid extracts were applied as a band 2.5 cm wide on freshly activated plates of silica gel H (20 x 20 cm, 250 microns, Applied Science Laboratories). Lines were scribed between samples to prevent sample migration. Reference samples of pure cholesterol and a cholesteryl ester (cholesteryl olate) (Applied Science Laboratories) were also chromatographed. Chromatograms were developed using a hexane, diethyl ether, acetic acid (80:20:2) (v:v) solvent system which separates major lipid classes (17). The solvent front was allowed to rise to within 3 cm from the top of the plate. Chromatograms were air dried and regions corresponding to cholesterol esters and cholesterol were visualized with iodine vapor and by reference to the standard samples ($r_f$ values cholesterol 0.25, cholesterol olate 0.97). As expected, $^3$H incorporation was limited exclusively to cholesterol and cholesteryl ester, and no incorporation was observed in the other plasma lipid fractions. Silica gel from these regions was scraped into scintillation vials. Silica gel samples and associated lipids were suspended in scintillation gel (Aqua sol 2 plus water) and counted. Unstained areas of similar size to lipid zones were removed and counted as blanks. All samples were corrected for quenching by use of quenched standards; results are expressed as disintegrations per minute (dpm). Experimental results were analyzed by Student's t test (18).

RESULTS AND DISCUSSION

Copper deficient rats failed to grow as well as pair fed control animals; by 181 days the mean growth was depressed by 20% ($P < 0.05$). At 181 days the mean plasma copper concentration of copper deficient rats was reduced by 95% as compared to pair fed control animals (Table I). Copper deficient rats exhibited a 130% increase in plasma cholesterol concentration as compared to pair fed control animals ($P < 0.001$) (Table I), with no overlap between the experimental and control groups. This marked difference was apparent despite the feeding of cholesterol free diets. Four hours after $^3$H-mevalonate injection the percent dose incorporation of $^3$H-mevalonate into total plasma lipids, plasma cholesteryl esters and plasma cholesterol was elevated 100%, 80% and 162% respectively in copper deficient rats as compared to control animals (Figure 1), all differences being statistically significant. The percent dose of $^3$H-mevalonate incorporated into plasma lipid fractions was
TABLE I

Plasma Cholesterol Concentration and Specific Activities of Copper Deficient and Pair Fed Control Rats Four Hours After \(^3\)H-Mevalonate Injection at 181 Days, X ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Copper Deficient Rats</th>
<th>Control Rats (Pair Fed)</th>
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<tbody>
<tr>
<td>Plasma Cu, µg/ml</td>
<td>0.08 ± 0.02</td>
<td>1.58 ± 0.16</td>
</tr>
<tr>
<td>Plasma cholesterol,</td>
<td>109.6 ± 7.9</td>
<td>48.9 ± 4.8</td>
</tr>
<tr>
<td>mg/dl</td>
<td></td>
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</tr>
<tr>
<td>Specific activity,*</td>
<td>5.04 ± 0.49</td>
<td>4.33 ± 0.51</td>
</tr>
<tr>
<td>plasma free cholesterol</td>
<td></td>
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</tr>
<tr>
<td>Specific activity,</td>
<td>8.01 ± 0.78</td>
<td>12.44 ± 1.38</td>
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<tr>
<td>plasma cholesteryl esters</td>
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*Expressed as (dpm x 10^3)/mg.

calculated assuming a plasma volume of 40.4 ml/kg for the rat (19). Plasma copper concentrations of copper deficient rats showed a significant correlation with % dose of \(^3\)H-mevalonate incorporated into plasma cholesterol \((r = -0.77, P < 0.033)\) and plasma cholesteryl esters \((r = -0.70, P < 0.07)\). A significant decrease in the specific activity of the plasma cholesteryl ester fraction \((35.6\%, P < 0.001)\) was observed with copper deficient rats (Table I), and perhaps reflects the increase in plasma cholesteryl ester pool associated with copper deficiency. Despite the marked increase in the % dose of \(^3\)H-mevalonate incorporated into plasma free cholesterol in copper deficiency, no significant difference was observed in the specific activity of plasma free cholesterol between copper deficient and pair fed control rats.

Mevalonate is an obligatory metabolite in cholesterol synthesis (20), and \(^3\)H incorporation into plasma lipids following \(^3\)H-mevalonate injection is a measure of net cholesterol influx to and efflux from the plasma pool during a given interval. Changes in \(^3\)H incorporation in the plasma pool are dependent upon cholesterol synthesis, clearance to the plasma, degradation to bile acids in the liver with subsequent biliary excretion, and the uptake of cholesterol by peripheral (extra-hepatic) tissues. The amount of mevalonate injected in these studies, 0.16 µg mevalonate/100 g body weight, represents approximately 10% of the estimated pool of 1.43 µg mevalonate/100 g body weight (20), an amount unlikely to perturb cholesterologenesis significantly.

The marked increase in both plasma cholesterol concentrations and \(^3\)H incorporation into plasma lipids in response to copper deficiency (Figure 1) suggests a role for dietary copper in regulating the clearance of newly synthesized cholesterol from the liver to the plasma pool. Since liver constitutes a major site of cholesterol synthesis (21) the enhanced \(^3\)H incorporation into plasma lipids would suggest an increase in cholesterol synthesis in copper deficient rats. However, previous reports have noted no
INCORPORATION OF $^3$H-MEVALONATE INTO PLASMA LIPIDS, $\bar{x} \pm$SEM

$^3$H incorporation into plasma lipids 4 hours after $^3$H-mevalonate injection in copper deficient and pair fed control rats at day 181. Results are expressed as percentage of the administered dose appearing in the total plasma volume (plasma volume calculated according to 19).

significant differences in liver cholesterol concentrations (4), or in the rate of in vitro cholesterol synthesis (3) in copper deficient rats despite significant differences in plasma cholesterol concentrations. Furthermore, initial studies show that the incorporation of $^3$H-mevalonate into liver lipids of copper deficient rats, raised on an identical diet, is reduced by 18% ($P < 0.04$) (Table II). Despite a small, but not significant, reduction in liver cholesterol in copper deficient rats the specific activity of the liver cholesteryl ester fraction was reduced by 38% (Table II). This further suggests an enhanced clearance or turnover of hepatic cholesterol in copper deficiency.

The transport of hepatic cholesterol in the plasma is dependent upon plasma lipoproteins. Plasma low density lipoproteins (LDL) transport cholesterol from the liver to peripheral (extra-hepatic) tissues where a cellular LDL receptor mediated uptake ensures that these tissues utilize cholesterol of hepatic origin for their metabolic needs (22). Recent evidence suggests that high density lipoproteins (HDL) transport cholesterol from the peripheral tissues to the liver for catabolism to bile acids (23).
TABLE II

Specific Activity and Percent Dose of $^3$H-Mevalonate Incorporated Into Liver Lipids Four Hours After $^3$H-Mevalonate Injection in Copper Deficient and Pair Fed Control Rats at 181 Days, $X \pm SEM$

<table>
<thead>
<tr>
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<th>Copper Deficient Rats</th>
<th>Control Rats &lt;br&gt;(Pair Fed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver cholesterol, mg/g wet liver</td>
<td>$2.44 \pm 0.16$</td>
<td>$2.78 \pm 0.11$</td>
</tr>
<tr>
<td>Percent dose $^3$H-mevalonate incorporated into total liver lipids*</td>
<td>$12.7 \pm 0.73$</td>
<td>$15.4 \pm 0.90$</td>
</tr>
<tr>
<td>Specific activity liver free cholesterol+</td>
<td>$2.19 \pm 0.28$</td>
<td>$1.98 \pm 0.19$</td>
</tr>
<tr>
<td>Specific activity liver cholesteryl esters</td>
<td>$5.35 \pm 0.59$</td>
<td>$8.59 \pm 0.79$</td>
</tr>
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</table>

*Percent dose, 100 g body weight, incorporated into total liver lipids.

+Expressed as (dpm x $10^8$)/mg.

Conversion of cholesterol to the bile acids, with subsequent biliary excretion, represents the only significant route for cholesterol excretion (24). The influence of copper nutrition on the incorporation of $^3$H-mevalonate into plasma and liver cholesterol and cholesteryl ester fractions suggest that copper may influence cholesterol turnover or the clearance of cholesterol from liver to plasma. Changes in cholesterol metabolism due to copper deficiency may be of importance in the etiology of ischemic (coronary) heart disease. The concentration of cholesterol in plasma is predictive of risk of ischemic heart disease (6) and recent analysis of human diets in the United States has shown that both institutional (8,10,11) and self-chosen diets (9) provide considerably less than the 2 mg of copper thought to be the daily requirement of adults (12,13).

Although the results reported in this study were obtained with cholesterol-free diets, the observations may also apply to diets commonly consumed in the United States which are typically high in cholesterol. Both exogenous (dietary) and endogenous cholesterol contribute to body pools (21). Dietary cholesterol is transported to the liver by chylomicron remnants (25) and enters the hepatic pool, and copper nutrition may also influence cholesterol metabolism when diets are both high in cholesterol content and low in copper.

The results also demonstrate the necessity of adequate and well-defined amounts of dietary copper in studies of cholesterol metabolism in both laboratory animals and people.
REFERENCES

19. ANON., In: Respiration and Circulation, Committee on Biological Handbooks, Federation of American Societies for Experimental Biology (FASEB), Bethesda, Maryland, 1971, p. 381.