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David Bayard Milne for the Doctor of Philosophy
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SPECIES AND DIET

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Paul H. Weswig

Copper in blood and liver can be divided into several fractions. Copper in blood is about evenly divided between erythrocytes and plasma. Normal concentrations for most species range between one and two parts per million. Plasma copper is mainly a copper protein, ceruloplasmin and a loosely bound form, reacting directly with Na diethyldithiocarbamate (DR Cu). In livers of normal rats (ca. 18 ppm Cu) the distribution is: debris, 12.8%; mitochondria, 13.5%; microsomes, 17.9%; and soluble fraction, 54.8%. On the other hand, in cattle with higher liver copper levels (ca. 69.5 ppm) the distribution is: debris, 22.2%; mitochondria, 12.3%; microsomes, 14.6%; and soluble fraction, 50.9%. The distributions are influenced by dietary copper in rats fed rations containing from 1 ppm to 200 ppm Cu. Erythrocyte and DR Cu appeared to be directly related to the copper intake, with values ranging from 0.95 to 2.04 $\mu\text{g}/\text{ml}$ and 0.19 to 1.52 μg respectively. Ceruloplasmin values were depressed in the low ration (6.6 IU) and remained constant with Cu intakes greater than 10 ppm (35 IU).

Liver Cu levels were depressed in the low (1 ppm intake) group (10 ppm), "normal" in the range of 10-100 ppm intake (20 ppm) and

elevated with 200 ppm intake (69 ppm). The distribution in the liver fractions was affected by copper content, with the soluble fraction being the most sensitive to changes in liver copper. The debris increased relatively little in copper content.

The copper status of the rat greatly influenced the metabolism of injected ^{64}Cu . In all cases, radiocopper was removed rapidly from the plasma and concentrated in the liver. The incorporation of copper-64 in the liver was directly related to the copper status of the rat (low, "normal", high). There was a subsequent increase in blood activities and a reduction of liver copper after four hours in the low and "normal" groups. The increase in the blood radioactivity corresponded to an increase in ceruloplasmin activity in rats maintained on a low copper ration. The relative distribution of radioactivity in the liver fractions of "normal" rats at all time intervals was as follows: soluble > debris > mitochondria > microsomes. There were significant deviations in rats on low or high copper rations.

Pathways for the movement of copper in the blood and liver in the various species are discussed.

Metabolism of Copper in Blood and Liver
As Influenced By Species and Diet

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Professor of Agricultural Chemistry
in charge of major



Chairman of Chemistry Department



Dean of Graduate School

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Typed by Virginia Milne for David Bayard Milne

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Metabolism of Copper in Blood and Liver
As Influenced by Species and Diet

INTRODUCTION

Copper has been associated with plant and animal tissue for over a century (102, p. 48). Only within the last 40 years copper has been shown to be essential for the normal functioning and well-being of many animals.

Underwood (102, p. 2) defined an essential element as follows:

1. Repeated and significant responses in growth or health to dietary supplements of the element.
2. Development of a deficiency state on diets otherwise adequate and satisfactory, i.e., containing all other known dietary essentials in adequate amounts and proportions and free from toxic properties.
3. With some elements the deficiency state has been further correlated with the existence of subnormal levels of the element in the blood or organs of the deficient animals.

Several workers have demonstrated responses to dietary supplements of copper in different species. In early investigations on the role of copper in preventing anemia in rats on milk diets, Schultze, Elvehjem and Hart (87) demonstrated responses in both growth and hemoglobin production with added copper. More recently Mills and Murray (63) in studies with a semi-purified solid ration showed that 50 μ g of copper per gram produced a significant growth response in rats. Significant growth responses have also been demonstrated in cattle (25, 65), chickens (23), swine (98) and sheep (102, p. 85).

The symptoms of copper deficiency are varied. The most common is impaired iron utilization and low hemoglobin values, as copper is

involved somehow in hemoglobin synthesis. Different workers have shown that copper and iron fed together can help cure nutritional anemia in rats (36, p. 804-811), children (52, 80), dogs (30), chickens (37), swine (22; 58, p. 100-105) and cotton rats (Sigmondon hispidus texianus) (66).

It was noticed that anemia and hair depigmentation were related in both rats and cats (30). Both of these symptoms were corrected by adding copper to the diet. Depigmentation and anemia, which could be corrected by copper administration, have been observed in black and hooded rats (47), rabbits (92), cattle (102, p. 80), sheep (102, p. 80) and chickens (37). Smith (92) showed that in rabbits the greying of hair was a more sensitive index of copper deficiency than reduced hemoglobin values. In addition to dietary anemia, Teague and Carpenter (98) noted that in swine a bone disorder was produced which could be reversed by added dietary copper. In cattle and sheep a brittleness of bones and osteoporosis leading to fractures was noted (16). Bone deformities have also been observed in chicks and dogs (102, p. 75-76).

Cunningham (16) also recognized an ataxia associated with a degeneration of the myelin in the nervous system of sheep and cattle on low copper forages. As the ataxia develops, incoordinated movements of the hind limbs, a stiff staggering gait and a swaying of the hind quarters were observed in copper-deficient lambs. In calves the ataxia develops after exercise in the form of loss of muscular control of one or both hind limbs. This symptom has not been associated with copper deficiency in other species.

In most cases copper levels in the blood, liver and other tissues are influenced strongly by the diet (102, p. 50-66). After a discussion of some of the biochemical roles of copper and interrelationships with other elements, a study of the influence of species and diet on the levels and movement of copper in the blood and liver will be presented on a subcellular basis.

SOME BIOCHEMICAL FUNCTIONS OF COPPER

Copper in Hemoglobin Synthesis

One of the earliest known functions of copper is its role in hemoglobin synthesis and iron utilization. Hart (36), in his studies on iron metabolism, was the first to note that copper had a stimulatory effect on hemoglobin production in the rat. About the same time, Elden (20) showed that copper and iron fed to dogs had an unusually favorable effect on increasing hemoglobin production. Feeding iron alone (21), or copper alone (20), had only a moderate effect on increasing hemoglobin production, but in most cases the combination of the two produced favorable results.

Salts of several different elements were tried in place of copper for the prevention of nutritional anemia (47, 101, 105). In most cases copper was the only element studied that had the ability to supplement iron in the prevention of nutritional anemia in rats (74). Titus (100) claimed that manganese alone had almost the same effect as copper in hemoglobin synthesis, and that a manganese copper complex gave a quicker response from the standpoint of hemoglobin synthesis than either copper or manganese alone. This claim, however, was disproved by later workers (47, 101).

The function of copper in hemoglobin synthesis appeared to be connected with the incorporation of inorganic iron into the hemoglobin molecule (24). When hematin (as a source of "organic iron") was added to the diets of anemic rats, in the presence of copper, there was a partial cure of the anemia (21), but the regeneration of hemoglobin

was not as complete, or as fast as the regeneration obtained when ferric chloride was added to the diet. Josephs (45) showed that in rats copper increases the proportion of hemoglobin iron in the blood, but not the proportion of iron in the other tissues.

The portion of iron that is influenced by copper is the mobile portion of the non-hemoglobin iron (45). Gubler et al. (32) demonstrated in swine fed a low copper diet that besides the inability to utilize iron for hemoglobin synthesis, there was an impaired ability to absorb iron from the gastrointestinal tract and incomplete mobilization of iron from the tissues. Cohen et al. (13) presented evidence which indicated that the influence of copper on iron absorption was not due to the simultaneous administration of copper but was correlated with the copper already present in the tissues. Elvehjem (24), on the other hand, showed that iron fed in the absence of copper would not change the hemoglobin content of the blood, but would increase the stored iron in the liver. When iron was replaced by copper in the diet (24, 67), the iron already stored in the liver was used for hemoglobin synthesis.

Recently Osaki et al. (76) implicated ceruloplasmin¹, a copper protein in serum, in the utilization of iron for hemoglobin synthesis. They pointed out that ceruloplasmin oxidizes Fe^{++} more rapidly than any other substrate and that the ferroxidase activities of various sera correlated precisely with p-phenylenediamine oxidase activity. This led Osaki (76) to suggest that a biological role for ceruloplas-

¹To be discussed in detail later.

min in serum would be to promote the rate of iron saturation of transferrin, thus stimulating iron utilization.

Copper in Cytochrome Oxidase

It has been found that copper is present in cytochrome c oxidase and is necessary for activity. In early studies with anemic rats Cohen and Elvehjem (13) noted that in rats fed only milk there was a marked decrease in the A component of the cytochrome system. Also, the amount of oxidase in the liver was greatly reduced. When the rats were given copper and copper plus iron, both the A component and the oxidase activity were increased (13). Later Schultze (85, 86) showed that copper was necessary for cytochrome oxidase activity in the heart, liver and bone marrow of rats. Iron deficiency, on the other hand, where copper was fed, did not affect the cytochrome oxidase activity. The reduction of cytochrome c oxidase activity has also been connected with copper deficiency in swine (34, p. 535), cattle (65) and lambs (42, 64). Both Howell (42) and Mills (64) noted that both the copper content and cytochrome oxidase activity of the brains and livers of swayback lambs were greatly reduced.

Ever since the early work of Keilin and Hartree (48), copper has been associated with cytochrome oxidase. They rationalized that cytochrome oxidase could be a copper protein because: (a) copper salts were the only single metal salts known that could oxidize all of the components of cytochrome; (b) the addition of copper to nutrient media for yeast and for copper deficient rats increased the concentration of cytochrome a; and (c) the intensity of the indophenol reaction of

various tissue preparations correlated with their copper contents. Waino et al. (106) established that there was a good correlation between the copper content, the heme content and the activity of cytochrome oxidase in a purified preparation. It was therefore concluded that the cytochrome c oxidase is a copper enzyme which contains a heme and a lipid.

Vander Wende (103, 104) showed that copper was tightly bound to the protein and could be removed only by dialysis against strong copper chelating agents. Recently Nair and Mason (68) confirmed that copper was necessary for cytochrome oxidase activity. They were able to remove more than half of the copper with bathocuproine disulfide under anaerobic conditions in the presence of reduced cytochrome c. The copper depleted enzyme was relatively inactive. However, upon replacement of the copper with Cu^+ -acetonitrile, the original activity was regained.

It was suggested by Lemburg (51) that copper could play a role in the biosynthesis of the heme a moiety of the cytochrome oxidase, since the heme a content of heart muscle from copper-deficient swine was depressed severely. Matrone (61) noticed that there was also a decrease in the heme a content of the livers from copper-deficient rats. In the bone marrow of the copper-deficient rats, where the hemoglobin is synthesized, the cytochrome oxidase activity was depressed. It is possible that if copper plays a role in synthesizing hemes, both the cytochrome oxidase and hemoglobin would be influenced by the copper status of the animal. To substantiate this, Gallagher (28) pointed out that in rats where the copper intake was limited there was

a reduction of the rate of protoheme synthesis along with the other symptoms of copper deficiency.

Copper and Elastin Synthesis

Copper deficiency in chickens and swine can result in death due to aortic rupture (15, 73). Hunt and Carlton (43) showed in copper-deficient rabbits an elastin defect consisting of loss of elastin fibers and calcification and fragmentation of the internal elastic membrane in muscular and elastic arteries. Savage et al. (83) studied copper deficiency in turkey poults. Histological sections of aortas from copper-deficient turkeys showed a fragmentation of elastic lamellae and an accumulation of non-elastin material between the lamallae.

Starcher, Hill and Matrone (95) raised chicks on diets containing less than one part per million copper and chicks on a control ration, with a 25 ppm copper supplement. In both cases the elastin content of the aortas increased with age. However, the elastin content of the aortas from copper-deficient chicks increased more slowly. After 49 days, the elastin content of aortas from control chicks was 12%, wet weight, while the elastin content of aortas from copper-deficient chicks was only 8.1%. The addition of copper to the diets of 27-day old chicks resulted in an increase in aortic elastin concentration to that of the control chicks after the sixteenth day of supplementation.

O'Dell et al. (72) investigated the effect of copper deficiency upon the composition of aortic tissue of chicks. After four weeks on

copper-deficient and control (50 ppm copper) diets, the aortas of the chicks were fractionated into soluble collagen, insoluble collagen, and elastin. These fractions were then analyzed for nitrogen, hydroxyproline, and hexoseamine. The nitrogen content, as percent wet weight, of the saline extract was about twice as high in the copper-deficient aortas as that in the controls. Also, hydroxyproline and hexoseamine concentrations were significantly higher in the extracts of copper-deficient aortas. These increases were not entirely explained by an increase in soluble collagen, so the authors (72) concluded that there must be an increased concentration of an unidentified soluble protein. The percentage of nitrogen removed by autoclaving and by sodium hydroxide extraction was somewhat greater in the deficient aortas, but it was not collagen. The control aortas contained 47% elastin (dry weight basis), and the deficient about 26%.

Amino acid analysis of the elastin from control chicks and copper-deficient chicks (62, 72, 95) showed that the elastin from copper-deficient chicks had a much higher lysine content, and a lowered desmosine and isodesmosine content.

Desmosine and isodesmosine are structural isomers (62) composed of a pyridinium ring with four side chains located at positions 1, 3, 4 and 5 (desmosine) and positions 1, 2, 3 and 5 (isodesmosine). Each side chain has a carboxyl terminal and an α amine terminal group. It has been proposed that either isomer could serve as a cross link between as many as four polypeptide chains.

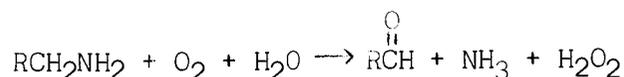
Miller et al. (62) studied factors influencing the formation of aortic elastin cross links (desmosine and isodesmosine) in chicks in

vivo and in isolated embryonic chick aorta in tissue culture. They found a marked increase in the lysine and a decrease in the desmosines in the alkali insoluble elastin from copper-deficient chicks. They suggested the presence of an unidentified intermediate in normal elastin whose synthesis is copper dependent. In tissue culture, the incorporation of lysine-¹⁴C into desmosine and isodesmosine was inhibited by β aminopropionitrile and by penacillamine (62). These agents may prevent the formation or condensation of the intermediates which normally produce the cross links, since it is believed that the above agents chelate copper and hence make it unavailable.

Hill and co-workers (38) found that the amine oxidase activity of chick aorta is negligible at hatching and if the diet contains copper, amine oxidase activity would appear within three days. However, if there was no copper in the ration the amine oxidase remained undetectable. Using ¹⁴C-labeled lysine they were able to show that the incorporation of the label into desmosine was increased by the addition of amine oxidase to the preparation. From these results Hill (38, 49) implicated amine oxidase as one of the biochemical mechanisms in which lysine is converted to the desmosine.

Copper in Amine Oxidase

As noted above, copper deficiency in the chick (38, 49) and in the pig (6) results in reduced or undetectable amounts of plasma amine oxidase. Amine oxidases are widely distributed in plants, animals and bacteria (69). This group of enzymes catalyzes the oxidation of primary amines to aldehydes as follows:



Most of the amine oxidases purified from various sources contain copper (69). In both plasma (108) and beef liver mitochondrial amine oxidase (69), the specific activity of the preparation was directly related to the copper content. Yamada (108) was able to remove the copper from plasma amine oxidase by dialysis against sodium diethylthiocarbamate in acidic buffer. The inactive enzyme then could be reactivated by dialysis against cupric copper (69, 108).

Role of Copper in Pigmentation

As mentioned previously, one of the symptoms of copper deficiency can be a decoloration of the hair coat. Thus, Flesch (26) suggested that copper plays an important role in mammalian pigmentation. He showed that in vitro, the oxidation of dopa, a precursor of melanin, was strongly catalyzed by cupric ions. This reaction was catalyzed more strongly by cupric ions than by any other heavy metal. Flesch (26) also noted that the black and grey hair of rats, guinea pigs and rabbits generally, but not always, contained more copper than the white hair from the same animals. Idris (44, p. 78-86) demonstrated that there was a relationship between copper status, pigmentation and copper content of the hair of different strains of rats and hamsters.

Tyrosinase, or polyphenol oxidase as it is sometimes called, a copper-containing enzyme, was found to play a role in the conversion of tyrosine to the melanin pigments (56, p. 58-60). Tyrosine is oxidized to dopa, which is then oxidized to the corresponding quinone.

This quinone is rearranged and oxidized to **hallachrome** which is then converted to one of the melanin pigments (56, p. 58-60). It may be noted that in all of the oxidations of **this enzyme, tyrosinase** behaves as a **terminal oxidase** as it utilizes **molecular oxygen**.

Therefore, it was suggested (56, p. 58-60) that this system could be compared with the cytochrome oxidase system.

INTERACTIONS WITH OTHER METALS

The presence of excessive amounts of some other elements such as molybdenum and zinc can severely alter normal copper metabolism. In most cases high levels of molybdenum in the diet will produce clinical symptoms of copper deficiency (102, p. 117), e.g. anemia, depression of growth, hair depigmentation and swayback in lambs, all of which could be alleviated by addition of copper to the ration.

The interactions between copper, molybdenum and sulfate are quite complex with respect to copper levels in the blood and liver of different species (102, p. 115-119). In rats (31), when copper stores were low on a copper-deficient diet, small amounts of molybdenum produced toxic symptoms which were intensified by simultaneous addition of sulfate. On the other hand, when copper stores and intake were adequate, larger amounts of molybdenum were required to produce molybdenosis, and sulfate completely prevented the harmful effects of molybdenum. Chronic copper poisoning was observed, with high liver copper levels in sheep, under conditions of moderate levels of copper intake and very low levels of molybdenum and sulfate (102, p. 115-119). On the other hand, depletion of the sheep's copper reserves to the point of copper deficiency has been reported (102, p. 115-119) on normal copper and high molybdenum and sulfate intakes.

High levels of zinc in the diet can induce anemia and depressed growth rates (102, p. 181). This is accompanied by high zinc levels in the tissues, subnormal copper concentration in blood and liver, low iron concentration in the liver and reduced levels of cytochrome oxidase and catalase in liver and heart. These symptoms can largely be

overcome by supplements of copper.

FRACTIONS OF COPPER IN THE BLOOD AND LIVER

BLOOD COPPER

The distribution of copper in the blood between the serum or plasma and erythrocytes varies somewhat between species. In humans the copper level of the serum is higher, with a value of 114 μg . of copper per 100 milliliters compared to 89 μg . per 100 milliliters of packed red blood cells (11). In cattle, this distribution is about even for normal animals with whole blood and plasma levels at about 1.00 μg . of copper per milliliter (1). Differences were observed in cattle fed low copper forages and in cattle on forages with an unfavorable copper-molybdenum ratio (1). There is little information about the distribution of copper between the serum and erythrocytes for other species.

Serum Copper

Copper is present in serum in two different forms. A blue copper containing protein, ceruloplasmin, accounts for most of the copper (about 93% in humans) (11). The copper in ceruloplasmin is tightly bound and will not react directly with chelating agents like sodium diethyldithiocarbamate, without first destroying the molecule. The rest of the copper in the serum is loosely bound and will react directly with sodium diethyldithiocarbamate and is therefore termed "direct reacting" copper (33). The direct reacting fraction accounts for about 7% of the serum copper in man (11), 1% in rats and 42% in swine (33). It is believed that this loosely bound copper is the form

in which copper is transported in the blood, while the ceruloplasmin is an oxidase enzyme (11).

Ceruloplasmin

Ceruloplasmin was first isolated and characterized by Holmberg and Laurell in 1948 (39). It is an α_2 globulin with a molecular weight of about 151,000. Human and porcine ceruloplasmin contain eight copper atoms per molecule while induced chick ceruloplasmin was found to contain only five copper atoms per mole (93, p. 37; 94).

This protein behaves as an oxidase enzyme with copper as the active group (40). It can oxidize readily the following types of compounds: aromatic polyamines and polyphenols, e.g. *p*-phenylenediamine; enediols, e.g. ascorbic acid; and several inorganics, e.g. Fe^{++} , $\text{Na}_2\text{S}_2\text{O}_4$, NH_2OH and $\text{K}_4\text{Fe}(\text{CN})_6$ (76, p. 560). Since ceruloplasmin is an oxidase with copper involved in the active site, its activity can be inhibited by KCN, NaN_3 , NaOCN, KSCN and sodium diethyldithiocarbamate.

The copper in ceruloplasmin can be removed by either treatment with urea (57) or strong chelating agents (57, 58), like BAL or EDTA. This has been described as an "all or none" process under these conditions (2, 58). Scheinberg and Morell (84) found that in vitro about half of the copper in ceruloplasmin is capable of exchanging with ^{64}Cu , but only when the molecule has been reduced with ascorbate. This was confirmed more recently by Marriott and Perkins (57), who found that six of the eight copper atoms of human ceruloplasmin are exchangeable with free cuprous ions if oxygen is excluded. However, no evidence has been obtained to indicate that this exchange occurs in

vivo (10, 96). Sternlieb et al. (96) indicated that in vivo ^{64}Cu is incorporated only at the time of synthesis of the ceruloplasmin molecule in humans.

It is interesting to note that in spite of the amount of work that has been done in the last twenty years and the amount learned about the ceruloplasmin molecule, the biological role of this protein still remains in question. Recently, however, Osaki et al. (75) suggested that since ceruloplasmin oxidizes Fe^{++} more rapidly than any other substrate, it could play a role in iron metabolism. They proposed that a biological role could be in promoting iron saturation of transferrin, thus stimulating iron utilization. However, more evidence must be obtained before a definite biological function is established.

Several species differences in both ceruloplasmin levels and properties have been noted (93, 94). Differences range from different copper contents, as mentioned above, to different electrophoretic mobilities (46, 60). Martin and co-workers (60) found that ceruloplasmin from different animal species differed in their electrophoretic mobilities in starch gel as follows: goat > sheep > rabbit > horse = cat > rat > monkey > human > pig. Species differences were also noted in the abilities to oxidize different substrates (29). Rat serum oxidized preferentially p-phenylenediamine, while sera from other species could oxidize both p-phenylenediamine and N,N dimethyl p-phenylenediamine, although at different rates (29). Seal (89) made a survey of ceruloplasmin levels, as p-phenylenediamine oxidase activity at pH 6.0, of 115 different species representing 38 orders from seven

vertebrate classes. He found very large species differences, with the pig having the most active serum, ranging down to undetectable amounts in sixteen different species.

Erythrocyte Copper

Most of the copper in human erythrocytes is contained in the copper protein, erythrocytin (91). The copper in this protein accounts for about 60% of the total red cell copper (11). Another copper-containing fraction has been separated from erythrocytin in red cells (91). This copper fraction is fairly labile and exchanges readily with the plasma while the copper in erythrocytin is more tightly bound. Recently Newmann and Silverberg (71) suggested that this more labile fraction is in the form of copper amino acid complexes.

Normal copper levels in erythrocytes have been presented for humans and swine. They are 89 and 110 micrograms of copper per 100 milliliters of packed red cells, respectively (11, 50).

LIVER COPPER

Beck (4) has shown that the normal liver levels of most species is within the range of ten to fifty parts per million on a dry basis, with a high proportion of these between fifteen and thirty parts per million. There are some species, including cows, sheep and domestic ducks (4; 102, p. 54-56) in which the liver copper levels are not confined to a narrowly defined "normal" range. In these species, levels of 100 to 400 parts per million may be encountered in normal animals. Beck (4) suggested that these species apparently have a different

mechanism regulating liver copper storage than those species with more narrowly defined normal copper levels.

Brinkman and co-workers (9) fractionated rat liver by differential centrifugation. They showed that the copper was distributed as follows: 15% of the total copper in debris; 20% in the mitochondria; 10% in the microsomes; and over 55% in the supernatant, or soluble fraction. Other workers (19, 99) have studied the distribution of metals in rat liver under slightly different conditions and have found essentially a similar relative distribution. However, there is little data available on the normal copper contents of liver fractions for other species.

Since information is scant with respect to normal distribution of copper in the above-mentioned fractions of blood and liver for different species, we decided to investigate the distribution in several laboratory animals and ruminants.

METHODOLOGY

Blood and liver samples were obtained from freshly sacrificed animals. The livers were frozen for subsequent analysis. Blood samples were immediately centrifuged and the plasma and erythrocytes separated. The erythrocyte volume was noted. The erythrocytes and plasma were then analyzed for copper. Direct reacting copper and ceruloplasmin were determined in the plasma as described below.

Copper Analysis

Copper was analyzed by a modification of the Eden and Green method (18) as follows:

The plasma or erythrocyte sample (three to ten milliliters) was treated with two milliliters of concentrated sulfuric acid and three milliliters of 60 percent perchloric acid. The mixture was heated in pyrex digestion tubes over a low flame until the organic matter was charred to finely divided particles in a freely boiling solution. When the mixture had cooled, five milliliters of concentrated nitric acid were added. If the nitric acid was added earlier considerable foaming occurred. The mixture was heated over a low flame until a colorless solution remained. Digestion was continued over a high flame until only the sulfuric acid remained in the solution and all of the excess nitric and perchloric acids had boiled off, as evidenced by the sulfuric acid refluxing on the sides of the tube.

When the digestion was complete, five milliliters of double glass distilled water and 2.5 milliliters of a 40 percent ammonium citrate

solution were added. The purpose of the ammonium citrate was to form a complex with the ferric ions present to prevent them from reacting with the color reagent. After the solution had cooled, ten milliliters of concentrated ammonium hydroxide were added to bring the pH to 8.5-9.0

The samples were then transferred to separatory funnels for the color development. Ten milliliters of a 0.1 percent solution of sodium diethyldithiocarbamate were added to each sample. This formed a yellow complex with the copper with an absorption maximum at 440 m μ . This complex was extracted with two ten-milliliter portions of redistilled carbon tetrachloride. The carbon tetrachloride layers were drawn off, filtered through anhydrous sodium sulfate to remove any water, and pooled. These pooled carbon tetrachloride layers were then brought to a final volume of twenty-five milliliters.

The optical density at 440 m μ , of the final solution, was determined against a reagent blank with a Coleman model 14 spectrophotometer. The corrected optical density values were used to compute the copper content of the sample with the following equation:

$$\text{copper in } \mu\text{g/ml.} = \frac{\text{corrected E} \times \text{K}}{\text{ml. of sample}}$$

where K is the calculated slope of the standard curve.

Direct Reacting Copper

Direct reacting copper was determined by a method of Gubler et al. (33) as follows:

One half of a milliliter of saturated sodium pyrophosphate and

2.5 milliliters of double glass distilled water were added to one milliliter of plasma. The optical density (E_1) at 440 $m\mu$ was measured. Two tenths of a milliliter of a 0.1 percent sodium diethyldithiocarbamate solution was added and the optical density (E_2) at 440 $m\mu$ was determined after five to ten minutes. The optical density (E_3) of a reagent blank containing 3.5 milliliters of double glass distilled water, 0.5 milliliters of sodium pyrophosphate solution and 0.2 milliliters of sodium diethyldithiocarbamate solution was determined. The amount of direct reacting copper was then calculated with the following equation:

$$\text{DR copper in } \mu\text{g/ml.} = (E_2 - (E_1 f + E_3)) \times K$$

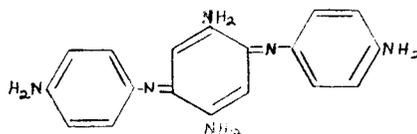
where $f = \frac{\text{volume before added carbamate}}{\text{volume after added carbamate}} = 0.952$ and K is a constant

derived from a standard curve.

Ceruloplasmin (p-phenylenediamine oxidase) Assay

Ceruloplasmin was assayed for as the p-phenylenediamine (PPD) oxidase activity as described by Houchin (41). One tenth of a milliliter of plasma was added to one milliliter of freshly prepared 0.1 percent PPD in acetate buffer (ionic strength 1.2, pH $5.2 \pm .05$) at 37°C. After fifteen minutes incubation the reaction was stopped by adding five milliliters of 0.02 percent sodium azide solution. The optical density was then measured at 525 $m\mu$ on a Coleman model 14 spectrophotometer against a reagent blank. The optical density E was converted to International Units (81) by the following equation: International Units = $E(349)$ where 349 is a factor converting the

absorbancy of the oxidation product to micromoles of Bandrowski's base formed per minute per liter of plasma under the above conditions.



Bandrowski's Base

Bandrowski's base is an oxidation product of para phenylenediamine with the same absorption spectrum as the ceruloplasmin oxidation product (81).

Liver Fractionation and Copper Determination

A small portion of the liver was reserved for copper analysis and about five grams were fractionated as follows:

The liver was homogenized in nine parts of a 0.25 M sucrose solution. This homogenate was centrifuged at 700 x g for ten minutes. The particles were resuspended in the sucrose solution and recentrifuged at 700 x g for another ten minutes. This fraction was termed debris. The combined supernatants were then centrifuged at 5,000 x g for ten minutes, the particles resuspended and centrifuged at 5,000 x g another ten minutes to obtain the mitochondria. The combined supernatants from this step were centrifuged at 75,000 x g for 30 minutes to obtain the microsomes. The supernatant from this step was termed either "supernatant" or the soluble fraction.

Portions of the intact liver, debris, mitochondria, microsomes and an aliquot of the supernatant fraction were analyzed for copper by

a procedure similar to the one mentioned above. The sample of liver, or liver fraction, was placed in a preweighed digestion tube. The wet weight was noted. Then the sample was dried to constant weight at one hundred degrees centigrade and the dry weight of the sample determined. Two milliliters of sulfuric acid, three milliliters of perchloric acid and five milliliters of nitric acid were added and the samples were digested as above. The above procedure was then followed for the digestion and color development. The copper content was calculated as follows:

$$\text{copper in } \mu\text{g/g.} = \frac{\text{E sample} \times K}{\text{sample weight}}$$

where K is the calculated slope of the standard curve. While the whole liver copper content was expressed as micrograms of copper per gram of liver, dry weight, the copper content of the fractions was expressed either as percent of the total or as micrograms of copper per fraction per gram of liver, wet weight.

RESULTS AND DISCUSSION

The distribution of copper in blood fractions for different laboratory animals, cattle and sheep are summarized in table one. Age and strain, as well as species, apparently influence this distribution.

Idris (44, p. 59-70) found differences in blood and liver copper levels between the Wistar albino rat and a Oregon State University brown strain of rats (resulting from crosses between Evans-Long, Sprague Dawley and hooded rats), maintained on the same commercial laboratory ration² with a copper content of 14.5 ppm (44, p. 39).

Results in table one tend to confirm this observation. Differences are noted in all fractions except the direct reacting copper. However, more work would have to be done with several different strains and cross-bred strains to determine if there are any real genetic influences on copper metabolism and on copper levels in blood and liver fractions.

In lambs four to eight weeks old there is no detectable direct reacting copper in the plasma. On the other hand, in six-month lambs and adult ewes about twenty-five percent of the plasma copper will react directly with sodium diethyldithiocarbamate. Ceruloplasmin values, however, are not significantly different between the ewes and lambs. One possible explanation could be that the young lambs are still on a predominantly milk diet and milk is generally a very poor source of copper (102, p. 66). As will be discussed in the next section, the direct reacting copper in rats appears to be directly

²Purina Laboratory Chow

TABLE I. Summary of Average Copper Levels in Blood Fractions for Different Species

Species: Strain or age	Rat		Cotton Rat	Hamster	Sheep		Cattle
	O.S.U. Brown	Albino	(<u>Sigmondon hispidus</u>)	(<u>Mesocricetus auratus</u>)	Adult	Four-Wk.	
Whole Blood Cu $\mu\text{g/ml}$	1.31	0.83	1.10	1.63	1.09	0.89	0.97
Erythrocyte Vol. ml/100 ml	48.0	47.3	52.8	45.4	43.0	35.6	46.7
Erythrocyte Cu $\mu\text{g/ml}$	1.22	0.90	1.37	1.60	1.10	1.01	0.97
Plasma Cu $\mu\text{g/ml}$	1.40	0.77	0.78	1.66	1.09	0.76	0.96
Direct Reacting Cu $\mu\text{g/ml}$	0.19	0.20	—	0.71	0.25	n.d.	0.18
Ceruloplasmin I. U.	39.4	47.1	—	5.9	39.5	37.0	24.4

related to copper intake. This observation could also apply to sheep in the above case. The ceruloplasmin levels would not necessarily differ between the lambs and ewes since lambs are born with high liver copper stores (102, p. 54) and, as Owen (79) indicated, ceruloplasmin is synthesized in the liver.

In most cases, blood values were near one $\mu\text{g}/\text{ml}$. copper. Hamsters had somewhat higher blood copper levels ($1.63 \mu\text{g}/\text{ml}$.) even though they were maintained on the same ration (14.5 ppm Cu) as the rats and cotton rats. These higher blood copper levels were reflected in both the erythrocytes and plasma. The hamsters were also somewhat different in that almost half of the plasma copper was in the loosely bound form and that the ceruloplasmin activities were much lower. In most of the species studied, with the exception of hamsters and lambs, the direct reacting copper was the same (about $0.20 \mu\text{g}/\text{ml}$.). This accounts for about 20 to 25 percent of the total plasma copper. In all cases, the level of direct reacting copper was highly variable and deviated approximately 50 percent.

As mentioned previously, animals can be placed into two classes with respect to liver copper. Most species have liver copper levels in the range of ten to thirty parts per million. These include common laboratory species with liver copper levels as follows: albino rat, 16.6 ppm liver copper (dry weight); brown rat, 18.3 ppm; cotton rat (Sigmondon hispidus), 17.3 ppm; and hamster (Mesocricetus auratus), 25.8 ppm. Again, it is worth mentioning that these animals were maintained on identical commercial stock rations. Some species, particularly ruminants, generally have higher and more variable liver copper

contents. Primary examples are cattle, with liver copper contents ranging from 24 to 485 parts per million, and sheep, with values of 74 to 1000 parts per million copper in the liver (102, p. 54-55), dry weight.

Table II illustrates the distribution of copper in livers from brown rats, representing the first class of animals, and from cattle, representing the second class of animals. In both cases, most of the copper is found in the soluble fraction. The only significant difference in these distributions is a higher percentage of copper appearing in the debris fraction of bovine liver.

TABLE II. Average Relative Distribution of Copper
In Rat and Bovine Liver Fractions

Whole Liver Cu (ppm dry wt.)	<u>Rat</u>	<u>Bovine</u>
	18.3	69.5
<u>Distribution</u>	%	%
debris	12.8	22.2
mitochondria	13.5	12.3
microsomes	17.9	14.6
soluble	55.8	50.9

The distribution of copper in bovine liver fractions was as highly variable as the whole liver copper levels as indicated in Table III. The relative amount of copper in the debris and mitochondria increased with increasing liver copper levels, while the relative amount of copper in the soluble fraction decreased. Apparently the particulate fractions in bovine liver have an increased ability to

bind copper. This might explain why cattle and sheep can maintain constant blood copper levels while the concentration of copper in the liver can change by a factor of ten.

TABLE III. Effect of Liver Copper Levels on the Distribution of Copper in Bovine Liver Fractions

Whole Liver Cu (ppm dry wt.)	<u>24.1</u>	<u>45.7</u>	<u>139</u>
<u>Distribution</u>	%	%	%
debris	16.1	21.7	28.8
mitochondria	6.7	14.2	15.9
microsomes	12.9	16.9	13.9
soluble	64.3	47.2	41.4

INFLUENCE OF DIETARY COPPER
ON COPPER-CONTAINING FRACTIONS IN THE BLOOD AND LIVER

The blood and liver copper levels are strongly influenced by the diet. When albino rats were fed abnormally high levels of copper (8), the blood copper was raised to a maximum of two to five times the normal values, while the liver levels increased up to 300 times normal concentration. Similar effects on feeding large amounts of copper to cotton rats (Sigmondon hispidus texianus) were observed (66). The blood values were increased to about twice the normal levels and the liver copper concentration was from ten to a hundred times normal. This phenomenon has also been recognized in cattle (97), sheep (90) and swine (27).

The opposite effect was noted in animals receiving diets deficient in copper. Dempsey and co-workers (17) showed that when adult rats were restricted in their dietary intakes of copper, there was a rapid decrease in the liver copper. When these rats were fed copper there was a subsequent increase in both serum and liver copper concentrations. Schultze (88) and Lahey (50) observed decreases in the blood copper levels of pigs placed on a copper-deficient diet. Again, when copper was fed there was a subsequent increase in the blood copper levels. Wintrobe et al. (107) noted that in most cases the red cell copper remained essentially constant while the plasma copper varied somewhat more. However, erythrocyte copper levels were depressed in copper deficiency (107). In copper-deficient sheep, Starcher and Hill (94) observed a reduction in the ceruloplasmin levels as well as the total serum copper.

Since little has been reported to the effects of dietary copper on the various fractions of the blood and liver, the following experiment was designed.

METHODOLOGY

Design

Fifty 21-day old rats were randomized according to the following procedure:

1. Only litters with five or more pups of the same sex were used.
2. Five littermates of the same sex from each of ten litters were distributed at random between five groups.
3. Twenty-five females and 25 males were used.

Diets

The five groups of rats were fed a basal ration supplemented with different amounts of copper. The composition of the basal ration (Table IV), a modification of a ration used by Mills and Murray (63), was as follows: 20 percent casein, 65 percent Cerelose, ten percent Wesson oil, four percent mineral mix (Table V) and one percent vitamin mix (Table VI). Also included in the basal ration were vitamin A acetate, four milligrams per kilo, vitamin D, 200 micrograms per kilo and α tocopherol, 0.2 grams per kilo of diet. The basal ration was analyzed for copper by the Eden and Green method (18) as described earlier. It was found that this ration contained approximately 1.0 μg of copper per gram. This diet was supplemented with various amounts of copper, in the form of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, as follows: group I, no supplement; group II, 0.0392 grams per kilo; group III, 0.1960 grams per kilo; group IV, 0.392 grams per kilo; and group V, 0.784 grams of copper sulfate per kilo. This would bring the copper contents of the

TABLE IV. Basal Ration

	<u>g/Kg diet</u>
Casein	200
Cerelose	650
Wesson Oil	100
Mineral mix	40
Vitamin mix	10
Vitamin A	4 mg.
Vitamin D	200 γ
α tocopherol	0.2 g.

TABLE V. Mineral Mix

	<u>grams</u>
NaCl	292.5
KH_2PO_4	816.6
MgSO_4	120.3
CaCO_3	800.8
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	56.6
KI	1.66
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	8.45
ZnCl_2	0.5452
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.0476

TABLE VI. Vitamin Mix

Thiamine-HCl	40 mg
Riboflavin	25 mg
Pyridoxin	20 mg
Ca-D-pantothenate	200 mg
Choline Chloride	10 g
Niacin	1 g
Menadione	10 mg
Folic acid	20 mg
Biotin	10 mg
Vit B ₁₂ (1 %)	1 g
	<hr/>
	12.325 g
Lactose	<u>87.675 g</u>
	100 g

different rations to approximately 1.0, 10, 50, 100 and 200 parts per million respectively.

Methods

The rats were maintained on the above rations for eight weeks, during which growth rates and feed intakes were recorded. At the end of eight weeks the rats were anesthetized with CO₂ and most of the blood was removed by a heart puncture. Livers were removed, blotted, weighed and frozen for subsequent analyses. Liver, red blood cell and plasma copper, direct reacting copper, and ceruloplasmin activities were determined as described earlier. Three livers were picked at random from each of the five groups, fractionated and analyzed for copper as described in the previous section.

RESULTS AND DISCUSSION

The growth rates were influenced by the level of copper in the ration as indicated in Figure 1. The rats on the unsupplemented diet had better growth rates than any of the rats in the supplemented groups. This was apparently due to a decreased feed consumption as the copper levels increased (Table VII). As would be expected, the male rats in most of the groups grew faster and larger than the females. The only exception was in the group of rats receiving 200 ppm copper, where there were no differences in eight-week gains between males and females.

TABLE VII. Growth and Feed Intakes
As Influenced by Copper Supplement

<u>group</u>		<u>copper supplement</u>	<u>eight week weight gain</u>	<u>average daily food intake</u>
		ppm	g	g
I	M	0	192	12.7
	F		153	12.2
II	M	10	189	11.5
	F		143	10.7
III	M	50	134	9.1
	F		124	9.3
IV	M	100	85	7.0
	F		74	6.6
V	M	200	61	5.9
	F		62	6.1
LSD 5%			20.1	0.4

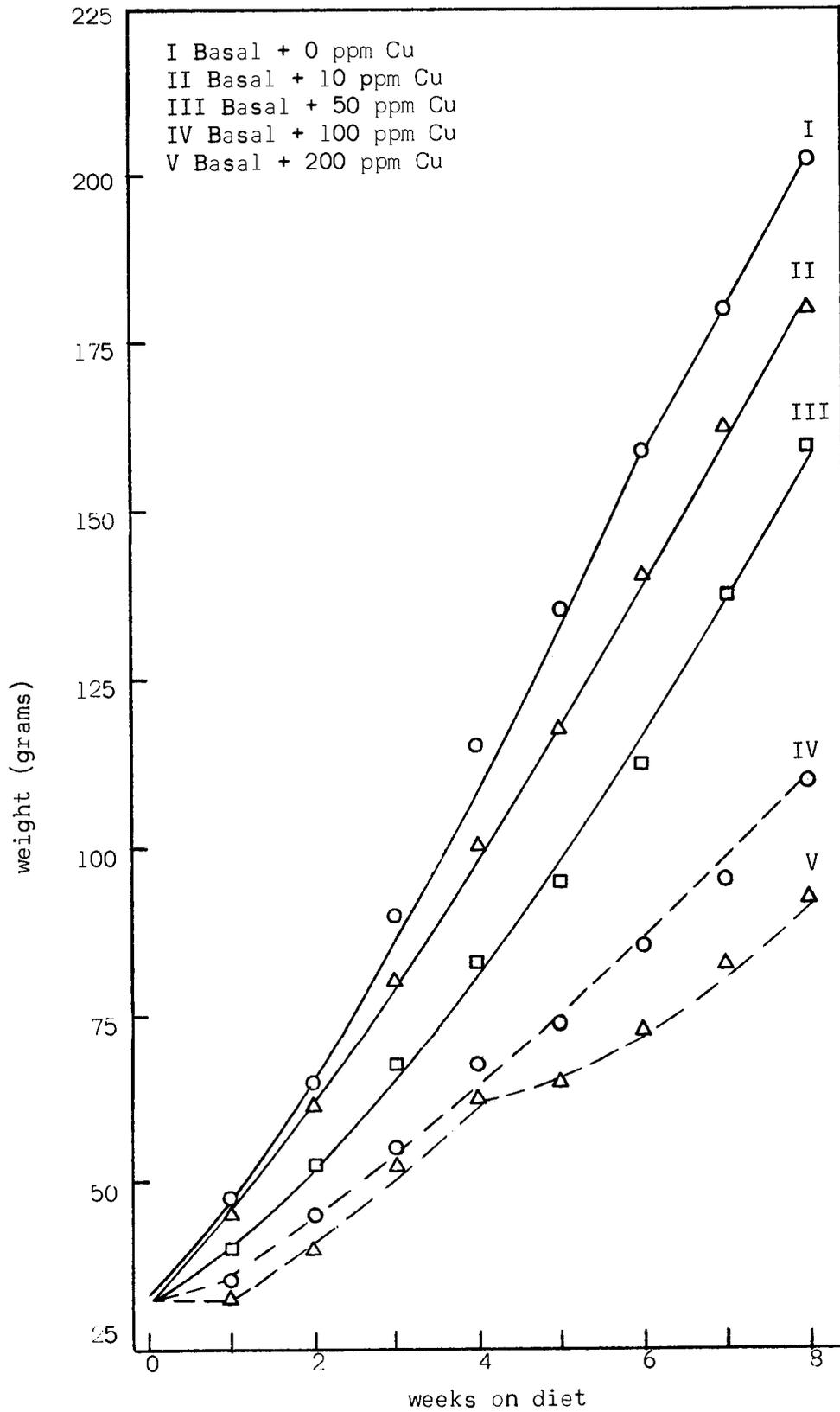


Figure 1. Growth of Rats on Different Copper Supplements

All of the rats that had two hundred parts per million in their ration suffered from severe diarrhea, were unthrifty and most of them appeared highly nervous. During the first week on the high copper diet three rats died, apparently because they refused to eat. There were no mortalities in the other groups. The only gross symptom of copper deficiency observed in the rats receiving one part per million copper was a depigmentation of the hair coat (Figure 2). The low level of copper, however, did not affect growth or the rats' ability to reproduce under the conditions of this experiment.

In a separate experiment to determine the effect of low levels of copper on the rats' ability to reproduce, four 16-week old female rats maintained on a low copper (one part per million) ration since weaning were used. Two were mated with a male rat raised on the same ration. The other two were mated with a proven sire taken from the stock colony. One of the females that was mated with the proven sire did not become pregnant. The other became pregnant and bore six young. Two days later all of the young were eaten by the mother. Both of the females mated with the "low copper" male became pregnant and bore young. One, a mother of five, ate her young in the first day after birth. The other has successfully raised her litter of four. The average litter size of normal stock animals is about eight, with about 70 percent of the females that are placed with the males mating. The cannibalism noted above is not normally observed in stock rats.

Effect of Dietary Copper on Blood Fractions

All of the blood fractions were influenced by dietary copper, as

Figure 2. Effect of Copper Supplementation on Pigmentation. Darker rat above received a 10 ppm Cu supplement while lighter rat received a one ppm dietary copper basal ration.

noted in Table VIII. The erythrocyte volume was depressed significantly in rats receiving 100 and 200 parts per million copper. This was due to some breakdown and hemolysis of the red blood cells. Underwood (102, p. 90-93) stated that hemolytic jaundice is one of the symptoms of copper toxicity. Copper levels in the erythrocytes reflected copper intakes, with the low copper rats having significantly lower erythrocyte copper levels and the rats receiving 100 and 200 ppm copper having significantly higher levels of copper in the erythrocytes.

Plasma copper levels increased with increasing amounts of dietary copper. The direct reacting copper seemed to directly reflect dietary levels better than any other fraction. This would indicate that the copper is transported from the intestine to the tissues in a more loosely bound form. Ceruloplasmin activities, on the other hand, were significantly depressed in the group receiving the unsupplemented ration. No significant differences from normal values were found in the ceruloplasmin activities of the four groups receiving copper supplements. Copper, then, is apparently necessary for inducing activity, but excessive amounts of copper will not inhibit ceruloplasmin as p-phenylenediamine oxidase activity in vivo.

Influence of Dietary Copper on Liver Copper

The influence of dietary copper intake on whole liver copper levels is shown in Figure 3. Rats receiving diets low in copper have significantly reduced liver copper stores. With supplementation, liver copper levels remain normal until intake of about 1000 μg of

TABLE VIII. Average Copper Content of Blood Fractions as Influenced by Dietary Copper

Group	I	II	III	IV	V	LSD	P
Dietary copper ppm	0	10	50	100	200		
Erythrocyte Volume ml/100 ml	49.6	46.4	47.5	41.8	39.6	5.44	<.01
Erythrocyte Copper $\mu\text{g/ml}$	0.95	1.63	1.45	1.89	2.04	0.37	<.05
*Plasma Copper $\mu\text{g/ml}$	0.62	1.13	1.16	2.34	—	—	—
Ceruloplasmin Activity I.U.	6.6	40.5	34.6	31.1	35.2	7.7	<.05
Direct Reacting Copper $\mu\text{g/ml}$	0.19	0.35	0.54	1.17	1.52	0.62	<.05

*Pooled samples

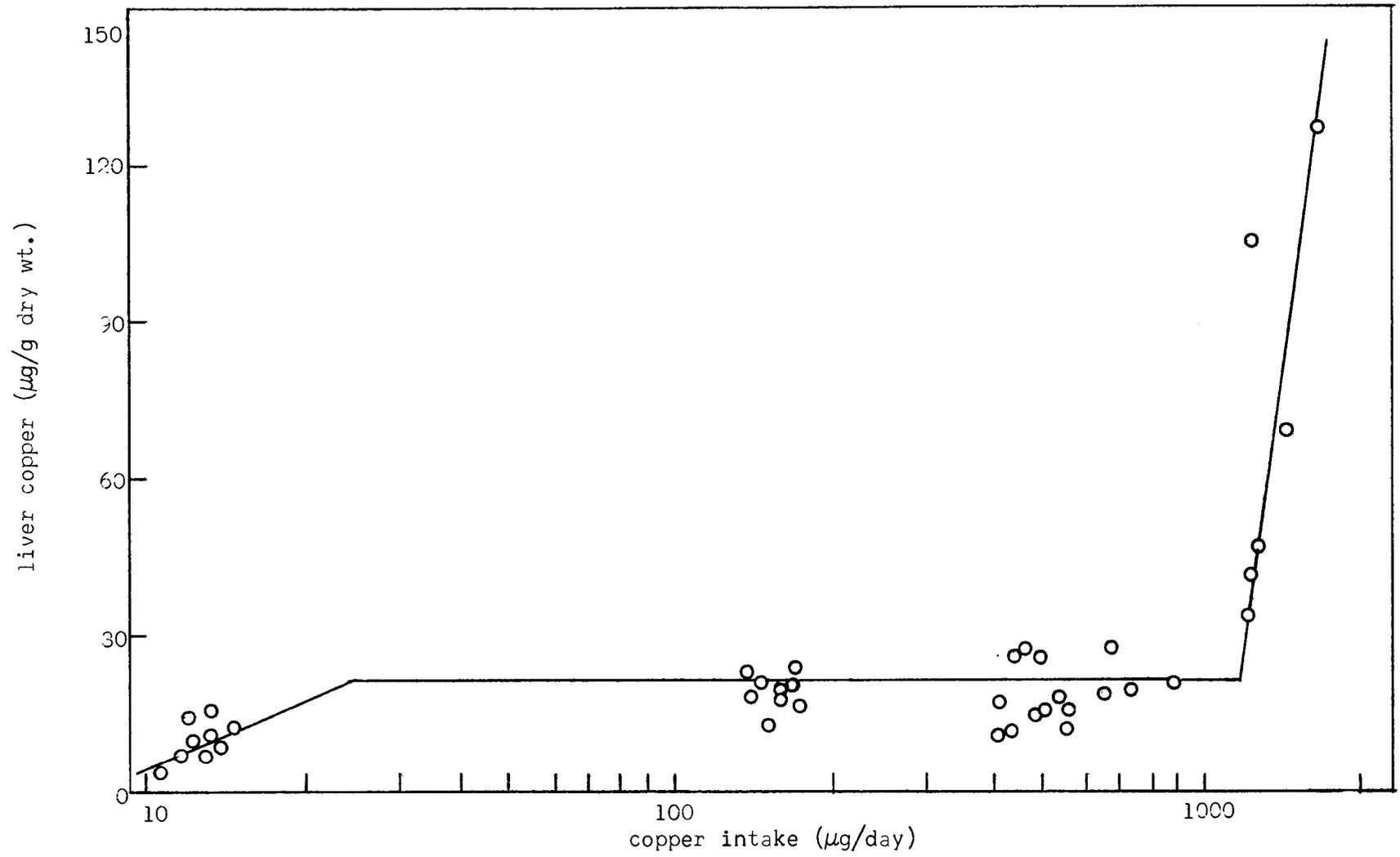


Figure 3. Influence of Copper Intake on Liver Copper

dietary copper per day is reached. This corresponds to approximately 200 parts per million copper in the ration (see Table VII). When this threshold is reached the liver stores of copper increase rapidly, suggesting an overloading of the excretory capacity of the rat. This threshold differs from species to species. Fifty parts per million of dietary copper is sufficient to elevate the liver stores in cotton rats (66), while this threshold is not reached until somewhere between 70 and 130 parts per million dietary copper in swine (5).

Cattle and sheep present an entirely different picture. In both cases the blood values reflect copper intakes only at subnormal levels (1, 55). When normal blood levels were reached, the liver copper contents ranged from 30 to 1000 parts per million in cattle with no significant change in blood copper values (1) and from about 50 to 4000 parts per million in sheep with little change in blood levels (55). Beck (5) suggested that these species differences were due mainly to differences in the excretory mechanism. However, it would be difficult to use this hypothesis to explain why the blood copper levels in ruminants remain relatively constant while liver stores change drastically. Apparently there must also be an increased ability to bind copper in the liver so as not to elevate blood levels.³

Table IX summarizes the average distribution of copper between liver fractions in the five groups of rats. The relative amount of copper in the soluble fraction and mitochondria remained essentially constant. The only apparent differences were a decrease in the

³ Refer to the previous section.

TABLE IX. Percent Distribution of Copper in Liver Fractions

Group	Copper Supplement	Whole Liver Cu ppm (dry)	Debris	Mitochondria	Microsomes	Supernatant
I	0	8.7	% 20.3	% 12.4	% 13.9	% 53.4
II	10	19.9	14.3	12.7	17.1	55.9
III	50	18.2	15.2	11.9	20.5	52.4
IV	100	19.7	14.1	15.1	18.1	52.7
V	200	68.5	14.3	11.0	23.8	50.8

relative amount in the debris with an increase in the relative amount of copper in the microsomes from the depleted group to the supplemented groups.

There was a very good correlation between the amount of copper in each fraction, per gram of liver, and the copper content of the liver (Figure 4). The soluble fraction by far was the most sensitive to changes in liver copper while the copper content of the debris fraction changed relatively little. Copper levels changed about the same in both the microsomes and mitochondria with liver copper concentration. It was apparent that most of the excess copper was stored in the soluble fraction and that there was an equilibrium between the copper in the soluble fraction and the copper in the mitochondria and microsomes.

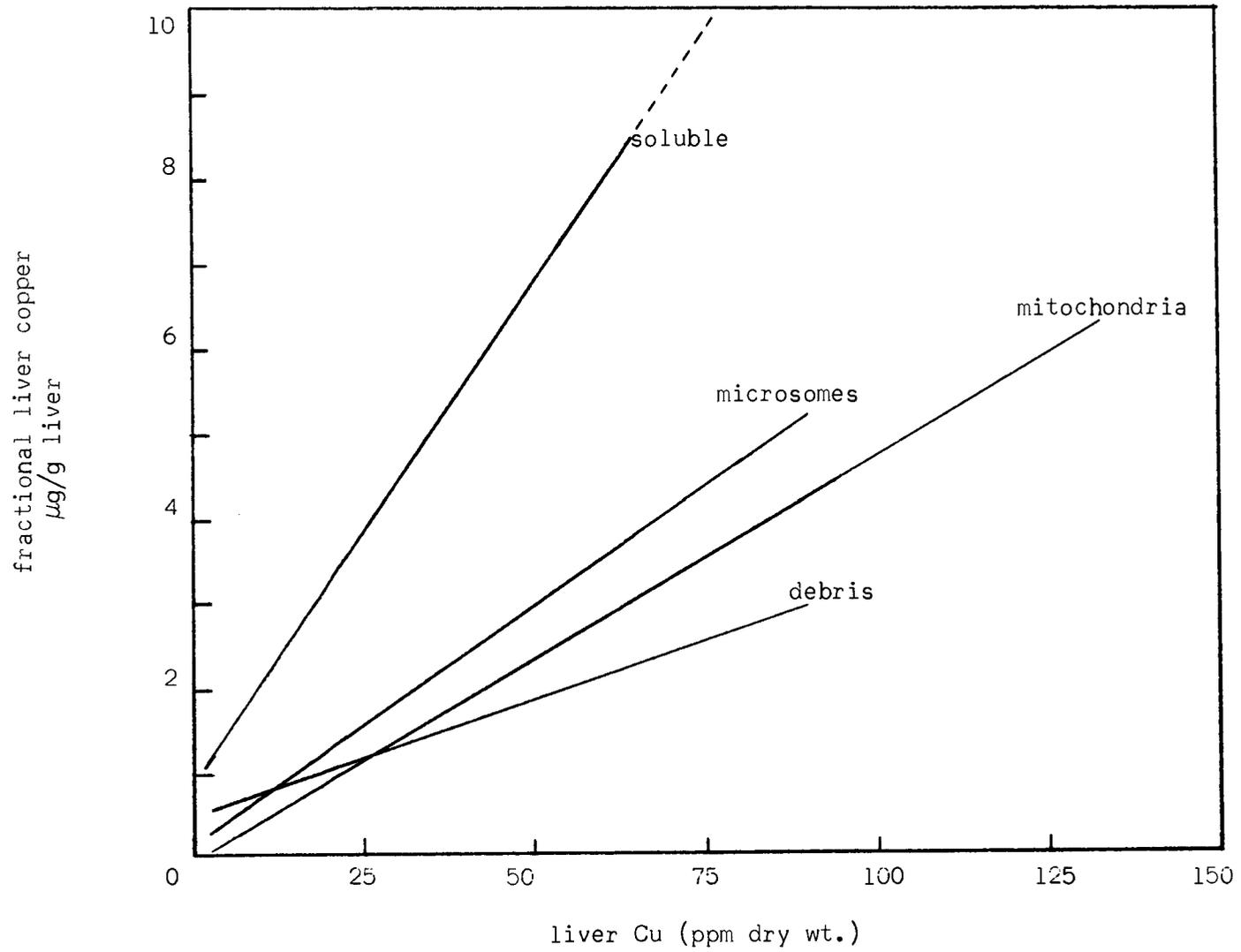


Figure 4. Relationship between Liver Copper and Copper in Fractions

INFLUENCE OF COPPER STATUS ON COPPER-64 METABOLISM

Owen (78) found that an intravenously injected dose of copper-64 acetate was transported rapidly to the liver, kidneys and intestine in rats. More than fifty percent of the dose appeared in the liver within four hours (78). In cattle, Comar et al. (14) showed that about 32 percent of an injected dose of radioactive copper reached the liver within a few hours. After the maximum was reached, the copper was slowly released from the liver: first it was returned into the plasma as ceruloplasmin and then it was principally excreted via the bile into the intestine and feces (77, 78).

In the species that have been studied, rats (77, 78), cattle (14), pigs (7) and rabbits (54), injected radiocopper was cleared rapidly from the plasma. Initially, most of it was associated with albumin (54), but later the proportion associated with ceruloplasmin and the globulin increased (7, 54, 78).

Bush and co-workers (10) found that radioactive copper was taken up rapidly by human and dog erythrocytes, both in vivo and in vitro. This was recently confirmed by Newmann and Silverberg (71), who found that a portion of the erythrocyte copper was in equilibrium with the direct reacting fraction of copper in the plasma.

Since dietary copper influenced the copper levels in the different fractions of the blood and liver, the question was raised concerning the influence of the copper status of the animal on the metabolism of injected copper. Also, there was no information showing how copper is metabolized by the subcellular fractions of the liver. The

following experiments were designed to answer these questions.

MATERIALS AND METHODS

Experiment A

Twelve 28-day old male rats were divided into three groups. They were fed the basal ration (Table IV), supplemented with different amounts of copper as follows: group I, no supplement; group II, 10 parts per million copper; group III, 200 parts per million copper. These levels represented low, normal, and toxic amounts of copper respectively. At the end of eight weeks approximately 50 microcuries of copper-64, as the acetate⁴, were injected into the tail vein of each rat. One rat from each group was sacrificed at one, two, four or eight hours after injection by CO₂ anesthesia and blood removal via heart puncture. The livers were removed, rinsed in physiological saline, blotted and weighed.

Counting and Half-Life Corrections

All samples obtained were counted in a Technical Associate well-type, thallium-activated, sodium iodide crystal scintillation counter. Since copper-64 has only a 12.9 hour half life, the time was noted when each sample was counted. The counting rates were then corrected back to the calculated time at which the injected dose would equal fifty microcuries for each rat.

⁴A sterile solution of ⁶⁴Cu acetate was obtained from Cambridge Nuclear Corporation.

Sample Preparation

Blood samples were centrifuged and the plasma was separated from the erythrocytes. The erythrocytes were then washed with physiological saline. Aliquots of whole blood, red blood cells and plasma were counted.

Ceruloplasmin ^{64}Cu and non-ceruloplasmin ^{64}Cu were separated on DEAE cellulose by a method described by Owen (78).

Three milliliters of plasma were dialyzed for eight to ten hours against 0.05 M acetate buffer, pH 5.5. The dialyzed plasma was then passed through an eight-millimeter by six-centimeter column of DEAE cellulose, which had been equilibrated with the acetate buffer. The sample was eluted from the column with the 0.05 M acetate buffer until the light brown band passed through and the eluant was clear. The ceruloplasmin was then eluted from the column with 0.5 M NaCl. Figure 5 shows a typical elution pattern of the radioactivity. Peak A represents the non-ceruloplasmin ^{64}Cu while peak B represents the ^{64}Cu associated with ceruloplasmin. Ceruloplasmin activities were also determined in each plasma sample.

A sample from each liver (0.1-0.3 gram) was counted prior to fractionation. The livers were then fractionated by the procedure described earlier. Aliquots of each fraction were then counted.

Experiment B

This experiment was designed because some of the results in the previous experiment appeared anomalous and data for a shorter time

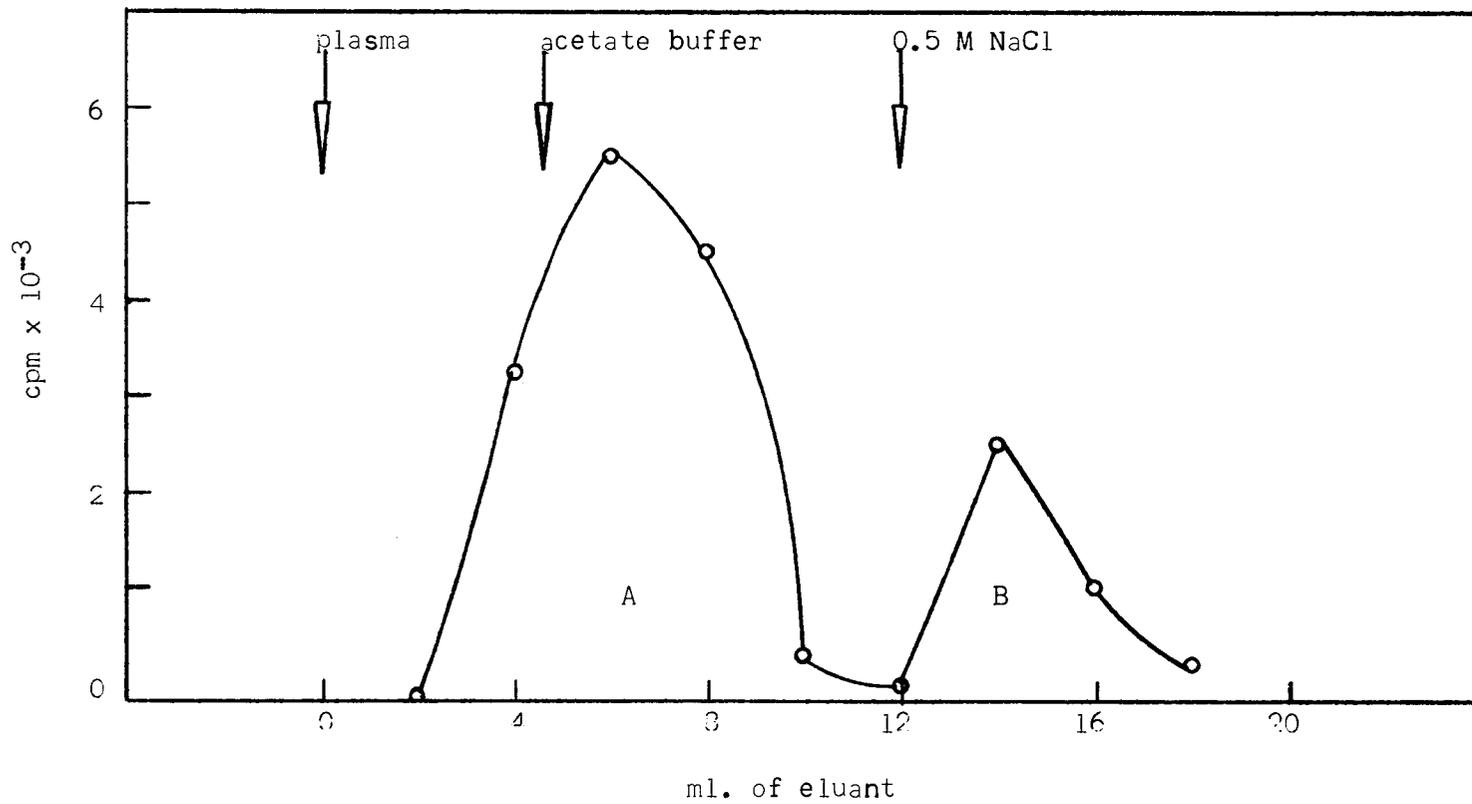


Figure 5. Elution of Plasma-⁶⁴Cu from DEAE Cellulose

interval was desired. The experiment above was repeated with the following changes:

1. Because of a delay in obtaining the radioactive copper, the rats were maintained on the rations for nine weeks instead of eight.

2. Ten milligrams of copper wire were irradiated in the Oregon State University reactor to obtain copper-64. This wire was dissolved in a drop of nitric acid and a milliliter of glacial acetic acid was added. The mixture was heated until the brown NO_2 fumes were driven off. The solution was then neutralized with sodium hydroxide and made up to ten milliliters. Two tenths of a milliliter, corresponding to $200 \mu\text{g}$ of Cu^{++} , was injected into the tail vein of each rat. At the time of injection, the dose per rat was about twenty microcuries. However, standards of experiment A and experiment B were corrected to a fifty microcurie basis for purposes of comparison.

3. One rat from each group was sacrificed 30 minutes after injection. Also, one rat each from group II and group III and two rats from the unsupplemented group were sacrificed four hours after injection.

4. An aliquot of the liver soluble fraction was counted after dialysis overnight against double glass distilled water.

It was found from the four-hour group of rats that the two experiments were equivalent since the distributions and corrected counting rates did not appear significantly different.

RESULTS AND DISCUSSION

In many aspects the copper status of the rat influenced the metabolism of copper-64 during the first eight hours after injection. In all cases, the radiocopper was removed rapidly from the plasma in the first four hours (Figure 6). Most of the dose was accumulated in the liver (Figure 7), with the larger proportion appearing in the livers of rats maintained on the 200 ppm copper ration (Figure 8).

After four hours there was a subsequent increase of copper in the plasma in rats maintained on unsupplemented and ten ppm copper-supplemented rations. This corresponded to a definite increase in the ceruloplasmin activity in rats that were maintained on the unsupplemented ration (Table X).

TABLE X. Effect of Time and Diet on Ceruloplasmin Activity

<u>Group/Time (H)</u>	<u>1</u>	<u>2</u>	<u>4</u>	<u>8</u>
I (0) I.U.	0	2.1	0	23.1
II (10) I.U.	32.1	31.2	38.4	37.2
III (200) I.U.	37.9	14.3	29.3	18.7

In order to determine the validity of the apparent increase of activity in the rat receiving no copper supplement at eight hours after injection, the following experiment was run.

One hundred micrograms of copper, as copper acetate, in 0.1 milliliter aqueous solution, was injected into the tail vein of each of two male rats and two female rats maintained on the unsupplemented ration.

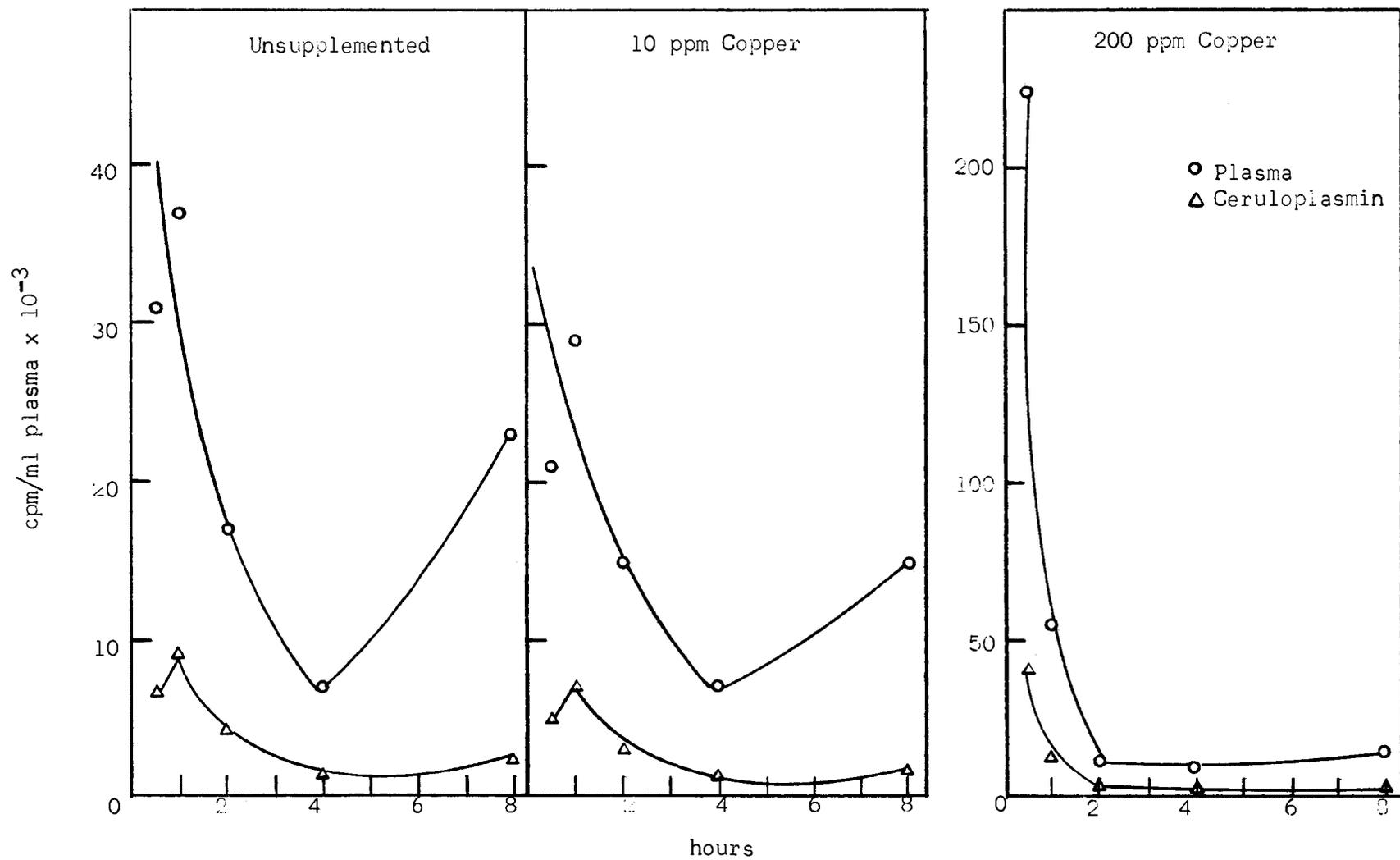


Figure 6. Changes of Plasma and Ceruloplasmin- ^{64}Cu Activity with Time

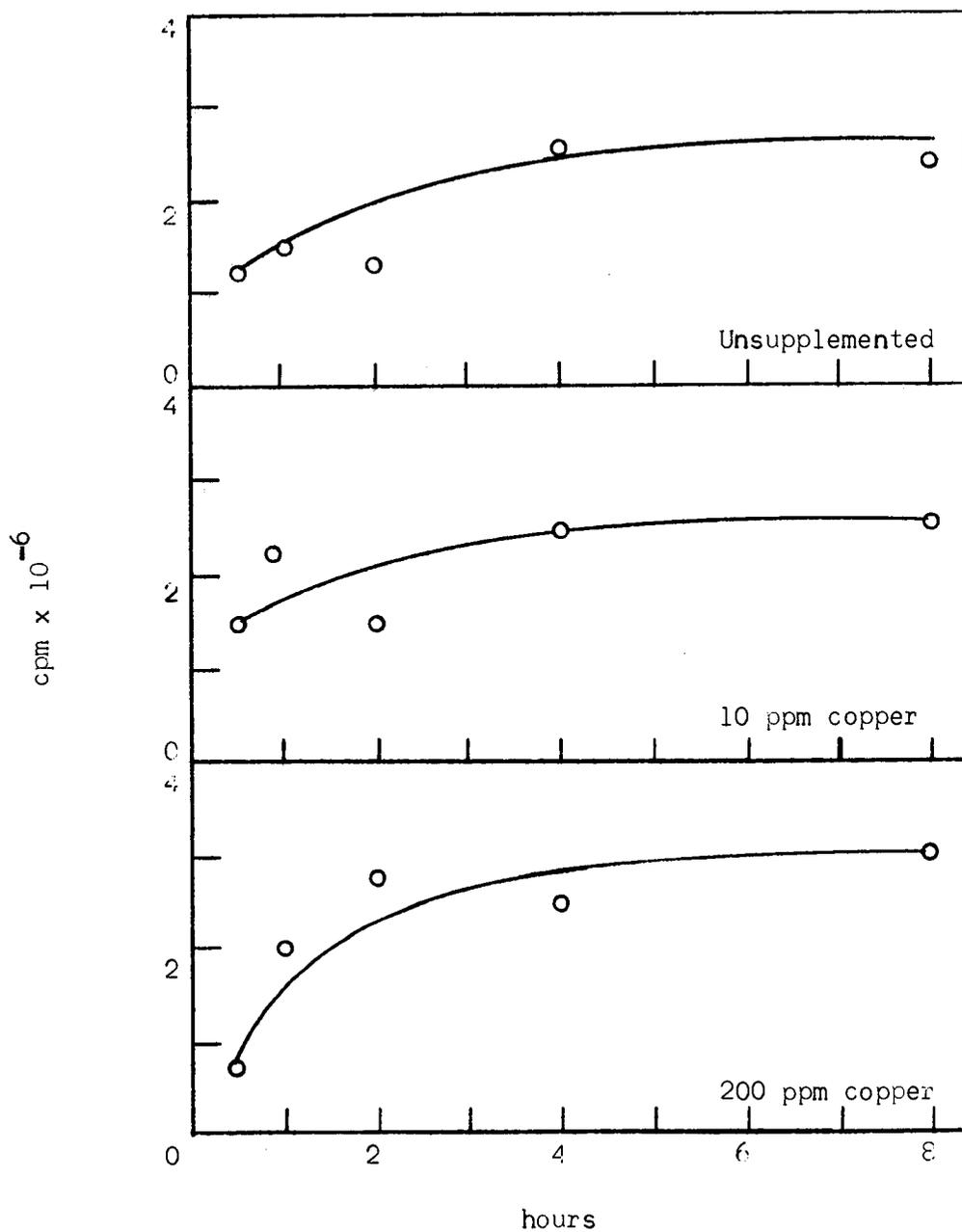


Figure 7. Changes in Liver Copper-64 Activity with Time

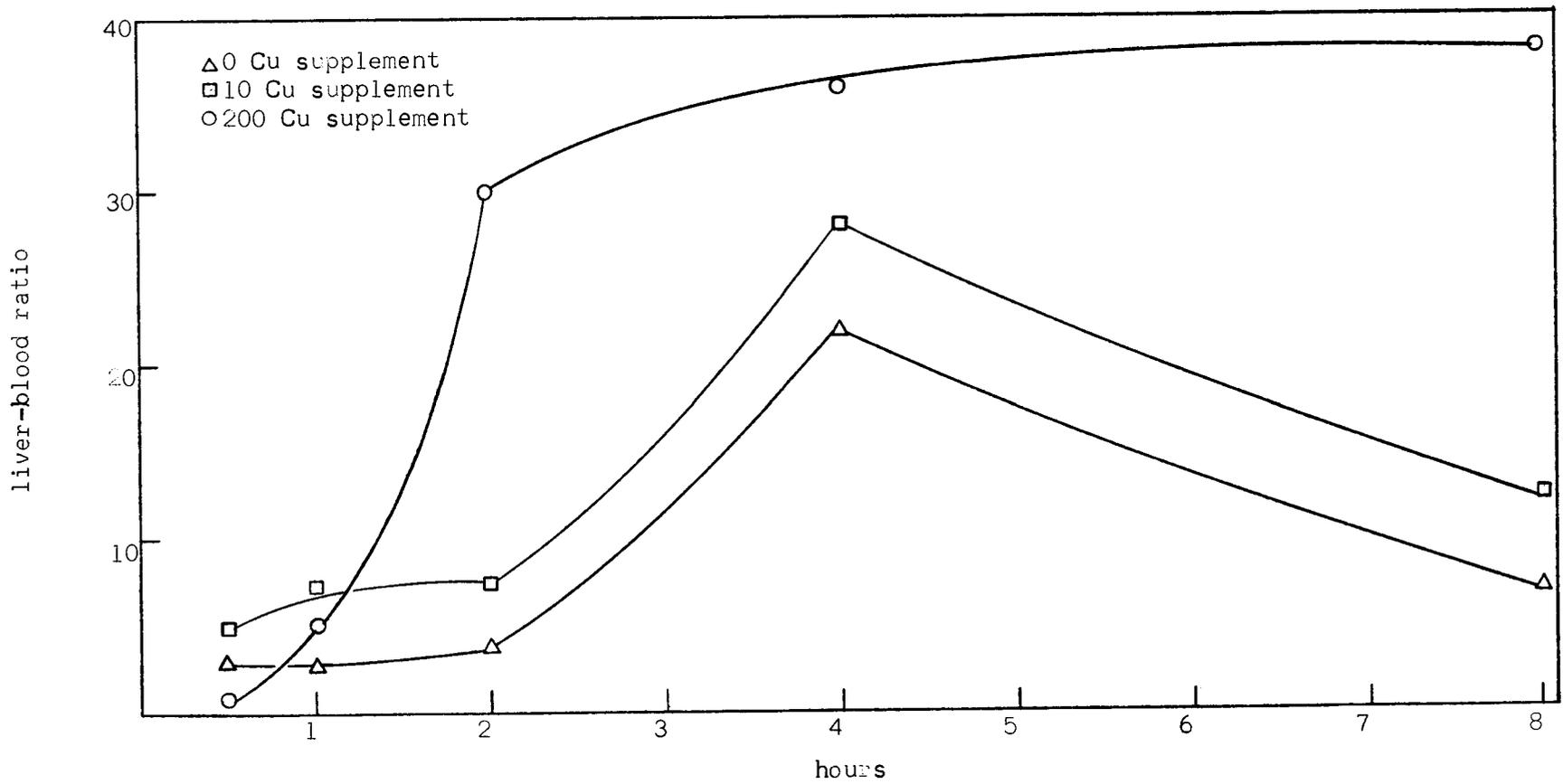


Figure 8. Ratio of Liver to Blood ^{64}Cu with Time

Two controls, one male and one female, were injected with 0.1 milliliter of double glass distilled water.

One milliliter of blood was removed from each rat via heart puncture prior to injection and the ceruloplasmin activity in the plasma was determined. After 8, 24, 48, 72 and 96 hours, blood samples were removed from each rat for the determination of ceruloplasmin activities.

As seen in Figure 9, there was a dramatic increase of the ceruloplasmin activities in the rats injected with copper. The control rats showed essentially no change in activity. The activity in general appeared more greatly influenced in the injected female rats than in the injected males. This could be explained by the fact that the males were larger than the females (275 g. versus 230 g.). Thus, the dose in micrograms per kilo body weight would be smaller (364 versus 435 $\mu\text{g}/\text{K}$). Also, if the same amount of ceruloplasmin were produced, the apparent activity would be diluted in the larger blood volume of the males.

Hemoglobin levels were not affected by the injected copper. Osaki (75) suggested that ceruloplasmin could play a role in hemoglobin synthesis. However, these results do not tend to confirm this hypothesis. The hemoglobin values, in all groups, dropped from 13.5 ± 1.5 mg/100 ml. blood at the time of injection to 9.0 ± 0.3 mg/100 ml. of blood after 48 hours. This decrease was probably due to repeated bleeding. No differences were observed between the control or injected groups at any of the time intervals observed.

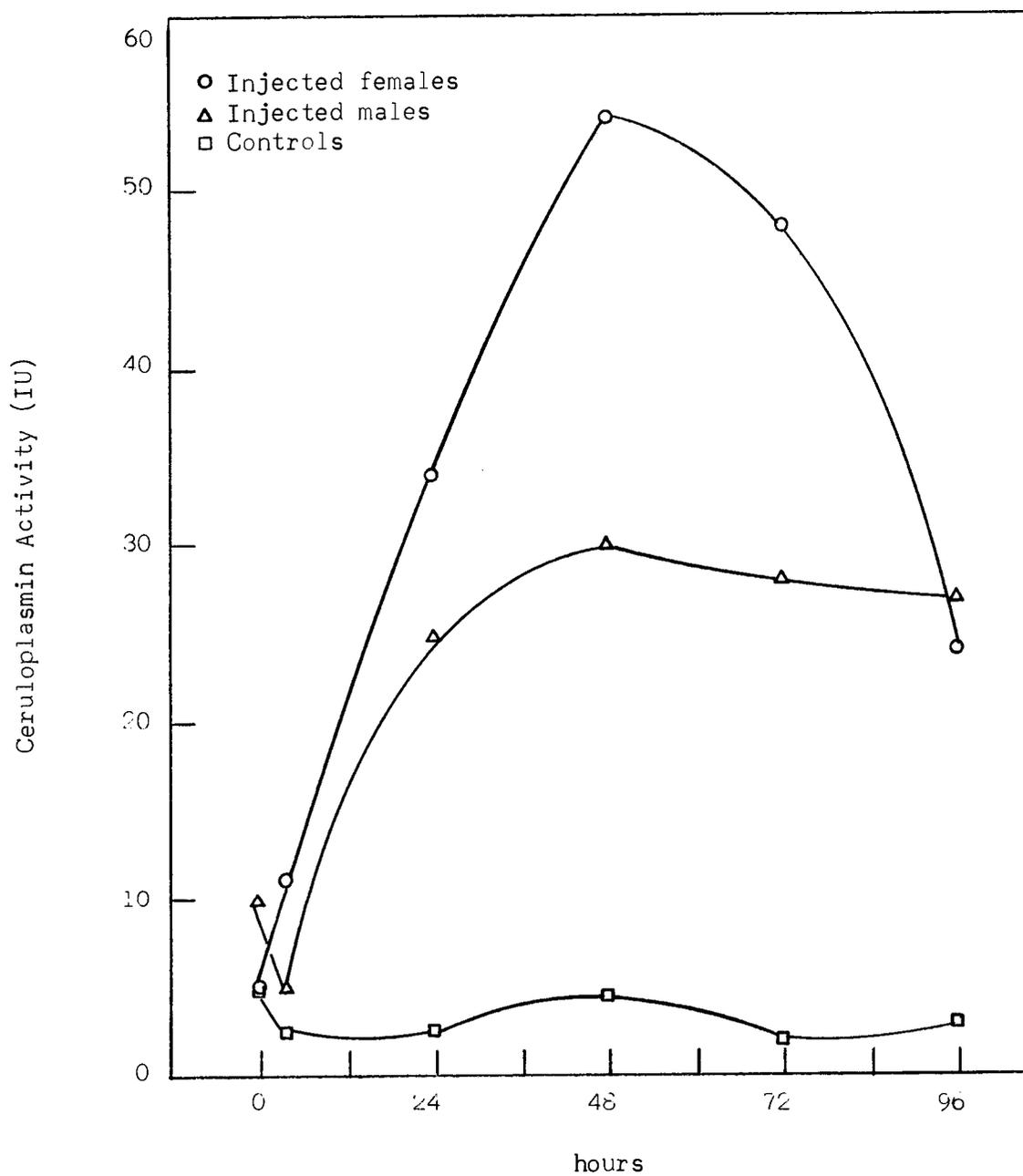


Figure 9. Induction of Ceruloplasmin Activity by Injected Copper

When the incorporation of copper-64 into ceruloplasmin was studied, an anomaly appeared. The incorporation of radiocopper into the ceruloplasmin fraction appeared to parallel the radioactivity in the plasma during the first four hours (Figure 6). The slight increase in ceruloplasmin-⁶⁴Cu at eight hours after injection did not appear significant and could not account for the large increase in the PPD oxidase activity or the large increase in the plasma copper in the low and "normal" groups.

It had been believed (96) and assumed (78) that there is no exchange in vivo of ceruloplasmin copper with ⁶⁴Cu in the plasma. The above results suggest, however, that there is some incorporation of copper into the ceruloplasmin fraction either by exchange or incorporation by binding onto the ceruloplasmin molecule. Also, copper is apparently necessary for the induction of ceruloplasmin, for PPD oxidase activity, but not entirely as an integral portion of the ceruloplasmin molecule.

There was rapid incorporation of copper-64 into the red blood cells (Figure 10). Apparently there was an equilibrium between the red cells and the plasma during the first few hours since the activity in the erythrocytes and plasma paralleled each other until the erythrocyte copper reached a minimum at two hours. There was then a slow increase of activity up through eight hours.

These results are similar to those obtained by Newmann and Silverberg (71) in normal humans. They suggested that the initial rapid uptake reflected the uptake into the more labile fraction in the erythrocytes. When the initial peak is reached there is an

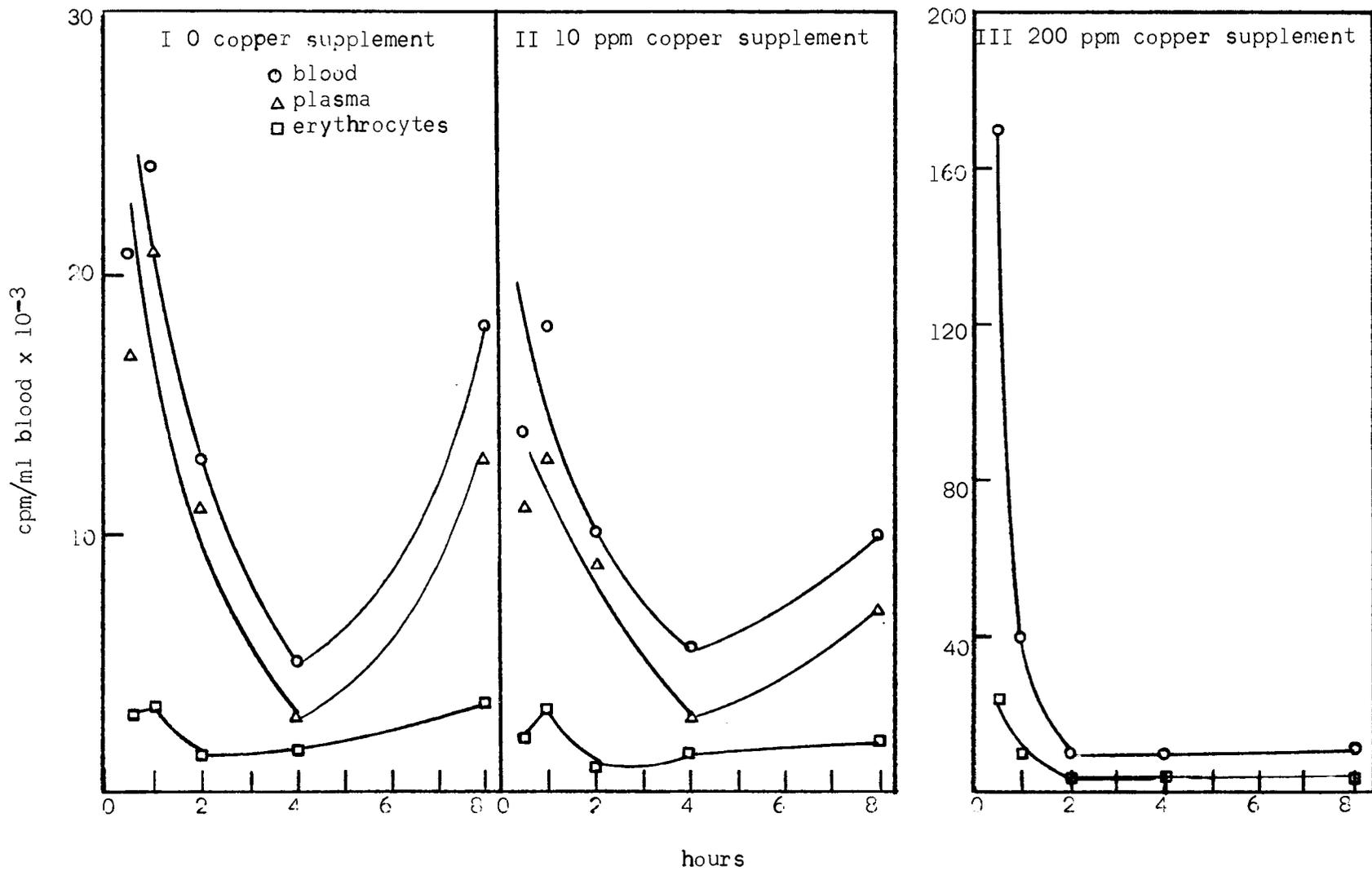


Figure 10. Changes in Blood, Plasma and Erythrocyte Activities with Time

apparent equilibrium between the plasma copper and the erythrocyte copper until the minimum is reached. The secondary and more gradual increase would indicate a more complex equilibrium between the more stable, tightly bound copper in erythrocyte, the "labile" copper in erythrocytes and the loosely bound copper in the plasma.

Newmann and Silverberg (71) presented evidence indicating that there is a third fraction of copper in the plasma, consisting of copper-amino acid complexes. They suggested that the labile fraction of copper in the erythrocytes was also in the form of copper-amino acid complexes and that it is these fractions which are in equilibrium. In addition, it was suggested (71) that there is an equilibrium between the copper in the labile fractions and in erythrocyte, within the erythrocytes, with the equilibrium strongly toward the erythrocyte-bound copper.

The metabolism of copper within the liver was found to be complex. The amount of copper-64 taken up by the liver seemed to be directly related to the copper status of the animal (Figure 7). The activities after eight hours expressed as counts per minute per gram of liver were as follows: low copper group, 220×10^3 ; normal copper status, 256×10^3 ; and high copper status, 525×10^3 cpm per gram of liver. Since the sizes of the rats in the different groups differed and consequently the liver sizes, the activities were calculated on a whole tissue basis. The results showed the same pattern after eight hours as follows: group I, 2.34×10^6 ; group II, 2.36×10^6 ; group III, 2.98×10^6 cpm per whole liver. The maximum, however, was reached in the unsupplemented and "normal" groups after four hours and showed a

different pattern as follows: unsupplemented group, 2.68×10^6 , and "normal" copper status, 2.52×10^6 cpm per whole liver. The reduction in the liver activity in these two groups, between four and eight hours, corresponded to an increase of copper-64 levels in the blood.

Apparently, since the tissues are presumably saturated with copper in the rats on the high copper ration, some detoxification mechanism is rapidly clearing the copper from the blood and holding it in the liver for subsequent excretion in the bile. It has been established in several species (77; 102, p. 67-70) that the bile is the main excretory pathway of absorbed or injected copper. In the other groups, similar mechanisms work to clear the excess copper from the blood. However, there evidently is a greater demand for copper by the tissues in the animals of lower copper status. Therefore, some of the copper is then released back into the blood to help satisfy these needs.

The distribution of the injected copper-64 in the liver varied somewhat, both with time and copper status. An equilibrium between the various liver fractions was apparently reached rapidly in rats on 10 ppm copper ration, since the relative distribution of copper in the various fractions changed very little between thirty minutes and eight hours (Figure 11). At all time intervals, the relative distribution of activity was as follows: soluble > debris > mitochondria > microsomes. In all of these, the activities (cpm/fraction per gram of whole liver) paralleled the whole liver activities. There was, however, an increase in the activity of the soluble fraction between four and eight hours while the radiocopper did not change significantly in

the particulate fractions.

This picture is somewhat different in rats of low copper status and in rats of high copper status. Figures 12, 13 and 14 show the changes in the distribution of radiocopper in the fractions of livers from these groups. In both cases the particulate fractions demonstrated the most dramatic changes. The debris fraction and the mitochondria took up most of the dose in the liver, in the rats on the high copper ration (Figure 13). Uptakes in both the soluble fraction and the microsomal fraction were highly variable in this case. It was therefore difficult to determine if there were any definite trends in either of these fractions.

The microsomes followed the same pattern of uptake in the low copper status livers as in the "normal" situation, with very little change with time. On the other hand, there were very pronounced changes in the debris and in the mitochondria in the unsupplemented group (Figure 12). There was a very significant increase in the radiocopper levels of both of these fractions between two and four hours, and a subsequent drop by eight hours. After rapid incorporation of radiocopper into the soluble fraction of the liver, in the low group, there was a drop in the activity until after two hours. Then there was a gradual increase of radiocopper in the soluble fraction up through eight hours. The similarity between the copper-64 uptake pattern of the red blood cells and the liver soluble fraction in the low and normal groups suggested a partitioning of the copper in the liver soluble fraction analogous to that found in the erythrocytes (71).

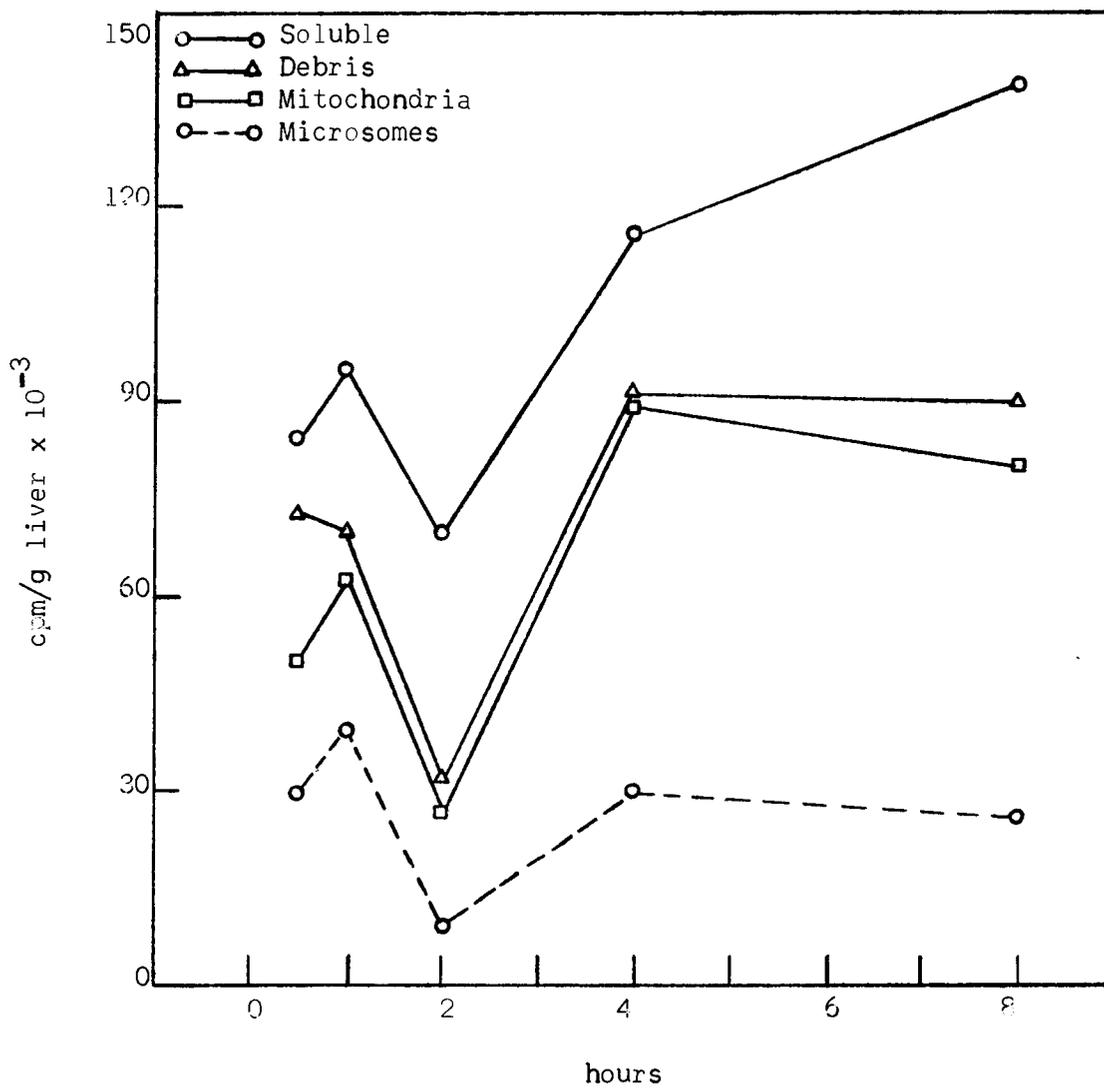


Figure 11. Distribution of ^{64}Cu in Liver Fractions of Rats on 10 ppm Cu Ration

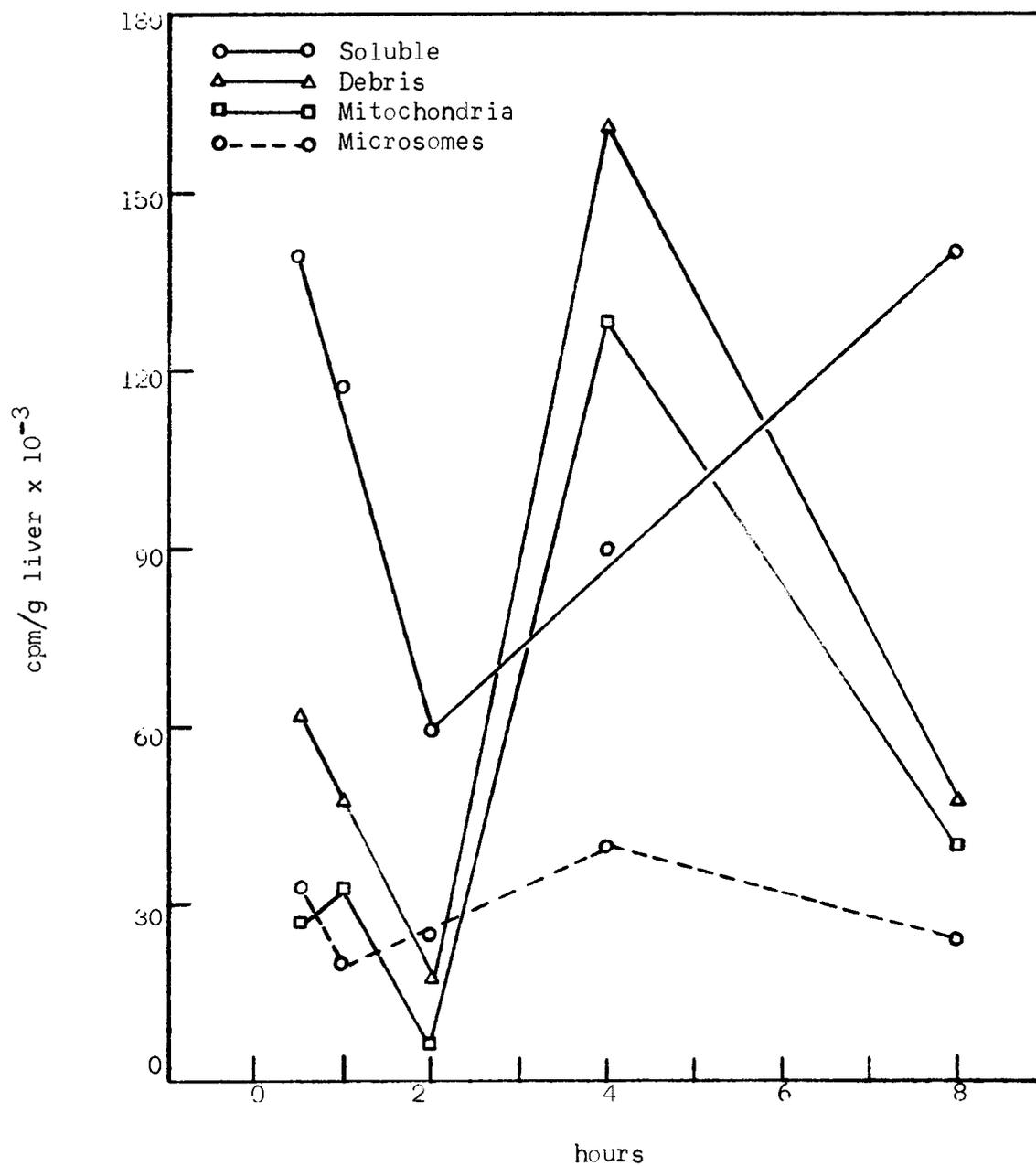


Figure 12. Distribution of ^{64}Cu in Liver Fractions of Rats on Unsupplemented Ration

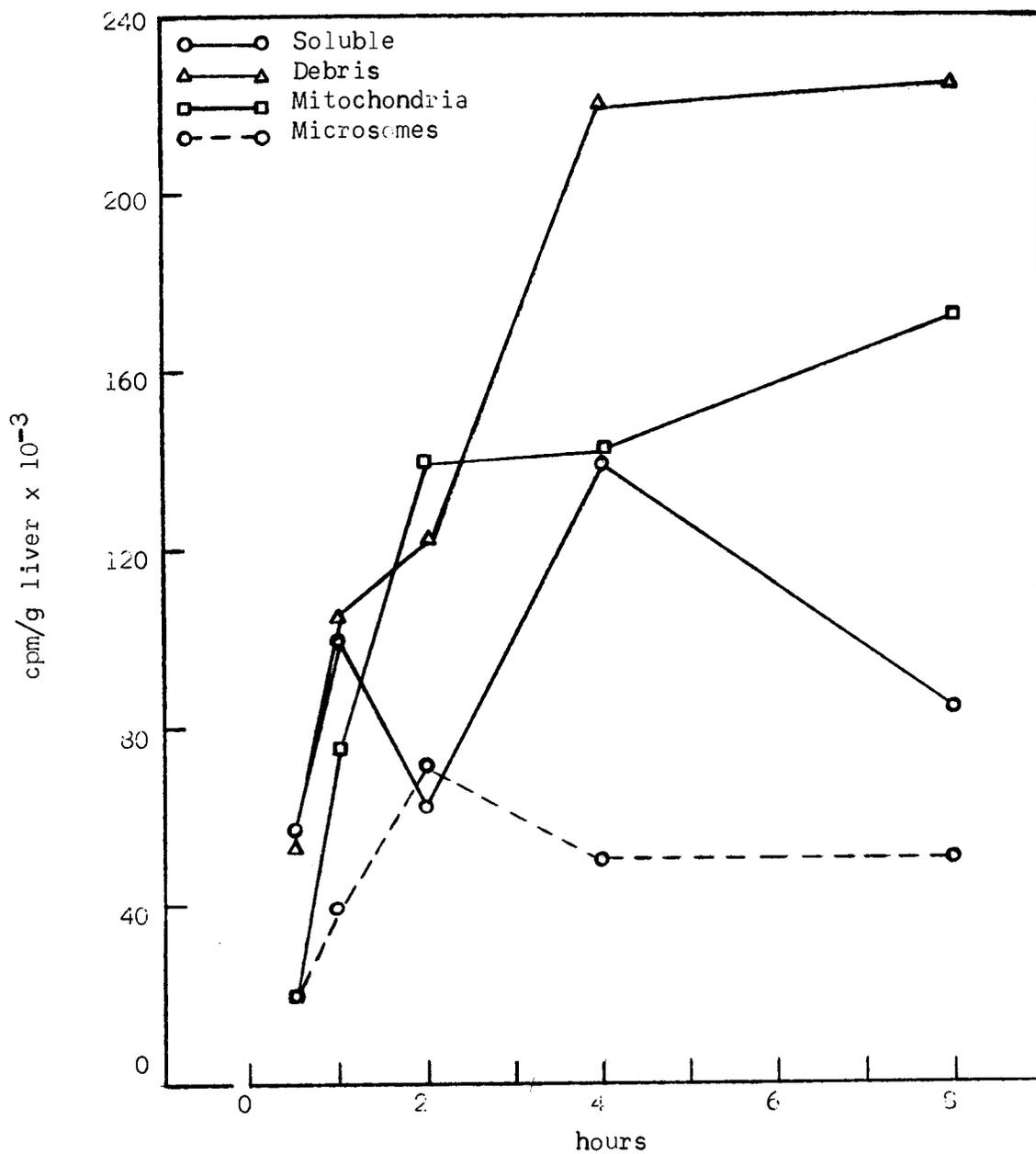


Figure 13. Distribution of ^{64}Cu in Liver Fractions of Rats on 200 ppm Cu Ration

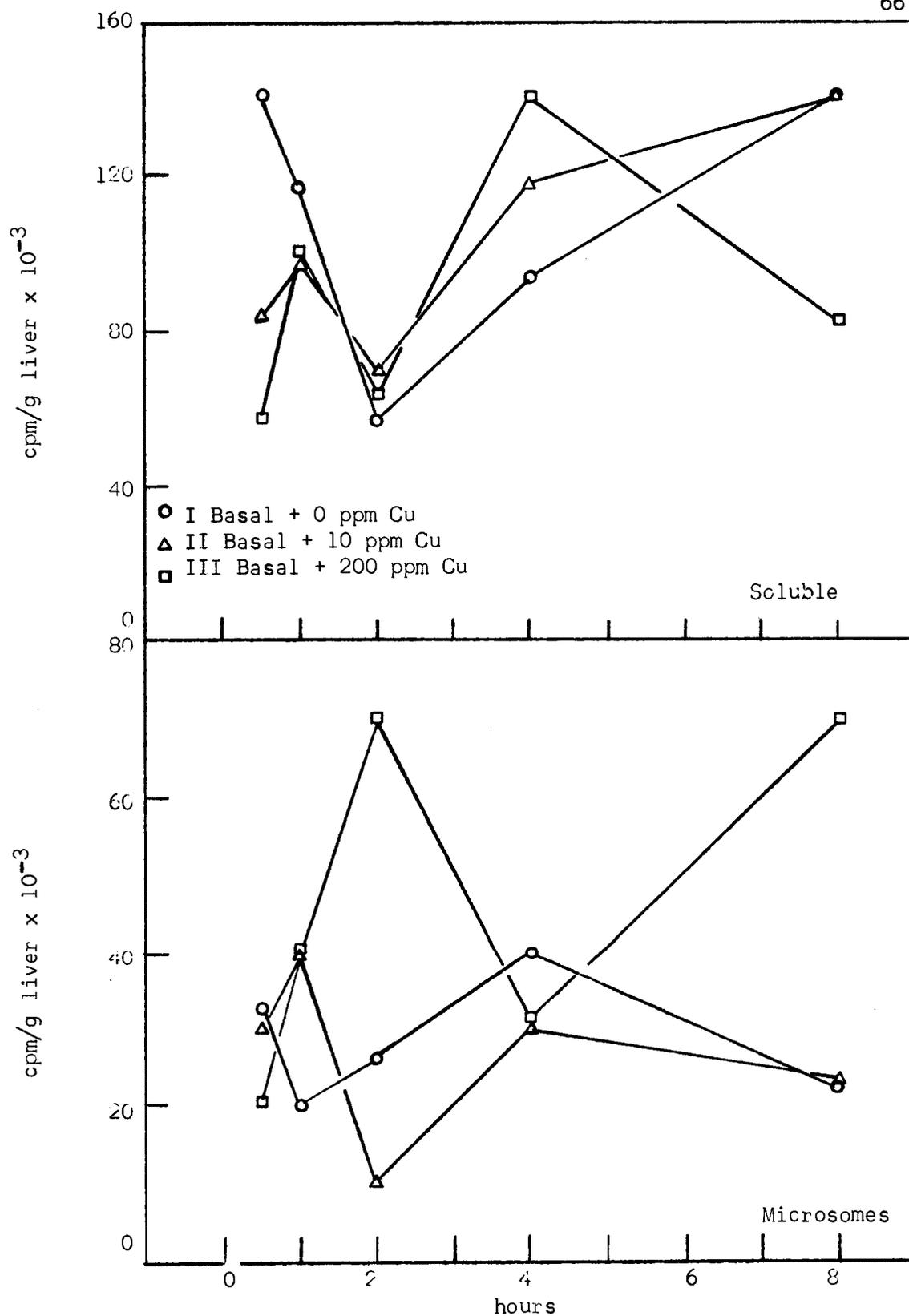


Figure 14a. Changes in Activity with Time in Different Rat Liver Fractions

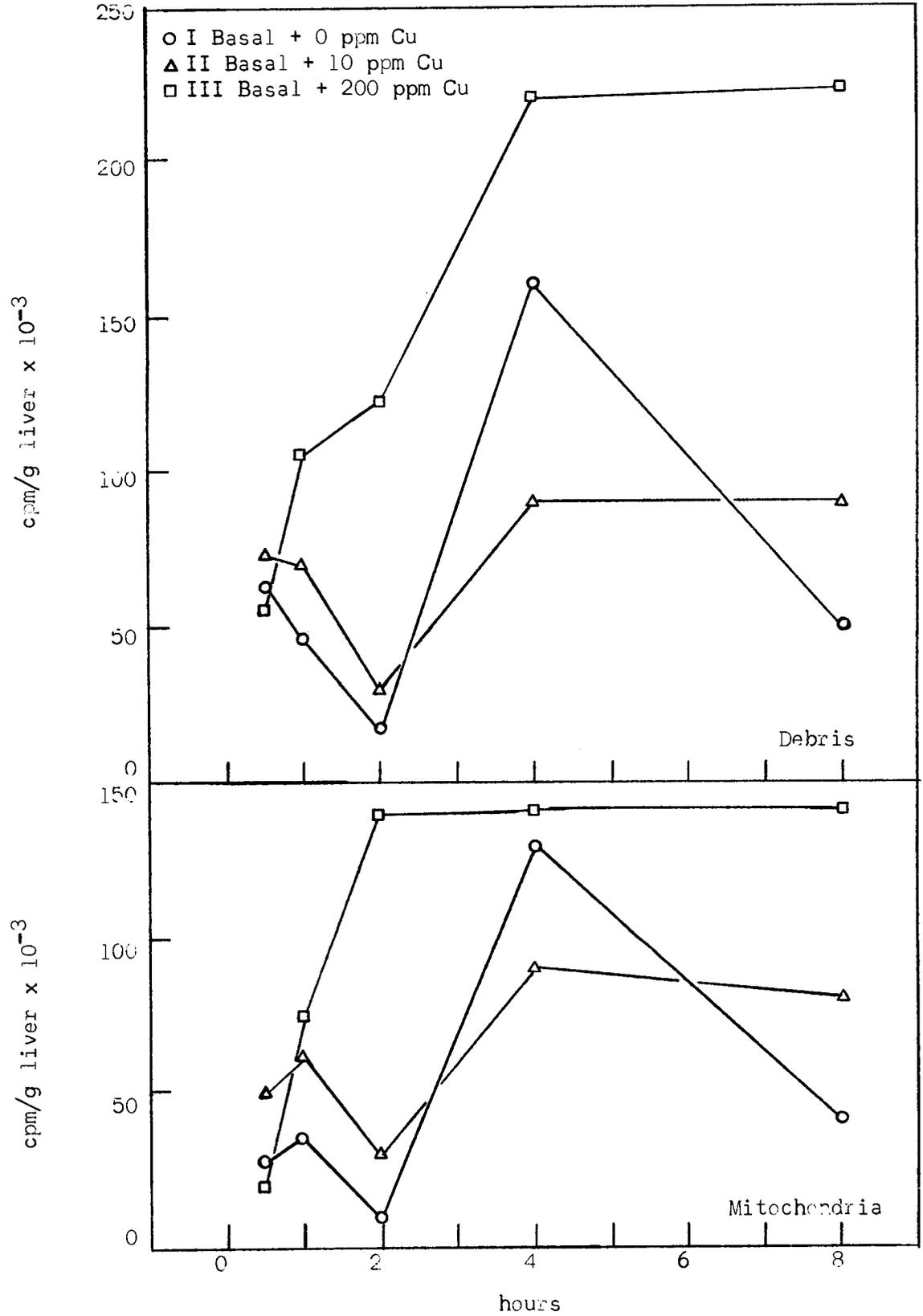


Figure 14b. Changes in Activity with Time in Different Rat Liver Fractions

To test this hypothesis, aliquots from the liver soluble fractions of the four-hour rats on the three different rations were dialyzed overnight against double glass distilled water. Eleven and a half percent of the activity was dialyzable in the ten ppm group and 4.25 percent dialyzable copper-64 was found in the unsupplemented group. No apparent differences were observed between the dialyzed soluble fraction and the non-dialyzed soluble fraction in the rats on the high copper ration. These results suggest the existence of at least two forms of copper in the liver soluble fraction. A stable, more tightly bound form of copper accounts for most of the copper in this fraction while there is a smaller, more labile form of copper that is easily dialyzable. An equilibrium could exist between this labile fraction and the direct reacting copper in the plasma in a similar manner to that existing between the labile copper in erythrocytes and the loosely bound copper in plasma.

GENERAL DISCUSSION AND CONCLUSIONS

A scheme for the movement of copper in the blood and liver is proposed in Figure 15. The fraction of plasma copper, commonly referred to as direct reacting copper, is represented by three species in equilibrium. Most of this copper is bound to serum albumin while a small portion is complexed with amino acids. Newmann and Silverberg (71) and Sarkar and Kruch (82) have presented evidence indicating that a small portion of the loosely bound copper in plasma is in the form of amino acid complexes.

Reaction one is indicated as an equilibrium between the loosely bound copper in the plasma and the intestine because the copper is absorbed in the upper part of the small intestine. In addition, Owen (78) found that about seven percent of injected radiocopper rapidly became incorporated in the walls of the intestinal tract. Newmann and Silverberg (70, 71) showed that amino acids, particularly histidine, enhanced the uptake of copper by the different tissues (reactions two, three and four). Results presented above suggest the partitioning of the liver into the particulate fractions and two forms of copper in the soluble fraction. There apparently is a "labile" fraction of loosely bound copper, in the soluble portion of the liver, that is in equilibrium with the "labile" copper in the plasma. This more loosely bound copper is dialyzable against double glass distilled water and accounts for less than ten percent of the copper in the soluble portion of the liver. The remaining copper in the liver soluble fraction is more tightly bound and apparently is the fraction where most

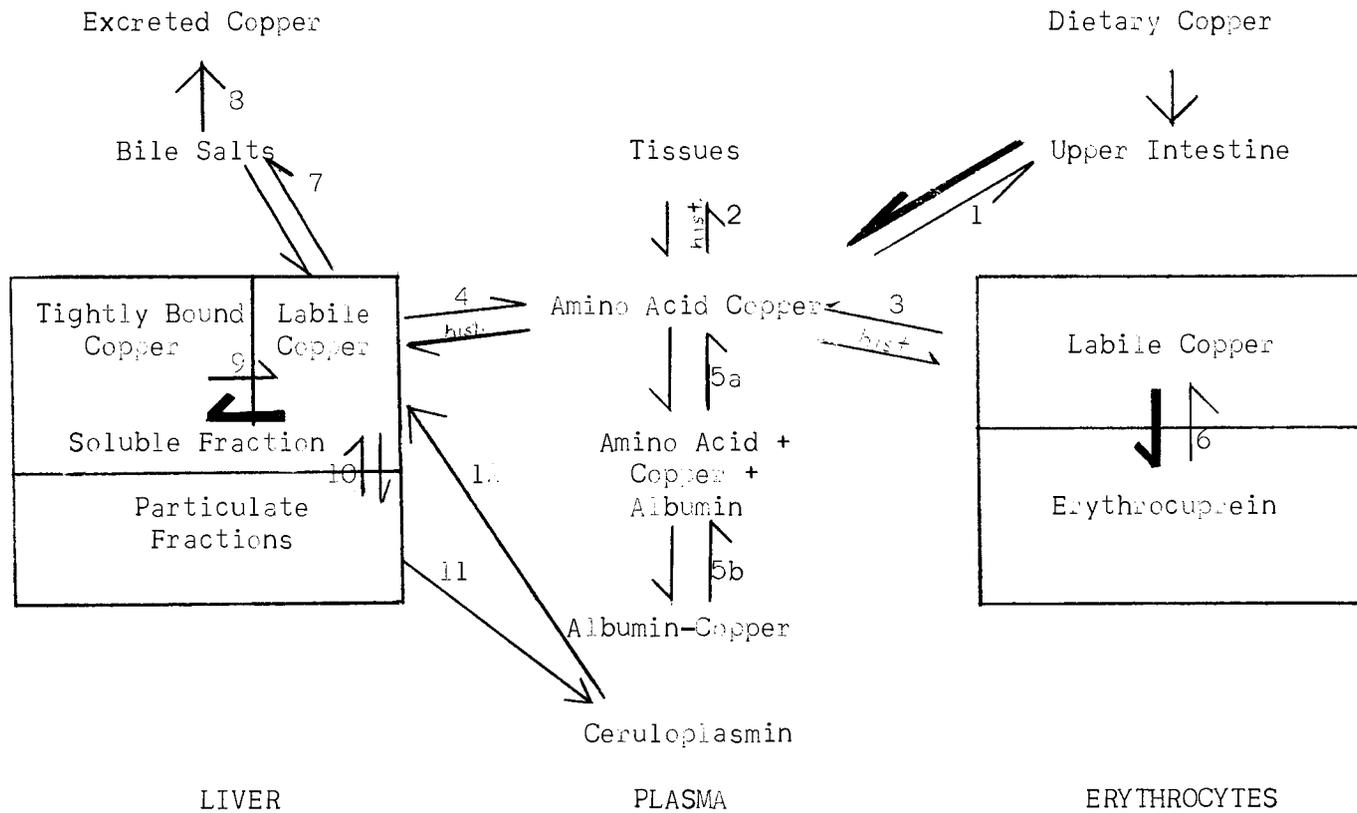


Figure 15. Proposed Scheme for the in vivo Movement of Copper in Blood and Liver

of the copper is stored in the liver. The equilibrium of reaction nine would be toward the more tightly bound copper.

Newmann and Silverberg (71) suggested a similar partitioning in the red blood cells. As mentioned earlier, copper in the erythrocytes has been separated into two forms, erythrocuprein and a more labile copper that is in equilibrium with the plasma. The equilibrium in reaction six is toward the more tightly bound erythrocuprein (71).

Owen (79) has demonstrated that ceruloplasmin is synthesized by the liver (reaction eleven). It has been postulated that this takes place in the microsomes (79). However, there is yet no clear-cut evidence supporting this hypothesis. The average biological half life of ceruloplasmin was found to be about 56 hours (3). Aisen et al. (3) noted that most of the copper from labeled ceruloplasmin appeared in the liver and about five percent was found in the bile and excreted in the feces. This breakdown of ceruloplasmin and subsequent excretion is illustrated in reactions twelve, seven and eight.

Species Differences in Metabolism of Dietary Copper

The scheme in Figure 15 can be used to explain several phenomena related to copper metabolism. As noted earlier, there are several species differences in the metabolism of copper, particularly between ruminants and non-ruminants. The copper in the blood, especially direct reacting copper in rats, can be highly variable, depending on the diet, while liver levels remain constant over a wide range of dietary copper levels. On the other hand, in cattle or sheep, the blood copper levels remain relatively constant over a wide range of

liver and dietary copper levels.

In rats, if the copper intake (reaction one) is increased but the uptake of copper by the liver remains constant (reaction four) and equal to the excretion of copper via the bile (reaction eight), the amount of direct reacting copper in the plasma, erythrocyte and tissue copper levels would increase. In this case, liver copper levels would remain constant. The metabolism of ceruloplasmin (reactions eleven and twelve) would not have to be influenced in this case, but in copper deficiency the synthesis of ceruloplasmin (reaction eleven) would be depressed.

In the case of copper toxicity, the tissues become saturated (reaction two). If reactions seven and eight, the excretion of copper, are rate-limiting, when the copper intake passes a certain point, or threshold, liver copper levels will naturally increase. The phenomenon of liver copper levels remaining constant until a certain level of dietary copper is reached has been observed above and elsewhere (5, 66) in non-ruminants.

On the other hand, if the equilibrium in reaction four is shifted more toward the liver and is equivalent to the difference between the copper intake and the tissue demands, the amount of direct reacting copper would remain constant. If the uptake of copper by the liver exceeded the excretion by the bile and if the copper were more tightly bound in the liver, liver copper levels would increase while the blood levels of copper would remain relatively constant. This is the general picture in ruminants such as cattle and sheep.

During periods of low copper intake the tissues, including blood and liver, would eventually be depleted of copper, assuming the excretion of copper (reaction eight) exceeded the rate of copper absorption (reaction one).

Metabolism of Injected Copper

When copper is injected into the blood, there is a very rapid uptake by the labile fractions in the erythrocytes and liver. In the case of animals of low or "normal" copper status, some of this liver and erythrocyte copper is released back into the blood, either in the loosely bound form to meet tissue demands or as ceruloplasmin.

Evidence presented earlier also indicates a secondary, more gradual uptake of copper by both the liver soluble fraction and the erythrocytes. This would correspond to the incorporation of copper into erythrocuprein and the more tightly bound portion in the liver soluble fraction. Both of these species of copper are relatively stable, and the equilibriums of reactions six and nine are toward the more tightly bound forms.

If copper is injected into rats whose tissues are already essentially saturated with copper, as in the case of copper toxicity, there is a rapid uptake of copper from the plasma by the liver. Since the tissues and probably the tightly bound copper in the liver soluble fraction are saturated, the copper apparently is bound more to the particulate fraction in the liver (reaction ten) until it can be excreted from the body via the bile and feces (reactions seven and eight).

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APPENDIX

TABLE A. Slopes and regression coefficients for liver fractions.
(Figure 3)

	<u>Slope</u>	<u>Regression Coefficient</u>
debris	0.024	0.833
mitochondria	0.050	0.931
microsomes	0.055	0.903
soluble	0.112	0.933

Raw Data⁵ for ⁶⁴Cu Metabolism Experiment

TABLE B. Whole liver cpm x 10⁻⁶ (Figure 7)

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	1.15	1.54	1.09	3.41	2.77	2.34
10	1.20	2.28	1.47	3.19	1.85	2.36
200	0.76	2.01	2.76	2.56	1.90	2.98

TABLE C. Blood cpm x 10⁻³

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	354	484	268	60.6	106	332
10	258	323	194	90.6	80.5	180
200	1,083	414	94.3	77.6	49.4	78.5

⁵Analysis of variance was not performed because of the apparent interaction between time and copper supplement.

TABLE D. Liver: Blood (Figure 8)

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	3.24	3.18	4.43	17.3	26.3	7.06
10	4.68	7.05	7.61	35.2	22.9	13.1
200	0.70	4.86	29.9	33.0	38.5	37.9

TABLE E. Blood cpm/ml $\times 10^{-3}$ (Figure 10)

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	21.5	26.1	12.7	5.6	6.1	17.8
10	14.2	18.4	9.6	4.7	4.2	9.6
200	168.5	41.7	8.7	9.0	7.4	8.2

TABLE F. Plasma cpm/ml Blood $\times 10^{-3}$ (Figure 10)

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	17.4	21.7	11.3	3.7	4.6	13.3
10	10.9	13.8	8.7	3.7	2.7	7.5
200	142.2	33.2	7.4	7.7	4.1	6.8

TABLE G. Erythrocytes cpm/ml Blood $\times 10^{-3}$ (Figure 10)

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	4.1	4.4	1.4	1.9	1.5	4.4
10	3.3	4.6	0.8	1.0	1.5	2.1
200	26.2	8.5	1.3	1.3	3.3	1.5

TABLE H. Liver cpm/g x 10⁻³

Cu Supplement/Time (H)	.5	1	2	4a	4b	8
0	189	47	109	426	497	220
10	172	334	136	315	249	256
200	182	341	381	548	442	525

Changes in ^{64}Cu Distributions in Liver Fractions as Percent Total with Time

TABLE I. Percent distribution of ^{64}Cu in livers of unsupplemented rats (Figure 12)

Fraction/Time (H)	.5	1	2	4a	4b	8
debris	24.3	22.0	14.5	40.7	36.5	19.3
mitochondria	10.3	15.0	6.2	28.5	31.9	15.2
microsomes	12.6	8.8	24.0	10.2	9.3	9.3
soluble	52.8	54.2	55.4	20.6	22.3	56.2

TABLE J. Percent distribution of ^{64}Cu in livers of 10 ppm Cu supplemented rats (Figure 11)

Fraction/Time (H)	.5	1	2	4a	4b	8
debris	30.7	25.6	23.1	26.9	28.8	26.9
mitochondria	20.6	23.4	20.4	24.8	31.7	24.0
microsomes	12.7	15.5	6.8	7.7	12.3	7.7
soluble	36.0	35.5	49.7	40.6	27.2	41.4

TABLE K. Percent distribution of ^{64}Cu in livers of 200 ppm Cu supplemented rats (Figure 13)

Fraction/Time (H)	.5	1	2	4a	4b	8
debris	36.7	32.8	31.0	43.3	38.4	40.6
mitochondria	12.4	23.4	35.2	22.2	33.9	31.4
microsomes	12.9	13.1	18.2	3.3	10.0	12.6
soluble	38.0	30.7	15.7	31.3	17.7	15.4