INTERACTION OF LIPIDS WITH COPPER

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The release and uptake of copper was studied in many tissues of the rat in vivo and in vitro in copper deficiency and loading experiments. Severe to moderate depletion of the metal was observed in the tissues of deficient animals. Liver, heart and blood were found most sensitive to lack of dietary copper. Skeletal muscle was severely, although slowly, depleted; brain exhibited the greatest resistance to depletion. Plasma ceruloplasmin activity was decreased. Prolonged administration of cuprous or cupric copper in rats of various ages resulted in heavy deposition of the metal in liver and kidney. Hair, spleen, blood, brain and heart were also affected but skeletal muscle (and testes and lungs, in some cases) retained its normal content of the metal. Elevated values of ceruloplasmin activity in plasma were observed.

Copper was found and measured in the lipid extracts of the rat brain, liver, kidney and heart. The uptake of the metal by lipids was studied in vivo and in vitro. Liver and kidney lipids of the copper-loaded rats had increased copper content. Refined and extracted lipids exhibited binding ability for copper ions; this was higher for nitrogenous lipids.

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PREFACE

Copper is a trace metal of the animal organism shown to be essential for life. It plays an important role in many enzymic systems and its excessive deposition in human tissues is responsible for the clinical manifestations of Wilson's disease, or hepatolenticular degeneration.

Despite the efforts of many investigators, the metabolism of this metal is not clearly understood. The mechanisms of copper absorption and secretion have not been elucidated and only a few copper compounds have been isolated from liver, brain and blood. The state of most of the element in the animal body has not been studied at all.

One of the most obscure aspects of copper metabolism is related to its transport. Thus, although the major portion of copper in blood plasma is part of the ceruloplasmin molecule (the remainder is loosely bound to albumin), tissues contain the metal bound to proteins, such as cerebrocuprein, hepatocuprein and erythrocuprein. Therefore, copper undergoes a change of protein "medium" during transport, the mechanism and the location of which remain unknown.

In a series of experiments presented in this thesis, the comparative release and uptake of copper by the tissues of the rat are studied under conditions of copper deficiency and copper loading respectively. The purpose of this study is to provide information about the behaviour of each particular tissue with respect to copper, and correlate the evidence obtained, with its physiology and structure.

Assuming the participation of the cell membranes in the voyage of copper from blood to tissues, the interaction of structural

lipids with copper is studied in another set of experiments, *in vivo* and *in vitro*.

Thus, the present research, is a broad examination of the differential behaviour of tissues in respect to copper.

I. REVIEW OF LITERATURE

A. BIOLOGY OF COPPER

1. The copper content of animals

Bucholtz and Meissner (1), one hundred and fifty years ago were the first to detect copper in biological materials. A specific role for copper was accepted only when its presence was shown in the molluscan blood and in the tail feather pigment of the turaco bird.

Despite the increased number of biological materials found to contain copper, the element was accepted as a definite physiological constituent only at the beginning of this century and since then, copper was established to be an essential nutrient, the absence of which leads to severe derangements in growth, physiology and metabolism.

Many data have been published pertaining to the distribution of copper in animals and plants. Almost every tissue has been found to contain some of the metal. Thus, liver, heart, kidneys, hair, skin and brain contain usually the highest concentration of copper; lungs, spleen, muscle, bone and blood are in an intermediate level, while pituitary, thyroid and thymus have the lowest concentrations. Digestive fluids, spinal fluid, saliva and urine contain copper, although in variable concentrations. The metal concentration in feces depends on the diet.

In general, the average concentration of copper in the body of adult vertebrates is of the order of 1.5 to 2.5 μg/gm fat-free tissue (1). The comparative biochemistry of the blood and liver of vertebrates regarding their copper concentration, has been studied by Beck (2) and variations were shown in the blood copper, not necessarily parallel to the phylogenetic relationships. Pig, among placental mammals, had the highest copper content in blood (140-150 μg %); rat,

sheep, ox and man were intermediate (about 100 μg %), while guinea pig and rabbit had the lowest (50-70 μg %). Even lower concentrations of copper in blood were found in large marsupials (30-40 μg %), while those of the fowl and domestic turkey were the lowest (20-30 μg %). Fish, reptiles and amphibians were in the same range as most higher mammals.

The concentration of copper in liver of most species was found to be below 15 μg/gm fresh tissue. In some cases (ruminants, duck, frog and certain fish) higher values (25-100 μg/gm fresh tissue) have been found.

Copper has been also measured in a large variety of species other than vertebrates such as insects (adult, larva and pupa). Copper is now recognized as an essential constituent of most microorganisms, but the dependence of the tissue concentration of the metal upon that of the nutrient medium has made it difficult to define the "normal" content.

Very few reports are concerned with the distribution of the metal among cells or within the cell. Histochemical methods have been applied only in cases where the copper content in certain tissues is abnormally high. Chemical determinations of copper in different parts of the normal human brain showed a greater copper content in the grey matter relative to the white matter (3). A striking finding was a very high copper content in substantia nigra and locus ceruleus (4).

Recently Colburn and Maas (5) have found that copper in synaptic vesicles and nerve endings in the brain of mouse was concentrated several-fold relative to its concentration in the rest of the brain. According to these authors both quantity and intracellular loaction of copper suggest that copper is present in structures associated with

norepinephrine rather than with unrelated proteins in the synaptosomes.

The distribution of copper in the liver of rats has been studied by Thiers and Vallee (1) and it was found to be as follows: connective tissue 140, nuclei and cell residue 86, mitochondria 160, microsomes 120 and the clear supernatant 560 μg copper/gm nitrogen. Administration of Cu^{64} in mouse resulted in a differential distribution between nucleus and cytoplasm. The cytoplasmic uptake of the element in liver, kidney, spleen and lung was greater than that of the nucleus.

2. Copper compounds in the animals organism

Only a small percentage of the total copper in the animal body has been identified as part of purified copper compounds. These compounds are divided into the following main groups:

a) Copper porphyrins

The copper porphyrin Turacin contains 5.9% copper, has a molecular weight 1095, and is found in the tail feather pigment of the turaco bird.

b) Cuproproteins

Among the cuproproteins are the hemocyanins (1) of molluscs and arthropods with a copper content of 0.24 - 0.26 and 0.15 - 0.19%, respectively. Their molecular weight varies between 400.000 to 1.000.000.

Cerebrocuprein I is a copper-containing protein of unknown function, isolated from bovine brain by Porter and Folch (6) and human brain by Porter and Ainswirth (7). It contains 0.25 - 0.30% copper and its molecular weight is of the order of 30.000 to 40.000. By differential centrifugation of brain homogenate, cerebrocuprein I has been shown to occur in the supernatant fraction. Hepatic copper proteins of unknown function have been isolated by Mann and Keilin, and by Mohamed and

Greenberg from the horse liver, and by Morel et al. (1) from the liver of normal cases and patients with Wilson's disease. No difference in the composition of the amino acids of the proteins from these two groups was observed. However, the copper content of preparations of this protein from patients with Wilson's disease was almost ten times as high as in normal liver.

Hemocuprein or erythrocuprein is another protein discovered by Markowitz et al. (8) in red blood cells; it contains 0.34 % copper.

c) Curpoenzymes

i. Ceruloplasmin

In 1948 Holmberg and Laurel (9) isolated ceruloplasmin from the blood plasma. 95% of the copper circulating in the blood plasma is tightly bound to this specific protein. The remaining 5% is loosely bound to albumin. The molecular weight of the blue coloured ceruloplasmin is 151.000 and its molecule contains 8 atoms of copper. There is strong evidence that 4 copper atoms are in the cupric and 4 in the cuprous state. Ceruloplasmin exerts enzymatic activity, oxidizing p-phenylenediamine (10), epinephrine, norepinephrine and 5-hydroxytryptamine (11). In addition, ceruloplasmin has been found to have an authentic and distinctive ascorbate oxidase activity (12).

By chromatography on hydroxylapatite ceruloplasmin from normal human plasma splits into two components (13), identical in copper content, absorption spectrum and oxidase activity. However, the chromatographic properties and electrophoretic mobility differ.

The formation, metabolism and functional role of this copper enzyme in the organism, have been studied mainly by using radioactive copper. The investigations have indicated that orally administered Cu^{64},

after absorption in blood as free copper, is rapidly incorporated into the copper-containing liver protein discovered by Morel and his colleagues. The copper bound to this protein is transferred to ceruloplasmin. The specific activity of copper in ceruloplasmin approaches but never exceeds that of copper in the liver protein. Eventually, either in the liver or in the intestinal wall, copper is liberated from ceruloplasmin and excreted in the feces.

Regarding the functional role of ceruloplasmin, two main theories have been developed.

α. Copper transport protein

Bearn and Kunkel (14), using Cu^{64} detected the short half life of ceruloplasmin. This led to the assumption that ceruloplasmin acts as a copper transport protein. It has been found that half of the copper in ceruloplasmin can be exchanged with ionic copper in vitro when ceruloplasmin is reduced by ascorbic acid (15). It is unknown to what extent this mechanism takes place in vivo.

In addition, an influence of ceruloplasmin on the route of copper excretion through the bile has been proposed.

In conclusion, there is some evidence that ceruloplasmin contributes to the regulation of net absorption of copper in the body, but the exact mechanism is still unknown.

β. The enzymatic function of ceruloplasmin

A terminal oxidase activity for ceruloplasmin has been shown by using p-phenylenediamine as well as physiological substrates such as aromatic amines. Epinephrine and norepinephrine are oxidized to the indoloid structures adrenochrome and noradrenochrome. However, because of the low enzymatic activity, the physiological significance of these

conversions is questionable. 5-Hydroxytryptamine is transformed to a new indolic substance in the presence of ceruloplasmin (11).

In addition, Walaas and Walaas demonstrated that cellular metabolites such as reduced di- and triphosphopyridine nucleotides (DPNH, TPNH) are oxidized by ceruloplasmin in the presence of norepinephrine and epinephrine as well as dopamine (16). In this reaction, the aromatic amines act as reversibly oxidized and reduced mediators, norepinephrine being the most active in this respect. The action of ceruloplasmin results in the initial formation of free radicals of catecholamines. Such radicals are very reactive and may participate in polymerization as well as in oxidation reduction reactions. During the action of ceruloplasmin on catecholamines, the latter reduce reversibly the cupric ions of the enzyme.

In summarising all known facts about ceruloplasmin, it is admitted that conclusive evidence regarding its function is lacking.

ii. Tyrosinase

This cuproenzyme is necessary for the first step in the chemical conversion of tyrosine to melanin pigment in the melanocytes. This is the production of 3,4-dihydroxyphenylalanine and its quinone.

Tyrosinase contains 0.2% copper, in the cupric state. It has been postulated that copper is reduced to the cuprous form by the substrate and then reoxidized by molecular oxygen.

iii. Dopamine-β-hydroxylase

It has been shown by Levin et al. (17) that this enzyme catalyzes the conversion of dopamine to norepinephrine according to the following equation:

$$\text{Dopamine} + O_2 + \text{ascorbate} \xrightarrow{\text{fumarate}} \text{l-norepinephrine} + \text{dehydroascorbate} + \text{water}$$

The enzyme is not specific for dopamine, catalysing also the side-chain hydroxylation of many analogues of phenylethylamine; the requirements for ascorbate and fumarate are not specific.

Dopamine-β-hydroxylase, a mixed function oxidase or monoxygenase, contains 0.65 to 1 μg copper per mg of protein (4 to 7 μmoles per μmole). It has been shown by Friedman and Kaufman (18) that of the total copper, 2 μmoles per μmole of enzyme are in the cupric state, while the amount of cuprous copper is variable. According to the authors the cupric ions may be more specifically related to the hydroxylase activity of the enzyme than cuprous ions; the cupric ions may be the site of reduction of the enzyme by ascorbate in the following proposed scheme where E stands for enzyme and RH for substrate:

$$E\begin{matrix}Cu^{2+}\\Cu^{2+}\end{matrix} + \text{ascorbate} \longrightarrow E\begin{matrix}Cu^{+}\\Cu^{+}\end{matrix} + \text{dehydroascorbate} + 2H^{+}$$

$$E\begin{matrix}Cu^{+}\\Cu^{+}\end{matrix} + O_2 + RH \longrightarrow E\begin{matrix}Cu^{2+}\\Cu^{2+}\end{matrix} + ROH + H_2O$$

It has also been suggested that copper plays an important part in binding substrate to an enzyme surface. Charge transfer complexes have been observed (19) between enzyme-bound copper and substrate, and it has been proposed that this is the function of the cuprous copper present in several proteins, but no direct evidence for this proposal has been obtained for dopamine-β-hydrolase (20).

iv. Uricase

Uricase is present in the liver of mammals other than primates

and it contains one atom of Cu^{++} per mole. This enzyme promotes the oxidation of uric acid to allantoin.

v. β-Mercaptopyruvate transsulfurase

This enzyme catalyzes sulfur transfer from the mercaptopyruvate to cyanide to form thiocyanate and pyruvate and it contains one atom copper per molecule.

vi. Cytochrome oxidase

Cytochrome oxidase is known to contain 0.006 % copper. The role of copper in the molecule of this enzyme is unknown (1).

vii. Tryptophan pyrrolase

Studies by Maeno and Feigelson (21) on bacterial tryptophan pyrrolase in vitro suggested that this enzyme might be copper-dependent; as the purification of the enzymatic preparation increases, the content in copper increases too. In addition, chelating agents, specific for copper, inhibit the activity of the enzyme whereas non-specific agents have no significant effect.

d) Ribonucleic acids

Preparationsof ribonucleic acid from phylogenetically diverse sources were found to contain significant concentrations of metals including copper (22). Thus, RNA obtained from calf thymus and Euglena grocilis contained 76 and 190 μg copper/gm RNA respectively. The authors suggest that the metals may serve to link the nucleicacid to the protein moiety of ribose nucleoprotein.

e) Lipids

It has already been reported by Gregoriadis and Sourkes (23) that lipid extracts from rat brain, liver, kidney and heart tissue contain traces of copper. This was confirmed later by Maas and Colburn

in 1965 (24) who found minimal amounts of copper in the lipid extract of synaptosome from mouse brain.

3. Binding of Cu^{++} in vitro by compounds of physiological importance

a) Catecholamines

Cupric ions in the presence of oxygen promote the non-enzymatic oxidation of epinephrine to adrenochrome and norepinephrine to noradrenochrome (25). It has been shown that this conversion is dependent upon the formation of a Cu^{++} ion complex of catecholamines which is autoxidable. Cu^{++} ion complexes with catecholamines have been studied by Walaas et al. (16) using their absorption and electron-spin resonance spectroscopy in oxygen-free solution.

Colburn and Maas (5) used potentiometric techniques for the study of co-ordination chemistry of copper among other metals with norepinephrine (NE) at physiological pH and it was found that NE essentially chelated the metals present even at equimolar metal concentration. The authors found it of interest to titrate ATP with copper in the presence and absence of norepinephrine since ATP has been found concentrated in synaptosomes and associated with norepinephrine storage in many studies (26), (27). A reasonable hypothesis in this case seemed to be that ATP, copper and norepinephrine may form an ATP-Cu-NE ternary complex and that such complexes might be of particular importance in the reaction mechanism of catecholamines. Experiments in vitro confirmed the formation of such a complex (24).

If now ternary complexes of the type ATP-Cu-NE structure are formed in vivo, a reasonable hypothesis would seem to be that such complexes represent a mechanism for storage or binding of catecholamines. The existing evidence supports this hypothesis (5).

b) Catecholamines, Phospholipids, ATP.

When Maas and Colburn (24) titrated phosphatidyl-serine (PS) or phosphatidylinositide (PI) in the presence of Cu^{++} ions, co-ordinations complexes were obtained from the organic phase. In the presence of ATP, a ternary Cu-ATP-phospholipid co-ordination complex was formed not incorporated in the organic solvent. In addition, lipids extracted from synaptosomes showed similar behaviour (incorporation of copper in organic solvent and prevention of this by ATP) suggesting the formation of ternary complexes in situ between synaptic lipids, copper and ATP. Further experiments suggested the existence of ternary complexes between PS, copper and d,l-NE.

Thus, a mechanism was proposed by the authors, by which a neurotransmitter such as NE, might be released from the presynaptic terminal. The NE-Cu-phospholipid complex has altered solubility characteristics such that a polar NE molecule becomes more soluble in an organic solvent, or in other words, the energy barrier to the movement of NE into an organic solvent is reduced by complexing; (the partition of NE in the organic solvent through the formation of the ternary complex is prevented by the addition of ATP).

This might be of significance in understanding a mechanism where-by a polar molecule such as NE moves across the potential energy barrier of the lipid membrane. The following hypothesis regarding synaptic membrane function and NE was suggested (24): in the resting membrane, the presence of ATP increases the energy barrier to the movement of NE across the internal surface of the membrane. If ATP is removed as by an ATPase, either unfilled co-ordination positions on the copper-phospholipid moiety become available to NE, or the removal of ATP from a quaternary complex occurs and a ternary phospholipid-

Cu-NE complex remains. In either case the electronic structure of this ternary complex is such that its solubility in the aqueous phase is decreased whereas in the lipid phase (the cell membrane) its solubility is increased. The net result is an increase in movement of NE across the cell membrane. Displacement of NE by ATP from a metabolic source in vivo or an exogenous source in vitro, would bring about a resumption of the usual resting membrane. It is noted that by this mechanism, the membrane ATP will tend to prevent loss of NE from the vesicle.

It is felt by the authors that there is true uptake of NE against a gradient owing to the presence of copper and iron within the granules, but that in addition, the presence of magnesium and ATP diminishes the rate of outflow of NE from the interior of the vesicle.

In connection with the role played by copper as well as by aromatic amines in the metabolism and functioning of the extrapyramidal system, discussed recently by Sourkes and Poirier (28), the formation of complexes between copper, aromatic amines and phospholipids, and the fact that aromatic amines can serve as substrates for copper containing enzymes may be of great importance.

c) Peptides

Titration and spectral measurements of sperm whale metmyoglobin and apomyoglobin (29) in the presence of Cu^{++} ions indicate that the metal ion may form chelates with imidazole groups and some other proton-bearing groups such as peptide amides. On the other hand, the titration and spectral properties of complexes of certain histidine-containing peptides have proved to be similar to those of protein (30). In addition, the optical rotatory dispersion curves for various forms of some simple dipeptides and their complexes with Cu^{++} ions have also been measured.

The copper complexes of polypeptides have been studied extensively by many workers because of their biological significance in enzyme reactions. It is generally believed that formation of a copper complex involves displacement of the peptide hydrogen.

4. Factors influencing the concentration of copper in tissues

Three important factors influence the level of copper in the blood and tissues of different animal species in the normal state: diet, age and hormones or pregnancy.

The content of copper in the diet, its chemical form and presence or absence of other cations or anions can influence the levels of copper in the animal tissues. Abnormally high or low copper content generally causes corresponding changes in the copper concentration in the liver and in some cases in the blood.

The copper content of human tissues at various ages, has been measured and found increased in the first 3 months of life. Liver for example, from 0 to 3 months, contains about 80 μg copper/gm fresh tissue. From 3 months to one year contains about 6 μg and after one year there is no significant change. This is observed only in liver; other tissues do not show such a pronounced decrease (31). In general, the total body content of copper in newborn animals is greater than that of the adult.

During pregnancy, copper in serum was found to increase gradually, begining at the 8th and having a maximum during the 32nd and 34th week. The copper level returns to its normal value in 6 to 10 weeks post partum (32). Copper and ceruloplasmin levels in serum increase after administration of ethynylestradiol (33). Apart from pregnancy, increased copper levels in plasma have also been found in hepatic cirrhosis.

Both these conditions are associated with hyperestrogenism and it is assumed that estrogens are directly responsible for this increase.

Severe thyrotoxicosis results in increased copper concentration in serum. Rabbits and frogs treated with antithyroid drugs have a low copper content in whole blood, increasing to the normal levels by discontinuation of the drugs (34).

5. Absorption of copper

There is no doubt, that copper is absorbed from the upper alimentary tract, because oral ingestion of Cu^{64} results in a prompt rise in the radioactivity of plasma (35).

Nothing is known about the mechanism, if any, regulating the absorption of copper. It is likely that copper homeostasis is accomplished by an adjustment of the rate of excretion to that of absorption (36). Quantitative studies on copper absorption in the mouse suggest that two mechanisms are involved (37). There is an apparently active transport mechanism which is saturated when doses of 25 μg are given. A second mechanism, assumed to be passive, allows for the absorption of copper, directly proportional to the dose presented. Approximately a tenth of the ingested amount of copper is absorbed.

The absorption of copper by the gastrointestinal tract is greatly influenced by the form of the copper. Thus, it has been found (1) that neutral or anionic organic complexes are more readily available to copper-deficient animals than ionic copper given as the sulfate salt. On the other hand, very tightly bound copper such as cupric sulfide, is not absorbed.

6. Excretion of copper

In a normal animal, the major pathway of the excretion of copper

is the bile. This was first suggested by Flin and Inouye (1) after experiments in the cat and confirmed later in the humans. Intraveneous administration of copper was followed by an immediate rise of the copper concentration in the bile (38). Experiments with Cu^{64} administered to dogs with complete biliary obstruction, with the flow of bile diverted into the urinary bladder and in normal animals, showed that 7-10 % of the radioactivity is excreted in the bile, 1.5% is passed directly through the intestinal wall and about 0.6% is excreted in the urine. When large quantities of non-radioactive copper are injected daily for 32 days, the excretion of copper in the urine is increased in dogs with bile duct ligation but not in normal animals (39).

The rate of urinary copper excretion is associated with the concentration of the non-ceruloplasmin copper in serum. Administration of Cu^{64} to humans results in an increased urinary excretion in the first two hours. The close relationship between the non-globulin Cu^{64} concentration and urinary excretion of Cu^{64} suggests that copper loosely bound to albumin is the main source of urinary copper (40).

B. COPPER DEFICIENCY IN ANIMALS

1. Techniques

a) Copper-free diet

This technique is recommended for experiments the purpose of which is the study of the effect of the copper deprivation on the function of different organs of the animal and in general the physiology of the body. It is also used for the study of enzymes suspected to contain copper in their molecule or to be copper dependent. Since no other factors are involved in this technique, it can be concluded that the induction of the activity of a certain enzyme is due to the absence of copper.

The usual diet in copper deficiency experiments is liquid or dry milk, both of which are poor in copper. Being also poor in other metals and vitamins, the milk is supplemented with necessary nutrients. Raw minced beef muscle and semipurified diet have been also used to produce copper deficiency in mice and rats (41).

b) Copper-free diet supplemented with ions alone or in combination with certain chemicals

In studies of cation antagonism, diets poor in copper in combination with supplements of the studied cation are given. When the symptoms of copper deficiency are accentuated in the presence of the cation, the latter is accounted for as antagonistic to copper. Several ions have been found to antagonize copper.

i. Copper-molybdenum, sulfate

A disease of cattle known locally as "teart" and characterized by severe diarrhea and loss of condition was found by Ferguson et al. (42) to be due to molybdenum intoxication. Copper deficient cattle exhibited the same disease and this led to the empirical and successful treatment of "teart" by giving copper sulfate to the diseased animals.

The discovery by Dick (43) that dietary sulfate intensifies the harmful action of molybdenum in the copper nutrition of sheep, introduced another element into the copper-molybdenum interrelationship. On the other hand, the observation by Gray and Daniel (44) that methionine alleviates molybdenum toxicity in the rat and that sulfate is as effective cannot be explained as a species difference, because of the diametrically opposite effects.

Gray and Daniel (45) in a study of molybdenum-sulfate or molybdenum-methionine effect on rats, postulated that molybdenum has

two different actions: in copper-depleted rats consuming a copper deficiency diet, molybdenum produced a greater copper deficiency which was exarcerbated by sulfate and prevented by copper. In rats with normal copper stores receiving adequate dietary copper, molybdenum did not induce a copper deficiency but caused some other dysfunction in metabolism (slower growth than normal) which was prevented partially by methionine and completely by sulfate.

ii. Copper-zinc

The antagonism between copper and zinc was shown thirty years ago by the observation of Sutton and Nelson (1) that diet rich in zinc produced anemia in rats in addition to subnormal growth and reproductive failure. In subsequent works, it has been found that anemia could be partially reversed by copper (46) and that the feeding of excessive dietary zinc to the rat markedly reduced the liver cytochrome oxidase and catalase activities. The enzymatic levels were restored to normal by the administration of small amounts of copper sulfate (47).

Diets containing high amounts of zinc suppress the concentration of copper in liver if the copper intake is marginal. On the other hand, when copper is given in excess, the level of zinc in liver decreases (1).

According to Hill et al. (48), the effect of zinc in reducing the cytochrome oxidase activity is explained by the fact that zinc can potentially attain the 4 co-ordination tetrahedral of the monovalent copper existing in the enzyme molecule. Thus, from this line of reasoning, zinc and any other metal which can attain this co-ordination, would be expected to be a copper antagonist. Cadmium which can attain the 4 co-ordination tetrahedral of monovalent copper, was found to be copper antagonist in the chick (49).

iii. Copper-silver

The mentioned theory has been used to explain the antagonistic effect of silver in chicks (48). When copper-free diet plus silver sulfate was given, the chicks lost weight and developed anemia. Controls receiving copper supplement remained normal in this respect. It was presumed by the authors that the univalent cation of silver which was added to the diet was in equilibrium with the divalent ion species of silver by the redox systems of the intestinal tract and of the body. Divalent silver would also be expected to be a copper antagonist because it has the potential of attaining the configuration of divalent copper complexes.

iv. Penicillamine hydrochloride

This well-known copper chelating compound used for the treatment of patients with Wilson's disease has also been used to produce copper deficiency in rats but without significant effect (50).

v. Reducing agents

It is known that in those oxidase enzymes containing copper, such as laccase, ceruloplasmin and possibly cytochrome c oxidase, that part of the metal component which exist in the +2 valence state, undergoes reduction and subsequent reoxidation during the course of the enzymatic reaction (51). This dependence upon oscillating valence states for function, suggested the possibility that reducing agents might influence copper metabolism. Thus, it was found that dietary ascorbic acid reduced the uptake of Cu^{64} by the liver, whether the isotope was given orally or intraperitoneally (52).

2. Manifestations of copper deficiency in animals

a) Clinical observations

i. Growth retardation, alopecia, achromatotrichia etc.

Rats made copper deficient showed in 60 days growth retardation (50). At this state, the conjuctivae and the skin of the paws appeared extremely pale, the colour of the pupil, which normally possessed a red tinge, was yellower in appearance. Textural changes in the hair were a common feature in these rats. Growth retardation has also been observed in rabbits fed whole milk powder for 13 weeks (53).

It has been reported that a deficiency in copper produced a graying of the hair of black rats and both depigmentation and baldness have been observed in cats when fed a copper-deficient diet. This syndrome was to a certain extent corrected by feeding copper and zinc.

Loss of crimping of the wool fibers has been observed in sheep. This change, while accompanied by a decrease in copper content, has also been correlated with an increase in the sulfhydryl groups and a decrease in the disulfide linkages of the wool (54).

ii. Neurologic disease

An original observation thirty years ago by Bennet and Chapman (55) that a severe copper-deficiency in pregnant ewes can produce a neonatal ataxia (swayback) in their lambs, has been verified by many workers with animals under natural or experimental conditions. More recently, Howell and Davison have shown that the copper content of the brain and the liver of lambs with swayback was significantly lower than that in normal animals (56).

The neurologic disease is characterized by inco-ordination of gait, paralysis and death. Examination of the central nervous system shows a diffuse symmetrical cerebral demyelination with secondary degeneration of the motor tracts of the cord. The administration of copper

to the ewe completely prevents the development of the disease in the lamb.

The nervous system disorder has been observed only in the second generation of copper-deficient animals. It is believed that manifestations of the deficiency begin in utero and clinical signs may not show up until the lambs are a few weeks or months of age. Experimental copper deficiency in rats, rabbits, chickens or pigs, failed to produce demyelination of the central nervous system.

In experiments to ascertain the factors which underlie demyelination of the central nervous system in copper deficiency, Gallagher et al. (57) concluded that the decrease in phospholipids synthesis and the loss of cytochrome oxidase activity, both observed in copper deficiency have a bearing on the myelin status and are particularly likely to lead to lesions of amyelination or demyelination if severe at the critical period, when myelin is being laid down most rapidly.

iii. Cardiovascular disorders

Experimentally induced copper deficiency in pigs and chickens leads to the death of these animals usually as a consequence of rupture of the coronary artery, the ascending segment or arch of the aorta or the pulmonary artery (58). The earliest observed histological lesion in the vessels is fragmentation of the internal elastic lamina and the affected vessels have a lowered elastin content (59). It is therefore probable that a defect in elastin leads to the cardiovascular lesions which cause the death of the animals. Isolation of mucopolysaccharides from the aortas of pigs raised on a copper-deficient diet and from control animals, showed that the copper-deficient aortas contained about 3 times as much polysaccharide as the controls (60).

iv. Anemia

Anemia as a result of copper deficiency has been demonstrated in rats, rabbits, chickens, pigs, dogs, sheep, goats and cattle. In true copper deficiency the peripheral red blood cells are predominantly hypochromic in all species except in dogs, where they are normochromic and normocytic (61).

Copper deficiency results in a decrease of the absorption of iron from the gastrointestinal tract and in a reduction of the survival time of R.B.C. (62). The total body iron is reduced in copper-deficient animals and the capacity of bone marrow to produce erythrocytes is limited.

It has been generally hypothesized that copper acts merely to catalyze the introduction of iron into hemoglobin. Another role of copper was also indicated, namely the stimulation of erythropoiesis and/or stimulation of the release of erythrocytes from the bone marrow. The blood of copper-deficient chicks promotes less incorporation in vitro of radioglycine into heame than does that from well-nourished fowl. The addition of copper to the blood of deficient animals restored the rate of glycine incorporation toward normal (63). It has also been suggested that one block in heame synthesis may be in the conversion of delta-aminolevulinic acid to protoporphyrin (64).

v. Skeletal abnormalities

Copper-deficient cattle, lambs, dogs and pigs showed gross deformities of the extremities with bowing and spontaneous fractures.

b) Biochemical observations

i. Copper content of tissues

Rats fed more than two months with a milk diet low in copper

had low content of copper in their tissues (50). The most depleted tissue was liver. Depletion in copper occurred in kidneys, spleen, spinal cord and brain, although to a moderate degree in that order.

The copper concentration in blood is also decreased during copper deficiency in rabbits (53). Depletion of copper in the liver has also been observed in chick and sheep.

ii. Enzymatic activities

A number of enzymatic activities have been studied in the tissues of the copper-deficient rat by Gallagher et al. (65). Cytochrome oxidase is reduced in moderate to advanced depletion and the mitochondria from these animals are very susceptible to "aging". The oxidative capacity of mitochondria is lost in extreme copper deficiency, the loss being ascribed to a decrease of cytochrome oxidase activity and aging. Extreme copper deficiency led also to a decrease of DPN-cytochrome reductase and a marked increase of isocitric dehydrogenase. Diminished cytochrome oxidase activity was also observed in the heart and liver of copper-deficient pigs (66). The mean catalase activity in the liver of copper-deficient animals was significantly lower than that of controls.

iii. Phospholipid synthesis

Considerable depression of mitochondrial phospholipid synthesis in liver but not in brain has been observed by Gallagher et al. (57) in the copper-deficient rat. The authors were able to demonstrate a possible failure of acylcoenzyme A to condense with α-glycerophosphate to form phosphatides and this was considered to be the primary lesion in the decreased synthesis of phospholipids, perhaps related to the neurological damage observed in copper-deficient sheep.

3. Copper deficiency in man

The average dietary uptake of copper by adults has been esti-

mated to be of the order of 2 to 5 mg (67). Adult subjects are in copper balance and therefore the total amount of body copper does not increase or decrease over time. It seems that a deficiency of copper of any magnitude would not be likely to occur in adults, because even a diet of mediocre quality contains at least 2 mg of the metal, widely distributed in foodstuffs. Therefore, a copper deficiency could occur as a result of failure to absorb the element of to retain it within the body, or more likely as a result of a combination of both.

Since hypocupremia is the sine qua non of copper deficiency in animals and one of the earliest detectable manifestations of copper depletion, the occurrence of copper deficiency in humans in the absence of hypocupremia would seem unlikely. About 95% of the serum copper is normally bound to ceruloplasmin and therefore hypocupremia might result from one of two distinctly different mechanisms:

a. Inability to synthesize ceruloplasmin

An inability to synthesize ceruloplasmin at the normal rate, could result from a deficiency of copper induced by a dietary lack of the metal, decreased absorption, failure to utilize, increased excretion or combination of these. The inability mentioned could also occur in the presence of adequate amounts of copper as a result of the inability to synthesize the protein portion (apoceruloplasmin) of the enzyme, due to a specific metabolic effect or to a generalized protein deficiency.

b. Excessive loss of ceruloplasmin

Hypocupremia could occur if large amounts of ceruloplasmin were lost across the intestinal wall or into the urine or bile. Other serum proteins of equal or smaller molecular weight would also be lost and

thus the hypocupremia might be associated with hypoproteinemia.

C. COPPER POISONING

Supplementation of experimental animals with large amounts of copper has been carried out by several investigators for two main purposes: (1) the study of the function of different tissues when copper is loaded and (2) the setting up of experimental model of Wilson's disease. The methods followed include administration of copper as a copper salt or in combination with another ion. In both cases, the supplements are given to the animal either mixed with the basal diet or by intraperitoneal, intramuscular or intravenous injection.

1. Effects of copper poisoning

a) Clinical observations

Wolff (68) studied the deposition of copper in the albino rat. Various amounts of copper were administered by daily intraperitoneal injections for 236 consecutive days. Necropsy showed excessive deposition of copper in liver and kidney but not in brain.

Goldfish, kept by Vogel (69) for several weeks in water to which copper sulfate had been added, developed in some cases signs of distress, becoming inactive and breathing from the surface of the water. In addition, their swimming motion was fluttery, gyrational or backward. Concomitantly, most developed hemorrhages in the bases of the pectoral and pelvic fins, in the gills and occasionally at the base of the dorsal fin and at the roots of the scales. Chemical analysis of their brains, livers and kidneys disclosed deposition of copper in concentrations comparable to those that occur naturally in Wilson's disease.

Hemolytic episodes have also been observed in copper-poisoned animals. Todd and Thompson (70)showed that repeated injections (twice

daily for 2 to 3 days) caused hemolytic episodes in sheep similar to the crisis of chronic copper poisoning. The hemolytic crisis in sheep is preceded by some 24 hours or more by an increase in the concentration of copper in the blood stream, but whether copper acts directly as an hemolytic agent in this case is not clear.

b) Histological observations

Groups of inflammatory cells, areas of degeneration, hyalinization and periportal fibrosis, all compatible with early cirrhosis were consistent findings in the liver of copper-poisoned rats (68). In addition, large amounts of the metal were found by histochemical methods in the cytoplasm of hepatic parenchymal cells. The same rats showed degeneration and sloughing of the epithelial cells of the proximal renal tubules and specific stains revealed that copper was localized in the epithelium of the proximal convoluted tubules.

Cytologic alterations in the central nervous system of experimentally copper-poisoned animals are not usually observed. However, histochemical studies in the goldfish by Vogel (69) made clear that much copper had accumulated within the large neurons, principally in those of telencephalon and anterior horn region of the spinal cord. The intraneural deposition of copper was regularly associated with cytologic changes, notably contraction and hyperchromaticity of the nerve cells with tortuosity and fragmentation of the axis cylinders and lysis and loss of neurons.

c) Biochemical observations

i. Copper in tissues

In very few copper-poisoning experiments has copper been measured in the tissues of the loaded animal. The existing data includes

copper values in liver, kidney and brain. Gubler et al. (71) found that rats receiving a basal diet containing 0.1 % copper sulfate for 86 days, had accumulated copper in their livers and kidneys but not in brain. The copper in liver was increased by eight-fold and in kidneys by three-fold. The plasma copper was also increased by 20 %. The copper excreted in urine was doubled.

Copper-overloading in rats with Cu^{64} by Scheuer and Barka (72) disclosed that the retention of the metal by the liver is not due to saturation or impairment of the normal excretory mechanism but rather to diversion of part of the copper from the normal excretory pathway.

Single injections of 80 to 160 mg copper in sheep (70) resulted in a 20- to 30-fold increase of blood copper after half an hour. The animals survived for only a few hours. On analysis, the liver and kidneys were found to contain 230 and 80 ppm of copper, respectively in the wet tissue. Intravenous injections in sheep of 40 mg copper, tolerated by the animal without effect, resulted in a 4-fold increase of copper in blood in half an hour, falling to near normal figures within a few hours.

ii. Hematological examinations

Chronic copper poisoning in calves (73) produced marked increase in packed cell volume (P.C.V.) and hemoglobin (Hb) at the onset of the clinical phase. However, after the first day of the crisis, both P.C.V. and Hb values fell very rapidly below the normal levels. At the height of hemolysis, free Hb in the plasma reached a concentration of more than 1 gm/100 ml and marked methemoglobinemia occurred at the hemolytic crisis.

Sheep injected intravenously with 80 mg copper as acetate showed after 17 hours hemoconcentration. In the same experiment, with multiple injections of copper at concentrations insufficient to cause any

acute symptoms, the blood appeared deeper in colour on the second day and this was taken as evidence of a slight degree of hemolysis. On the third day methemoglobinemia and jaundice occurred. Minimum values of P.C.V. and Hb below the normal levels were recorded after 5 to 6 days. Again, as in calves, methemoglobinemia occurred in all animals at the time of hemolysis. Anemia did not occur in rats fed high amounts of copper for 120 days (71).

iii. Enzymatic activities

Sheep maintained for 3 months on a ration containing 250 ppm copper and then given a single injection of 80 mg copper as the acetate salt, showed elevated levels of serum lactic dehydrogenase (L.D.H) and glutamic-oxaloacetic transaminase (G.O.T). Only slight increases in these enzymes were noted in the sera from animals on normal rations injected with the same amount of copper (70).

L.D.H. and G.O.T. in the sera of poisoned calves were also increased one week before the hemolytic crisis and during the clinical phase. It appears that at the time of crisis, widespread damage of liver takes place; also, plasma activities of these enzymes increase abruptly. The chain of events which is characterized by the hemolytic syndrome may be regarded as resulting from the liver injury (73).

Studies on the effects of copper and other metals on some enzymes in Neurospora crassa by Nicholas and Commisiong (74), showed that the activity of cytochrome c oxidase was enhanced by adding copper. A similar result was obtained by Van Reen (47) in the rat where added copper alleviated the effect of zinc in depressing cytochrome c oxidase in the liver. In the same experiment with Neurospora crassa, copper depressed the activities of nitrate reductase, cytochrome c reductase

and acid phosphatase.

2. Effect of other ions on the toxity of copper

a) Manganese

The clinical and pathological similarity between the manifestations of chronic manganese poisoning in man and the changes which occur in Wilson's disease led Gubler et al. (71) to determine whether or not the administration of large amounts of manganese influences the metabolism of copper in rats. It was found that high dietary copper plus excessive manganese caused a marked increase of copper in the body, particularly in the liver and spleen, but also in the plasma and brain. However, neurologic abnormalities, corneal pigmentation, aminoaciduria and histologic alterations in the brain were not observed.

b) Molybdenum and sulfate

Chronic copper toxicosis was observed in sheep fed a ration low in molybdenum, low to normal in copper, and probably normal in sulfate (75).

c) p-Dimethylaminobenzene (DMAB)

Fare and Woodhouse (76) found that when rats are fed DMAB there is a gradual increase in the concentration of copper in liver, reaching a value 35% above normal after 380 days on a diet of maize containing 0.09% DMAB. An equivalent decrease in the copper content of the kidney was observed.

When 0.5% of cupric oxyacetate hexahydrate was fed in addition to the DMAB, a good degree of protection was given against the development of liver tumors, and the copper content in liver increased enormously to 40 times normal after 380 days, but not to any appreciable extent in kidney.

3. Effect of copper on the toxicity of other agents

a) Cysteamine and N,N-Diethylcysteamine

It has been shown by Stern et al. (77) that if 10 mg/kg body of copper sulfate is injected intraperitoneally 15' after N,N-diethylcysteamine in mice, then all mice died, while none of those which were injected only with N,N-diethylcysteamine died. This observation of an interaction between copper and a tremor-producing agent, is of interest in relation to Wilson's disease, where elevated brain copper and intention tremor frequently occur.

The effect of copper upon the tremor in mice produced by cysteamine and N,N-diethylcysteamine and upon the toxicity of these substances, has been investigated by Curzon and Schnieden (78). The toxicity of N,N-diethylcysteamine was shown to be markedly potentiated by copper sulfate while the toxicity of cysteamine was only affected slightly, possibly because of the insolubility of the cysteamine-copper complex that is formed. Though enhancement of N,N-diethylcysteamine tremor by copper was shown, it was not possible to demonstrate an enhancement of cysteamine tremor.

Copper chelates very strongly with cysteamine. Cysteamine-copper complexes with charge transfer properties have been reported, and these may well possess high biological activity (79). It is of interest that cysteamine causes catecholamine release from the adrenals and that the catecholamine dopamine potentiates N,N-diethylcysteamine tremor (77). There is some evidence that disturbed catecholamine metabolism may be causally related to the tremor occurring in various extrapyramidal diseases (28) and if a cysteamine-like substance were demon-

strated in brain, then the copper-potentiated tremor might be a close model of the intention tremor of Wilson's disease (78). While cysteine is not decarboxylated to cysteamine by amino and decarboxylases, other pathways of cysteamine formation in vivo have been suggested (80), though direct evidence is at present lacking.

4. Copper poisoning in Man - Wilson's disease or Hepatolenticular degeneration (HLD)

In his original monograph in 1912, Wilson described the disease, which is now named after him, as familial in the sense that frequently more than one member of a family is affected with it.

Subsequent studies by Bearn (81) have indicated that the disease is hereditary and is transmitted in autosomal recessive fashion by parents who never develop the illness. This implies both that Wilson's disease appears in those subjects who are homozygous for the "Wilson's disease gene" and that each parent of such a subject must be a heterozygote who possesses one of these abnormal genes and one of its normal alleles.

a) Clinical and pathological picture

HLD is characterized by involuntary movements, usually of the nature of tremor, dysarthria, muscular weakness, spasticity and contractures, with progressive emaciation. These may be associated with emotionalism and certain symptoms of a mental nature. HLD is progressive and, after a longer or shorter period, fatal.

Pathologically it is characterized by bilateral denegeration of the lenticular nuclei; cirrhosis of the liver is constantly found. Rings of brown or grey-green pigmentation in Descement's membrane near the outer margin of the cornea,regarded as pathognomonic of Wilson's disease (Kayser-Fleischer rings), have been observed in nearly all cases.

b) Biochemical picture

i. Copper in tissues and fluids

Positive copper balance is a central feature of this disorder with a resulting accumulation of copper in all the tissues of the body and consequent intoxication of the cells of these various tissues, particularly the brain, liver and kidneys. Deposition of copper in these organs is associated with degenerative changes in (a) the brain, affecting most often the basal ganglia and leading to the tremor-rigidity syndrome; (b) the liver, leading to cirrhosis and (c) the kidneys, leading to abnormalities of renal tubular function.

The intracellular distribution of copper in brain and liver, both from Wilson's disease and from normal subjects, has been studied by Porter (82). The largest proportion of the copper in brain was found in the subcellular soluble fraction. The yield of copper in this fraction from brain of HLD case was 18 times that obtained from normal brains. According to the author, the excess of copper becomes bound to a number of different brain proteins which are normally copper-free. A significant portion of the pathological copper in liver is found in the mitochondrial fraction, accounting for 35 % of the total tissue copper and containing more than 40 times as much copper as in the mitochondria of normal adult human livers.

Uzman (83) in his study of the nature of copper-binding in normal and HLD brain concluded that the excess copper deposited in HLD is of the direct-reacting variety available for chelation. Whether this copper is fixed <u>in situ</u> as a complex with proteins or with peptides is still an open question. In addition, Uzman found that the copper is deposited in high concentrations in discrete sites within

glial cells, but is absent in nerve cells and ground substance.

Deposition of copper in Descemet's membrane of the cornea is responsible for the development of the Kayser-Fleischer rings.

Finally, the concentration of loosely bound non-ceruloplasmin copper in serum of Wilson's disease patients is increased and the ceruloplasmin-copper is decreased, the net result being a decrease in serum copper. The urinary excretion of copper is increased. The urinary copper is non-dialyzable, probably deriving from direct-reacting copper that has been excreted with albumin and/or polypeptides into the urine (84).

ii. Ceruloplasmin

Wilson's disease has been associated with a quantitative deficiency of the plasma copper protein ceruloplasmin. Almost all patients with HLD have less than 20 mg of ceruloplasmin per 100 ml of serum and some have even too little to be detectable. Normal values are 20-35 mg per 100 ml.

iii. Aminoaciduria

Since the report of Uzman and Denny-Brown (85) that Wilson's disease is associated with an aminoaciduria, several studies of this aspect of the disease have appeared. The amino acid distribution in 24-hour speciments of the urine of patients with HLD has been investigated by Stein et al. (86). The highest levels of excretion relative to the normal were reached by threonine and cystine, which were elevated 20-fold. The levels of serine, glycine, asparagine, valine, tyrosine and lysine were increased 5 to 10-fold, while histidine, ornithine and phenylalanine occurred at 2-4 times the normal level. Two amino acids not encountered in normal urine, namely proline and citrulline appeared in considerable quantities in many cases. Finally, the amounts of

aminoadipic acid, methionine, isolencine, leucine and arginine seemed to be the least abnormal, while on the average, there appeared to be a diminished excretion of taurine, 1-methyl-histidine and 3-methyl-histidine.

Amino acids in conjugated linkages occur in HLD urine in quantities about double those obtained from normal urine.

In Wilson's disease, the level of urinary amino acids is quite sensitive to the protein uptake and fasting urine has a very different amino acid composition from that found for the 24-hour speciment. The aminoaciduria of Wilson's disease is said to result from a renal lesion. Other renal abnormalities found in HLD include glycosuria, phosphaturia, hypouricemia and uricosuria.

c) Primary lesion in Wilson's disease; theories

i. Defective synthesis of ceruloplasmin

According to Scheinberg (87), in Wilson's disease the deficiency or absence of ceruloplasmin in the serum is due to a specific inherited inability to synthesize this protein at a normal rate. The serum copper, bound loosely to serum albumin rather than tightly to ceruloplasmin, is excreted in the urine and deposited in tissues where the affinity for copper is greater than that of serum albumin. Accumulation of copper in brain, liver and kidney leads to the observed disturbances.

Nevertheless, some workers find the relation between deficiency of ceruloplasmin and the abnormal copper metabolism hard to accept, because of reports of persons with abnormally low concentration of the copper protein who do not have HLD and of patients with normal concentration of ceruloplasmin who unquestionably have the disease (88). Furthermore, it has been known for several years that in some patients

with HLD, moderately deficient in ceruloplasmin, pregnancy (89) and administration of estrogens can raise the serum concentration of ceruloplasmin into the normal range, if not above it. In such patients it would not be surprising if necrosis or inflammation of hepatic parenchyma had the same effect, particularly since hepatic dysfunction has been shown to impair degradation of estrogenic hormones. Thus, this may have played a role in the raised ceruloplasmin concentration of some patients with HLD.

ii. Formation of proteins with high avidity for copper

Liver tissue homogenates from Wilson's disease patients have, according to Uzman et al. (90), increased avidity for copper. Electrophoretic separation of the liver extracts contain an extra protein fraction. The authors suggest that the high copper-binding of this fraction results in the increased avidity for copper in this disease. It is also suggested that the mechanism of copper deposition in the liver (and possibly in brain) in HLD is due to a genetically determined abnormality in protein metabolism which leads to the formation of proteins with high avidity for copper. The disturbance in ceruloplasmin synthesis is explained by the fact that copper is not available for the incorporation into the apoprotein in the liver.

d) Treatment of the disease

The methods of treatment introduced to counteract the disordered copper metabolism in HLD can be divided in three groups:

i. Increase of the serum ceruloplasmin

Sufficient amounts of purified ceruloplasmin have been administered intravenously in order to restore the serum oxidase to a normal level but without success (91). Estrogens have also been administered

to patients with Wilson's disease and, although an increase in the copper protein was found in some of them, the course of the disease was unchanged.

ii. Restriction of the absorption of copper

Oral administration of potassium sulfide and of ion exchange resins in an attempt to prevent absorption of intestinal copper resulted in a negative copper balance in patients with HLD.

iii. Elimination of the excessive copper deposited in the body tissues

2,3-Dimercaptopropanol (BAL), ethylenediamine tetraacetate (Versene) and β,β'-dimethylcysteine (penicillamine) (92), all chelating copper and excreted in the urine, have been employed for this purpose.

D. CHEMICAL RELATION BETWEEN CATIONS AND LIPIDS

1. K^+, Na^+, Ca^{++}, Mg^{++} and lipids

The binding of inorganic ions by lipids was suggested by some of the earliest investigators of lipid chemistry. Thus, Koch (93) reported the binding of alkali and alkaline earth metals by phosphatides; Blix obtained the potassium salt of sulfatides; and ether-soluble calcium phosphatides have been extracted from cabbage leaves.

Since then, a great deal of work has been done on the identification and isolation of metal-lipid complexes. Folch showed that the potassium and sodium content of isolated phosphatidyl serine was in a ratio 1:1 with phosphorus and these cations were bound in a salt linkage (94). In addition, diphosphoinositide has been isolated as salts of sodium, potassium, magnesium and calcium (95). In another paper (96) Folch again demonstrated that brain lipid fractions may contain alkali and alkaline earth metals bound simply as salts to acidic lipids and

that the cations are interchangeable. At the same time, it was found that the sodium salt of synthetic phosphatidic acid is soluble in ether or chloroform (97).

In addition to phospholipids, peptide- or protein-containing lipids were also found to contain metals. Hakamori (98), reported the isolation of metal-bound lipid-peptide complex from ox-brain, and Papahadjopoulos (99) demonstrated the requirement of calcium in the formation of a lipoprotein complex exhibiting full activity as a prothrombin activator.

Lipids have been found not only to contain metals but to bind them in vitro as well. Various authors have found that certain cations react in vitro with aqueous cephalin suspensions, but Christensen (100) was the first to perform a quantitative study of sodium- and potassium-binding by cephalin. Calcium, magnesium, potassium and sodium are transferred from an aqueous system into a lipid solvent in the presence of cerebrosides, gangliosides and acidic lipids respectively (101, 102).

The mechanism of this transfer has been studied extensively with gangliosides in the presence of mineral salts, by Quarles and Folch (103). They were able to show that sodium, potassium and magnesium do not affect the solubility of gangliosides in an aqueous phase, although the presence of a wide range of calcium results in a shift of most of the lipid into the organic phase, transferring back to the aqueous by adding higher concentrations of calcium, sodium or magnesium. Competitive effects between the cations were also demonstrated. The explanation given was that a change in the physical state of the ganglioside molecules, such as an alteration in micellar form occurs in high concentration of salts.

The effect of salts on the behavior of proteolipids in diphasic systems was studied by Webster and Folch (104). They were able to show that the stability of proteolipids is influenced by the pH and ionic strength of the medium, the splitting off of the lipid moiety being roughly proportional to the log of ionic strength. Monovalent ions had higher splitting capacity than divalent.

Various theories have been suggested to account for the ability of lipids to attract, in vivo or in vitro, cations of great physiological importance. Vandenheuvel (105), in a schematic description of lipid-protein interaction in a hydrated lipoprotein layer, underlines the role of calcium and other polyvalent cations by binding lipid and protein groups; he suggests that there is competition of inorganic ions for charged groups in both lipid and protein.

Furthermore, the possible role of lipids in the transport of ions in tissues has been the subject of much experimental work and discussion. It is thought, for example, that phosphatidic acids are involved in sodium transport in tissues (96), and that acidic lipids play a role in the electrolytic balance of nervous tissues (106). Indirect evidence implicates gangliosides in cation transport (107) and synaptic transmission in the central nervous system (108).

2. Trace metals and lipids

Very few reports in the available literature are concerned with the binding of physiologically important trace metals. Ward and Fautl (109) in 1963 reported that manganese reacts in vitro with phosphatidic acids giving a ratio 1:1 with phosphorus.

Copper and iron were reported recently by Colburn and Maas (24) to form complexes with phosphatidyl serine and phosphatidyl inositide

as well as with lipid extract from mouse brain.

E. PARTITION OF LIPIDS

All known methods for the extraction of lipids from animal tissues and fluids can be divided historically into two main groups. The first group includes those used before the introduction of Folch's methods, whereas in the second group belong those developed by Folch.

The justification of this distinction lies in the fact that the newer methods offered simplicity and high extracting power, and provided the basis for the discovery of some new important lipids. These methods effected a revolution in the study of the chemistry of brain and nerve.

1. Old methods

Ether was one of the first organic solvents used for extraction of lipids from animal tissues. However, it was soon understood that ether alone does not extract a great part of the phospholipids (110), but ether extraction followed by treatment of the residue with warm alcohol resulted in removal of the remaining phospholipids.

The important discovery by Zuelzer (110) in 1899 that phospholipids were insoluble in acetone, introduced as a first step of the extraction procedure, the treatment of the tissue with acetone which would extract the neutral fats, fatty acids and water. The residue was then re-extracted with ether and alcohol to obtain the phospholipids.

By the beginning of this century extraction consisted of the following steps:

a) Drying of the fresh tissue: The drying was carried out either in vacuo or by treatment with alcohol, acetone or anhydrous salts. Obviously acetone treatment was the best technique because this solvent

could eliminate neutral fats and fatty acids besides water.

b) Extraction and isolation of lipids from dried materials: Ether and alcohol were used for the extraction, in mixture or one at the time.

In 1914, Bloor (111) in his method for the determination of fat in blood, standardized the alcohol-ether mixture to 3:1 composition (approximately 5:1 on a molar basis), but as he himself has stated, the extraction of lipids was incomplete. In addition, some of the inorganic constituents were also extracted. Their elimination was carried out by evaporation of the ether-alcohol extract and extraction of the residue by petroleum ether in which inorganic materials are insoluble. However, it was shown later by Folch and Van Slyke (112) that the petroleum ether solution thus prepared, contained water-soluble extractives, soluble in petroleum ether by means of lipid-salts soluble in organic solvent. Folch and Van Slyke proposed a solution to this problem by precipitating proteins and lipids of blood together, removing water-soluble extractives from the precipitate by washing with water and extracting the lipids from the washed residue (112).

The phosphorus content of the lipid extract has been used as criterion for a quantitative yield of lipids. Thus, Boyd (113) proved that Bloor's solvent mixture extracts a maximal yield of plasma lipids at room temperature. Phosphatides were found the most difficult lipids to extract and therefore a quantitative extraction of phosphatides affords evidence for a complete extraction of the other lipids. Evidence was also given that Bloor's extract does not contain any significant proportion of phosphorus in forms other than the phosphatides, by adding tracer amounts of $Na_2HP^{32}O_4$ to plasma and finding that less than 0.05%

of the p32 was extracted (114).

Relatively little attention has been paid during the early period of techniques for the extraction of lipids, to the possible decomposition of the biological sample before and during manipulations, resulting in an altered state of some lipids. Brante in 1949 (115), reviewing the difficulties of the extraction of lipids concluded that the lipids in the tissues should be rendered fully accessible to the subsequent extraction and that undivided portions or the formation of clusters should be avoided, their centers being very inaccessible to the solvent effect.

Some of the requirements initially put on the extraction procedure were: applicability to fresh tissues, completeness and selectiveness in regard to lipids, and simplicity of technique. When fresh tissues are extracted, some sort of dehydration preceding the extraction is implied for most of the more selective solvents. Thus, the extraction may be carried out with water-miscible lipid solvents such as acetone or alcohol which cause, in addition, denaturation of proteins and splitting of the protein-bound lipids. It has also been found that hot ethanol or methanol in excess is capable of a quantitative release of most lipids from tissue, particularly when they are present in mixtures so that there is a mutual increase in their solubility. Brante himself has adopted and routinely used a primary extraction with alcohol-ether, 3:1, followed by a re-extraction with hot chloroform which was also used to release any lipids remaining in the tissue residue after the alcohol-ether extraction.

McKibbin and Taylor (116), in 1949, to minimize artifacts produced by post mortem enzymatic hydrolysis of the tissue lipids, extracted lipids from frozen tissue ground with dry ice. The ground tissue

was refluxed for 1 1/2 hrs. with a large quantity of Bloor's mixture, filtered, evaporated and the residue extracted in a Soxhlet extractor with chloroform for 6 hrs. Then the combined alcohol-ether and chloroform extracts were concentrated under reduced pressure in a stream of nitrogen and the precipitate obtained was dissolved in chloroform.

Using any of the known methods, a certain quantity of non-lipid tissue constituent is always obtained in the extract. Thus, efforts have been made to prevent such contaminations and up to 1951, the following methods for the elimination of impurities were mainly used:

a) Addition to the alcohol of a more selective lipid solvent which may also contribute to the dissolving power of alcohol.

b) Special pretreatment of the tissue, resulting in the removal of some non-lipid water-soluble constituents. Precipitation of proteins with colloidal iron or tungstate-sulfuric acid and subsequent washing of the precipitate with water, or dialysis of the tissue homogenate at low temperature, were some of the most commonly used pretreatments.

In conclusion, the method of Bloor, either in its original form or with slight modifications, has been a standard procedure for the preparation of lipid extracts from tissues.

2. New Methods

It was early recognized that in the case of nervous tissue, not all the lipids present are extracted by Bloor's procedure. Thus, the introduction of a subsequent extraction with another solvent of higher power, usually chloroform, was found necessary. This resulted in time-consuming and complicated methods.

In 1951 Folch, Ascoli, Lees, Meath and Le Baron (117), published a simple method for the extraction of lipids from brain tissue. By this

method, the fresh tissue is homogenized with a 2:1 chloroform-methanol mixture (approximately 1:1 on a molar basis), in the proportion of 20 ml of mixture per gm of tissue. Usually, 3 minutes suffice for complete extraction. The method was found to be applicable to every tissue. A comparative study of extraction by the methods of Brante (115) and McKibbin and Taylor (116) showed a significant increase of the total solids in the crude extract obtained by the Folch method, a fact underlining higher extracting power (117).

The elimination of the non-lipid water-soluble tissue constituents was achieved by placing the clear lipid extract in contact with a large amount of distilled water. The high efficiency of this "washing procedure" does not depend for its mechanism of action on simple diffusion across a stable interface, but on a complex sequence of events that can be summarized as follows: as soon as the water comes in contact with the extract, methanol starts diffusing into the water, thus leaving chloroform in the extract. The chloroform, being heavier than the rest of the extract, flows toward the bottom of the container, a new layer of fresh extract replacing it at the interface. At the same time, the methanol-water mixture that has moved to the water side, being lighter than water flows upward and is replaced by new water. After equilibrium has been reached, two layers are observed with an interfacial fluffy material. The upper water-methanol layer contains all the impurities, while the fluffy material and the lower chloroform phase contain the washed lipids. Addition of methanol to the interfacial "fluff" and chloroform layer results in a monophasic clear solution.

The described time-consuming washing procedure was later substituted by Folch and his colleagues (101) by a more accurate and rapid

technique involving partitioning of the extract with 0.2 volume of aqueous solution of salts and centrifugation of the mixture to obtain a clear chloroform layer containing all extracted lipids except strandin, a newly discovered lipid of high molecular weight, which could be isolated from the upper water-methanol layer by dialyzing out all other solutes.

The development of these methods resulted in the discovery and isolation by Folch's group of two very important lipids: proteolipids (118) (119) and strandin (120). Proteolipids were found later to be the main constituent of myelin while strandin is assumed to be polymerized form of gangliosides in gray matter.

Treatment of the brain tissue with Folch's mixture results in the extraction of nearly all lipids. However, some di- and tri-phosphoinositides remain tightly bound to the brain proteins and are released only by extraction with acidified chloroform-methanol mixture. Dittmer and Dawson (121) obtained tri-phosphoinositides by extraction of the tissue with chloroform-methanol mixture and re-extraction of the residue with slightly acidified chloroform-methanol mixture.

Recently, it was found that the extractibility of the white matter proteolipids into the Folch's mixture is dependent on the initial pH of the tissue homogenate. Wolfgram (122), studying the role of pH in the extraction of proteolipids, concluded that more proteolipid is solubilized at acid pH than at neutrality and conversely less is solubilized in the alkaline pH range. The additional proteolipid that becomes soluble at acid pH is trypsin-digestible and has a different amino acid composition from the trypsin-resistant proteolipid of Folch and Lees (118). On partitioning the extract with 0.2 volume of water,

the new proteolipid remains in the lower phase as does the Folch-Lees proteolipid.

II. METHODS AND MATERIALS

A. CHEMICAL

1. Determination of copper

Two steps are involved in any method for the determination of copper in biological materials; a) the liberation of the metal from its organic complexes and, b) the determination of inorganic copper by a micromethod.

The following procedures are used mainly for the first step:

a) Liberation of the metal from its organic complexes:

i. Dry-ashing

The biological sample is ashed at 600-700° C and determination of copper ions is carried out by one of the known micromethods. Although dry-ashing is an accurate method, it is time consuming and open to the criticism that copper may be lost by spattering or volatilization as a mineral salt.

ii. Wet-ashing

The organic matter is oxidized to CO_2, H_2O and NH_3, using strong oxidizing reagents such as HNO_3, $HClO_4$, conc. H_2O_2 or different metallic catalysts at a relatively low temperature between 100 to 235° C. At the end of the oxidation, the solution contains the salts of the inorganic constituents of the sample and residual reagents.

iii. Mild treatment with acid

Copper in blood is bound mainly to proteins and attempts were made to break the bond. It was recognized early that the copper-protein bond is very sensitive to low pH.

Proteins of plasma are precipitated with cold or hot CCl_3COOH (123, 124, 125), and free copper is extracted from the supernatant.

All these methods are unsuitable for the determination of copper in red blood cells.

in 1939 Schmidt (126) demonstrated that the protein-bound copper in plasma could be completely released by treatment with dilute HCl and two years later Holmberg (127) found that whole blood or plasma copper is liberated by incubation with HCl 6N. A systematic study by Gubler et al. (128) of the effect of various concentrations of the acid on the copper-protein bond, revealed an optimal normality of 2 (128).

The only other organ examined by this technique is brain (129) but the proposed method fails to give reproducible results.

iv. Relative merits of the above methods

The advantages and disadvantages of these methods for the liberation of copper, have been studied in order to choose the most convenient one for the determination of copper in tissues and fluids in experiments presented in this thesis.

Incubation at low pH, to release copper, was excluded because, according to present knowledge, this method is applicable only to biological fluids.

Some of the most important advantages of the wet-ashing procedure in comparison with the dry-ashing include rapidity, oxidation at low temperature, exclusion of volatilization of copper salts and trapping of ammonia in H_2SO_4 with the formation of $(NH_4)_2SO_4$. The last one was the most decisive reason for the choice of the wet-ashing method, because determination of nitrogen was included in some of the experiments.

b) Determination of inorganic copper:

Dry- or wet-ashing or incubation at low pH yield copper in an ionic form which is more convenient for a chemical, physical or physico-

chemical determination. But even after its liberation, copper still co-exists in the final sample with other inorganic ions commonly found in biological materials.

Iron, calcium, potassium and phosphates occur in all biological samples and interfere with the determination of copper. Some of them react with reagents added for the colorimetric determination of copper or interfere in methods like flame or atomic absorption spectrophotometry.

Therefore it is often necessary to eliminate the interfering ions or to isolate the copper ions. However, some colorimetric methods are highly specific for copper and no coexisting inorganic ion interferes.

The best known methods for the determination of copper in biological materials are divided into four groups.

i. Colorimetic methods

ii. Flame spectrophotometry

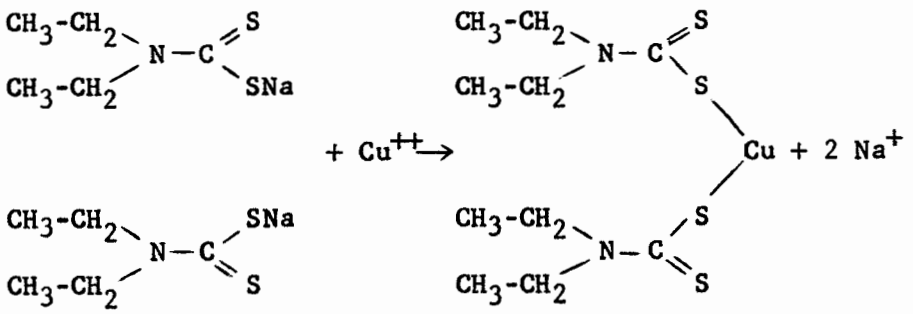
iii. Atomic absorption spectrophotometry

iv. Neutron activation analysis

i. Colorimetric methods

a) Sodium Diethyldithiocarbamate or DDTC method

The method is based on the yellow product formed in the following reaction:



Iron gives a brownish yellow complex with DDTC and calcium precipitates as phosphate salt at the alkaline pH used. Upon the addition of a large quantity of sodium pyrophosphate and NH_3 iron deionizes, forming an iron-ammonium pyrophosphate complex and the pH becomes strongly alkaline.

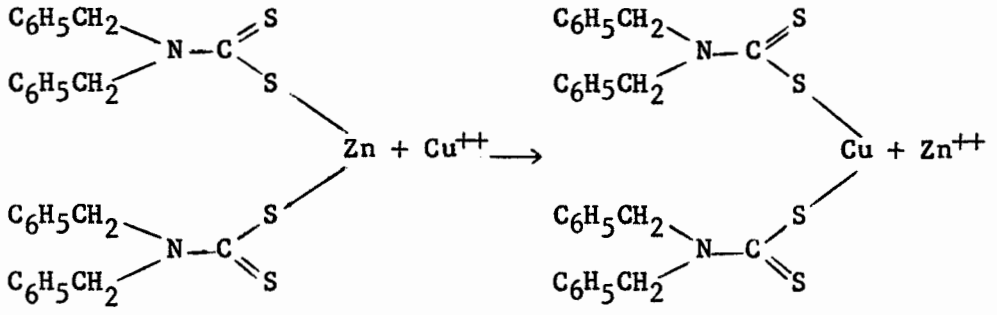
Deionization also occurs when citrate is used to prevent precipitation of calcium phosphate. Ferric ions are completely deionized in the presence of sufficient ammonium and citrate salts and it is not necessary to use pyrophosphate for deionization when citrate is already present.

Therefore, for samples relatively low in calcium and phosphorus but high in iron, such as blood or soft tissues, the pyrophosphate and citrate methods are equally good. For materials of unknown Ca and P content, deionization of iron is carried out with ammonium citrate.

After the elimination of the interfering factors, DDTC is added and the developed complex is extracted with amyl alcohol and measured in a colorimeter.

b) Zinc dibenzyl-dithiocarbamate or DBDC method

Cu^{++} ions in a highly specific reaction with DBDC give a yellow complex (130):



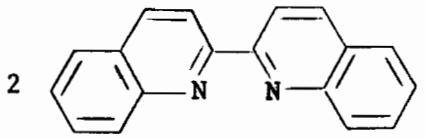
DBDC has been used for the determination of copper in urine.

c) Oxalyldihydrazide method

Various workers have described the use of oxalyldihydrazide and acetaldehyde for the colorimetric determination of copper. When in aqueous ammoniacal solutions, these reagents give a blue-red colour with Cu^{++} (131).

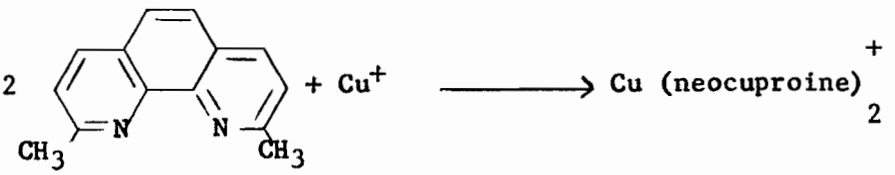
d) 2,2-Biquinoline or cuproine, 2,9-dimethyl-1,10-phenanthroline or neocuproine, and 2,9-dimethyl-4,9-diphenyl-1,10-phenanthroline or bathocuproine methods.

Cuproine gives an intense purple colour with cuprous ions (132).



The reaction is specific for Cu^{+}. Although it forms no colour, iron interferes by reducing the transmittancy, thus giving erroneously high values for copper.

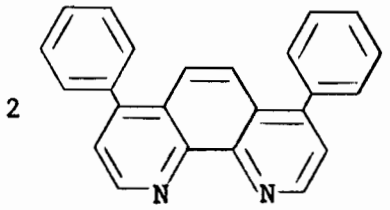
Neocuproine is also a specific reagent for Cu^{+} forming a bright orange soluble compound (133).



No other cation is known to give coloured complexes with neocuproine. An orange yellow precipitate is formed with large amounts of nitrate, perchlorate and the halides, but this causes no interference because the copper-complex is quantitatively extracted.

Ions such as nitrate, which react with hydroxylamine (added for the reduction of Cu^{++} to Cu^{+}) must be eliminated.

Finally, bathocuproine is thought to be superior to all other copper reagents in sensitivity and selectivity (134).



ii. Flame photometry

When Cu^{++} ions are introduced into a flame of sufficient temperature to excite them, they emit radiation at a characteristic wavelength of 3247 Å (135). Ions such as phosphate and sodium, usually present in much greater quantities than copper, increase the background radiation. Preliminary extraction of copper by dithizone into CCl_4 eliminates the interference (136).

iii. Atomic absorption spectrophotometry

This method, first described in 1955 (137), was applied to the determination of copper in 1960 (138, 139). The solution of the sample is sprayed into an air-acetylene or air-coal gas flame and the absorption of light by the copper atoms in the flame is determined. A hollow cathode lamp with a copper or brass cathode which emits the line spectrum of Cu is used as a light source.

Unlike other metals, copper has the most sensitive line (3247 Å) in a region of the spectrum where emission from the flame is affected by a number of elements. Sodium and potassium emit a continuum in the region of 3300 Å and calcium seems to depress the flame emission at this wavelength. Again, all these interferences are eliminated when copper is first extracted into an organic solvent.

iv. Neutron-activation analysis

Even without the use of the highest available neutron fluxes, the thermal-neutron activation analysis is convenient for determining trace metals with much greater sensitivity than that of any current technique. Additionally, high specificity may be obtained since interfering nuclear reactions are usually unimportant.

A method was proposed in 1964 (140) for the determination of trace metals, including copper by thermal neutron irradiation for 7 days. After irradiation, chemical separation of trace metals and elimination of elements of no interest is necessary. Copper is measured in a γ-ray spectrometer.

The value of the method for the biological field lies not only in its accuracy. Even more interesting is the fact that quantities of the order of 10^{-3} μg copper can be measured, provided that extreme precautions against contamination are taken.

v. Discussion

Accuracy, sensitivity, simplicity and specificity were the factors among others to be accounted in choosing a method.

With the exception of the neutron-activation analysis, all described methods present the same order of accuracy and sensitivity. Because all methods include the tedious step of the copper liberation procedure, the simplicity is again more or less the same. The cuproine, neocuproine and bathocuproine methods present the higher specificity but they lack simplicity for the fact that reduction of cupric copper to cuprous copper is needed.

Flame and atomic absorption spectrophotometry have been excluded because expensive instruments are needed. These two methods are likely most useful when the determination of more than one trace metal is required. Moreover, at the present time the latter method does not offer significantly greater sensitivity than the colorimetric. The time consuming and expensive neutron activation analysis is obviously not a method for routine purposes.

Finally, the DDTC method was chosen because it has been tested for years in many laboratories and it was proven simple, sensitive and accurate, giving reproducible values for copper.

c) Methods finally adopted for determination of copper

The method of Eden and Green (141), which includes wet-ashing and reaction with DDTC, was adopted for reasons cited above. Nevertheless, minor modifications were made to this method in an attempt to overcome difficulties arising from the nature of some samples. All glassware used had been treated with a mixture of $K_2Cr_2O_7$ and H_2SO_4 and rinsed thoroughly with tap and distilled H_2O successively. Reagents were A.R. grade. Details about reagents used throughout the experiments presented in this thesis are given with each procedure.

Digestion of tissues or fluids

A known quantity of the sample was placed in a 30 ml Kjeldahl flask and 1 ml conc. H_2SO_4, 2-4 ml $HClO_4$ 60% and two glass beads to avoid bumping, were added. Eventually, a drop of octyl alcohol was added to prevent frothing, especially when a lipid solution was to be digested. The digestion was carried out in an electric burner (Laboratory Construction Company). and a series of 12 samples was usually treated at the same time. When lipids were to be digested, 2-3 ml of nitric acid were added in addition to the other reagents for the following reasons:

Perchloric acid itself under special circumstances is a very explosive reagent. However, used carefully and in the right way it is not dangerous. During digestion with the acid, complicated organic structures are broken down to a series of fragments or degradation units which in turn are oxidized finally to CO_2, H_2O and NH_3, the latter being trapped by the sulfuric acid present.

When lipids are digested, polyhydric alcohols among the degradation units, form explosive esters with perchloric acid and in order to prevent explosion, it is necessary to oxidize the alcohols. An assurance involves the use of perchloric acid with the addition of nitric acid. In the presence of nitric acid, sugars, polyhydric alcohols and cellulose among other substances are destroyed. When oxidation is complete there is no further evolution of brown oxides of nitrogen. At this time, the high boiling perchloric acid volatilizes and displaces the boiling nitric acid. Finally the 60% perchloric acid is concentrated by heating to 72.5% water azeotrope.

H_2SO_4 was mainly used to raise the temperature in the last stages of combustion and drive off most of the residual perchloric acid. A clear colourless liquid was obtained at the end of the digestion. If the oxidation is complete, it remains colourless when it is cooled down. A blank containing all reagents was treated simultaneously throughout the whole procedure.

When nitrogen and phosphorus in addition to copper were to be determined, the digest was made up to 10 ml with distilled H_2O. A suitable portion of this solution was pipetted into a 50 ml tube and enough H_2SO_4 was added to bring the total amount of the acid to 1 ml. Ten ml saturated sodium pyrophosphate and 5 ml NH_3 (3 ml to neutralize

the acid and 2 ml to bring the pH up to 9-10) were added and the development of the colour was achieved by the addition of 2 ml 0.5% sodium diethyldithiocarbamate. The copper-DDTC complex was extracted with exactly 5 ml normal amyl alcohol and it remained stable for several hours at room temperature. The amyl alcohol layer was dried with anhydrous Na_2SO_4 and its optical density was read against the blank in a Coleman junior spectrophotometer at 440 mμ. The values for copper were calculated from a curve made by digestion of samples containing known amounts of copper sulfate.

2. Determination of nitrogen

The method of T.S. Ma and G. Zuaga (142) for the micro-Kjedahl determination of nitrogen was adopted. After digestion, the nitrogen is obtained as ammonium sulfate. Ammonia is collected after addition of excess of NaOH, in a dilute solution of boric acid, containing two drops of bromocresol green and methyl red mixture. The boric acid solution changes from bluish purple to bluish green as soon as it comes in contact with ammonia. When all ammonia is distilled, the solution is titrated with HCl 0.01 N until the bluish colour disappears. Again, the values for nitrogen were obtained from a curve made by digesting solutions containing known amounts of ammonium sulfate. Values were expressed as μg of nitrogen.

3. Determination of phosphorus

The Fiske and SubbaRow (143) method was used because of its simplicity and accuracy. A certain volume of the digest solution was transferred to a 10 ml flask and was neutralized with NaOH 2N using phenolphtalein 1% in alcohol as an indicator. This step, not included in the original method, became necessary since experience proved that

even a small excess of acid interferes with the development of the colour in the later steps of the method.

The phosphomolybdic acid formed by the addition of 1 ml ammonium molybdate in H_2SO_4 5N, was reduced to a blue complex by 0.4 ml of 0.25% 1,2,4-aminonaphtholosulfonic acid solution containing sodium bisulfite and sodium sulfite. The volume was made up to 10 ml with distilled water and the O.D. read against the blank (from the same blank digest used for copper) in a Coleman spectrophotometer. The phosphorus values were obtained from a curve made from standard solution of phosphate run throughout the whole procedure. Values were expressed as μg of phosphorus.

4. Determination of ceruloplasmin activity

The colorimetric method of Ravin (144) was adopted for the measurement of the ceruloplasmin activity in the plasma. In the original method, Ravin uses 0.1 ml of human plasma. It has been found in this laboratory that the same quantity of rat plasma gives an extremely low value. Therefore 0.2 ml were used in all determinations.

Two 0.2 ml plasma portions were pipetted into two 15 ml test tubes. 1 ml of 0.5% p-phenylenediamine.2HCl substrate and 2 ml acetic acid-sodium acetate buffer pH = 6 were added to each tube, plus 1 ml 1% sodium azide to one of them (used as blank). After 60' incubation at 37° C, another 1 ml 1% sodium azide was added to the second tube and 6 ml of 3% NaCl to both of them. The lavender colour produced in the test was measured against the blank in a Coleman spectrophotometer at 530 mμ. The O.D. values obtained were used as units of the enzyme activity.

Several plasmas from control rats were measured for ceruloplasmin activity, their O.D.'s being used as control values.

5. Blood hemoglobin

Hemoglobin was measured by the method of Clegg and King (145). Heparinized blood, 0.05 ml, was pipetted into 4.95 ml NaOH 0.1 N. The solution was placed for 5' in boiling water and the colour measured against NaOH 0.01 N in a Coleman spectrophotometer at 540 mμ. The values for hemoglobin were calculated from the O.D. of a standard solution of NaOH 0.1 N containing 37.5 mg pure hemin/lt.

B. EXTRACTION AND WASHING OF LIPIDS

1. Extraction of lipids

The method of Folch et al. (117) was adopted for reasons cited in Section I. Organs were taken out immediately from the animals killed and kept frozen, if they were not to be used the same day. The fresh tissue was weighed and homogenized with $CHCl_3$:CH_3OH 2:1 mixture (19 ml/gm fresh tissue) for 3' at 4° C in a VIRTIS "45" homogeniser. The homogenate was filtered through a lipid-free filter paper, the precipitate washed with a few ml of mixture and the filtrate made up to a volume corresponding to 25 ml/1.5 gm fresh tissue. The lipid extract was kept at 4° C.

2. Physical partition of extracted lipids

Two methods of Folch are described for the elimination of the non-lipid constituents from the lipid extract. The second one, partition dialysis method, was originally used by Folch for the isolation of Strandin (120).

a) Diffusion between phases in contact (117)

A certain portion of the lipid-extract was equilibrated with five- to ten-fold its volume of distilled water according to the following procedure: 25 ml lipid-extract were pipetted into a 30 ml beaker placed on the bottom of 1 lt. beaker. The lipid-extract in the small beaker was covered very carefully with distilled water. More distilled water was added to the larger beaker to cover the small one and nearly reach

a level of 5 inches above the surface of the lipid-extract. As soon as the water was added on the surface of the lipid-extract, methanol started diffusing into the water phase carrying out all the non-lipid constituents.

Finally, three phases were obtained; the water phase containing all the non-lipid constituents plus water-soluble lipids, the chloroform layer containing chloroform-soluble lipids, and an interfacial fluff layer containing the majority of proteolipids.

b) Partition dialysis method (120)

25 ml of lipid-extract was placed in a cellophane bag and the system was dialyzed for 3 or 4 days against H_2O, saturated with chloroform in a cold room. The outside water was changed twice a day. Here again, three phases were formed inside the bag; a water layer containing water-soluble lipids, a chloroform layer with all chloroform-soluble lipids and a fluff made up of the majority of proteolipids.

A special technique was developed for the separation of the three phases. The dialysis bag was nicked at the top and the water layer was pipetted off. The cut end was tied again and a very small hole was opened on the bottom of the bag with a pin. Chloroform started dropping slowly into a beaker. When no further drop was obtained, only "fluff" unable to pass through the hole, remained inside the bag. The bag was then turned upside down, cut at the top and methanol was added to dissolve the fluff.

C. ANIMALS

All rats used throughout the experiments presented in this thesis were male Sprague-Dawley albino rats of various ages.

To carry out the experiments in vitro presented in this thesis, it was necessary to have a picture of the copper pattern in the tissues of the normal animal. Thus, the metal was determined in a large variety of tissues from Purina- or milk-fed rats.

1. Deficiency of copper

In a typical experiment, 32 weanling albino rats, distributed in 8 cages, were used for the production of copper deficiency. The animals were divided into two groups 8 days after beginning on the deficiency diet. One group was used as control and was given Cu^{++} in addition to the daily diet.

a) Diet

i. Basal diet

Evaporated milk diluted with distilled water 1:1 and powdered milk were used. The chosen brands of milk were those with the lowest content of copper. Milk from three brands for evaporated and three for powdered milk was analyzed for copper.

Brand	Evaporated Milk μg Cu/ml	Powdered Milk μg Cu/gm
Carnation	2.3	0.4
Farmer's wife	0.0	- -
Morning	1.3	- -
Crino	- -	0.1
A. and P.	- -	0.6

The chosen milk had the following labeled composition for each case:

	Evaporated Milk %	Powdered "Skimmed" Milk %
H_2O	78.0	3.5
Butter fat	4.0	1.0 (fat)
Protein	6.9	36.8
Sugar milk	9.6	51.0
Minerals	1.5	8.1
Vitamin D	133 i.u.	
Vitamin C	14 mg	

ii. Supplements

Vitamin (Ostoco drops, Frosst Co.). 0.1 ml of the solution was diluted to 10 ml with distilled H_2O. 0.1 ml of this solution was given per os to each rat/day. Vitamin composition/ml : Vit. A : 10.000 i.u., Vit. D : 1.600 i.u., Vit. C : 120 mg, Vit. B6 : 2.5 mg and NaI : 0.16 mg. 50 µg Mn^{++} (as $MnSO_4$ solution) and 400 µg Fe^{++} (as $FeSO_4$ solution) per os to each rat/day.

iii. Analysis of diet and water for copper

Copper was determined in diet and water used for the dilution of the evaporated milk, immediately after the preparation of the food and few hours later after the food had been kept in the bottles used to feed the animals. No difference in copper content was found, its values being similar to those obtained by analysis of the untreated food.

b) Precautions taken to avoid copper contamination during the experiment.

The sources of the copper contamination during the deficiency were included in:

1) atmosphere

2) food and supplements

3) cages

4) handling of the rats

The following precautions were taken in order to avoid contamination:

1) Atmosphere: all cages were covered with a plastic material.

2) Milk that was low in copper by analysis was used (p. 59). Glass distilled water was used to dilute the evaporated milk. Vitamins contained no copper and all copper, iron and manganese salts were A.R.

3) All cages were made of plastic material and galvanized iron. Galvanized parts were covered with silicon. In addition, the cages were washed every day and rinsed with distilled water.

4) Clean plastic gloves were used to handle the rats, the deficient group being handled first.

c) Measurements made

Every 7 or 8 days during the course of the deficiency, 2 or 3 rats from each group were anaesthetized, and as much blood as possible was removed from the abdominal aorta. The animals then were killed and brain, heart, kidney, liver and muscle tissue were removed. The tissues were washed in saline, dried on filter paper, weighed and frozen.

The following determinations were carried out in the biological samples:

1) Copper in tissues and blood plasma

2) Ceruloplasmin in plasma

3) Hemoglobin in blood.

In addition, the body weight of all rats was recorded throughout the copper-deficiency experiment.

2. Copper-loading experiment with rats

a) Diet

During the copper-loading experiment, all rats, control and loaded were fed Purina Rat Chow and were drinking tap water.

b) Experimental plan

Rats of various ages were used. The animals were divided into control and supplemented groups as follows:

Table Ia

	Control	Group I Supplemented with Cu^{+}	Group II Supplemented with Cu^{++}	Body weight grams
Weanling	3	6	6	35 - 45
Adult	3	4	4	150 - 390
Aged	2	3	3	480 - 600

The supplemented groups I and II were given cuprous and cupric copper respectively in amounts increasing during the course of the loading. The body weight of the rats was recorded every 4 or 5 days throughout the experiment.

Administration of supplements: divalent or monovalent copper, as a $CuSO_4$ solution (500 μg Cu/ml saline) or as CuCl suspension (500 μg Cu/ml saline), were injected intraperitoneally every day or two days throughout the experiment, depending on the health of the animal. The amounts of copper injected are seen in table Ib.

Table Ib

	Group I				Group II		
	Wean-ling	Adult	Aged		Wean-ling	Adult	Aged
	$\mu g\ Cu^{+}$				$\mu g\ Cu^{++}$		
1st to 10th day	50	200	200	1st to 20th day	200	1000	1000
11th to 14th "	100	400	400	21st to 42nd "	500	1000	1000
15th to 24th "	200	400	400	43rd to 66th "	750	1000	1000
25th to 34th "	400	800	800	67th to 134th "	1000	1000	1000
35th to 49th "	500	800	800				
50th to 76th "	750	1000	1000				
77th to 134th "	1000	1000	1000				

The rats were killed in 3 intervals with the exception of two animals which died during the experiment. The first group of eight rats was killed the 42nd day, the second group of nine rats the 70th day, and the third group the 134th day.

Blood was removed as in the copper deficiency experiment, the animals were killed and liver, brain, kidney, spleen, heart, lungs, upper limb muscle and testes were removed, washed, dried in a filter paper, weighed and frozen. A portion of hair from the back of the animal was cut, washed, dried and preserved for analysis.

c) Measurements made

The following determinations were carried out in the tissues obtained:

1) Copper in all tissues and blood plasma

2) Copper in the lipid extract from liver and kidney

3) Ceruloplasmin activity in plasma

4) Hemoglobin in blood

3. Other species

Lipid-extracts, used in experiments presented in Section V, were obtained from the brain of monkey Rhesus.

III. COPPER CONTENT OF THE LIPID EXTRACTS FROM RAT TISSUES

A. INTRODUCTION

It has already been shown in Section I that copper is found in animal tissues and fluids mainly bound to proteins. In the blood serum copper is tightly attached to apo-ceruloplasmin; a small part is loosely bound to albumin. Hematocuprein contains a large portion of the metal in erythrocytes and human or bovine brain copper is partly attached to cerebrocuprein I. Liver also contains copper proteins. Some enzymes, such as tyrosinase and dopamine-β-hydroxylase, have been described as copper proteins.

However, the quantity of copper represented by all known copper compounds is only a small percentage of the metal present in the animal body. Tissues like muscle, skin and bones contain the bulk of the copper but nothing is known about its state there. Furthermore, organs of great physiological importance, such as heart, kidney and lungs, are known to contain copper but have not been studied in respect to copper status. Therefore, it is not an exaggeration to conclude that only a small part of the copper present is of known state and an even smaller part, of roughly known function. The latter includes some enzyme systems recently studied (18).

To understand the role of copper in the physiology of the body, we need more information about the copper compounds, and their location and function. Regarding the transport, absorption and excretion of the metal, the existing data fail to give a full description of any of these processes. There is also no explanation for the high avidity for copper of certain tissues in copper-accumulation experiments, and for the exclusion of excess of the metal in the brain region. Finally, one of the

puzzling questions regarding Wilson's disease is the priority of the two events: (a) ceruloplasmin deficiency (resulting according to Scheinberg (87) in excess of free copper available for deposition in tissues) and (b) deposition of large amounts of copper in tissues (owing to high avidity for copper according to Uzman (90) and thus resulting in a lack of free copper for the formation of ceruloplasmin).

The study of the transport of copper through the cell membrane may be of great importance to understand some of these unexplained phenomena. It has been suggested that lipids may play a significant role in the transport of certain cations, such as sodium, potassium, magnesium and calcium (105) and many lipids have been found to contain these metals.

Furthermore, trace metals have been recently implicated in the metal-lipid interaction and roles have been assigned to iron and copper in the cell membrane structure and function (24).

The possibility that lipids extracted from tissues may also contain copper led the writer to detect and determine the metal in lipid extracts from various organs of the rat. The extraction was carried out by the method of Folch as it is described in Section II, B. Preliminary experiments, using small quantities of brain tissue showed that if copper exists in the lipid fraction, it must be present in very low concentration not measurable by any of the routine methods. Thus, large quantities of tissue were extracted and the total extract, varying from 500 to 1500 ml, was treated with extreme precautions against copper contamination.

B. TECHNIQUE

50 to 150 gms. fresh or frozen tissue from adult, purina-fed,

albino rats (Section II, C) were homogenized with 17 volumes of Folch's mixture in a homogenizer that had been very carefully washed with distilled water and $CHCl_3$-CH_3OH 2:1 mixture. The homogenate was filtered on a prewashed filter paper and the filtrate concentrated by heating to eliminate all organic solvents. The remaining emulsion of lipids and water was digested in small portions in a Kjeldahl flask. An equal volume of Folch's mixture, run throughout the whole procedure (including 'homogenization') was used as blank. The described procedure was followed for all tissues studied, and because of its difficulty, only a few determinations for each tissue have been done.

C. RESULTS

The values of copper found are shown in Table III.

Table II

Organ	Total solid of the lipid extract (% of fresh tissue)	Copper content of $CHCl_3$-CH_3OH extract (% of total Cu)
Brain	9.10	1.7
Liver	6.30	4.2
Heart	4.13	0.5
Kidney	6.86	10.0-17.2

The following can be concluded:

1) The lipid extracts of all organs studied (brain, liver, heart and kidney), contain trace amounts of copper.

2) Kidney and liver extracts contain more of the metal than the brain extract and the latter more than the heart extract. In addition, the kidney lipid extracts show, in some cases, relatively

high values in copper.

3) There is no relation between the lipid content of a tissue and the copper content of the lipid fraction. Brain, for instance, with the highest content of lipids has a relatively low copper content in its extracted lipids.

D. DISCUSSION

The detection of copper in the lipid extracts does not necessarily mean the presence of the metal in the lipid fraction of tissues in situ. As is shown in Section V, lipids bind large amounts of ionic copper in vitro. Thus, there is the possibility of an internal contamination during the extraction procedure, provided that ionic or loosely bound copper exists in tissues. The existing evidence suggests that the bulk of the metal in tissues is protein-bound. Furthermore, the great affinity of proteins for copper would not leave much of the free cation. In addition, it can be easily calculated that the blood present in the tissues (and known to contain about 5 μg copper % loosely bound to albumin), cannot provide more than 10% of the metal in the lipid extracts. Finally, there is evidence from experiments presented in Section V that free copper in tissues would more likely bind to proteins than to lipids during the extraction.

Useful information could probably be obtained by injecting the animal with Cu^{64} and then preparing radioautographs of tissue slices. The cell membrane containing much lipid, should present black silver spots in excess of other areas.

In conclusion, discovery of copper in the lipid extracts of brain, liver, kidney and heart, suggests the existence of a new copper compartment in the animal body. Since the majority of lipids in these

tissues are part of the cell membrane structure, it can be assumed that copper is a membrane constituent.

It is of interest that organs like brain and heart, where no deposition of copper is observed during overloading of rats (Section IV) show the lowest content of copper in the lipid fraction and that liver and kidney, where heavy deposition of the metal occurs, present the highest copper concentration in lipids. Thus, the lipid pattern in the cell of these tissues might play an essential role for both the content in the lipid fraction and transport of the metal through the membrane. If the lipids in the membrane play a definite role in the transport of copper, the fact that more of the metal is found in the cell membrane of liver and kidney might underline a high "traffic" of copper across. In fact, the turnover of copper in these two tissues should be higher since both are the main sites of the excretion of the metal.

Differential binding of copper by lipids could also give a chemical basis for variations in the copper content of lipid extracts. It is therefore strongly desirable to know the behavior in vitro of individual lipids known to exist in cell membranes and of lipids extracted from tissues, toward copper. Furthermore, it would be of interest to study the rate of copper deposition and depletion in a number of tissues in relation to the copper content and to the lipid pattern of the cell membranes.

Finally, interaction of lipids in vivo with copper could be studied by providing animals with excess copper and studying the copper content of the lipid extracts of various tissues.

IV. COPPER CONTENT OF RAT TISSUES

A. THE COPPER CONTENT OF TISSUES OF RATS ON COMPLETE DIET

The comparative study of copper depletion and overloading in the rat tissues, requires knowledge of their metal concentration under usual dietary conditions. Thus, the copper content of many tissues of rats of various ages were measured by the method of Eden and Green. The results of analysis of blood and organs are shown in Tables IIIa and IIIb.

1. Results and discussion

a) Rats fed Purina diet

Hair, heart, kidney, liver and brain in all three age groups had the highest copper content, while testes, spleen, lungs, plasma, muscle and red blood cells (RBC) had the lowest (Table IIIa) . Among weanling, adult and aged rats, differences are observed in the order of the tissues found to be rich in copper. Thus, while heart holds the first position in weanling and adult rats, hair predominates in aged animals. Although only a small number (2) of hair samples were analyzed in adult rats and no analysis of this tissue was done in young animals, hair must be tentatively regarded as the richest tissue in copper. The much higher values for hair copper found by Owen, although they cannot be explained at the present, support the above hypothesis.

The copper content of liver is higher in young rats than in adult and aged. In contrast, the kidney values increase with age. It is known that newborn animals contain increased amounts of copper in their liver, thus, weanling rats have probably not reached the normal value found in the liver of adult animals. With the exception of liver, a small gradual increase of the copper content of all tissues studied

Table IIIa

Copper content of rat tissues

Tissue		Purina-fed rats - body weight			
		35-45 gm	150-390 gm	480-600 gm	200-350 gm (Owen,146)
Liver	n	4	7	5	10
	Mean*	**4.32±0.33	3.82±0.11	3.80±0.19	4.38±0.16
	Range	3.67-5.20	3.53-4.24	3.20-4.13	-
Kidney	n	4	8	5	10
	Mean	3.12±0.31	4.26±0.15	5.05±0.17	5.10±0.22
	Range	2.55-3.63	3.20-5.23	4.70-5.67	-
Heart	n	4	15	5	4
	Mean	4.51±0.62	5.65±0.24	5.02±0.16	4.54±0.37
	Range	2.67-5.22	4.30-7.20	4.60-5.22	-
Brain	n	4	9	5	4
	Mean	1.87±0.11	2.44±0.10	2.10±0.07	2.88±0.15
	Range	1.57-2.19	1.90-2.88	2.00-2.24	-
Spleen	n		5	4	5
	Mean		1.29±0.28	1.04±0.09	1.80±0.27
	Range		0.88-2.40	0.90-1.31	-
Testes	n		5	4	4
	Mean		1.49±0.11	1.51±0.39	1.96±0.22
	Range		1.09-1.80	0.87-2.50	-

(Cont'd.)

Table IIIa (Cont'd.)

Tissue		Purina-fed rats - body weight			
		35-45 gm	150-390 gm	480-600 gm	200-350 gm (Owen,146)
Lungs	n		9	5	4
	Mean*		**1.38±0.13	1.22±0.17	1.57±0.21
	Range		1.00-1.70	0.80-1.83	-
Leg muscle	n	4	7	4	5
	Mean	1.00±0.03	1.17±0.07	1.37±0.22	1.41±0.12
	Range	0.96-1.03	0.87-1.40	0.98-2.00	-
Hair	n		2	5	3
	Mean		4.80	6.36±0.63	31.7,21.9,44.8
	Range		4.30, 5.30	5.62-8.40	-
Plasma	n	4	2	5	10
	Mean	1.43±0.10	1.57	1.52±0.04	1.23±0.04
	Range	1.20-1.62	1.60, 1.55	1.40-1.64	-
Red blood cells	n	4	2	5	
	Mean	0.96±0.09	0.76	1.08±0.13	
	Range	0.80-1.20	0.87, 0.65	0.72-1.52	

* ± Standard Error.

** μg Cu/gm fresh weight of tissue or μg Cu/ml plasma or packed red blood cells.

is observed in growing rats. Spleen is not included because of its variable content of blood. In addition, heart and brain show a decline in the aged animals. The gradual increase of the copper concentration in some tissues through the life of the rat, can be easily seen in muscle and kidney. Finally, no differences are observed through aging in the plasma and RBC copper content.

b) Rats fed liquid milk diet

On discussing the results obtained from rats fed liquid milk (Table IIIb) , the following facts should be kept in mind: (1) Both groups of rats (young and adult) were maintained on the milk diet from the time of weaning (weight: 35-45 gm); (2) the young group includes rats being on this diet for 8-15 days although the adult group was drinking milk for 21-68 days. It is therefore expected that any alteration of the copper content in the tissues of the animals, owing to the new diet, might not be obvious in young rats. Nevertheless, 8-15 days were enough to bring about an increase of a more than 100% of the copper concentration in kidney. A small part of this increase is obviously due to aging, although the fact that copper was given in ionic form could explain the rest of the increase. In fact it is suspected that the state and the amount of the metal given as well as milk itself might be responsible for the observed variations in milk-fed rats. These animals received copper as $CuSO_4$ solution or mixed with milk. It has been shown by Mills (1) that there is a difference in rate of absorption between ionic and non-ionic state of copper. In addition, copper was given to the rats either by mouth or dissolved in milk; in both cases less or more of the metal could be obtained by the animals because of an imperfect swallowing or because being most of them sick, their

Table IIIb

Copper content of rat tissues

Tissue		Milk-fed (Cu supplemented) rats - body wt.		
		35-65 gm	100-300 gm	180-200 gm (Warren,50)
Liver	n	9	19	4
	Mean*	**5.15±0.34	4.80±0.24	***3.17±1.41
	Range	4.00-6.83	4.00-8.81	2.12-3.70
Kidney	n	10	12	4
	Mean	5.08±0.83	6.80±0.55	4.65±1.75
	Range	2.74-9.56	4.30-9.91	3.60-5.60
Heart	n	6	8	
	Mean	4.30±0.48	5.33±0.09	
	Range	2.67-5.22	4.80-5.91	
Brain	n	9	13	4
	Mean	2.08±0.13	2.23±0.08	2.62±1.19
	Range	1.72-3.03	1.81-2.67	1.87-3.15
Leg muscle	n	6	12	
	Mean	1.13±0.08	1.00±0.05	
	Range	0.96-1.40	0.72-1.20	
Plasma	n	2	10	
	Mean	1.56	1.34±0.05	
	Range	1.70, 1.42	1.20-1.65	
Red blood cells	n	6	8	
	Mean	0.94±0.18	1.09±0.06	
	Range	0.80-1.25	0.94-1.33	

* ± Standard Error.

** µg Cu/gm fresh weight of tissue of µg Cu/ml plasma or packed red blood cells.

***Corrected to µg/gm fresh wt. of tissue from the original results of Warren.

consumption in milk was greatly varied. The same explanation could be given for the increase of the copper concentration in the liver of the milk-fed rats.

Other tissues, such as heart, muscle and brain do not show significant differences in copper content from those observed in Purina-fed rats.

B. THE COPPER CONTENT OF TISSUES OF COPPER DEFICIENT RATS

Many attempts have been made in the past by various workers to produce copper deficiency in animals. As discussed in the introductory section, the general purpose of these experiments was to obtain information about the effects of the copper depletion of the organism, on the biological activity of various tissues as well as the rate at which copper is eliminated from various organs. The copper deficiency experiments presented in this thesis were carried out for the following additional purposes: (1) comparative study of the rate of elimination of copper from various organs, a subject on which many workers in this field have failed to provide data, and (2) the study of effects produced on the formation of ceruloplasmin and hemoglobin.

1. Results and discussion

Several experiments in copper deficiency have been carried out in rats fed copper-low diet by the technique described in Section II. In all these experiments, copper was determined at time intervals in a large variety of organs including heart and muscle tissue. The copper content of these organs during development of copper deficiency has not been reported at all in the past. Hemoglobin was measured in blood, ceruloplasmin in plasma and the average body-weight of both groups in one of the experiments during the course of the deficiency is shown in

Fig. 1. Apart from the biochemical studies, clinical observations have been also done. During a typical copper deficiency experiment, the following were observed:

1. All animals, control and deficient, developed diarrhea 2-3 days after the beginning of the experiment. It is almost certain that this was due to the liquid milk diet, because in another experiment, rats receiving dry milk diet had almost no diarrhea.

2. Fifteen to twenty days later, most of the deficient rats lost a large amount of hair, especially around the neck. Alopecia has been frequently observed in copper deficient animals (Section I).

3. At about the same period of time, the pink colour of the eyes of most deficient animals changed to pale pink. This change usually coincided with the decrease in hemoglobin content of blood.

4. The body-weight of the deficient rats increased more slowly than that of the control groups. Copper is known to be part of some enzyme systems. A deficiency in copper would affect the levels of copper-dependent enzymatic activities thus resulting in disturbances of the general health of the animal.

5. A 10-20% mortality was observed in both groups, the deficient one having the higher percentage. It is unlikely that the mortality observed was the result of an extreme copper deficiency, because many deaths were also recorded in the control animals. Diarrhea, leading to dehydration and loss of the electrolyte balance, is probably the main reason for the mortality in both groups. The fact that more deficient rats died, underlines the effect of the copper deprivation in making the animals weaker and more likely to die.

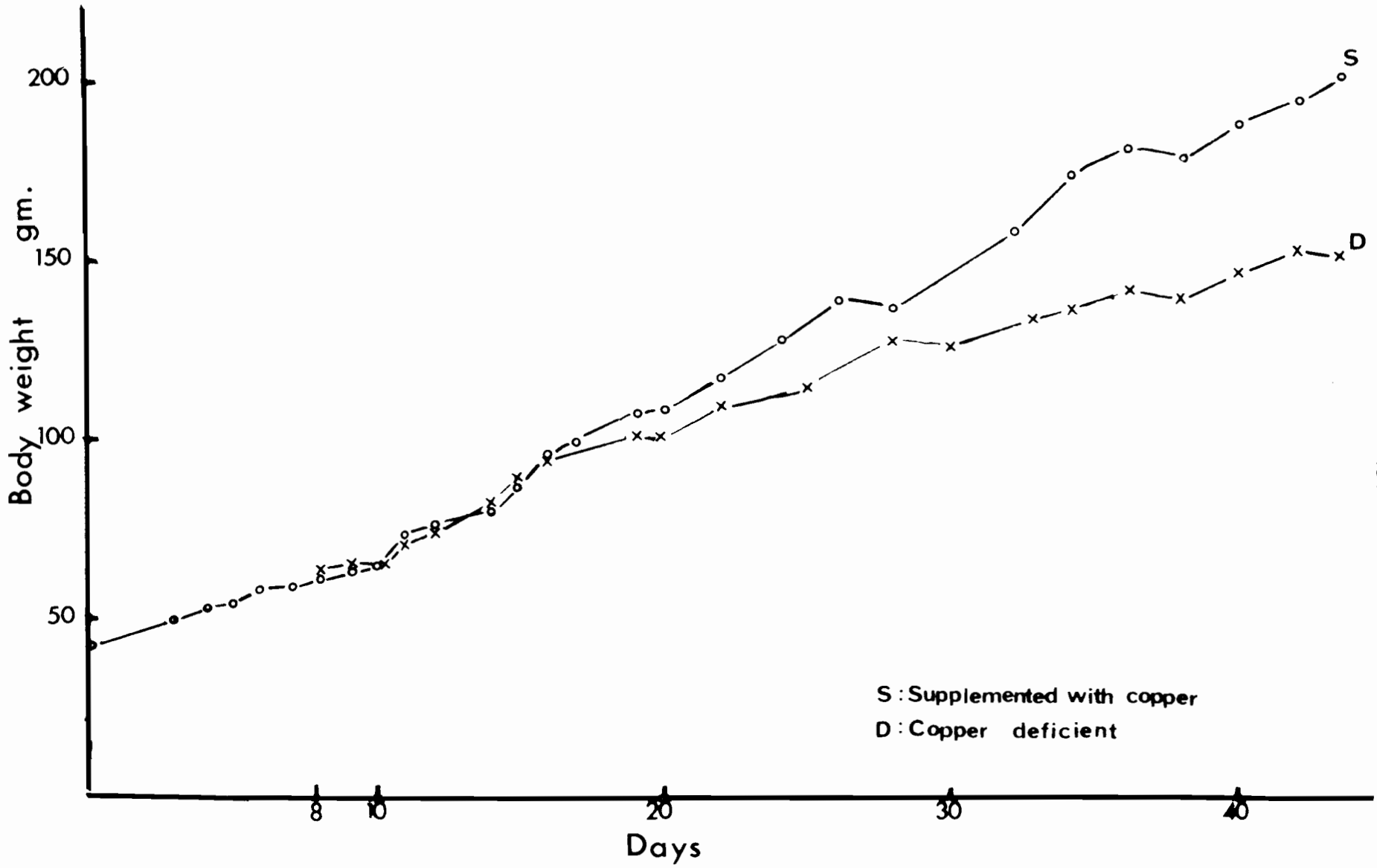


Fig. 1: Average body weight of control and copper deficient rats in the course of copper deficiency.

In evaluating the findings of the copper deficiency experiments, each tissue is examined separately: liver and heart appear to be the most sensitive tissues in copper deprivation, in terms of fast copper depletion (Tables IVc, IVd). Assuming that 5 μg/gm fresh tissue is the average normal concentration of copper in heart, the values found after 15 days deficiency in experiment 1, represent 15% of the normal value. This drop is maintained up to the 44th day. On the other hand, liver with a normal value around 4 μg/gm fresh tissue, retains in 15 days 25% of its total copper. By the end of 44 days, the copper content drops a little more.

The same rate of decrease of the copper content in liver and heart is observed in experiment 2. Experiments 3 and 4, although run for 68 and 51 days, do not show any further decrease of copper in liver than that observed in 35 days in experiment 2. Actually, the values obtained at the 15th day of the experiment 1, although slightly higher, show that significant liver deficiency is achieved in 2 weeks. It is reasonable to assume that the animals, despite the extreme precautions against copper contamination, manage to satisfy their needs in copper to a certain degree. In conclusion, 2 weeks in a very carefully conducted experiment, are enough for the study of biological functions of liver and heart in the state of a moderate copper deficiency.

Milk-fed control rats in experiment 1 and 2 have a copper content in their kidneys (Table IVb) varying from 4 to 10 μg/gm fresh tissue, in contrast to a lower range for the same tissue in Purina-fed rats (Table IIIa). Possible explanations for this were given in page 72. The deficient animals contained a considerable amount of copper in their kidneys up to the 23rd day, even in experiment 1 which must be considered

as the most successful one, being carried out in a perfectly isolated small room.

Brain tissue loses copper slowly but constantly (Table IVd). In 44 days, a 30% deficiency is obtained and it may be or not satisfactory depending on the studied copper-enzyme. Thus, such a moderate deficiency does not affect the synthesis of phospholipids in brain catalyzed, according to Gallagher and colleagues (57), by an unknown copper-dependent enzyme. Leg muscle (Table IVe) is another tissue losing copper constantly and in 44 days a 90% deficiency is achieved. Obviously, muscle tissue, being the largest copper pool in the animal body, provides the needed metal through the course of deficiency. Accidental contamination does not influence the muscle tissue, since other tissues such as liver and heart have a priority in using this extra copper. This explains the stability of muscle tissue in copper content and the steady decrease in its copper concentration in the course of deficiency.

Red blood cells (Table IVf) contain and lose less copper than plasma, the decrease being progressive, although in plasma (Table IVf) a large drop in copper is already obtained during the first 20 days. Plasma is sensitive to variation in the copper content of the diet, although red blood cells are not. In a study of the distribution of copper between plasma and cells after injection of copper (70), the trends in whole blood and plasma copper were similar in that maximum concentrations were attained soon or immediately after injection and fell rapidly thereafter. Red cell copper, although increasing markedly during the first hour after injection, continued to increase slowly for at least a further 5 hours. Thus, an accidental copper contamination

during the course of the deficiency would affect largely the plasma copper concentration rather than that of erythrocytes. In addition, in a deficiency state, accidental excess of copper would be primarily captured by other depleted tissues and only a small amount would be probably available for a plasma-red cell copper equilibrium, if such an equilibrium exists.

Blood hemoglobin (Table IVg) in young control rats, seems to have a low concentration of the order of 8 gm % and it is increased as the animals grow. Anemia due to copper deficiency, is achieved at the end of a month and it can be followed by observing the colour of eyes becoming paler throughout the course of deficiency.

Ceruloplasmin values (Table IVg) vary greatly among control animals, reaching in some cases an optical density (O.D.) equal to zero. In addition, ceruloplasmin in the plasma of Purina-fed rats has in general higher values than in milk-fed control rats. In 15 days of copper deficiency, there is no marked difference in ceruloplasmin between control and deficient groups. However, in 45 days, the deficient rats tend to have lower values than the controls.

Table IV

Effect of copper deficiency in rats on tissue copper concentration*

IVa : Brain

IVb : Kidney

IVc : Liver

IVd : Heart

IVe : Leg Muscle

IVf : Blood

IVg : Hemoglobin and Ceruloplasmin

*Rats weighing 35-45 gms. were used. They were fed copper deficient diet for 0-8 days before being divided into control (copper-supplemented) and deficient (copper-low diet) groups.

Five copper-deficiency experiments were carried out as follows:

Date	Composition of diet	Number of days before division
Expt. 1 Feb. 10-Mar. 25/64	Evaporated milk supplemented with vitamins, Manganese, Iron (controls also fed copper)	8
Expt. 2 Apr.6 - May 11/64	"	0
Expt. 3 May 13 - Jul.20/64	"	0 (Table IVc : liver, Table IVd : blood)
Expt. 4 Jul.27 - Sept.16/64	"	0 (Table IVc : liver)
Expt. 5 Nov.14 - Dec.21/65	Dry skim milk suppl'd. with vitamins, Manganese, Iron (controls also fed copper)	0 (Table IVg, applies only to ceruloplasmin)

Values in the tables, except where otherwise stated, represent μg of Cu/gm fresh weight of tissue. Each value represents an individual rat.

Abbreviations: Defic. = rats in copper deficiency
Suppl. = rats supplemented with copper.

Table IVa: BRAIN

Expt. 1			Expt. 2		
Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.
8 days	1.79*, 1.80		0 days		2.19*, 1.72
	1.85, 1.92				1.57, 2.01
15	1.30, 1.76	2.21, 1.72	7	1.87, 2.25	3.03, 2.21
	1.48	2.09			
23	1.75, 1.75	2.09, 2.48	14	1.87, 1.62	2.67, 2.58
30	1.39, 1.54	2.12, 1.94	21	1.77, 1.81	2.15, 1.94
37	2.35, 1.68	2.63, 2.22	28	1.53, 1.68	2.45
44	1.38, 1.43	1.81, 1.93	35	-	-
	1.46, 1.48				

*µg Cu/gm fresh weight of tissue.

Table IVb: KIDNEY

Expt. 1			Expt. 2		
Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.
8 days	2.88*, 2.83		0 days		2.55*, 3.93
	3.11, 3.22				2.74, 3.27
15	3.01, 2.57	6.94, 9.51	7	3.57	8.74, 6.15
	2.34	4.64			
23	2.93, 2.47	5.27, 8.20	14	3.30, 3.37	9.56, 7.21
30	1.97, 1.95	7.20, 4.30	21	3.73, 2.48	9.91, 6.86
37	3.80, 3.63	6.81, 5.19	28	2.06, 2.13	9.87, 7.90
44	2.71, 3.65	5.48, 4.70	35	-	-
	3.37, 3.79				

*µg Cu/gm fresh weight of tissue.

Table IVc: LIVER

Expt. 1			Expt. 2			Expt. 3			Expt. 4		
Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.
8 days	2.09*		0 days		4.42*	0 days		4.10*	0 days		4.00*
	3.82				5.20			4.20			4.18
	2.63				3.67			3.98			
	2.40				4.00						
15	1.27	4.40	7	2.68	6.83	7	2.83	4.15	8	2.50	4.10
	1.34	4.33		2.87	5.62		2.90	-		2.80	5.06
	1.10	-									
23	1.44	4.00	14	2.48	4.75	21	2.00	4.85	16	1.80	-
	1.17	4.50		1.51	6.90		2.02	-		1.90	-
30	0.92	4.44	21	2.17	4.23	68	1.20	-	26	1.50	5.10
	0.98	4.66		1.35	8.81		1.05	-		1.40	4.80
										1.38	4.00
37	1.15	4.10	28	1.15	5.02				51	1.20	-
	0.92	4.19		1.10	5.17					1.15	-
										1.40	4.40
44	1.06	4.99	35	1.11	4.90						
	1.11	4.50		1.03	4.70						
	0.90										
	1.18										

* µg Cu/gm fresh weight of tissue.

Table IVd: HEART

Expt. 1			Expt. 2		
Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.
8 days	3.40*, 2.48		0 days		5.22*, 5.18
	1.98, 3.30				5.00, 2.67
15	1.58, 0.73	1.90, 2.85	7	3.28, 3.70	2.98, 4.80
	0.70	1.27			
23	2.86, 2.05	5.08, 3.85	14	3.10, 2.66	5.75, 4.82
30	1.44, 1.34	3.30, 2.70	21	3.73, 2.42	4.80, 5.91
37	3.29, 3.36	4.92, 5.66	28	2.55, 2.87	5.15, 5.64
44	1.01, 0.96	6.50, 4.21	35	2.31, 2.80	5.20, 5.43
	1.19, 1.05				

*µg Cu/gm fresh weight of tissue.

Table IVe: LEG MUSCLE

Expt. 1			Expt. 2		
Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.
8 days	1.19*, 0.90		0 days		1.01*, 1.01
	0.89, 0.94				0.96, 1.03
15	0.67, 0.42	0.90, 0.61	7	0.84, 0.84	1.38, 1.40
	0.54	0.76			
23	0.47, 0.47	1.05, 0.98	14	0.88, 0.67	0.96, 1.06
30	0.55, 0.38	0.72, 0.88	21	0.82, 0.68	1.10, 1.17
37	0.61, 0.70	1.00, 0.98	28	0.70, 0.62	1.20, 1.15
44	0.15, 0.25	1.11, 0.77	35	0.60, 0.57	-
	0.16, 0.21				

*µg Cu/gm fresh weight of tissue.

Table IVf: BLOOD

Expt. 2					Expt. 3				
	Plasma		R.B.C.			Plasma		R.B.C.	
Deficiency period	Defic.	Suppl.	Defic.	Suppl.	Deficiency period	Defic.	Suppl.	Defic.	Suppl.
14 days	1.25*	1.70	1.15*	1.65	0 days		1.20*		0.90
	1.18		0.76				1.62		0.95
							1.28		0.80
							1.62		0.97
21	0.34	1.50	0.50	1.33	7	1.30	1.30	1.10	1.20
	0.16	1.35	0.41	1.30		1.25	1.42	0.85	1.25
28	0.31	1.25	0.70	1.03	21	0.45	1.65	0.65	1.15
	0.50	1.27	0.45	1.06		0.43	1.20	0.68	1.15
35	0.34	1.30	0.37	0.98	30	0.28	1.30	0.42	-
	0.32	1.20	0.41	0.94		0.21	1.45	0.40	
					50	0.20	-	0.35	-
								0.27	
					68	0.18	-	0.40	-
								0.28	

*µg Cu/ml plasma or packed cells.

Table IVg: HEMOGLOBIN and CERULOPLASMIN

Expt. 1			Expt. 2			Expt. 5		
Blood hemoglobin			Blood hemoglobin			Plasma ceruloplasmin		
Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.	Deficiency period	Defic.	Suppl.
37 days	6.0*	13.0	0 days		8.5*	15 days	0.130**	0.260
	11.0	14.3			8.4		0.150	0.075
					6.4		0.100	
					12.7		0.000	
44	6.6	14.0	7	9.7	10.5	43	0.040	0.000
	8.2	14.8			8.4		0.025	0.100
	8.8						0.000	0.135
	10.4						0.025	0.090
							0.010	0.190
							0.000	0.120
								0.080
								0.060
								0.070
								0.000
			14	11.4	13.1			
				8.6	11.1			
			21	8.9	10.8			
				10.4	12.5			
			28	9.8	13.5			
				11.5	13.1			
			35	10.1	13.6			
				11.2	12.8			

* gms per 100 ml whole blood.

** Optical density read in a Coleman spectrophotometer at 530 mμ.

C. THE COPPER CONTENT OF TISSUES OF COPPER-LOADED RATS

The copper-loading experiment presented in this thesis, was carried out to study the following processes:

i. Rate of copper accumulation in many tissues.
It has been shown in Section I, C, that very few copper-loading experiments have been done in which the metal has been measured in tissues.

ii. The effect of copper-loading on the production of ceruloplasmin.

iii. Difference in biological action between cupric and cuprous ions. So far, all experiments dealing with copper-loading have been uniformly done with cupric copper. Cuprous copper which is twice as toxic as cupric (147) seems not to have been used in such experiments. It has been shown in Section I that cuprous copper is the active state by which the metal acts in some copper containing enzymatic systems. In addition, nothing is known about the proportion of cuprous copper relative to the total copper in the animal body. Regarding the transport of the metal through membranes of the cell or cell particle, it is not known whether this is carried out in the monovalent or divalent state of copper. In view of this lack of evidence concerning the physiological behaviour of cuprous ions, it was hoped that administration to rats of excessive amounts of monovalent copper, would show interesting differences between the two states of the metal.

iv. Effect of copper-loading on the copper content of the tissue-lipids.

It has been shown in Section III that trace amounts of copper were found in the lipid-extract of liver, kidney, brain and heart. It has also been suggested that the presence of copper in lipids was re-

lated to the rate of copper transport through the cell membranes or to the mechanism of copper transport itself. Tissues overloaded with copper, would have an increased rate of copper transport and probably increased concentration of the metal in their lipid fraction.

v. Relation between age of the animal and copper effects. The loading experiment has been carried out in weanling, adult and aged rats.

1. Results and discussion

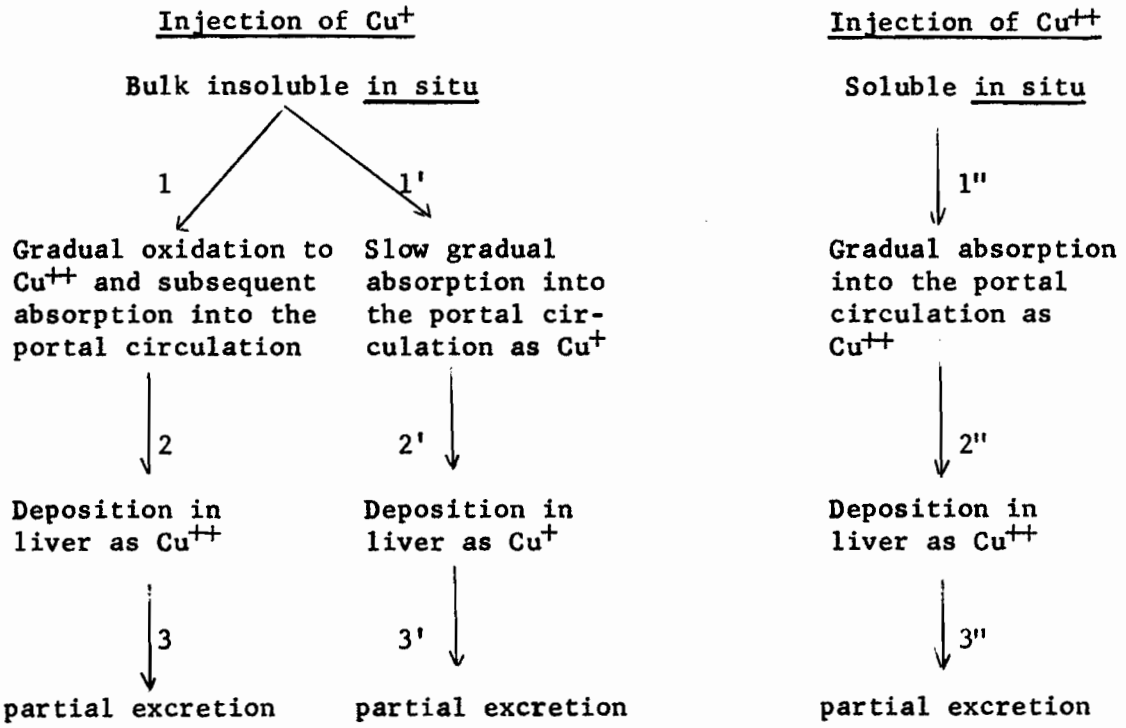
a) Copper accumulation in tissues

i. Weanling rats

On evaluating the values shown in Table Va, the tissues can be divided into 3 groups, depending on the accumulated amount of copper: liver and kidney, with relatively large amounts of copper, belong to the 1st group. In the second group, those tissues with moderate to low accumulation of the metal are included. These are: hair, spleen, heart, brain, erythrocytes and plasma. Leg muscle, testes and lung consist the third group that includes tissues with unchanged copper concentrations.

The copper content in the liver of the animals injected with cupric ions (group II), increased gradually up to the 70th day of the experiment. The same gradual increase is observed in the animals injected with cuprous copper (group I) but at a much lower level. In 134 days, the concentration of copper in the liver of group III rats decreased to levels similar to those observed in 42 days of copper overloading, although that of group I remained fairly constant. The difference between the two groups regarding the rate of accumulation of copper in liver may be due to the low solubility of cuprous copper. This metal was injected suspended in saline and its absorption could be much slower compared to that of

soluble cupric ions. It is unknown whether the monovalent metal injected was stored in liver in its original state as cupric copper or as both. The possible fate of both cuprous and cupric copper injected, is presented in the following diagram:



If the mechanism for the absorption of copper requires the divalent state of the metal, then step 1 is the most likely to occur. This would explain the lower concentration of copper in the liver of group I animals since a gradual oxidation of cuprous copper to cupric ions is required. On the other hand, it is known that ceruloplasmin contains in its molecule cuprous copper. Whether or not some of the copper, possibly absorbed as monovalent, participates in the equilibrium believed to occur between diffusible copper in intestinal plasma and ceruloplasmin copper (15), is unknown.

As soon as copper reaches the liver, part of it combines with the specific copper proteins, the bulk being excreted through the bile. In 134 days of overloading the copper content in the liver of group II was found decreased despite the continued injections of copper. This unexpected finding could be explained in at least two different ways:

1) The rate of copper uptake by liver is diminished because of: (a) reduced synthesis of some copper binding proteins, or (b) an inhibition of the enzyme(s) involved in the uptake of copper by the liver.

2) The rate of elimination of copper from liver is increased because of: (a) proteolysis of tissues heavily loaded with copper, or (b) adaptation of the enzyme(s) involved in the excretion of copper by the liver.

In addition, it could be argued that once weanling rats became adult in 134 days, there could result a changed behaviour of the organism toward copper. However, starting with adult rats an even higher rate of copper deposition in liver was observed (Table Vb).

Kidneys follow fairly the same pattern but their copper content is lower. The lower accumulation of copper in this tissue compared to that of liver, is in agreement with findings of other workers (70, 71). By the time copper reaches the kidney, it has already passed through liver where it has been transformed into ceruloplasmin and albumin copper. Thus, if ceruloplasmin production is not large enough, loosely bound copper increases and kidneys being a site of copper excretion, are exposed to relatively large amounts of free metal. However, in 134 days the copper content of kidney declines. This decrease parallels that of liver, and suggests a corresponding decrease of loosely bound copper.

Thus, it seems that of the two possibilities stated above, the second ascribing the decrease of copper in liver to a mechanism whereby enzyme(s) related to the excretion of copper are adapted, is the most likely to occur.

The concentration of copper in hair and spleen increases during the first 42 days and it remains constant thereafter. On the other hand, copper in heart (in some of the rats) and brain is increased toward the end of the experiment. However, it can be seen that heart and brain exhibit a higher resistance to copper deposition. In addition, no significant differences are observed between groups I and II. In the few copper-loading experiments in the rat, found in the available literature and discussed in Section I, no measurement of the copper in brain has been made. On the other hand, Vogel (69), in a similar experiment, found increased copper concentration in the brain of the goldfish. Whether this 50% increase of the copper in the rat brain described in this Section can be induced by prolonging the loading period, is a problem for the future. Whether or not the observed increase of copper in brain is related to its decrease in liver and kidney is unknown.

The copper content of plasma is moderately elevated. This is expected because of the induced excretion of the metal by the liver. Red blood cells, being in a medium with excessive amounts of copper, increase their content in copper. Increased copper concentrations were found in the testes and lungs of some animals. Muscle tissue, however, remained absolutely unaffected. The high resistance of muscle tissue for copper deposition in conditions under which even brain, considered to be "impermeable", accumulated small amounts of copper, is of obscure significance. Finally, the levels of hemoglobin in blood remained normal.

Regarding the growth of the animals in the course of the experiment, Figure 2 shows that the copper-loaded rats had a slower growth rate as compared with that of controls. At the end of the 134 days period, the average body-weight of both groups I and II was 40% lower than that of the control. The effect of copper-loading on the growth rate of the rats, although of unknown mechanism, can be attributed to one or more of disturbances resulted, such as poisoning of enzymatic systems related to protein synthesis, a decrease of the ATP production owing to the accumulation of copper in mitochondria.

Table V

Effect of copper-loading on the copper concentrations of the rat tissues*

Table Va : Weanling rats

Table Vb : Adult rats

Table Vc : Aged rats

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*Male albino rats weighing 35-45, 200-300 and 450-550 gms. initially were used. The animals were divided in groups according to Table Ia (Section II) and were injected intraperitoneally with cuprous or cupric copper as described in Section II, for 134 days. Uninjected rats of the respective groups were carried as controls. Rats were killed at 42, 70 and 134 days.

Diet:

All values, unless otherwise stated, represent μg of copper per gm fresh weight of tissue. Each column represents values of copper in tissues of a single rat.

Abbreviations:

Cu^{+} = Values of copper from rats injected with cuprous chloride.

Cu^{++} = Values of copper from rats injected with copper sulfate.

None = Values of copper from control rats.

Table Va: WEANLING RATS

Tissue	Copper	Duration of loading experiment (days)		
		42	70	134
Liver	None	4.0	4.2	3.8
	Cu^{+}	65.6	119.0	132.0, 95.1, 101.1, 72.5
	Cu^{++}	185.7	453.0	123.6, 112.0
Kidney	None	4.8	6.2	5.6
	Cu^{+}	20.8	47.3	53.1, 12.3, 17.5, 14.0
	Cu^{++}	48.9	49.3	17.2, 14.8
Heart	None	4.6	4.8	5.2
	Cu+	4.6	4.5	8.7, 7.8, 4.9, 10.1
	Cu^{++}	8.8	5.0	7.2, 10.2
Brain	None	1.9	2.0	2.1
	Cu^{+}	2.5	-	-
	Cu^{++}	2.2	-	3.0, 3.2
Spleen	None	-	2.3	1.0
	Cu^{+}	-	2.6	8.3, 6.4, 5.7, 7.2
	Cu^{++}	5.1	6.9	5.6, 6.5
Testes	None	-	1.8	1.8
	Cu^{+}	1.3	2.3	1.4, 2.8, 2.2, 2.3
	Cu^{++}	2.2	3.1	1.7, 1.8
Lungs	None		1.0	1.3
	Cu^{+}	1.4	1.8	1.5, 1.3, 1.3, 1.2
	Cu^{++}	1.3	3.7	1.6, 1.4

(Cont'd.)

Table Va: WEANLING RATS (Cont'd.)

Tissue	Copper	Duration of loading experiment (days)		
		42	70	134
Leg muscle	None	0.9	1.2	0.9
	Cu^{+}		1.4	1.1, 1.2, 0.9, 1.1
	Cu^{++}		1.2	1.4
Hair	None	-	5.3	6.7
	Cu^{+}	12.4	-	10.8, 9.9, 10.1
	Cu^{++}	14.2	12.4	11.0
Plasma	None	1.75*	1.55	1.52
	Cu^{+}	1.90	2.15	2.40, 2.65, 1.91
	Cu^{++}	1.83	2.3	2.85
Red blood cells	None	0.92*	0.65	1.16
	Cu^{+}	0.80	1.50	1.38, 1.48, 1.33, 1.44
	Cu^{++}	-	0.97	2.30, 1.36
Blood hemoglobin	None	14.8**	15.0	13.3
	Cu^{+}	13.2	12.7	12.4, 14.2, 14.6
	Cu^{++}	14.1	13.3	13.7, 13.3
Plasma ceruloplasmin	None		0.011***	0.025, 0.042
	Cu^{+}		0.036	0.140, 0.050, 0.050, 0.090
	Cu^{++}		0.055	0.055, 0.130

* µg Cu per 1 ml plasma or packed red blood cells.

** gms. hemoglobin per 100 ml whole blood.

***Optical density read in a Coleman spectrophotometer at 530 mµ.

ii. Adult rats

As shown in Table Vb, liver and kidney are the most affected organs. Hair, spleen, heart, brain, erythrocytes and plasma exhibit a moderate increase in copper and increases are observed in testes and lungs. Muscle tissue is not affected at all. The accumulation of copper in the liver of the adult rats has a different pattern from that seen in weanling rats. Injections of cupric copper in young animals resulted in a maximum concentration of copper in liver at 70 days. In contrast, adult rats have reached their maximum at 42 days, being fairly constant up to 70 days. Adult rats injected with cuprous copper showed the same observed in weanlings: gradual increase of the copper content in liver up to 70 days. However, it is noted that the maximum copper value, reached at 70 days, is almost four times higher than that of weanling animals. In conclusion, two differences are observed in the rate of copper accumulation in liver between adult and weanling rats:

1) Maximum copper concentration in adult animals is achieved much earlier.

2) More copper is deposited in the liver of group I adult rats during 70 days of overloading.

It is suggested that both differences are due to a higher efficiency of the young animals in excreting copper. A similar decrease to that seen in weanling rats, is observed in the liver copper by the end of the experiment. Kidney has the same pattern as liver and it differs from that of weanling rats in the same points described above, for liver differences. Regarding hair, spleen, heart, brain, erythrocytes and plasma and muscle tissue, no differences are observed either between weanling and adult rats or group I and group II animals.

Table Vb: ADULT RATS

Tissue	Copper	Duration of loading experiment (days)		
		42	70	134
Liver	None	3.8	3.6	4.1
	Cu^{+}	143.0	415.0	231.0, 90.9
	Cu^{++}	425.4	398.0	
Kidney	None	6.9	5.2	4.9
	Cu^{+}	43.5	101.5	99.2, 51.1
	Cu^{++}	125.8	130.0	
Heart	None	7.0	5.1	5.1
	Cu^{+}	5.0	4.6	9.9, 6.4
	Cu^{++}	5.3	6.1	-
Brain	None	1.9	2.2	2.1 2.1
	Cu^{+}	2.3	-	3.8, 2.9
	Cu^{++}	2.4	-	-
Spleen	None	2.4	1.3	1.2
	Cu^{+}	4.2	3.1	12.1, 10.5
	Cu^{++}	5.0	4.6	-
Testes	None	1.5	2.5	0.9
	Cu^{+}	1.8	1.4	3.6, 2.3
	Cu^{++}	1.8	2.2	-
Lungs	None	-	1.7	1.0
	Cu^{+}	2.0	2.8	2.8, 2.5
	Cu^{++}	2.0	3.6	-

(Cont'd.)

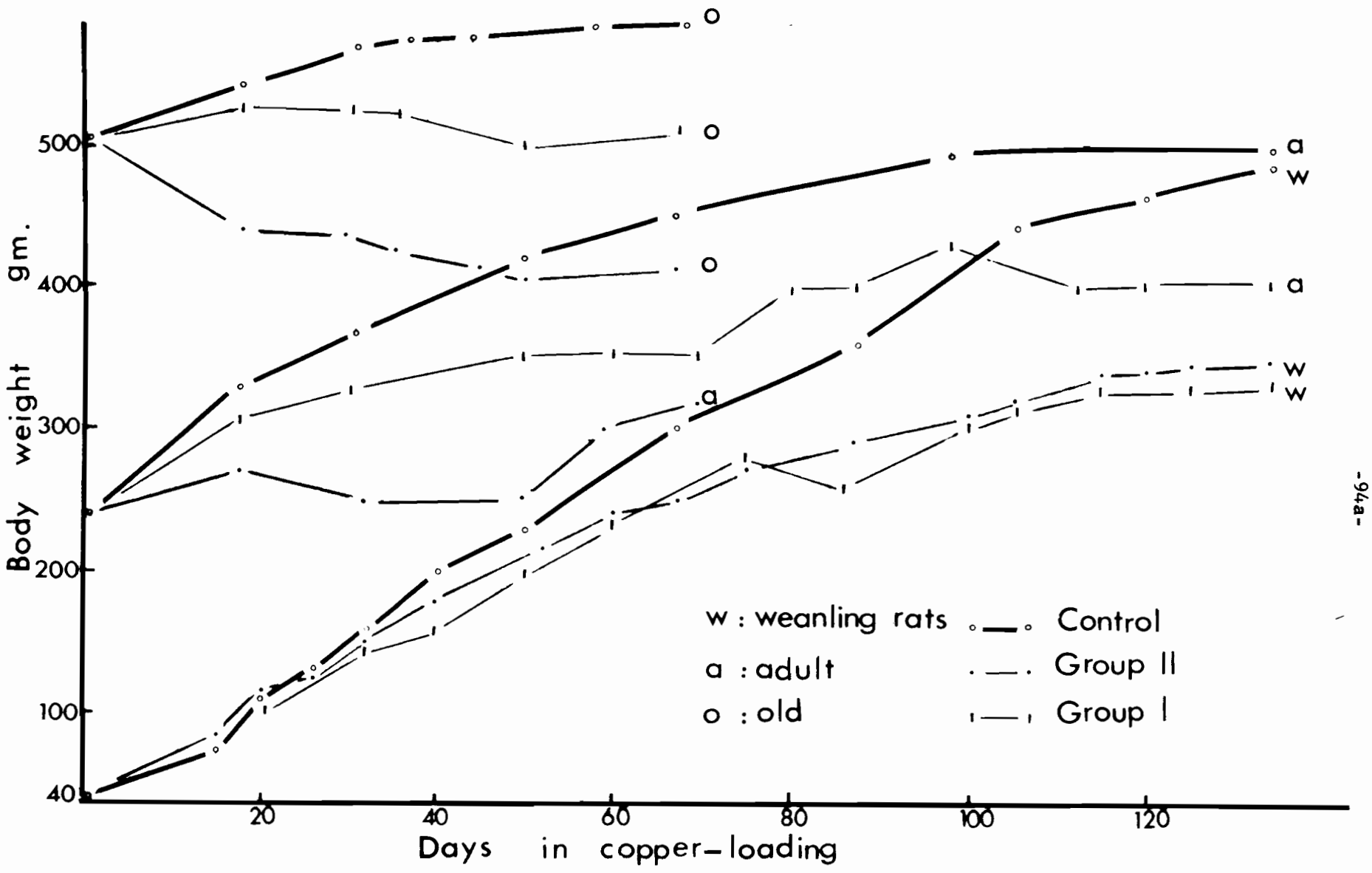


Fig. 2: Average body weight of control and copper-loaded rats in the course of copper-loading. Group I rats were injected with cuprous chloride and Group II with copper sulfate.

Table Vb: ADULT RATS (Cont'd)

Tissue	Copper	Duration of loading experiment (days)		
		42	70	134
Leg muscle	None	1.2	1.4	1.0
	Cu^{+}	-	1.5	0.9, 1.4
	Cu^{++}	-	1.0	
Hair	None	4.3	5.6	8.4
	Cu^{+}	10.3	9.6	8.9
	Cu^{++}	10.1	10.0	-
Plasma	None	0.93*	1.60	1.48
	Cu^{+}	2.56	1.90	-
	Cu^{++}	2.23	3.16	-
Red blood cells	None	0.87*	0.81	1.52
	Cu^{+}	1.10	1.10	1.74, 2.50
	Cu^{++}	1.41	1.10	-
Blood hemoglobin	None	14.8**	14.7	15.9
	Cu^{+}	13.3	12.7	8.5, 8.8
	Cu^{++}	7.4	13.3	-
Plasma ceruloplasmin	None		0.014***	0.035
	Cu^{+}		0.045	0.090, 0.095
	Cu^{++}		0.020	-

* μg Cu per 1 ml plasma or packed red blood cells.

** gms. hemoglobin per 100 ml whole blood.

***Optical density read in a Coleman spectrophotometer at 530 mμ.

Nevertheless, anemia was observed in some copper overloaded adult rats. Low hemoglobin values were found for one group II rat at 42 days and two group I rats at 134 days. Plasma ceruloplasmin was again found, as in weanling rats, increased.

The growth rate for group II rats up to 70 days of copper overloading is lower than that of either control or group I rats. In fact, the average body-weight of group II animals at 70 days is 32% lower than that of control group. On the other hand, the average body-weight of group I rats at 134 days is 20% lower than that of controls.

iii. Aged rats

These animals, over 3 years old, presented the same pattern as that of adult rats. However, a difference is observed in the rate of accumulation of copper in the liver of the group I rats (Table Vc). In adult animals, a maximal copper concentration was obtained at 70 days, and of the same level as for group I. In aged rats, the maximum is again seen at the same period of time but its level is only a third of that in group II. A slight degree of anemia was observed at 42, 70 and 134 days in the group II animals. Finally, in contrast with weanling and adult rats, the average body-weight of aged rats (group II) decreases in the course of the experiment although that of group I rats remains fairly constant (Fig. 2).

Having discussed the rate of accumulation of copper in the tissues of the injected weanling, adult and aged rats, two facts have imposed limitations on the evaluation of the results:

1) Animals which started receiving copper at an early period of their life, became older in the course of the experiment. Thus, their behavior as adults, toward copper may not be the same to that exhibited when weanling.

Table Vc: AGED RATS

Tissue	Copper	Duration of loading experiment (days)		
		42	70	134
Liver	None	4.5	4.1	-
	Cu^{+}	107.8	158.0	121.0
	Cu^{++}	413.9	470.0	128.1
Kidney	None	5.4	4.8	-
	Cu^{+}	27.3	136.0	40.1
	Cu^{++}	152.8	167.2	38.5
Heart	None	4.9	4.6	-
	Cu^{+}	5.2	4.1	5.7
	Cu^{++}	8.5	4.8	10.4
Brain	None	2.1	2.1	-
	Cu^{+}	2.5	-	-
	Cu^{++}	2.2	-	3.1
Spleen	None	-	1.0	-
	Cu^{+}	12.9	8.9	12.7
	Cu^{++}	14.4	7.3	-
Testes	None	-	1.8	-
	Cu^{+}	1.7	1.4	1.0
	Cu^{++}	1.6	1.9	1.2
Lungs	None	-	0.9	-
	Cu^{+}	1.9	2.2	1.6
	Cu^{++}	3.3	3.0	1.5

(Cont'd.)

Table Vc: AGED RATS (Cont'd.)

Tissue	Copper	Duration of loading experiment (days)		
		42	70	134
Leg muscle	None	1.1	2.0	-
	Cu^{+}	-	1.0	1.2
	Cu^{++}	-	1.2	0.8
Hair	None	-	6.6	-
	Cu^{+}	6.8	-	-
	Cu^{++}	11.8	10.4	9.0
Plasma	None	1.61*	1.41	-
	Cu^{+}	2.20	2.20	2.72
	Cu^{++}	2.10	2.40	1.92
Red blood cells	None	0.92*	0.72	-
	Cu^{+}	0.70	1.30	1.84
	Cu^{++}	2.35	1.70	1.12
Blood hemoglobin	None	14.9**	14.8	-
	Cu^{+}	15.6	14.2	15.2
	Cu^{++}	11.3	10.3	11.2
Plasma ceruloplasmin	None		0.016***	-
	Cu^{+}		0.051	0.380
	Cu^{++}		0.048	0.110

* μg Cu per 1 ml plasma or packed red blood cells.

** gms. hemoglobin per 100 ml whole blood.

***Optical density read in a Coleman spectrophotometer at 530 mμ.

2) Each copper value for 42 and 70 days in the course of the experiment, represents a single rat.

A general conclusion regarding the differential deposition of copper in the tissues and not affected by the limitations stated above, is that tissues directly related with the excretion of copper accumulate most of the totally deposited metal.

b) Copper content of the lipids extracted from liver and kidney

The administration of copper in rats, increased the absolute amount of copper in the lipid-extract of liver and kidney. Results are shown in Table VI.

i. Liver

The absolute amount of copper in the lipid-extract from the liver of copper-loaded rats was several-fold increased as compared to that of control animals (the absolute amount of copper for control animals was calculated from the percentage value shown in Table II, and the concentration of copper in the liver of normal adult rats shown in Table IIIa). When these values are expressed as a percentage of the total tissue copper, then the percentages of the copper-loaded rats are, with one exception, lower than that of controls. The relatively large variations are obviously due to the fact that each value represents a single animal. The control values were obtained from many animals the livers of which were pooled.

Strikingly low absolute and relative values were obtained from aged rats injected with cuprous copper. In fact the concentration of copper in the lipid-extracts was so low that its determination was impossible in the available small quantity of tissue. Relatively low values were also found for aged rats injected with cupric copper.

Table VI

Effect of copper-loading on the copper content of lipid-extracts from the liver and kidney of the rat

Age	Days of overloading	Liver						Kidney					
		µg Cu in lipids extracted from 1 gm fresh tissue			Cu content of $CHCl_3$-CH_3OH extract (% of total tissue Cu)			µg Cu in lipids extracted from 1 gm fresh tissue			Cu content of $CHCl_3$-CH_3OH extract (% of total tissue Cu)		
		Cu^{+}*	Cu^{++}*	None**	Cu^{+}	Cu^{++}	None	Cu^{+}	Cu^{++}	None	Cu^{+}	Cu^{++}	None
Weanling	42	3.42	0.89		4.90	0.48							
	70	0.86			0.72			1.50	4.00		3.06	7.50	
	134							2.10			3.80		
								0.75	2.61		4.10	13.10	
								0.71	0.52		4.82	3.40	
Adult	42	0.96	1.17		0.67	0.27							
	70	10.30	2.82	0.16	2.42	0.70	4.20		0.67	0.46-0.79		0.51	10-17.2
	134							1.26			1.25		
Aged	42	0.00	0.75		0.00	0.18							
	70	0.00	0.63		0.00	0.13		0.68	0.95		0.50	0.50	
	134							0.00	0.00		0.00	0.00	

*Rats injected with cuprous chloride or copper sulfate.

**Control rats.

ii. Kidney

As in liver, most of the animals showed higher absolute and lower relative values when compared with those of controls. Lipid extracts from kidneys of aged rats were very low in copper, especially in 134 days of loading. Assuming that the measured copper in the lipid fraction of liver and kidney has not resulted from internal contamination, it can be concluded that copper is attracted by the lipids of the cell or cell particle membranes during its transport. Preliminary experiments showed that the deposition of copper in liver has occurred at the subcellular level. Thus, the mitochondrial fraction contained almost half of the deposited metal, the rest being distributed among nuclei, supernatant and microsomes in that order. It is therefore reasonable to assume that the binding of copper by the structural lipids had occurred in all membranes involved in the structure of the cell.

c) Clinical manifestations

Most of the injected rats, especially weanling and adults of group II, developed chronic ulcerations at the site of injection, that is, the lower anterior abdominal wall. Death occurred in a few of them, preceded by general cachexia and muscular atonia.

The tissues of the animals were also much affected. Liver was found in most cases attached to the diaphragm, fibrous and lighter in colour. There was a general increase of the liver weight as compared with that of controls (Fig. 3). The kidney surface was rough, porous and matted, and membranes were observed strongly attached to spleen. In addition, a great increase of adipose tissue was found in some of the group II rats. Finally, the development of testes of some weanling rats was impaired.

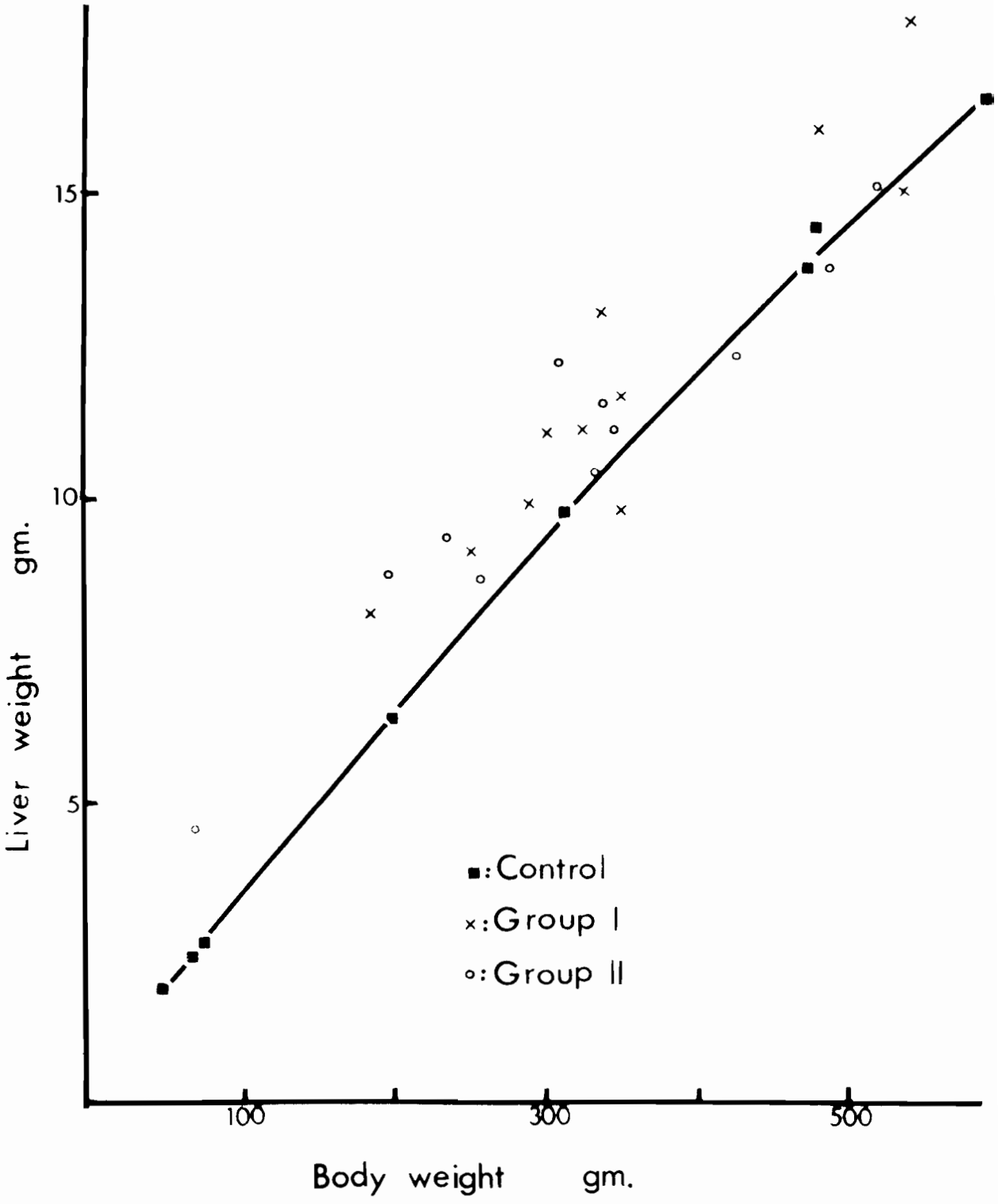


Fig. 3: Liver weight of control rats and rats injected with cuprous chloride (Group I) and copper sulfate (Group II).

V. BINDING OF COPPER IONS BY LIPIDS AND EXTRACTED LIPIDS

A. METHOD ADOPTED FOR THE PURIFICATION OF LIPIDS

The lipid extracts used in these experiments were obtained from rat or monkey tissues by the method of Folch described in Section II. The lipid extract contains all lipids (neutral fats, phospholipids, gangliosides, proteolipids, etc.), with the exception of certain phosphoinositides which are strongly bound to proteins and requiring acidified solvents for their liberation. The specific lipids used were analytical grade, when this was available; otherwise the highest grade of purity was purchased. Both lipid extracts and some of the specific lipids contain non-lipid contaminants which can be eliminated by special techniques. Two of these techniques, diffusion between phases in contact and partition by dialysis are described in Section II. The second technique, partition by dialysis, was chosen for the reason that it saves gangliosides; it involves dialysis of the extract against water at low temperature. During the dialysis procedure, two layers and an interfacial fluff are formed, the upper aqueous layer containing gangliosides, the lower chloroform layer containing all phospholipids, neutral fats, etc. plus chloroform-soluble proteolipid. Most of the proteolipids are incorporated in the fluff.

The distribution of lipids between the layers and fluff (when no copper has been added), is quantitatively constant provided that standard conditions are used (diameter of cellophan tubing, duration of dialysis, temperature, etc). It has been observed in this laboratory, that the distribution of lipids during dialysis was affected by the duration of the washing procedure: chloroform, being slightly soluble (1%) in water was dialyzing out, diminishing the volume of the lower layer and probably sweeping out a small amount of lipids. Decrease of

the volume of chloroform layer, resulted in a precipitation of lipids toward the interfacial fluff altering their distribution. To prevent the loss of chloroform, the outside distilled water was saturated with chloroform. This resulted in keeping the volume of the lower layer constant for at least 11 days. At the end of this period, analysis showed that copper distribution remained unchanged.

In a typical experiment for the study of the lipid-copper interaction, 0.5 to 1 ml of aqueous $CuSO_4$ solution was added to 25 ml lipid extract containing lipids extracted from 1.5 gm fresh tissue or to 25 ml solution of specific lipids. When the added volume of water was close to 1 ml, a milky solution was produced clearing easily on the addition of a few drops of methanol. The extra addition of methanol does not affect the solubility of proteolipids (138). The sample was then immediately dialyzed against water saturated with chloroform at 4° C for 3 days for the elimination of non-lipid contaminants and "freely-dialyzable" copper. Copper and, in some cases, phosphorus and nitrogen were determined in the two layers and the interfacial fluff.

B. BINDING OF EXOGENOUS COPPER TO SPECIFIC LIPIDS*

In order to derive some basic information about the ability of copper ions to bind lipids, the following commercial lipids of different degrees of purity were tested (source and grade in parentheses):

Tristearin	Cerebrin	(technical)
Batyl alcohol	Cephalin	(animal)
Methyl oleate	Lecithin	(animal, 90% pure)
Cholesterol	Lecithin	(vegetable)
	Lecithin	(egg)

*The author wishes to thank Drs. L.S. Wolfe and M. Spence of the Montreal Neurological Institute for their generous gift of ox-brain gangliosides.

β,γ-dipalmitoyl-d,l-α-lecithin (synthetic)

gangliosides (ox brain)

Solutions of the lipids, 1 mg/ml $CHCl_3$:CH_3OH 2:1 mixture (or 0.4 mg/ml for sphingomyelin and gangliosides) were prepared. To 25 ml portions 500 μg of copper were added. These samples were dialyzed in large glass cylinders for 3 days against chloroform-saturated water at 4° C in order to eliminate "freely-dialyzable" copper as well as non-lipid contaminants. At the end of this time the layers formed in the dialysis sac were carefully separated and analyzed.

1. Results and discussion

The results are set out in Table VII. In addition to the lipids shown there, tristearin, methyl oleate and batylalcohol were tested, but these did not bind any copper at all. The other lipids tested all bound some of the added copper and to varying extents. In the case of cholesterol, binding of copper was extremely small and may arise from attachment to some impurity in the product used.

As for the phosphatides, analysis for phosphorus and nitrogen indicated that some of the materials were quite impure, either because of co-extraction of non-phosphatide, partial hydrolysis of the phosphatides or both. Thus, the single sample of animal cephalin gave the theoretical analysis for phosphorus but was very low in nitrogen probably through degradation of the molecule. Synthetic lecithin was somewhat low in nitrogen and egg lecithin in phosphorus but the other two lecithins, animal 90% pure and vegetable, were impure. On the basis of P:N ratios the egg lecithin yielded the best analysis. The sphingomyelin used, also fell considerably short of the theoretical ratios of phosphorus and nitrogen to the pure lipid, although the P:N ratio was 0.62 (theory: 0.50).

Table VII: BINDING OF EXOGENOUS COPPER BY LIPIDS

Lipid*	Source, grade	Analysis μA/μM lipid			Copper bound*** after dialysis, μA						
					Total			$CHCl_3$ layer		Fluffy layer	
		P	N	P/N	Found	per μM lipid**	per μA of P	Found	per μA of P	Found	per μA of P
Cholesterol	Synthetic	0	0	-	0.11	0.002	-	0	-	0.11	-
Cephalin	Theory (MW = 735)	1.00	1.00	1.00							
	Animal	1.01	0.78	1.30	5.88	0.173	0.250	5.00	0.268	0.88	0.233
Lecithin	Theory (MW = 778)	1.00	1.00	1.00							
	Synthetic	1.03	0.90	1.14	1.41	0.044	0.058	0.45	0.061	0.96	0.056
	Animal	1.22	1.43	0.85	4.04	0.126	0.166	3.38	0.159	0.93	0.266
	Egg	0.94	0.99	0.95	0.71	0.022	0.040	0.62	0.034	0.09	0.074
	Vegetable	0.74	0.68	1.09	3.07	0.096	0.148	1.97	0.116	1.11	0.592
Sphingomyelin	Theory (MW = 731)	1.00	2.00	0.50							
		0.87	1.40	0.62	0.38	0.011	n.d.+	0.17	n.d.	0.21	n.d.
Gangliosides++					1.29			0	-	0.13	
Cerebrin	Technical	1.34%	6.47%	0.094	2.17			0.36		1.81	
	Recrystallized	0.46%	2.25%	0.092	2.88			0.63		2.20	
	Fraction insoluble in hot alcohol	1.50%	8.71%	0.078	1.02			0.39		0.62	

Footnotes, page 106.

* No copper was bound by tristearin, methyl oleate or batyl alcohol.

** The number of micromoles of lipid has been calculated using the molecular weights indicated in column 2. The ratio of total copper bound to lipid is based on the actual amount of lipid in the solution that was dialyzed, not on the amount of solids remaining after dialysis.

*** Copper sulfate solution providing 500 μg of cupric ions, nearly 8 μatoms, was mixed with 25 ml of the lipid solutions (1 mg lipid per 1 ml), and then dialyzed as described in the text. (For sphingomyelin and gangliosides the concentration was 0.4 mg lipid per ml.)

\+ n.d.: not determined.

\++ The only lipid to bind copper in the aqueous layer. μA Cu/μA N ratio for aqueous layer, 0.080 and for fluffy layer, 0.078.

Cerebrin lost about half its phosphorus and two-thirds of its nitrogen on recrystallization from ethanol. The fraction that was insoluble in hot ethanol was analyzed and found to be enriched in phosphorus and nitrogen, giving a ratio of these equal to 0.44. Theoretical ratio for sphingomyelin is 0.50.

Of the phosphatides studied, the cephalin, animal lecithin and vegetable lecithin bound large amounts of cupric ions. For these, as well as the synthetic lecithin (but not egg lecithin) about 0.9 - 1.1 μ-atoms of copper appeared in the fluffy layer derived from dialysis of 25 mg of lipid. The remainder of the bound copper was in the chloroform layer. The tendency of phosphatides, lipids containing both polar and non-polar groups, to move toward the interphase during dialysis is demonstrated in these results. Furthermore the ratio of Cu:P for synthetic lecithin in the fluff is very similar to the ratio in the chloroform layer (0.06) and this suggests that the same lipid is involved in both layers. The animal cephalin behaves similarly on dialysis with copper sulfate. Thus cephalin, which was not pure (P:N ratio:1.30), was able to bind large amounts of copper, more than any of the lecithins. This is to be expected, because the cephalins contain not only the phosphate residue but also the ethanolamine-NH_2 residue, serine (with its α-amino-α-carboxylic structure) and phosphoinositol.

Large discrepancies in the Cu:P ratios for the other lecithins probably stem from the presence of non-lecithin lipids and hydrolytic products of lecithin. Thus, if the sample of synthetic lecithin is regarded as a standard for the extracted lecithins used here, then under the experimental conditions used one can expect to have bound about 0.06 μ-atoms of copper per μ-mole of lecithin (Cu:P ratio). The animal and

vegetable lecithins yield much larger ratios, but egg lecithin has values of the same order: slightly larger for the fluffy layer, and only half as great for the chloroform layer.

For pure lecithin, the phosphate residue would seem to offer the best site for copper-binding. For partially degraded samples, glycerol-hydroxyl groups would become available (loss of fatty acids) or an additional site on the phosphate (loss of choline). In the case of egg lecithin the fraction remaining in the chloroform layer on dialysis seems to contain more material of the former type, i.e., able to bind copper but in linkages other than the phosphate. The sample of sphingomyelin did not have great copper-binding capacity. This material probably contained much ceramide phosphate, a composition that would provide a P:N ratio greater than 0.50.

Gangliosides bind much of the copper in the aqueous and none in the chloroform layer. On the other hand, owing to the same Cu:N ratio in both aqueous and fluff layer, it appears that a small part of the ganglioside-copper complex precipitates on the interphase. It is interesting that Quarles and Folch (103), in their study of the effect of calcium on the behaviour of peptide-free gangliosides in $CHCl_3$-CH_3OH-water biphasic system, found that the addition of 2 ml calcium solution of varying concentrations resulted in the quantitative incorporation of the metal into the $CHCl_3$ layer when the molarity was between 0.004 and 0.16M. In the experiment described here, working on the same amount of gangliosides (10 mg) and adding 1 ml of 0.078M copper sulfate (500 μg Cu), the absence of copper from the $CHCl_3$ layer underlines a difference in the behaviour of the two metals.

It is not easy to interpret the data of Table VII for cerebrin.

This is a crude extract of brain lipids which, on recrystallization from ethanol maintains its content of phospholipids (P:N ratio), with somewhat greater capacity to bind copper. The insoluble residue with a lower copper-binding ability did not provide a green precipitate in the fluff (as did the original sample but not the recrystallized material). A nitrogenous constituent is probably responsible for this green compound or complex observed in the fluff of the original sample.

C. EFFECT OF EXOGENOUS COPPER ON POLARITY OF PHOSPHOLIPIDS DURING DIALYSIS

Because of evidence of gross impurities in some of the phospholipids used (Table VII), certain experiments on the binding of exogenous copper were repeated using "washed" phospholipids. $CHCl_3$-CH_3OH solutions of the lipids were prepared as before, but after 72 hrs. of dialysis the aqueous layer was discarded and the remaining material was made up to 25 ml using methanol. Copper sulfate (containing 500 μg copper) was added as described previously, and the solution was dialyzed once more. To illustrate the procedure data in Table VIII may be consulted. Cephalin containing 1062 μg of P/25 ml of solution was dialyzed, during the course of which 237 μg of phosphorus were lost. This presumably represented dialyzable hydrolytic product present in the commercial cephalin. When the dialysis was carried out in the presence of copper, the same amount of phosphorus was lost, but less appeared in the aqueous phase than in the absence of added copper. The redistribution favoured the chloroform phase, as shown in the column of Table VIII for non-washed solutions. In the case of the washed solution of cephalin, the lipids of the aqueous phase were discarded, so that the solution, being dialyzed for the second time (the "washed" lipids), began with only

Table VIII

Effect of exogenous copper on polarity of phospholipids during dialysis

Lipid	Fraction	P content, μg			
		In fresh lipid sample	No copper added to non-washed soln.	Copper added to: non-washed solution	Copper added to: washed solution
Animal cephalin	Total	1062	825	820	587
	$CHCl_3$		563 (68%)	625 (76%)	537 (92%)
	Fluff		137	165	50
	H_2O		125	30	0
Synthetic lecithin	Total	1020		865	
	$CHCl_3$			265 (31%)	
	Fluff			600	
	H_2O			0	
Animal lecithin	Total	1208	945	900	725
	$CHCl_3$		700 (74%)	775 (82%)	600 (83%)
	Fluff		135	100	125
	H_2O		110	25	0
Vegetable lecithin	Total	729	730	750	700
	$CHCl_3$		685 (94%)	700 (93%)	575 (82%)
	Fluff		45	50	125
	H_2O		0	0	0
Egg lecithin	Total	937	875	900	810
	$CHCl_3$		825 (94%)	800 (89%)	775 (96%)
	Fluff		50	100	35
	H_2O		0	0	0

700 μg of phosphorus. A further loss of 113 μg P was sustained on the prolonged dialysis. Of the remaining lipid, most of it was in the chloroform layer, and none at all, judging from phosphorus analyses in the aqueous phase.

Similar results were obtained with animal lecithin, including an effect of copper in increasing the proportion of phospholipid in the non-polar phase (for both washed and non-washed solutions). On the other hand, the vegetable and egg lecithins which had P:N ratios close to the theoretical (Table VIII) suffered little loss in phosphorus content on dialysis, attesting in another way to the integrity of the phospholipid molecule in these particular products.

On summarizing the results obtained from the study of the interaction of copper ions with various specific lipids, the following can be concluded: 1) neutral lipids, such as tristearin or esters of fatty acids do not bind copper; 2) polar lipids form copper complexes. It seems that impure lipids bind more copper than refined or synthetic ones. This is attributed to hydrolytic products possessing extra sites for the binding of copper, i.e., splitting of lecithin results in extra OH groups in the phosphate moiety. Referring to phospholipids, animal cephalin binds more copper than any other lipid studied of this group. This is attributed to the fact that cephalin possesses besides the phosphate residue, ethanolamine-NH_2 residue, serine (α-amino-α-carboxylic structure) and phosphoinositol. Among the lecithin studied, the impure lipids had a greater affinity for the metal probably because of the reasons stated above. Synthetic lecithin binds copper giving a μA Cu/μM lipid ratio equal to 0.06 in both interface and chloroform phase. The finding that most of the copper was incorporated in the fluffy material,

implies insolubility of the synthetic lecithin-copper complex formed. Sphingomyelin binds small amounts of copper. Gangliosides were found the only lipid to provide lipid-copper complexes in the aqueous phase. Its μA Cu/μA P ratio was 0.08. Finally, cerebrin, a crude extract of ox-brain was found to attract large amounts of the metal. In addition, a green precipitate, probably proteinaceous, was observed in the fluffy interface upon the addition of copper. 3) Copper affects the polarity of phospholipids during dialysis by channeling phosphorus into the chloroform phase. The fact that this was observed only in animal cephalin and animal lecithin and not in vegetable or egg lecithin exhibiting a P/N ratio close to the theoretical value, underlines the contamination of the former pair of lipids with water-soluble phosphorus-containing lipids. On the other hand, the loss of considerable amounts of phosphorus during the dialysis of these two lipids in the presence or absence of copper, reinforces the hypothesis stated above of their being contaminated with hydrolytic products.

D. BINDING OF EXOGENOUS COPPER TO TISSUE LIPIDS

1. Equilibrium association of lipid and copper

The experiments with specific lipids and preliminary studies with tissue lipids indicated that the binding of copper was not stoichiometric. It was thought that for any given lipid able to bind copper, Lj, an equilibrium would be set up under specific experimental conditions that may be represented as:

$$Lj + Cu^{++} \rightleftharpoons (Lj - Cu)^{++} \rightleftharpoons (Lj - Cu)^{o} + 2H^{+}$$

The bracketed complexes would then represent the various forms in which ionic copper could be bound by functional groups of lipids,

i.e., carboxyl groups (phosphatidyl serine, gangliosides), phosphate residues (phospholipids including phosphoinositides), peptide and peptidic linkages (proteolipids, sphingolipids), vicinal hydroxyl groups (cerebrosides, sulfatides, inositides, gangliosides) sulfate residue (sulfatides) and others. Such an equilibrium would be established in a homogenous solution of lipids containing copper salt. Dialysis of the solution against water (saturated with chloroform) would upset the equilibrium, directing it to the left, with the passage of unbound or loosely bound, **cupric** ions. The term "loosely bound" must necessarily be empirical at the present stage, because the types of linkages for copper suggested in this paragraph (above) are all more or less readily reversed. However, keeping in mind this fact as well as the knowledge that some of these bonds withstand dialysis for as long as 11 days (p. 103) experiments were carried out with tissue lipids.

To five samples of chloroform-methanol extract of whole rat brain (25 ml each), 500 μg of copper (as the sulfate) was added. All samples were dialyzed for 3 days, at the end of which period, the aqueous and fluffy layers were removed and, together with one of the chloroform layers, were analyzed for copper. The remaining solutions were each made up to 25 ml with methanol, forming a clear solution, and were once more dialyzed. The whole procedure was repeated until the fifth dialysis period (for the last tube) had been completed. A similar experiment was carried out with the lipid extract of rat liver. The results are shown graphically in Fig. 4. During the course of dialysis, including changes of the fluid outside the sac, methanol is lost from the solution. The inward diffusion of water results in an upper aqueous layer and a lower chloroform layer, with a variable amount of frothy material (fluff) at

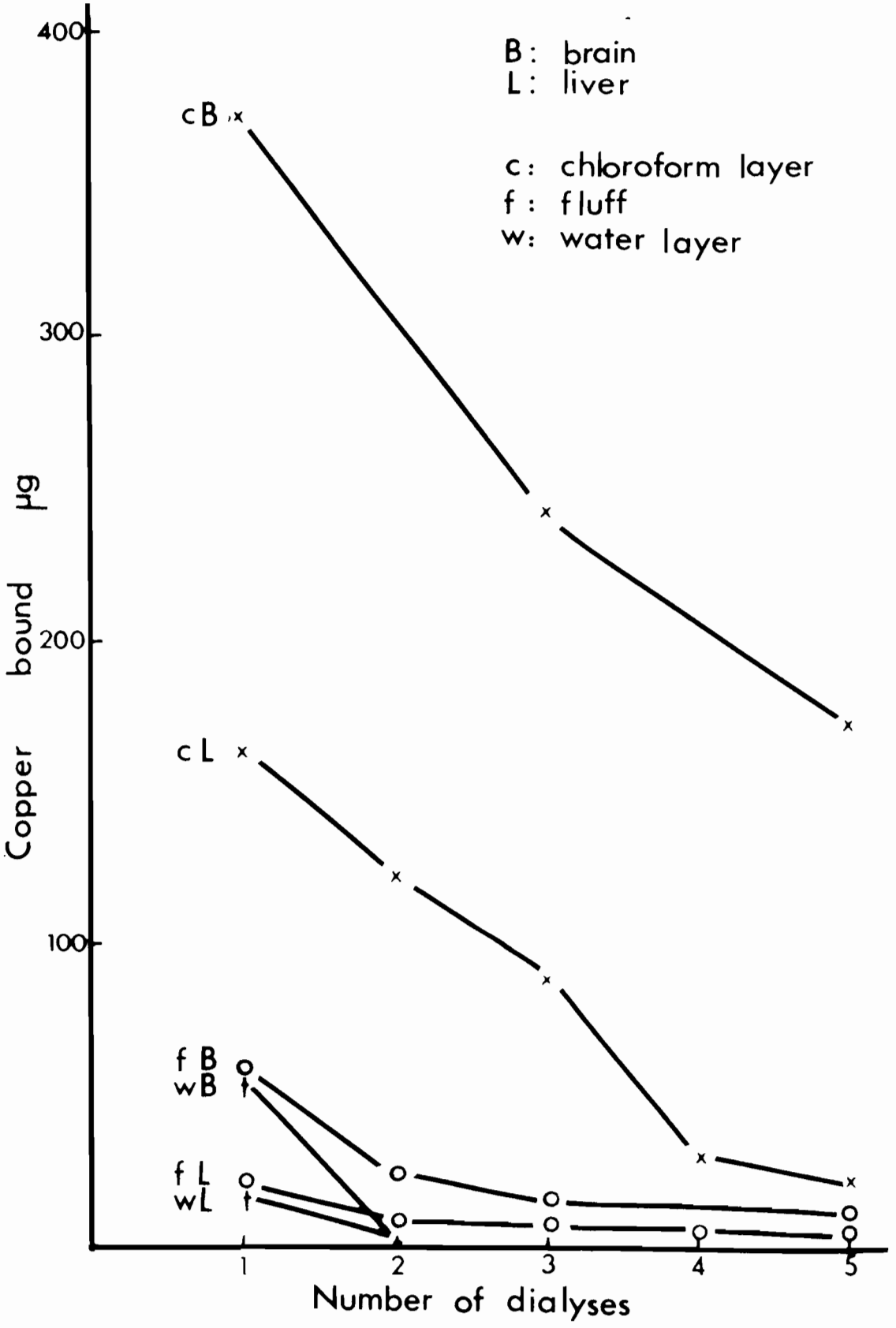


Fig. 4: Equilibrium association of rat brain and liver lipids with copper.

the interface. At the end of the first dialysis period, the chloroform layer contains substantial amounts of copper which, because of the non-polar character of chloroform, is probably largely present in a non-ionic form, although entrapped water in the lipid micelles would permit an ionic bond to exist. The copper found in the aqueous phase is probably associated with gangliosides, that in the fluff mainly with proteolipids. When the aqueous and fluffy layers are discarded before each successive dialysis and fresh methanol substituted, the lipids of the chloroform phase begin to dissociate, releasing a portion of the bound copper in a freely dialyzable form. With each dialysis a new fluffy layer appears, although progressively smaller in size and copper content. It can be assumed that the small amount of proteolipids dissolved in the chloroform layer as well as other substances, for example, phospholipids, gradually move up to the interface where they precipitate.

The curves for the chloroform layer in Fig. 4 provide evidence on the one hand, for an equilibrium-binding of exogenous copper to various lipids of brain and liver but on the other hand, demonstrate that at least some of the metal bound on mixing the copper salt with a chloroform-methanol solution of these lipids can be held very tenaciously.

In subsequent work, dialysis for 72 hours was used, i.e., corresponding to the first set of points in Fig. 4. The specific values for bound copper in the three phases varied with experimental conditions, some of which are now described.

2. Speed of association of copper with lipids

Several portions of lipid extract of rat brain were treated with 1000 μg of copper. The solutions were held at room temperature for various times to permit interaction, before they were subjected to

dialysis. The copper content of the phases was determined at the end of dialysis with results shown in Table IX. These demonstrated clearly that the copper-lipid interaction occurs very rapidly and no prolonged equilibrium period is needed to ensure binding of the metal.

Table IX

Dialysis of brain lipid-copper sulfate solutions after various times of interaction

Phase	Time of interaction				
	2 min.	1 hr.	2 hr.	3 hr.	24 hr.
Chloroform	305*	292	285	320	301
Fluff	132	125	120	138	123
Water	83	90	91	84	90

*μg of copper. In all the above experiments 1000 μg of copper (as copper sulfate) was added.

3. Copper-lipid interaction during extraction from tissues

It was of interest to study the affinity of lipids for the cation by varying the manner of presenting the copper. Thus, a homogenate containing 1.5 gm of rat brain was extracted with 25 ml chloroform-methanol mixture and copper was added in the usual manner to the filtered extract. A second portion of homogenate was extracted with chloroform-methanol mixture containing the same amount of copper. In this case the filter paper collected a green precipitate, presumably from the reaction of the cupric ions with proteinaceous substances in the tissue. The results of dialysis of these filtered extracts are compared in Table X.

Table X

Copper-lipid interaction during extraction from tissues

Phase	Extraction procedure	
	1.$CHCl_3$-CH_3OH 2.Cu	$CHCl_3$-CH_3OH-Cu
Total	1933*	468
Chloroform	610	360
Fluff	1210	65
Water	113	43

*μg of copper. The amount of copper used in these experiments was 5000 μg.

The most notable finding is the elimination of all but 5% of the copper bound in the fluff layer when the copper is allowed to contact all constituents of the brain tissue during the extraction. This decrease occurs also in the chloroform and aqueous layers but to a much smaller extent. It seems that the use of chloroform-methanol-copper as extractant first of all permits the reaction of much of the copper with substances that are insoluble in the organic solvent and secondly interferes with the extraction of proteolipids which are important components of the fluffy layer. Finally, prior reaction of copper with tissue and elimination of much of the added metal in this way, leaves a much smaller amount available for the chloroform-methanol soluble lipids. It would be noted that only one quarter as much copper was bound under these conditions as in the parallel method.

4. Effect of pH

The pH of chloroform-methanol extracts of rat organs is about 7, and the addition of the copper sulfate solutions (pH = 4.25

for a solution containing 5 mg/ml) brings this down appreciably. The effect of acidifying the chloroform-methanol extract on the ultimate distribution of bound copper was studied as shown in Table XI.

Table XI

Effect of acid on copper-binding by extracted lipids in rat brain*

Phase	Addition to $CHCl_3$-CH_3OH extract of Water	2N HCl
Total	1927**	361
Chloroform	607	77
Fluff	1210	187
Water	110	97

*To 25 ml of $CHCl_3$-CH_3OH extract of lipids of rat brain was added either 0.2 ml of water or of 2N HCl as indicated, followed by 1 ml water containing 5000 μg of ionized copper to each. Dialysis against chloroform-saturated water was carried out for 72 hours.

**μg of copper.

The outstanding effect of the initial acidification was the great reduction in binding of copper in the non-polar and fluffy layers. There is little change in the aqueous phase but the brain gangliosides that pass into this phase, containing carboxylic groups are probably substantially ionized at the pH attained with copper sulfate solutions, even without additional acid. Thus, their H^+ ion environment might by only minimally altered by the added HCl and its early removal by dialysis.

On the other hand, the added HCl causes important reversal of copper-binding by the less polar lipids (chloroform phase) and the

proteolipids (fluff). The effect on proteolipid-copper complexes parallels that seen on adding acid to ceruloplasmin; there is immediate release of copper from this protein.

5. Effect of washing of lipids on their binding ability for copper

So far, the affinity of lipids for copper, has been studied in non-washed extracts, containing all impurities and salts extracted along with lipids, for at least two reasons: washing, results in a small percentage of lost lipids. In addition, gangliosides are eliminated too. (The aqueous layer containing the gangliosides could be concentrated to a small volume and added again to the washed lipids, but there is the possibility that the concentration process may alter the nature of the water soluble lipids.) On the other hand, it seemed reasonable to study the effect of copper addition in the extract as it was obtained from tissue, containing natural constituents which might also affect the action of copper in vivo.

However, the following experiment has been performed to study the effect of washing on the binding ability of lipids: three samples of $CHCl_3$-CH_3OH extract from rat brain, in one of which 5000 μg copper (as copper sulfate) has been added, were dialyzed under the usual conditions. At the end of the dialysis period copper was measured in the phases of the copper-containing sample. The aqueous layers of the remaining two samples were removed, one of them discarded, and the other concentrated under vacuum at 40° C down to 0.5 - 1.0 ml and added back to the original sample. Methanol was added in both samples up to 25 ml and then 5000 μg copper. Dialysis followed for a second time. The results for copper values are shown in Table XII.

Table XII

Effect of prior washing of lipids on their binding ability for copper*

Phase	Crude extract	Washed extract	
		Aqueous layer discarded	Aqueous layer saved
Chloroform	660**	582	572
Fluff	1025	137	219
Water	87	34	72

*A $CHCl_3$-CH_3OH extract of pooled rat brains was treated as described in the text with copper sulfate. This was the crude extract. Two other portions of the $CHCl_3$-CH_3OH solutions were first dialyzed and their aqueous phases removed. One of them was discarded and the other after being concentrated, was added back. After addition of CH_3OH and copper sulfate, both were re-dialyzed. These were the washed extract.

**μg of copper.

The chloroform layer of both samples under the heading "washed extract" lost only some of its copper when compared to that of crude extract, but their fluffy layers held only one eighth (aqueous layer discarded) and one fifth (aqueous layer saved), respectively. On the other hand, less than half of the copper seen in the aqueous layer of the crude extract was retained in the case of washed extract (aqueous layer discarded).

The saving of the water-soluble gangliosides in a washed extract, results in a substantial increase of the copper in fluff but far lower than that expected. It seems that either the washing procedure profoundly affects the formation of lipid-copper complexes in-

corporated in this phase or an alteration occurs in the structure of water-soluble lipids during the concentration process. Gangliosides prepared by extraction of tissue with $CHCl_3$-CH_3OH mixture are protein-rich. Quarles and Folch (103) found a correlation between the peptide content of the ganglioside preparations and their tendency to accumulate at the interface in the presence of $CaCl_2$, and they suggested that the peptide may be responsible for the interfacial accumulation. Gangliosides prepared by the hot methanol extraction technique and known to be poor in α-amino acid nitrogen, did not behave similarly. Thus, it can be suggested that the concentration of the water layer under the conditions described above, resulted in a splitting or "denaturation" of the peptide moiety of gangliosides and an alteration of their affinity for copper. The fact that the water layer of the washed sample (aqueous layer saved) had essentially the "normal" concentration of copper, underlines the possibility that gangliosides themselves, retained their ability to bind copper.

6. Effect of other cations on the interaction of lipids with copper

Owing to the antagonism observed in the animal organism between copper and molybdenum, manganese or iron (Section I) it seemed of interest to study the effect of the presence of these cations on the affinity of tissue-lipids for copper. In four 25 ml samples of a $CHCl_3$-CH_3OH solution of lipids extracted from the monkey Rhesus brain, 1 ml 0.1 M $CuSO_4$ alone or mixed with 1 ml 0.1 M ammonium molybdate, manganous chloride or ferric chloride respectively was added. The values of copper found in all samples after dialysis are shown in Table XIII.

Table XIII

Effect of other cations on the interaction of lipids with copper

Phase	None	Molybdenum	Manganese	Iron
Chloroform	760*	1016*	713*	48*
Fluff	750	863	188	780
H_2O	44	60	42	29

*μg of copper.

Molybdenum seems to increase the copper bound in all phases. In connection with this, the known effect of cations on unmasquing functional sites of a protein has been discussed by Seals and Bynum (149). They found that the dye-binding capacity of casein increases if suitable agents, such as salts of copper, magnesium or zinc, are introduced to break intramolecular bonds, specifically the carboxyl-cationic nitrogen or hydroxy-cationic nitrogen bonds. In a similar mode of action, molybdenum could keep broken the intramolecular bonds of a certain kind of groups in lipid or proteolipids, thus giving a chance to Cu^{++} ions to compete and replace this cation.

Manganese on the other hand decreased the amount of copper incorporated in the fluff. Whether or not the competition takes place in the water-soluble lipids or in the proteolipids is unknown. Finally, iron binds with the $CHCl_3$-soluble lipids. This was shown by the detection of large amounts of iron in the yellow-coloured layer. The binding, results in the exclusion of copper from binding sites. How-

ever, the fluff copper remains unaffected.

7. Binding of copper by lipids extracted from various tissues of the rat

To several 25 ml samples of brain, liver, kidney and heart lipid-extracts and to an equal number of $CHCl_3$:CH_3OH 2:1 mixture samples, 0,50, 100, 150, 200, 400, 600, 800, 1000, 2000, 3000 and 5000 μg copper as $CuSO_4$ were added. All samples were dialyzed for three days. At the end of the dialysis chloroform and water layers and fluffs were analyzed for copper. The chloroform layers of the blank were found to contain 0 to 10 μg copper, depending on the amount of added metal, whereas water layers 0 to 20 μg. These values, although insignificant, were substrated from the corresponding values in the lipid samples. The results are presented in Fig. 5.

Regarding brain lipids, the increasing amounts of copper added seem to favor in the beginning capture of the metal by chloroform-soluble lipids. When the added copper reaches the range of 1000 or 2000 μg, the interaction favors the fluff lipids. This can be explained by assuming that there is a difference among lipids in binding ability. When the binding sites of a certain group of lipids in the chloroform phase are satisfied, another group, in this case proteolipids and/or protein-rich gangliosides, starts reacting. The water-layer content of copper remains constant for the range: 1000 - 5000 μg metal added.

The patterns of kidney and liver are quite similar. However, on comparing their chloroform layers, more of the metal is found in kidney. On the other hand, kidney contains less copper in the fluff. Finally, heart exhibits a similarity to brain probably because both these tissues contain large amounts of proteolipids. Heart water-layer

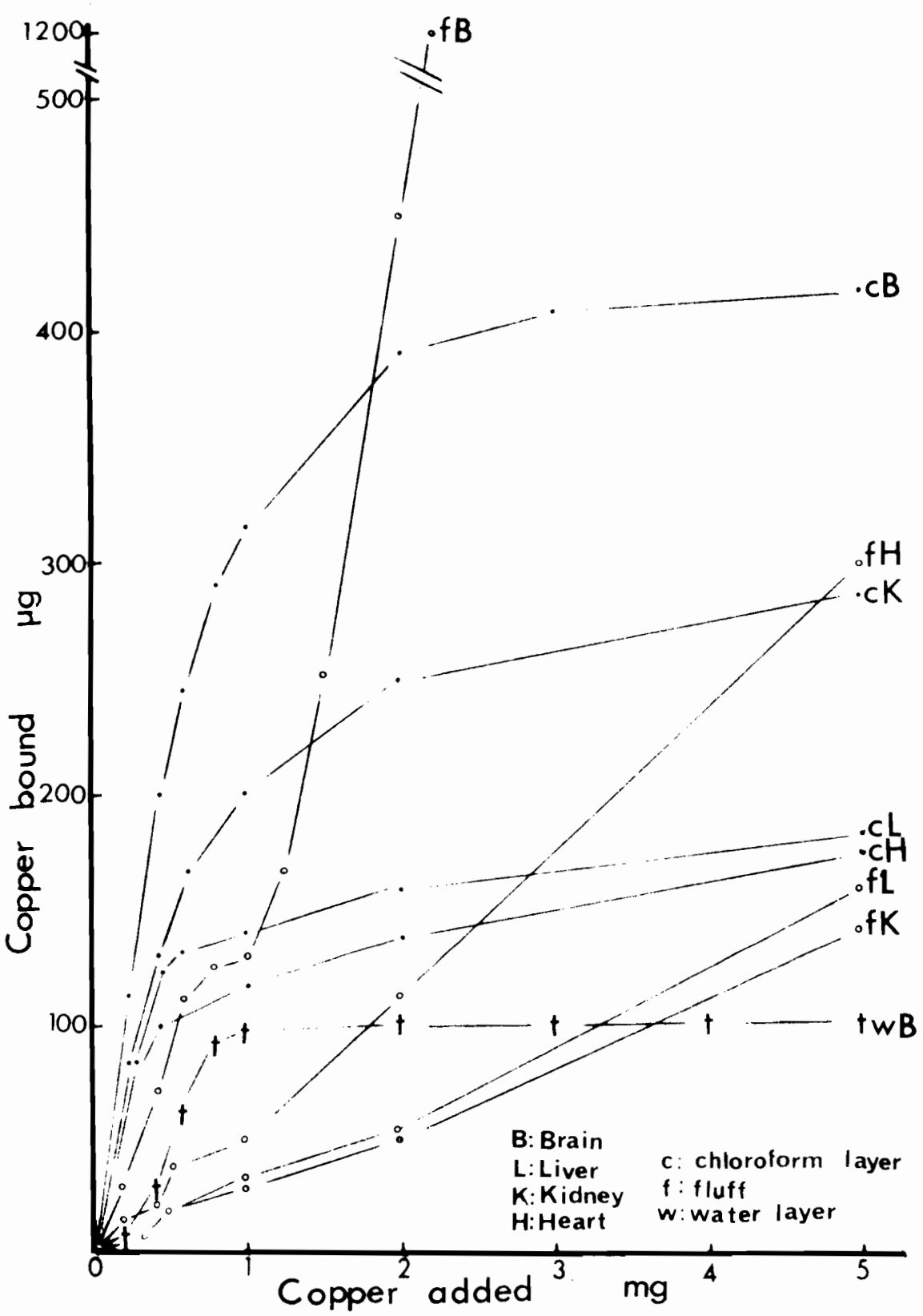


Fig. 5: Binding of copper by lipids extracted from brain, liver, kidney and heart.

as well as those of kidney and liver do not bind appreciable amounts of copper. Referring to the total bound copper, brain lipids attract more of the cation than the other tissues. Liver, kidney and heart lipids bind roughly the same quantity of copper. Looking at each particular phase, the following can be concluded. In all tissues studied, the copper content of the chloroform layer increases up to 2000 μg added metal and levels off thereafter. In fluff, however, there is a tendency of copper to plateau between 500 and 1000 μg added copper. When more of the cation is added, there is a dramatic increase of copper in the brain fluff. A similar increase is also observed in heart, although at a lower level, and in liver and kidney in that order.

8. Relation of bound copper to the nitrogen and phosphorus content of the lipids of rat brain

The addition of copper sulfate to the $CHCl_3$-CH_3OH extracts produces a greenish-blue color in the solution. It also causes a green precipitate to form. This precipitate becomes readily apparent with as little as 1000 μg of added copper per 25 ml of extract, and increases in quantity as the amount of exogenous copper is increased. On dialysis, the precipitate accumulates in the interfacial fluffy deposit. It is noteworthy that the washed lipids provided no such precipitate even with 5000 μg of copper. Webster and Folch (104) studying the behavior of proteolipids in biphasic systems, found that the addition of salts such as those of sodium, potassium, magnesium and calcium produces splitting of proteolipids into their nitrogenous and lipid components. The rupture of these linkages was roughly proportional to the logarithm of the ionic strength of the solution. The copper salt appears to achieve the same effect, but now the insoluble complex was coloured. Whether

the green precipitate resulted from the splitting of proteolipids or protein-rich gangliosides or both is unknown at the present.

The precipitate was prepared from brain lipids by the addition of 5000 μg of copper ions to 25 ml of $CHCl_3$-CH_3OH extract. The mixture was centrifuged at 2000 r.p.m. for 5 min. and the precipitate was removed, washed three times with a small amount of $CHCl_3$-CH_3OH mixture and finally dried by evaporation of the remaining solvent. The greenish-blue solid was insoluble in chloroform, carbon tetrachloride, benzene, ether, methanol, ethanol, dioxane and water. It could not be brought into solution with acid or alkali. Strong acids decolorized the complex, by dissociation of the copper, just as acid releases the copper of ceruloplasmin very quickly (127). At least part of the copper of the precipitate is loosely bound for on adding a solution of diethyldithiocarbamate a yellow colour appeared ("direct-reacting copper"). The addition of sodium hydroxide solution to the complex caused formation of a deep blue colour, probably as a biuret reaction. Analysis gave: copper, 12.0%; nitrogen, 7-8%; phosphorus, 0.21%. The composition of the precipitated complex was essentially the same when 25.000 μg of copper ions were used in its preparation. With 2.500 μg of copper, however, only half as much copper was found (5.8%) and more than twice as much phosphorus (0.50%).

In order to study the effect of the lipid-copper interaction on the distribution of phosphorus and nitrogen in the various phases obtained by dialysis, graded amounts of copper were added to $CHCl_3$-CH_3OH extracts of brain lipids and the solutions were dialyzed against chloroform-saturated water. Analyses were carried out for copper, nitrogen and phosphorus. The relation of the nitrogenous lipids in the $CHCl_3$-CH_3OH extract to the concentration of copper is revealed in Fig. 6. The addi-

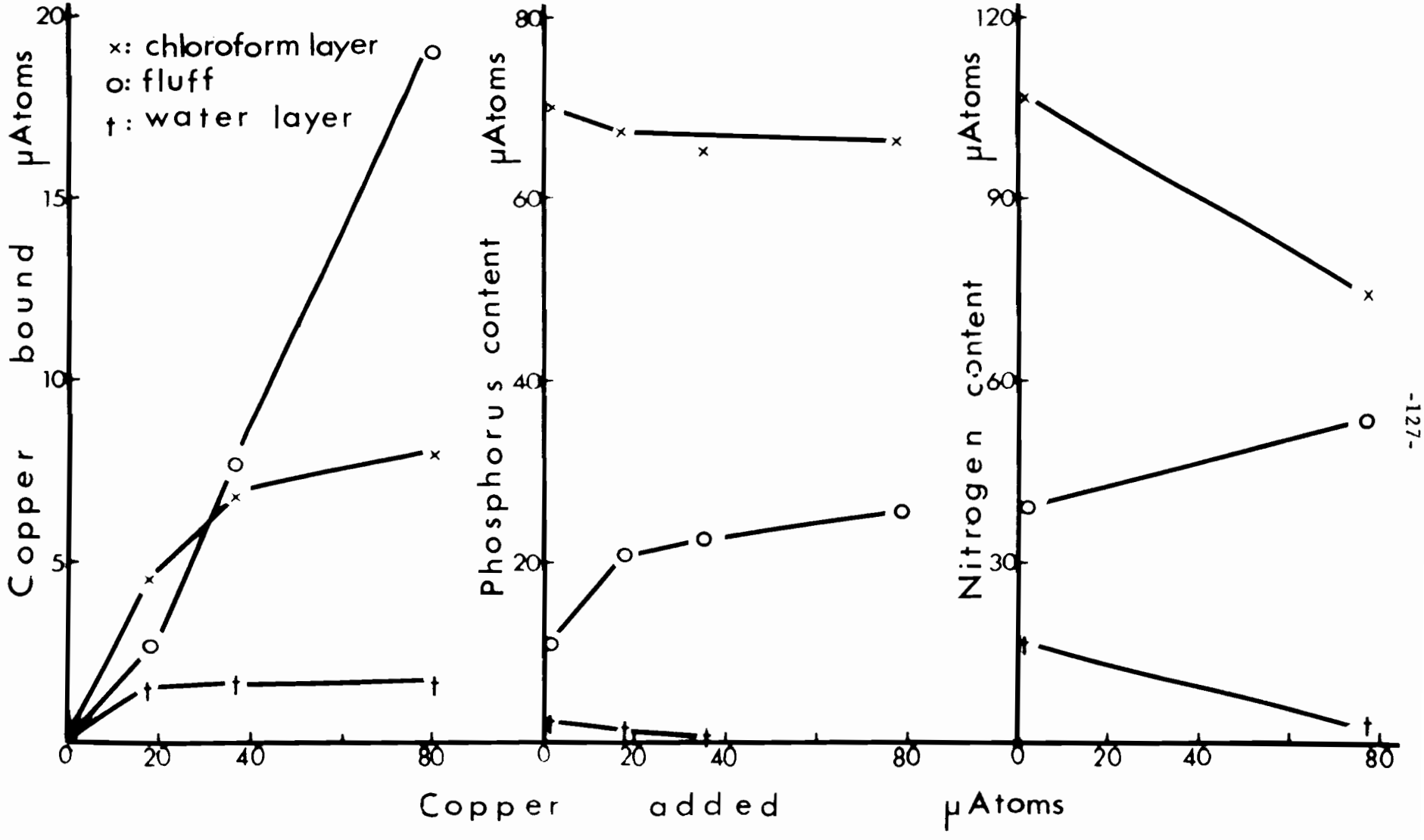


Fig. 6: The effect of the interaction of copper with lipids extracted from rat brain, on the distribution of phosphorus and nitrogen in the various phases obtained by dialysis.

tion of copper reduces their content in the aqueous and chloroform layers and increases them in the fluff. As there is a net loss of nitrogen on dialysis, it may be assumed that some non-lipid nitrogenous compounds are included in the $CHCl_3$-CH_3OH extracts, from which they escape on dialysis. In regard to the phosphate content of the fractions, a little is found in the aqueous layer in the absence of copper, and the addition of copper causes this small amount to be lost. This decrease occasioned by copper, and a larger one for the chloroform layer are made up by an increase in the phosphorus content of the fluff. This increase in phosphate amounts to 14 μM, the amount by which the nitrogen of the fluffy layer increases on addition of 5000 μg of copper and dialysis; the ratio for P:N of 1.0 suggests that phosphatides have moved into the fluff. From the results set out in Table VIII for non-washed lipid solutions, it seems unlikely that these could be responsible for the observed increase, but those data were obtained using only one tenth as much copper as in the present experiment. Moreover, the earlier studies showed that these substances do precipitate out in part at the interfacial layer (Table VII). As for other components of the fluff, an increase in proteolipids or protein-rich gangliosides would bring up the nitrogen content, and the P:N ratio of unity (for the change in fluff composition as a result of adding copper) could be restored by passage of more phosphoinositides into this phase.

Other rat organs were extracted and tested with copper sulfate for the formation of the insoluble copper complex. Extracts of heart, the only other tissue besides brain that contains important quantities of proteolipids, yielded the material, but extracts of liver and kidney did not do so.

9. Relation of the copper binding to the lipid-content of a tissue

The addition of copper sulfate to the lipid-extract obtained from any one of the tissues studied, revealed that not all copper added was bound to lipids, part of it diffusing out during the dialysis process. The amount of cation held in, was dependent on the tissue from which the lipids were extracted. The captured copper ions were distributed among the three phases and the level of the metal in each one seems to be in direct relation to those lipids which constitute each phase. There probably are some exceptions, such as that of protein-rich gangliosides, to which the addition of copper may result in the formation of insoluble lipid-copper complexes precipitating at the interface.

In Table XIV, the composition of lipids of rat and cattle tissues (values obtained from the literature) is compared to the values of copper distributed among the various phases obtained after the addition of copper sulfate in $CHCl_3$-CH_3OH extracts from the respective organs, and subsequent dialysis, as well as to the total bound copper. There is a rough relation between total amount of lipids in a tissue and total bound copper. Although heart contains the lowest quantity of lipids, its $CHCl_3$-CH_3OH extract binds copper as much as liver does, probably because of its higher content in proteolipids. Total phospholipids are more closely related to copper found in the $CHCl_3$-layer which holds this group of lipids. Lecithin has its lower concentration in brain and its maximum in heart. This is in contrast to the copper content in the $CHCl_3$-layer although it underlines a quantitatively minor importance of this lipid in connection with the copper binding. Cephaline and sphingomyelin on the other hand, as well as total lipid phosphorus are in a much better relation to the concentration of copper in the $CHCl_3$-layer.

Table XIV

Relation of the copper binding to the lipid-content of a tissue

Lipid	Rat brain	Rat kidney	Rat liver	Rat heart
Total lipids (150)	151.0*	54.0	54.0	34.5
Phospholipids	70-87	36.0	33-40	18-30
Lecithin	13.5-27	21.0	16-46	30.0
Cephalin	22-36	14.5	12-21	12.6
Sphingomyelin	10.5-24	4.5	3-6	2.1
Total lipid P (μm/1.5 gm fresh weight of tissue)	123.0	37.5	46.0	27.0

	Cattle brain		Cattle kidney	Cattle liver	Cattle heart
	White matter	Grey matter			
Proteolipids (118) (as proteolipid protein)	30-37	9.0	2.9	2.4	5.2
Strandin (120)	0.90-1.05*	9.00-10.5	< 0.15	< 0.15	< 0.15
Copper bound (in rat)					
Total	1.75**		0.45	0.39	0.40
$CHCl_3$ layer	0.65		0.29	0.20	0.17
Fluff	1.20		0.15	0.17	0.30
H_2O layer	0.10		0	0	0

* Corrected to mg/1.5 gm fresh weight of tissue from the original results of Collins and Shotlander (150) and, Folch and colleagues (118, 120).

**mg/1.5 gm fresh weight of tissue.

Proteolipids (data from cattle brain) and copper incorporated in the fluff are also in good agreement. Brain and heart which have the higher content in proteolipids have also the highest amount of copper in their fluffs. Because of the location of proteolipids in the myelin sheath (105), a structure that is incompletely developed in the rat at birth (151), addition of copper in lipid extract obtained from brains of newborn animals should result in a low copper content of the interfacial fluff. The results are shown in Table XV.

Table XV

Binding of exogenous copper by lipids of brain. Brains of neonatal and adult rats

Phase	Newborn	Adult
Chloroform	340*	540
Fluff	207	1110
Water	101	96

*μg of copper.

The pooled brains of 30 newborn rats were used to prepared a $CHCl_3$-CH_3OH extract. To each 25 ml of this and of the extract of adult brain, 5000 μg of copper ions were added, and then the solutions were dialyzed.

Copper in fluff is very much lower in the "newborn" fluff. Chloroform layer, also containing chloroform-soluble proteolipids, binds less of the metal. In addition, no green precipitate was formed.

Finally, looking again at Table XIV, strandin, known to exist in appreciable amounts in brain and thought to be polymerized form of gangliosides, is probably responsible for the presence of copper in the water-layer of brain.

VI. GENERAL DISCUSSION

Work on trace metals began during the last century, but it is only a few decades ago that their role in the physiopathology of the body appeared to be important and, in some cases, decisive. Copper, being a paradigm of this category of rare physiological elements is engaged in various roles; from an oxygen carrier in the blood of the molusks to an electron acceptor in some enzymes. The studies of copper metabolism were made more important by the discovery at the beginning of this century that abnormal metabolism of this metal leads to hepatolenticular degeneration (HLD) or Wilson's disease.

The bulk of studies on copper metabolism, as in similar cases, was carried out in three ways. 1) Using experimental animals and humans, the absorption, transport and excretion of the metal were studied. Research on the molecular level was also done by the isolation and purification of some copper compounds. 2) Studies were made on Wilson's disease patients for the understanding of the abnormal copper metabolism and discovery of pathognomonic chemical, clinical or histological characteristics. The setup of experimental models of HLD were also tried in animals. 3) Studies related to the isolation and purification of enzymes, indirectly contributed to the case by findings showing participation of copper in their activity or structure.

Despite the vast literature concerned with copper biology, the metal enters, acts in, and leaves the animal body in a manner that is not clearly established. Various theories discussed in Section I have been proposed for its absorption, transport and excretion in experimental animals and humans as well as in HLD patients. Nevertheless, the research on copper metabolism has led to some positive results which include iso-

lation and purification of the physiologically important copper compounds ceruloplasmin and erythrocuprein, and a large body of evidence about the significance of copper balance in various metabolic processes (hematopoiesis, growth and specific enzymatic activities). In fact, progress in understanding the function of copper, parallels that made in the electrochemistry of biological systems. This is not surprising since copper is associated with the electron transport.

However, nothing is known about the state of the major part of the copper in the body or the extent of participation of cuprous copper. Another problem is the transport of the metal to various parts of the body. Which is the copper transporting protein in blood, ceruloplasmin or albumin? Also, if copper is so loosely bound to albumin in a non-specific fashion, how can one explain the indifference of other sulfhydryl proteins toward this free copper?

It is the author's opinion that efforts should be made to elucidate the following oversimplified pathway where x, y and z stand for known or unknown moieties of a copper compound:

x - Cu (in plasma) $\rightleftharpoons$ y - Cu (in cell membrane) $\rightleftharpoons$ z - Cu (in tissues)

The above scheme poses three main problems:

1) The state of copper in blood.
2) Transport of copper through the cell membrane as y - Cu (or free copper?)
3) The state of copper in tissues.

In practical terms, the elucidation of this pathway could give an answer to the question of the deposition of copper in tissues in the course of Wilson's disease. It may also offer ways for an effective elimination

of the excessive metal from tissues. Two of the theories most accepted for the etiology of the great deposition of copper in the tissues of HLD patients, are involved in the first and the third stage described above. According to Scheinberg (87) excess of loosely-bound copper in blood results in a higher rate of copper penetration into the cell. His theory assumes that free copper ions are more available for transport. Yet, this contradicts the supposed copper-transporting role of ceruloplasmin, which some assume to bring the metal to the sites of cell penetration. The second theory, developed by Uzman (90), suggests an abnormally high affinity of tissue proteins for copper. It is not stated whether the inborn error of HLD leads to the formation of one abnormal protein common to all copper-loaded tissues or to several proteins each one specific for each tissue. Neither of these theories explains the failure of excessive accumulation of copper in the brain of experimental animals loaded with copper in prolonged experiments or the serious accumulation of the metal in their liver and kidney.

The second stage of the scheme described above is related to the copper transport inside the cell. The mechanisms here are obscure. Yet, there is no doubt that the rate of penetration of the cation is not uniform in different tissues. Brain, for example, is known for its resistance to the transport of various substances.

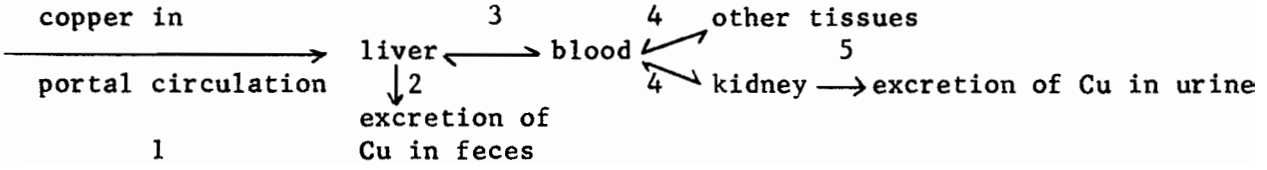
At this point, the justification of the present research lies in the following: copper during its transport into the cell or cell particles, crosses the cell or particle membranes. If the passage of copper is not carried out by simple diffusion through "pores", then the structural elements of the membranes must play a role. Various theories (105, 106, 107, 108) implicate phospholipids and gangliosides in the

transport of cations. Thus, it seemed reasonable to examine the concentration of copper in this well-defined compartment of the body: the cell membrane. Analysis of the lipid extracts from brain, kidney, liver and heart (Section III, Table II) showed that lipids contain trace amounts of copper and because the majority of these lipids are part of the cell or particle membranes, it was assumed that the copper measured is located in these membranes. Brain and heart extracts were found to contain less copper than those of kidney and liver. This was attributed to the fact that the latter tissues are ultimately concerned with the excretion of copper. If one assumes that the rate of copper transport through the cell membranes is related to its binding by the membrane lipids, then kidney and liver which exhibit high rates are expected to contain more copper in their extracts. The heart which is not a site of copper excretion and has the lowest total solids weight in its chloroform-methanol extract (Table II), has the lowest amount of copper in the lipid extract. Brain, known to have a very low rate of copper penetration, binds substantial amounts of copper probably because of its high content of lipids. It is conceivable that qualitative differences in the lipid pattern of the membranes of different tissues result in a differential binding of copper among the tissues.

Having shown the existence of copper in the tissue lipids of the rat, the next step was the comparative study of the overall rate of copper transport in a variety of tissues by carrying out experiments of copper deficiency and copper loading. The purpose of both groups of experiments was mainly the study of tissues in regard to their facility to loose or accumulate copper and the effect of disturbed copper balance on the copper content of lipids. To carry out these studies it was

necessary to have a picture of the copper pattern in the tissues of the normal animal. Thus, the metal was determined in a large variety of tissues from Purina-fed rats. Because in the copper deficiency experiments the animals were fed milk exclusively, a separate group of rats receiving milk and supplemented with copper (control group) was used to obtain copper values in tissues (Section IV, Tables IIIa and IIIb). Hair, kidney, liver, heart and brain were the richest tissues; testes and spleen had a moderate concentration of the metal; and lungs, blood and skeletal muscle were the poorest.

The tissues studied in conditions of deficiency and loading (Tables Va-g and Va-c) included liver, kidney, brain, heart, skeletal muscle and blood. Liver and kidney were the tissues most affected in both states. However, each of these tissues should be examined separately because although they are sites of copper excretion, the concentration of the metal in kidney depends on the circulating copper in blood and especially on the excretion of the metal by the liver. In other words, liver regulates in a way the amount of copper which can reach the kidney. In the copper deficiency state liver loses more copper and loses it much earlier than kidney. In loading, liver gains more of the metal than any other tissue. The following scheme, showing the fate of copper in a normal animal, explains the special position of liver in these abnormal conditions:



In copper deficiency, step 1 almost ceases to exist. On the other hand, liver continuously excretes copper in feces (step 2) and in blood (step 3) by which the metal is transported through the body (step 4). Thus, step 3, although diminished, operates as long as liver can provide the metal. In copper-loading, step 1 is enormously increased. Liver, with a high copper-storing potential and a great ability to eliminate copper through the bile (step 2), secretes into blood only a small fraction of the administered metal. One can assume from the increased values of ceruloplasmin in plasma (Tables Va, b, c) that the loosely-bound copper in blood is only slightly increased. Nevertheless, this excessive free copper accumulates in the tissues. The kidneys, either because they are sites of copper excretion or because they have a high ability to bind the metal, accumulate more copper than any of the other tissues, except liver.

Administration of cuprous chloride and copper sulfate, showed a difference in the deposition of copper in liver and kidney. This can be attributed either to the insolubility of the cuprous salt or to the need for its oxidation to the divalent state to be absorbed. Differences were also observed in the responses of rats of different ages. Weanling animals, for example, had a lower rate of accumulation of copper in liver and kidney. Whether this is due to a higher ability of the young organism to excrete excess of the metal or to a low binding ability of its tissues remains unknown. The amount of copper deposited in liver and kidney declined toward the end of the loading experiment. The physiological significance of this phenomenon as well as its mechanism remain obscure.

It is very difficult to classify the remaining tissues according

to their response in deficiency and loading. All tissues studied lost copper in the course of deficiency experiments, although some remained unaffected in loading. From this point of view, tissues which exhibited a negative and positive balance, respectively, will be discussed first.

Cardiac muscle is markedly depleted of copper in a deficiency state, but it shows a remarkable resistance in copper-loading. Brain exhibits the same resistance in accumulating or losing copper. This tissue is known to bind excessive quantities of copper in pathological conditions such as HLD, implying a high affinity of the brain proteins for the metal. But in the normal animal a blood-brain barrier seems to play a prominent role in preventing copper from penetrating the tissue. The 50% increase of the copper content of brain, measured after 134 days of loading, reveals first of all that an upset of the copper balance in this tissue is possible. Secondly, there is no need to believe that the deposition of copper in brain is directly related to the duration of loading. It is quite possible that the resistance of the tissue breaks down when certain conditions arising from the chronic poisoning are set up.

Erythrocyte copper followed a more or less similar pattern in both deficiency and loading. The hemoglobin content of the red blood cells was affected much more in deficiency than in the loading state. It is known that copper deprivation leads to a decreased hemoglobin formation for reasons stated in Section I. Whether the presence of excessive metal resulted in decreasing hemoglobin (in some cases), by acting on the sites of the formation of erythrocytes or by increasing the rate of their destruction is unknown.

In the category of unaffected tissues in the course of copper-loading experiment, skeletal muscle holds the first position. A low copper-binding ability of the proteins of this tissue or a selective permeability of the cells excluding the passage of copper could explain the lack of copper deposition there.

Lungs and testes were not examined in the deficiency state and the rate of copper depletion is unknown at the present. Both tissues were only slightly affected in the loading experiment. However, testes were atrophic in some of the rats which started receiving copper as weanlings.

Finally, plasma is examined last because the concentration and the state of copper there, seems to be decisive factor for the accumulation of the metal in tissues. As long as liver can function both by excreting copper in feces or by converting free metal to ceruloplasmin, the copper concentration in plasma remains at near-normal levels and its deposition in tissues is retarded. On the other hand, in the course of deficiency, plasma rapidly loses its copper because its supply from liver declines at an early stage and secondly because of an increased need of other tissues for the metal.

In conclusion, the comparative study of the overall rate of copper accumulation or depletion revealed great differences among various tissues, possibly due to the following:

1) Function of the tissue. (Liver and kidney for instance are directly related to the excretion or absorption of copper.)

2) Proteins with high or low binding ability for copper. (Liver and possibly, muscle tissue.)

3) Blood-tissue barriers. (Brain, muscle tissue?)

4) Mechanisms for absorption or excretion of copper, differing from tissue to tissue. (Liver or kidney).

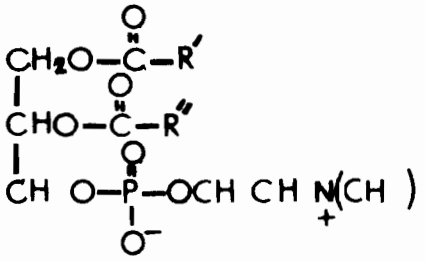
The last two factors could be directly related to the permeability shown by the cell membranes for copper. It is obvious, for instance, that liver has a special mechanism for the absorption of copper, which enables it to accumulate enormous amounts of the metal. This implies a high permeability of the cell membranes. Analysis of lipids extracted from livers and kidneys loaded with copper (Table VI) revealed increased amounts of the metal in the lipids of both tissues. However, the fact that liver or kidney lipids from aged rats did not contain copper as much as those from adult animals (despite the similar concentrations of copper in these tissues for both groups), raises the question whether lipids are directly related to the binding of copper. Unless a qualitative or quantitative difference exists in the pattern of lipids between adult and aged rats, the lower content in copper observed in the lipids of the latter group of animals is of unknown significance.

So far, the subject of copper passage inward or outward has been studied in two ways: the first one, of a static nature, dealt with the presence of copper in tissue lipids, presumably those of cell membranes; the second, a comparative study of the elimination or deposition of copper in tissues was of dynamic nature. For both cases, much information has been collected, enough to justify a more detailed study, partly at a molecular level, and related to the binding observed between copper and lipids. The interaction of copper and lipids in vitro, was studied by using either refined or extracted lipids. Various types of lipids were used such as neutral lipids, phospholipids, gangliosides, sphingomyelin and crude cerebrin. The partition dialysis technique that was

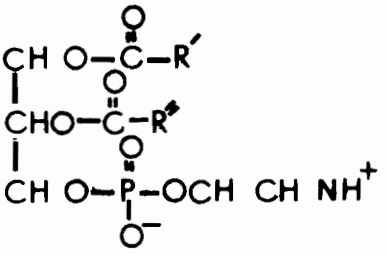
used had the advantage of (a) separating the solution of lipids into 2 or 3 distinct phases, each one containing lipids with specific solubility characteristics (this was especially useful for solutions of mixed lipids); and (b) a simultaneous elimination of the unreacted copper and other diffusible non-lipid contaminants. Thus, at equilibrium, the three phases contained only lipids and lipid-copper complexes.

With the exception of neutral lipids, all the lipids tested, and known to be structural units of the cell membrane, were found to bind copper. There is a variety of charged groups in the lipid molecules which may be attracting sites for copper. Figure 7 includes the chemical structure of some of the lipids studied. Functioning sites of phospholipids that may react with copper ions are the free hydroxyl groups of the phosphate for both cephalins and lecithins, the charged nitrogen of the choline moiety, the amino- or carboxyl group for the serine-cephalins and finally hydroxyl groups of the inositol moiety of phosphoinositides. Sphingomyelin has a phosphate group and the charged nitrogen which possesses a proton-bearing amido group able to bind cations (29). Gangliosides, on the other hand, present an even larger variety of charged groups: its ceramide moiety (amido group), its two hexoses (glucose and galactose with vicinal hydroxyl groups), its hexosamine (galactosamine) and its sialic acid moiety (amido and carboxyl groups) which appears to be N-acetyl neuraminic acid in brain gangliosides.

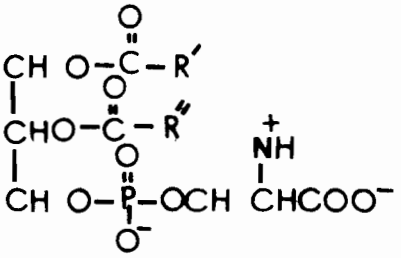
Among phospholipids, the relatively impure lipids used were found to bind more copper than the pure ones. For instance, "animal lecithin" had a P/N ratio much higher than that of synthetic lecithin. This was attributed either to contamination of the preparation with other lipids; or to hydrolysis during extraction or storage or both.



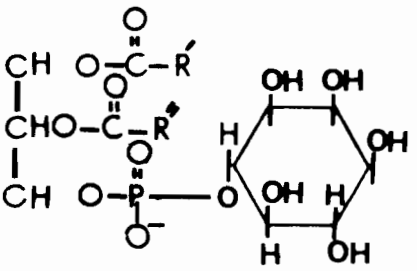
A Lecithin
(Phosphatidyl Choline)



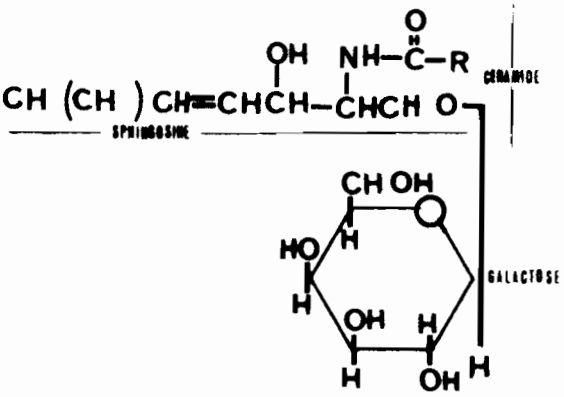
An Ethanolamine–Cephalin
(Phosphatidyl Ethanolamine)



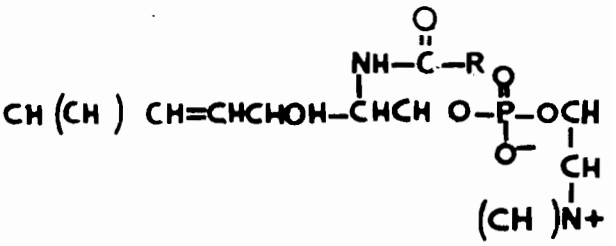
A Serine–Cephalin
(Phosphatidyl Serine)



A Phosphoinositide
(Phosphatidyl Inositol)



A Cerebroside



A Sphingomyelin

CERAMIDE–(HEXOSE)$_2$–HEXOSAMINE–SIALIC ACID

Ganglioside

Fig. 7: Phospholipids and nitrogenous lipids

In any case, it seems that these unknown impurities possess functional sites with far greater binding capacity than any one of the pure lipids studied. Preliminary experiments with synthetic cephalin (of the ethanolamine type) revealed a very low Cu/P atomic ratio, comparable to that of synthetic lecithin and far lower than that of animal cephalin. It is obvious then, that only a very high Cu/P ratio of the contaminant lipids could explain the increase of the ratio in animal cephalin and vegetable lecithin. There is no evidence on the nature of these impurities or their functional groups. However, technical cerebrin lost more than 60% of its nitrogen on re-crystallization. This strongly suggests a peptide impurity probably originating from coextracted proteolipids or peptide-rich gangliosides. Thus, it is possible that at least part of the extra copper bound in impure lipids is bound to peptides.

The nature of the bonds of the lipid-copper complexes is unknown. Evidence from experiments with refined as well as with extracted lipids suggests that the bonding is fairly weak. Thus, the data from the equilibrium association experiments (Section V, D) predicts a very low equilibrium constant. In addition, lowering of the pH decreased to a minimum the copper bound to extracted lipids (Section V, D, 4) and treatment with diethyldithiocarbamate resulted to the formation of the yellow complex, indicating the presence of "direct-reacting copper". In the case of synthetic lecithin (β-γ-dipalmitoyl-DL-α-lecithin), the only phospholipid assumed to be free of other contaminants, the Cu/P atomic ratio 0.058 implies a non-stoichiometric reaction. Therefore, one can postulate that a large number of phospholipid molecules are loosely combined in some manner with each atom of copper, forming an aggregate in which the polar heads of the lipid molecules are oriented

toward the copper. Similar aggregates could occur for cephalin, sphingomyelin or ganglioside-copper complexes.

The experiments with refined lipids thus established the existence of a lipid-copper non-stoichiometric interaction and a rough classification of lipids in terms of their binding ability was achieved. The study of this interaction was then extended to lipids extracted from various tissues of the rat. Brain lipids were found to bind about four times as much copper as liver, kidney and heart did. The metal was distributed in the chloroform and fluffy layers in all tissues studied, and in water layer only in the case of brain. The study of the copper uptake by the three phases suggests that initially the metal is bound by the chloroform-soluble lipids (or, at least, there is formation of chloroform-soluble complexes); as soon as these are saturated, other lipids react and the complexes formed accumulate in the fluff. Lipids of the water layer follow a rather independent pattern.

The exact mechanism of the reaction taking place is very difficult to understand because of the great variety of lipids involved and the disturbing fact that lipids which were dissolved in, say, the chloroform phase in the absence of copper could form complexes with copper that are insoluble in chloroform. In addition, some water-soluble lipids became soluble in the organic solvent in the presence of copper. Finally, proteolipids or peptide-rich gangliosides could be split into smaller fragments or micelles upon the addition of copper. The moieties formed, possessing different solubility characteristics from the original lipids and probably both containing copper, distribute themselves in different phases.

Combined evidence from copper, nitrogen and phosphorus values (Fig. 6) suggests the following:

1) Because addition of copper causes the nitrogen and phosphorus to decrease in both chloroform and aqueous layer and to increase at the interface, it seems that nitrogenous or phosphorus-containing lipids, form insoluble complexes with copper and precipitate in the interfacial fluff, thus increasing the proportion of metal there.

2) Chloroform layer contains and loses more nitrogen than phosphorus (in μatoms). Owing to the ratio μA N/μA P = 1 for phospholipids, the total precipitating nitrogen can be attributed to contaminating nitrogenous lipids or to lipids with a ratio greater than unity besides phospholipids. These are chloroform-soluble proteolipids, sphingomyelin or cerebrosides. The experiments with refined lipids revealed a partial incorporation of these compounds in the fluff upon the addition of copper. Proteolipids were not studied.

3) Aqueous layer also contains and loses more nitrogen than phosphorus. Water-soluble lipids such as free and peptide-rich gangliosides could complex with copper and account for this loss.

4) The presence of copper in both chloroform and aqueous layers underlines the fact that precipitation of copper complexes in the fluff is incomplete, and depends on the solubility of the complex (copper was found in the chloroform and fluffy phase of dialyzed solutions of synthetic lecithin; Table VIII).

5) There is no doubt that at least part of the copper in fluff originates from both chloroform and water-soluble lipids. The experiments with refined lipids showed that all compounds studied precipitated in part as copper-complexes in the interface. It is

also certain that the contribution of phospholipids, sphingomyelin and peptide-free gangliosides in this phase is very small. It should be noted that in the model experiments with refined lipids, 25 mg of each lipid were used. On the other hand, the dry weight of the lipid extracts of brain used to obtain the data of Fig. 6 was 136.5 mg. This weight accounts for total solids, to which phospholipids, according to Table XIV, contribute 70 - 87 mg. The evidence for a minor contribution of the lipids mentioned above, is as follows: (a) Synthetic lecithin, sphingomyelin and synthetic cephalin (preliminary experiments), present a low binding ability for copper. Gangliosides with higher binding potential contribute only a small amount of the lipid-copper complex to the fluff (Table VII). (b) Washing of the lipid-extract prior to treatment with copper, results in a dramatic decrease of the metal in fluff, corresponding to more than 50% of the total bound in all phases. In experiments to test the hypothesis that elimination of gangliosides during the washing procedure is responsible for this decrease (Table XII), it was found, unexpectedly, that restoration of these lipids to the solution does not result in the initial high values of copper in fluff. However, because no other lipid is thought to be lost during washing, the following suggestions are made to explain the observations: i) When copper is added to the non-washed lipid-extract a green precipitate is obtained with a nitrogen and copper contents of 7 - 8% and 12%, respectively. The precipitate gives the biuret reaction. An assessment of all its physical and chemical properties strongly suggests it to be a proteinaceous material. No precipitate is obtained from a washed-extract where gangliosides are absent. Thus, it can be assumed that the addition of copper results

in a splitting of the protein-rich gangliosides. Both moieties, protein and lipid, react with copper, the first one giving the precipitate, which accumulates in the fluff and increases enormously the copper content of this layer. ii) The process of restoration of gangliosides results in a splitting off of the protein moiety which becomes insoluble and therefore only the soluble lipid-moiety is restored to the solution.

Besides protein-rich gangliosides, proteolipids could also account for the presence of copper in fluff. Excessive copper could produce splitting of these lipids, resulting in the formation of green precipitate. It is difficult to explain on this basis, the failure of washed lipids to give increased copper concentration in the interfacial layer, unless one assumes that the removal of salts and, in general, the disturbance of the electrolyte balance results in the alteration of affinity of the proteolipid for copper. Such an alteration could occur by mutual interaction of the charged groups that become accessible to one another when ions are lost in washing.

To summarize the evidence presented for the participation of several groups of lipids in the formation of copper complexes, nitrogenous lipids (protein-rich gangliosides, proteolipids and free gangliosides) seem to contribute most of the binding ability. Phospholipids account for most of the chloroform layer and free gangliosides for the copper of the aqueous phase.

The interaction of lipids with copper is profoundly upset at low pH and in the presence of certain metals. Thus, manganese, inhibits the incorporation of copper in fluff, and iron decreases the concentration of the metal in chloroform layer. On the other hand,

molybdenum increases the copper content of all phases. It is obvious that manganese and iron antagonize copper, although each does this for a different group of lipids. The significance of this in relation to the transport of copper or to the effects of molybdenum and manganese on the deposition of copper in tissues, is unknown.

It is the author's view that the copper deficiency and loading experiments and the study of the interaction of lipids with copper are intimately related to copper transport. However, the evidence presented in this thesis, being only a reconnaissance of the whole subject, demonstrates that some further efforts are needed in the realm of the molecular biology of copper. Experiments that may be suggested are the continuation of those already presented:

1) Experiments on copper depletion or loading

a) Copper deficiency

i. Action of various drugs in accelerating the loss of copper from tissues. Besides the pharmacological value of this in connection with Wilson's disease, it is hoped that a more severe deficiency would result too.

ii. Detection of new enzymatic systems requiring copper. The focus of attention should be enzymes related to the metabolism of lipids.

b) Copper loading

i. Action of various substances in accelerating copper accumulation in tissues. Special attention should be given to an increased ratio of copper deposition in brain. To overcome the blood-brain barrier two measures are proposed: facilitation of the passage of copper by complexing the metal to an easily transported compound. Prior to this, experiments <u>in vitro</u> with monomolecular lipid layers would be of great

importance. Thus, the diffusion of copper through such a layer separating models of extracellular and intracellular fluids can be studied by using free ions and complexed to a series of substances. The second way is concerned with efforts to induce the permeability of the blood-brain barrier by altering its structure and especially by disturbing the lipid pattern of the cell membranes.

b) Study of the copper deposition on the subcellular level and the effect of the metal accumulated on various enzymatic functions.

2) Experiments on the interaction of lipids with copper

a) Identification of lipids which bind copper in vivo.

Owing to the extremely small amount of copper, thermal neutron irradiation of the lipid extract is necessary prior to separation of lipids by chromatography. By using lipid extracts obtained from copper-loaded animals, one can detect any difference in the distribution of the metal in conditions of positive copper balance.

b) Chemical methods or column chromatography to isolate lipid-copper complexes formed in vitro, for a comparative study of such complexes with those formed in vivo.

c) Studies on copper deposition in vivo and in vitro using various animal species known to differ qualitatively in the lipid structure of cell membranes.

SUMMARY

1.) Analysis of the lipids extracted from rat brain, heart, liver and kidney, revealed the presence of trace amounts of copper. Kidney and liver had the highest content of this metal and heart the lowest. For the tissues studied, the lipid-associated copper was 1.7 to 17.2% of the total tissue copper.

2.) Copper deficiency experiments carried out in rats resulted in the depletion of tissues of copper to a varying extent, depending on the duration of deprivation, the function and other characteristics of each tissue. Liver and blood (plasma) were depleted faster than any of the tissues studied, and heart and kidney less rapidly. Skeletal muscle lost more copper than any other tissue on a percentage basis; brain was depleted only to small degree. Ceruloplasmin activity in plasma and hemoglobin concentration in whole blood were also decreased.

Clinically, the deficient animals showed slower growth and alopecia.

3.) A large number of tissues was studied in the copper-loading experiment. As in copper deficiency, liver and kidney again were much affected. Very high concentrations of the metal were measured in these two tissues, but variations were observed among weanling, adult or aged animals and between cuprous and cupric copper injected. Other tissues analyzed showed a moderate increase or none at all in the metal, in the following order: spleen, hair, blood (erythrocytes and plasma). Heart and brain, exhibited a high resistance for copper deposition, but at 134 days of loading, a substantial increase of the copper content was observed. The metal concentration in lungs and testes was elevated

in some of the animals, although skeletal muscle remained unaffected. As in deficiency, the functional characteristics of each tissue were among the controlling factors for copper deposition.

The level of plasma ceruloplasmin was increased in the rats injected with either form of copper and low hemoglobin values were recorded in some of the animals. Excessive deposition of the metal resulted in a slower rate of the body weight increase of weanling and adult rats, compared to that of controls, and to loss of weight in aged animals. The copper content in the lipids extracted from the liver and kidney of these animals was markedly increased, thus suggesting the participation of the structural lipids in the transport of the metal.

4.) The interaction of copper with lipids in vitro was studied using synthetic and refined lipids as well as lipids extracted from the rat tissues. With the exception of neutral lipids, all others tested were found to bind the cation. Synthetic phospholipids had a lower binding ability, compared to that of refined, although gangliosides and technical grade or recrystallized cerebrin had the highest. The distribution of the lipid-Cu complexes upon dialysis of the solution against water saturated with chloroform, favoured the chloroform and fluffy layers. However, in the case of gangliosides, most of the copper was found in the aqueous phase.

5.) Addition of copper in lipid-extracts from brain, heart liver and kidney, resulted to the formation of lipid-Cu complexes which upon dialysis were distributed in the chloroform and fluffy layers and in the aqueous layer only in the case of brain. Brain lipids attracted

four times as much copper as lipids from other tissues did. The study of the distribution of nitrogen and phosphorus in the three phases before and after addition of copper, suggested that most of the metal found in the fluffy interface was bound to peptides or protein that are split from proteolipids or peptide-rich gangliosides upon the addition of the metal; fluff also contained smaller amounts of phospholipid-Cu complexes. The latter were thought to be mainly responsible for the presence of the metal in the chloroform layer. Finally, the copper found in the aqueous phase was attributed to gangliosides-Cu complexes.

The interaction of copper with lipids was found to be affected by a number of factors. Among them (a) low pH, (b) the presence of iron or manganese ions, and (c) washing of lipids prior to the addition of copper resulted in diminishing the quantity of metal bound to lipids.

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