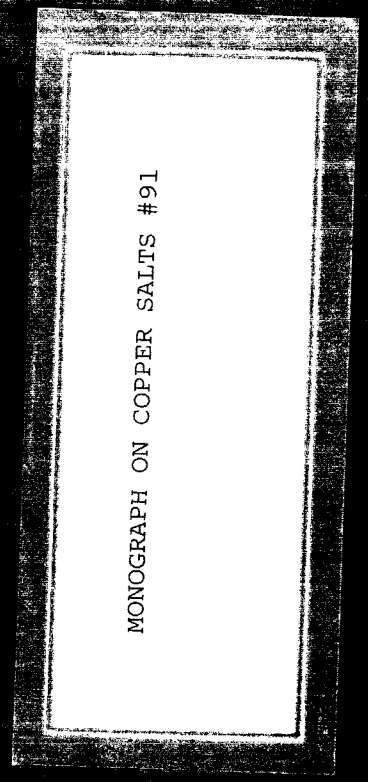
Monograph on **Copper Salts** #91 8/8/74



M O N O G R A P H

O N

C O P P E R S A L T S

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COPPER SALTS

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COPPER SALTS

Summary

A bulletin issued by the National Clearinghouse for Poison Control Centers in March-April, 1970, (269) authored by Richard Hall is extracted here making an excellent introduction to this monograph.

Copper poisoning from copper pots and pans has long been feared by housewives, not entirely without cause. Nearly two centuries ago, an article appearing in the Medical Transactions of the College of Physicians in London described several cases of copper poisoning, including that of a young woman who ingested 3 to 4 ounces of pickles heavily impregnated with copper after which she "slaked her thirst with vinegar" and died following ten days of incessant vomiting. Some interesting cases which have been reported during recent years are presented below.

1. Nine cases of copper intoxication with vomiting and diarrhea were caused by ingestion of apples boiled in a copper vessel at a school lunch kitchen.

2. An outbreak of nausea and vomiting occurred at a military base in the United States. It was caused by the use of a communion decanter whose worn silver plating left the copper base exposed. After a one hour contact with the worn vessel, grape juice used in the church service was found to have a pH of 3.1 and a copper content of 830-840 p.p.m.

3. Ten of fifteen persons attending a party exhibited weakness, abdominal cramps, dizziness, and headache after drinking a cocktail containing lemon juice, rye whiskey, sugar, and water. The cocktail had been chilled for two hours before serving in cocktail shakers whose inner plating appeared worn. The amounts ingested varied from one-half to three glasses which, on the basis of a subsequent test batch, represented ingestion of 5 to 32 mg of copper per person.

4. Fifteen of thirty-seven persons at a boat club luncheon exhibited nausea, vomiting, cramps, or diarrhea after drinking a punch containing carbonated soft drinks, club soda, vodka, and slices of lime and orange. The punch was being served from a three tiered fountain punch bowl made of chrome plated copper. The chrome of this fountain was eroded away in both the top and bottom tiers, and the punch was found to contain 50 p.p.m. of copper.

5. Several instances of nausea and vomiting have been reported from ingestion of carbonated beverages from post-mix vending machines. When the check valves on these machines become

defective, they allow carbon dioxide gas under pressure to leak into the water supply lines. If the water lines are copper, rather than plastic or stainless steel, the carbonated water dissolves some of the copper. Water from such machines has been found to contain 35 to 260 p.p.m. of copper.

6. In England, copper hot-water boilers (geysers) have been employed to heat water for tea. In one instance, twenty workmen exhibited nausea, vomiting, and diarrhea after drinking their morning tea. It was prepared with water which had been standing in a gas-heated geyser for 48 hours. This geyser had a blue-grey deposit of copper sulfate encrusted on the lid and float control arm. Water from it showed a brownish tinge due to suspended particles of matter which were found to be copper sulfate. The water was very slightly acidic (pH 6.7) and contained a total copper content in the suspension of 44 p.p.m.

Copper has been extensively mined throughout the world and used by mankind for thousands of years. Many of the copper mines of Europe have been worked since before the Roman Empire. Workings of copper in the oldest known copper mine associated with the smelting of this metal have been traced back to 4300 B.C. and archeological research indicates that the use of copper probably began about 5000 B.C. Moses who lived about 1200 B.C. recommended that copper vessels should be kept absolutely clean. Herodotus in the fifth century B.C. in writing of his travels in Egypt noted that the Egyptians "drink from cups of bronze and rinse them out every day, and not some only do this, but all." Thus copper containers for food and drink have been used since long before Christ, and are still in use today. Copper kettles are used in the canning industry and in the processing of evaporated milk. In many European countries, copper cookware is still commonly used by many housewives.

There appears to be no unanimity of opinion regarding the acute and chronic toxicity of copper and its salts. It is apparent that the acute oral toxicity will be more dependent upon the amount of copper or its soluble salts retained in the stomach than on the amounts actually ingested. Because of their irritant effects, even small amounts of soluble copper salts such as copper sulfate will cause vomiting in humans, and are indeed administered therapeutically for this purpose.

Copper sulfate intoxication causes a metallic taste in the mouth, a burning sensation in the epigastrium, eructations and repeated vomiting of greenish material. In severe cases, bloody diarrhea, shock, smoky urine, oliguria, anuria, uremia, enlarged tender liver, jaundice, coma, and death may occur.

In India, copper sulfate is a cheap, readily available substance which is quite commonly employed as a suicidal poison among people in the lower income group. A study of 53 adult patients

suffering from acute copper sulfate poisoning was made in New Delhi, India in 1964. Most of the patients were 16 to 25 years of age, and 12 of these 53 cases of copper intoxication were fatal. Statements obtained from the patients indicated that the amounts ingested varied from 1 to over 100 grams. Of the twelve fatal cases, seven died apparently either from shock and hypotension during the first 24 hours or from subsequent hepatic or renal damage with coma and uremia.

In 1896, a medical journal reported that human volunteers had consumed 20 to 30 mg of copper daily in copper preserves without any effect. Hoffman, in a Textbook of Inorganic Chemistry published in 1918, states that an adult can tolerate daily ingestion of 100 mg of metallic copper without harm. A dose of 250 to 500 mg of copper is stated to be the largest dose which can be taken unnoticed in food. Copper sulfate has been used in oral doses of 30 to 100 mg as an astringent, and 250 to 500 mg in a 1% solution as an emetic, although smaller amounts may also produce vomiting.

Chronic toxicity studies in animals showed that rats receiving ad libitum 500 p.p.m. of copper in their diet exhibited retarded growth, while 4000 p.p.m. caused starvation and death. Copper poisoning in animals leads to severe degenerative changes of the liver, kidneys, and spleen. Sheep having access to salt licks containing 5 to 9% (50,000 to 90,000 p.p.m.) of $CuSO_4$ over a 25 to 86 day period, and each ingesting an amount estimated to be not more than 50 grams, showed sudden onset of anorexia, hemolytic anemia, icterus, and hemoblobinuria followed by death in a day or two. Livers showed concentrations as high as 1.47 mg Cu per gram compared to a normal maximum of 0.04 mg/gm. Coppersmiths have reportedly absorbed so much copper that they exhibit green stains of the teeth, green tint of the hair, beard, and complexion, and "their bones in the churchyard are green". However, their morbidity and mortality was no greater than that of other industrial workers.

Although any water will take up traces of copper if allowed to stand long enough in contact with it, significant amounts of copper are not introduced into water from copper tubing until the pH falls below 5.6. At pH values of 5.0 and below, the amount of copper dissolved is increased. In the presence of CO_2, even higher concentrations of copper are dissolved into the acidic carbonated solution than are found with acid buffer solutions alone. With but few exceptions, the drinking water in the United States has pH values well above 5.0, with the usual limits for untreated natural waters being 5.5 to 8.5. In those few instances where it is lower, the acidity is likely to be caused by dissolved tannic acid from heavily forested areas. Tannic acid will form insoluble salts with copper, iron, and other metals.

Cases of poisoning have been reported from ingestion of certain foods and beverages which were processed or stored in contact with copper. Distilled liquors have been contaminated with copper dissolved from the copper worm piping of the condenser by the action of volatile organic acids such as acetic and citric. Acid foods including jellies, jams, and candies have been contaminated by being cooked in copper vessels. Plum jam, for example, is often cooked in copper vessels without causing any problems, but when the product is left too long in the vessels considerable quantities of copper compounds can be formed with the organic acids in the presence of atmospheric oxygen.

The French were reportedly the first to place on the market preserved food which was "greened" by addition of copper. This greening (coppering, verdissage, Fr.) of canned foods such as peas, beans, cucumbers, etc., also became popular in Austria, Germany, Italy, Switzerland, and England.

Canned fresh peas which contained 150 to 200 mg of copper per kg of food (150-200 p.p.m.) were reported to be perfectly edible from a standpoint of taste and absence of toxic symptoms. The amount of copper necessary to produce the desired amount of color is about 50 mg Cu/Kg of food (125 mg copper sulfate/Kg). The copper forms a firm compound with the chlorophyl of green vegetables which preserves their color. No effects were observed from prolonged consumption of greened foods corresponding to a daily intake of 12 to 15 mg of copper.

The significant statement appears in the last paragraph: "However, at the present time, no copper salts are approved for use as food coloring additives under the provisions of the U. S. Food, Drug, and Cosmetic Act".

The Washington Post on Sunday, June 2, 1974 published the following article by Jean Mayer (443):

Q. Some time ago I read an article that said copper kitchen utensils should not be used because they were hazardous. Since I regularly use a tea kettle with a copper bottom, I'm particularly concerned to know whether this is true.

A. During the 18th century, copper cooking utensils were widely used in some countries and copper poisoning was common. In fact, because of the high incidence of copper poisoning, the Swedish Senate, in 1753, prohibited their fleets and armies to cook with utensils made from anything but iron.

Today, however, few copper pots are used, and most of the ones that are used are lined with tin. So the food never comes in contact with copper anyway.

While we recognize that very excessive intakes of copper over long periods of time can be dangerous, little is known about whatever minimum levels of dietary copper can induce copper toxicity in normal individuals. Nor do we understand the influence of other dietary factors on the development of copper poisoning.

Copper toxicity is quite rare. It is only seen among people exposed to extreme industrial contamination, or among individuals with a rare inherited disorder of copper metabolsm called Wilson's disease.

There have been, however, extensive studies of copper toxicity in animals. Since it is believed that man handles copper in much the same way as other one-stomach species, it is estimated that man can tolerate 20 times his normal intake over a prolonged period before toxicity develops. In other words, there is a wide margin of safety between your normal intake from food, water, and your tea kettle and any amounts likely to produce toxic symptoms.

Two reports of particular interest have been included here, Davies (156), Chapter III, Copper - from his text "The Clinical Significance of the Essential Biological Metals", 1972, and Seelig (587), Review: Relationship of Copper and Molybdenum to Iron Metabolism", 1972. The latter report is devoted principally to molybdenum, but elucidates the role of copper in human metabolism. Both reports should be read.

For those readers who are not "Science Fiction" fans, the following was taken from a rather disturbing novel, "The Sheep Look Up" by John Brunner; the story describes a world of the future, polluted and ugly, and the author (British), curiously includes copper sulfate among the pollutants:

370 The Sheep Look Up

More smoke.
Local fallout.
Products accidentally vented from underground nuclear tests.

Oceanic fluorine.
Nitric acid.
Sulphuric acid.

Sewage.
Industrial effluents.
Detergents.

Selenium and cadmium from mine tailings.
Fumes from garbage incinerators burning plastic.
Nitrates, phosphates, fungicidal mercuric compounds from "compacted soils."

Oil.
Oil-derived insecticides.
Defoliants and herbicides.
Radioactives from aquifers contaminated by underground explosions, chiefly tritium.

Lead, arsenic, oil-well sludge, fly ash, asbestos.
Polyethylene, polystyrene, polyurethane, glass, cans.
Nylon, dacron, rayon, terylene, stylene, orlon, other artificial fibers.

Scrap.
Garbage.
Concrete and cement.
A great deal of short-wave radiation.
Carcinogens, teratogens and mutagens.
Synergistic poisons.
Hormones, antibiotics, additives, medicaments.
Drugs.

Solanine, oxalic acid, caffeine, cyanide, myristicin, pressor amines, copper sulphate, dihydrochalcones, naringin, ergot.
Botulinus.

Mustard gas, chlorine, Lewisite, phosgene, prussic acid.
T, Q, GA, GB, GD, GE, GF, VE, VX, CA, CN, CS, DM, PL, BW, BZ.

CO.
—to name but a few.

Various reports from India give that country a peculiar status with regard to a widespread use of copper sulfate in suicide, probably because of the easy availability and cheapness of the material. The section of this monograph devoted to "Acute and Chronic Toxicity" relates this aspect.

Chakraverty (117) made a statistical study of 729 cases of poisoning in India in the years 1957-58; about 20% of the cases were due to copper sulfate used as a suicidal agent. The mental and social conditions of the victims was found to be half-literate working class families; hemolysis was associated with the excessive doses, and moderate liver damage and toxic nephritis were also observed.

Chowdhury et al. (128) reported several years earlier (1961) on cases involving the ingestion of 0.5 to 4.5 ounces of copper sulfate. Transfusions and BAL (British Anti-Lewisite) were successful forms of treatment, and no fatalities were recorded.

Chuttani et al. (131) reported that a third (33.6%) of all poisoning cases (238) admitted to a New Delhi hospital in 1961 were due to acute copper sulfate poisoning. The amounts ingested were not known, but probably ranged from 1 g to 4 ounces. Some deaths resulted possibly due to shock and in later deaths, hepatic and renal complications played a role. Serum copper levels were much higher in these patients than in normal subjects.

Mital et al. (453) in a more recent (1966) report, supplements the earlier descriptions of copper sulfate poisoning with estimates of the amounts of copper sulfate ingested and the more important clinical complications encountered.

Singh and Singh (606) reported the biochemical changes occurring during acute copper sulfate poisoning. The main brunt of copper toxicity appears that borne by the erythrocytes, followed by liver and kidney reactions.

Papadoyanakis (507) described a case of suicide in Belfast, Ireland by copper sulfate. A 36-year-old man ingested 250 g copper sulfate, and survived 11 days of intensive hospital care. Death was probably caused by intestinal perforations and peritonitis. Within a day following hospitalization, jaundice appeared probably due to intense hemolysis and hepatocellular damage. Ashraf (21) in 1970 described 118 cases of acute

copper sulfate poisoning in Lucknow, India, with similar biochemical hepatic derangements, including jaundice and evidence of hepatocellular dysfunction.

Hypercupremia due to extended hemodialysis was reported by Klein et al. (377). The use of copper tubing in the dialysis equipment was apparently responsible for several cases of copper intoxication; chronic and acute orchitis were seen, as were myalagia, abdominal pain, diarrhea, acidosis, pancreatitis, methemoglobin formation and hemolysis - all signs of acute copper intoxication.

Copper poisoning due to water preparation in copper heaters for tea was described in an English report by Semple et al. (588). Severe gastroenteritis among workers in a Liverpool, England factory was traced to a boiler encrusted with copper sulfate. Other similar cases were noted.

An unusual case of copper poisoning due to copper plumbing was reported by Salmon and Wright (563). A 15-month-old infant hospitalized, showed signs of acrodynia (pink disease), suffering from prostation, misery, red extremities, hypotomia, photophobia and peripheral edema. Elevated serum copper levels and abnormal transferase levels indicated copper poisoning. An all-copper hot water system, in a new home during 3 months previous to the infant's hospitalization, had produced a chronic poisoning in the infant. The case is probably unique.

The use of copper sulfate in the treatment of various skin diseases is not uncommon. Small doses, 2 mg/day of copper sulfate in the treatment of leucoderma, led to a case of thrombocytopenia, reported by Pande and Gupta (506). An acquired sensitivity to copper sulfate was suggested as the reason for the reaction to copper sulfate, but the patient's refusal of further testing left the subject, moot.

Fairbanks (192) reported a rare case of hemolytic anemia in a 22-year-old woman who accidentally ingested an unknown quantity of a saturated copper sulfate solution. Treatment with BAL and other measures alleviated the symptoms of anemia. The author presents studies that have shown that the action of copper sulfate in the production of hemolysis is due to: inhibiting glucose-6-phosphate dehydrogenase competitively; penetrating the intact erythrocyte and inhibiting glycolysis; accelerating oxidation of nicotinamide-adenine dinicleotide phosphate; denaturing hemoglobin; and oxidizing glutathione.

The use of copper sulfate as an emetic has been recently challenged by several investigators. The recommendations of Karlsson and Noren (347), demonstrating the emetic properties of copper sulfate, have been questioned by Holtzman and Haslam (300), who suggested that hazards associated with copper sulfate render its use as an emetic questionable, and Decker et al. (159) showed in studies with rats, that copper sulfate which further poisons the liver and kidneys in treatment of many ingested poisons, could be extremely hazardous when used as an emetic in poison ingestion."

Nutritional studies showing the beneficial results of adding copper sulfate to the diet of pigs are represented by Brande and Ryder (84), and Thompson et al. (648). Growth of the animals is improved, and the unsaturated fats are increased by copper sulfate added to the diet up to 250 mg Cu/kg diet.

Elliot and Bowland (184) reported on efforts to use rat-feeding studies for information to improved diets in pigs, using copper sulfate supplements. The response of the rats to dietary copper sulfate was so different from that of pigs that the authors concluded that "the rat cannot serve as a suitable pilot species in which to study the effects of high levels of supplemental copper on fat metabolism in the pig".

Copper intrauterine devices for contraception are in very widespread use; Barranco (46) in 1972 reports a case of a 26-year-old woman who after insertion of such a device, showed pruritic dermatitis symptoms which were cleared-up only after removal of the device. Patch tests demonstrated a positive reaction to a 5% copper sulfate solution.

Metabolic disorders related to diseases involving copper have been described by Davies (156) in his Chapter (111) on Copper in a test dealing with the clinical significance of trace elements. Other studies included for their general interest but not abstracted in this monograph include: epilepsy, Brania, 1972 (92); leprosy, Khaire and Magor, 1972 (359); Wilson's disease, Sternlieb and Scheinberg, 1972, (621); "torsion dystomy", Herishamu and Loewinger, 1972, (288); Menke's kinky hair syndrome, Danks et al., 1972, (152); and Hodgkin's disease, Hrgovcic et al., 1973 (304).

The distribution of copper in rat tissues was reported in several papers by Lal and Sourkes (409; 410). Rats, injected intraperitoneally with up to 3.75 mg Cu/kg body weight as $CuSO_4$ in 0.9% NaCl solution, daily showed depressed growth and significant decreases in liver weight. Liver concentrations of copper were striking at the higher doses of

copper; kidney copper concentrations were elevated only by the higher copper doses. Liver copper concentrations were highest in mitochondrial fractions; increases were related to duration and dose of the copper sulfate.

Terai and Owen (643) in a recent study (1973) by the use of radioactive Cu^{67} as $CuCl_2$ injected intravenously in rats, reported that three fractional proteins were recovered from liver homogenate supernates: a molecular weight of about 150,000 corresponding to the reported values for ceruroplasmin; and lower molecular weights of about 31,000 and 11,000. Bile components showed two chromatographic components having molecular weights of greater than 800,000 and about 5,000.

Normal and Wilson's disease saliva and gastric juice were compared by Gollan et al. (242) with respect to the copper content. No significant difference was found in the values determined for normal and for Wilson's disease patients.

The role of dietary copper in hemoglobin metabolism was investigated by Evans and Abraham (190). Hooded Norway male rats were given copper-depletion diets followed by a copper-supplemented diet of copper, as $CuCO_3$. Hemoglobin, hematocrit, plasma ceruloplasmin oxidase activity, liver iron and copper were determined. The changes in liver iron concentrations and ceruloplasmin oxidase activity during copper depletion and repletion suggested that copper acts in hemoglobin metabolism to mobilize absorbed iron at the site of hemoglobin synthesis.

Thompson et al. (648) reported that a diet of 250 ppm copper (sulfate) given to pigs resulted in a significantly greater oleic/stearic acid ratio of outer subcutaneous fat and perirenal fat than in animals not given supplemental copper. Liver activity in supplemented animals was greatly increased.

COPPER SULFATE

Chemical Information

I. Nomenclature

A. Common Names

1. Copper sulfate
2. Copper sulphate
3. Bluestone (pentahydrate)
4. Blue vitriol (pentahydrate)
5. Roman vitriol (pentahydrate)
6. Salzburg vitriol (pentahydrate)
7. Dried cupric sulfate (monohydrate)

B. Chemical Names

1. Cupric sulfate
2. Copper sulfate
3. Copper (II) sulfate
4. Hydrocyanite and chalcanthite (minerals)

C. Trade Names

Following contain copper sulfate in addition to other substances: Ferrobid (Meyer) and Hemo-Vite (Drug Industries)

D. Chemical Abstracts Registry Number

007758-98-7 (cupric sulfate, anhydrous)

II. Empirical Formula

$CuSO_4$ (anhydrous), also monohydrate form and pentahydrate, $CuSO_4 \cdot 5H_2O$

III. Structural Formula

Not Applicable

IV. Molecular Weight

159.61 (anhydrous form)
249.68 (pentahydrate)

V. Specifications (Anhydrous)

From NAS/NRC Questionnaire: Pharmaceutical Grade

Appearance	White or grayish white powder
Identification	Meets NF tests for copper and sulfate
Assay	98-103% (USP)
Solubility	Meets NF requirements
Alkalies and alkaline earths	Meets NF test
Screens 100 mesh USS	95% thru minimum

Product shall be free from any off odors or flavors, impurities, or foreign matter of any kind. All process involved in the manufacture of this material shall be in accordance with good commercial practices and under strict sanitary conditions. The product shall meet all the requirements of the United States Pharmacopoeia.

VI. Description

A. General Characteristics

Grayish-white to greenish-white rhombic crystals or amorphous powder; nauseous, metallic taste. Pentahydrate, large, blue or ultramarine triclinic crystals, or blue granules, or light-blue powder.

B. Physical Properties

Anhydrous form highly hygroscopic; pentahydrate slightly efflorescent in air. Both soluble H_2O, practically insoluble in ethanol. The pentahydrate becomes anhydrous at 250 degrees; anhydrous form decomposes above 560 degrees.

C. Stability

Anhydrous form should be stored in tightly closed containers.

VII. Analytical Methods

From Food Chemicals Codex - Identification, General Tests; test for copper and for sulfate:

> Copper. When solutions of cupric compounds are acidified with hydrochloric acid, a red film of metallic copper is deposited upon a bright untarnished surface of metallic iron. An excess of ammonia T.S., added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-colored solution. Solutions of cupric

salts yield with potassium ferrocyanide T.S. a reddish brown precipitate, insoluble in diluted acids.

Sulfate. Solutions of sulfates yield with barium chloride T.S. a white precipitate which is insoluble in hydrochloric and nitric acids. Sulfates yield with lead acetate T.S. a white precipitate which is soluble in ammonium acetate solution. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

From AOAC (723), see analysis of copper, Sections 24.023 through 24.028.

Nadkarni and Haldar (474) reported in 1972 a "Neutron Activation Analysis of Copper by Substoichiometric Extraction With Neocuproine" which can be used "for the determination of trace amounts of copper in geological or biological materials with good degree of precision and in a very short time".

A neutron activation analytical method for the determination of manganese, copper and zinc in human blood serum and packed blood cells was described by Versieck et al. (675) in 1973. The results obtained by this method are reported for serum and blood cell values from 20 hospital patients tested. Kurz et al. (402) in 1972 described an atomic absorption spectrophotometric technique for determination of serum zinc and copper. Stone and Townshend (626) in the same year reported on the determination of nanogram amounts of copper by activation of insolubilized apo-polyphenol oxidase.

VIII. Occurrence

Occurs in nature as anhydrous form (hydrocyanite) and as mono- and pentahydrate (chalcanthite).

COPPER GLUCONATE

Chemical Information

I. Nomenclature

A. Common Name

Copper gluconate

B. Chemical Name

Cupric gluconate (Preferred name)

C. Trade Names

None found

D. Chemical Abstracts Registry Number

000527-09-3

II. Empirical Formula

$C_{12}H_{22}CuO_{14}$

III. Structural Formula

$Cu(CH_2OH(CHOH)_4CO_2)_2$

IV. Molecular Weight

453.85

V. Specifications

From Food Chemicals Codex:

Assay	Not less than 98.0% and not more than equivalent of 102.% $C_{12}H_{22}CuO_{14}$
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Lead	Not more than 10 ppm (0.001%)
Reducing substances	Not more than 1%

VI. Description

A. General Characteristics

As the hydrate (one molecule H_2O), light blue to bluish-green odorless crystals or crystalline powder; astringent taste.

B. Physical Properties

Very soluble H_2O (30 g/100 ml at 25 degrees); slightly soluble in alcohol and practically insoluble in most other organic solvents.

C. Stability

Should be stored in well-closed containers.

VII. Analytical Methods

From Food Chemicals Codex:

IDENTIFICATION

A. A 1 in 20 solution gives positive tests for Copper, page 926.

B. To 5 ml of a warm solution (1 in 10) add 0.7 ml of glacial acetic acid and 1 ml of freshly distilled phenylhydrazine, heat on a steam bath for 30 minutes, and allow to cool. Induce crystallization by scratching the inner surface of the container with a glass stirring rod. Crystals of gluconic acid phenylhydrazide form.

VIII. Occurrence

Prepared from reaction of gluconic acid ($C_6H_{12}O_7$) and basic cupric carbonate ($CuCO_3 \cdot Cu(OH)_2$.

Biological Data

I. Acute and Chronic Toxicity

All acute toxicity studies found were related to accidental or suicidal ingestion of copper sulfate: tabular materials are included in the document abstracts in this section.

Man

A review of the documents reporting acute poisoning by copper sulfate in humans reveals that India has for several decades been seriously concerned with the problem of both accidental and suicidal ingestion of copper sulfate, particularly among the poorer peoples of a number of Indian states.

Chakraverty (117) in 1963 performed a statistical study of 729 cases of poisoning admitted in Medical College Hospitals of Lucknow, Agra and Kanpur and Balrampur Hospital, Lucknow during the 2 calendar years of 1957 and 1958. Of this total number of cases, 134 or nearly 20% were due to copper sulfate. The types of poisoning reported were distributed as shown in the following table:

TABLE I

Showing the various types of poisoning in 729 cases

Types of poisoning	Total No.	Percentage	Deaths	Percentage
Copper sulphate	134	19·3%	16	12%
Alcohol	119	16·3%	1	·83%
Opium	96	14·1%	13	13·6%
Dhatura	86	12·64%	7	8·3%
Kerosene oil	70	9·6%	5	7·1%
Acid	31	4·2%	6	19·3%
Food poisoning	30	4·2%	3	10%
Bhang	16	2·2%	Nil	0%
Rat-killing poison	12	1·64%	,,	0%
Barbiturates	11	1·2%	1	9·39%
Lint. Camphor	7	·96%	1	14·3%
Arsenic	6	·82%	2	33%
Tobacco	4	·56%	Nil	0%
Pot. permanganate	4	·56%	,,	0%
Glass powder	5	·69%	,,	0%
D.D.T.	4	·56%	,,	0%
Corrosives	2	·22%	,,	0%
Castor oil	1	·14%	,,	,,
Petrol	1	·14%	,,	,,
Chloroform	1	·14%	,,	,,
Jamalgota	1	·14%	,,	,,
Turpentine oil	1	·14%	,,	,,
Cyanide	1	·14%	1	100%
List A.B.C.	1	·14%	Nil	0%
Undetermined	66	9·2%	6	9·99%

It will be seen for the above tabular statement that copper sulphate poisoning topped the list with nearly 20% of the total number.

A series of tests (urine, liver function, etc.) were made, and more detailed studies of 36 patients were performed. Copper sulfate was used most often as a suicidal agent; about half the cases were Hindus, the remainder, Muslims. Mental and social condition was found to be half-literate working class families. Hemolysis was observed infrequently, associated with excessive doses; moderate liver damage was also seen, as was toxic nephritis.

Chowdhury et al. (128) had reported earlier in 1961 on 20 cases of severe copper sulfate poisoning in Calcutta. Blood, liver and urine tests were carried out, and the following tables summarize the findings:

TABLE 2—SHOWING DETAILS OF THE CASES AND INVESTIGATIONS INCLUDING SERUM COPPER LEVEL. ALL THE CASES WERE CURED

Case No.	Sex, age (yrs.)	Clinical types	Quantity of copper sulphate (approx.)	Days of ingestion	Liver function tests							Haemolysis, jaundice. Blood sugar (B.S.)	Erythrocyte fragility	Urine	Serum copper level (μg. %)	Treatment with BAL (250 mg./amp.)
					Total protein (g./100 ml.)	Albumin (g./100 ml.)	Globulin (g./100 ml.)	Thymol turbidity (units)	van den Bergh: Direct	van den Bergh: Indirect (mg.%)	Alkaline phosphatase (K-A units)					
1	F 25	Mild	Small lump	3	6·8	4·6	2·2	3	Neg.	0·2	12					
2	M 22	Moderate	Small lump	3	6·1	3·0	3·1	12	Delayed positive	1·2	16	Jaundice				
3	F 25	Severe	Paste of a lump with mustard oil	2	Serum haemolysed							Haemolysis, jaundice		Alb. RBC		BAL four hourly
4	F 47	Moderate	Small lump	2									0·54%—0	Alb. RBC		BAL six hourly
5	M 40	Moderate	2 tea-spoonfuls	4	6·4	3·1	3·3	16	Neg.	0·2	12	Jaundice, haemolysis				BAL six hourly
				3		Haemolysis								Haematuria		
				4								No jaundice				
				5	7·2	3·5	3·7	10	Immediate positive	2·6	11					
				9										,,		
				14	6·0	3·2	2·8	9	Neg.	0·05	11					
6	F 25	Moderate	1 table spoonful	2	Serum haemolysed							Haemolysis, jaundice				
7	F 35	Severe	½ oz.	1												BAL 2 amp. six hourly
				6	Serum haemolysed							Haemolysis, jaundice		Alb. RBC Portwine Sugar ++ Few RBC		
				8												
8	M 23	Moderate	20 gr. ?	12	5·8	2·9	2·9	11	Neg.	0·4	21	No jaundice				
				2									0·54%—0	Alb.		BAL four hourly
				5								Jaundice		sugar +		
				7								No jaundice				
9	M 20	Moderate	Small lump	2	5·84	3·1	2·74	10	Neg.	0·3	20					
					Serum slightly haemolysed. Tinge of copper											
10	F 14	Mild	10 gr.	3	Haemolysis—mild							Haemolysis				
				4								Haemolysis		Alb.		BAL four hourly
									Delayed positive	Icteric index—12						
11	F 19	Severe	160 gr.	2	6·14	3·25	2·89	12				Haemolysis	0·54%—0	Alb. RBC	510	BAL four hourly
				3		Haemolysis						,,		Sugar—trace		Blood six bottles of 280 c.c. each —exchange transfusion
												B.S.—118 mg./100 ml.		Port-wine urine (0·14 mcg. cu in 12 mg. dried residue from centrifuged deposit		
				6					Delayed positive	2·4						

12	F 19	Severe	Drink of copper sulph.	1 3	5·85	2·7 Serum haemolysed	3·15	16	Delayed positive	1·2	25	Haemolysis, jaundice			260 (average of three normal —130)	BAL four hourly
13	M 14	Moderate	Two lamps	2	5·94	3·5	2·44	6	Neg.	0·2	16		All samples haemolysed		132 (normal control 125)	BAL six amps.
14	M 14	Mild	Small lamp	2	6·62	3·95	2·67	4	Neg.	0·05	14				200 (normal control 130)	
15	F 20	Mild	15 gr. ?	1	6·24	3·8	2·44	6	Neg.	0·1	13	Haemolysis slight	0·45%—0		158	
16	M 20	Mild	10 gr. ?	2 3	6·56 6·24	3·4 3·3	3·16 2·94	10 10	Neg. Neg.	0·2 0·2	16 18				240 200	
17	F 30	Severe	4½ oz.	1 2	7·65	4·14	3·51	6	Neg.	0·08	14			Alb. RBC sugar +	280	BAL six hourly
				4	7·38	3·65	3·73	10	Immediate positive	1·6	15				260	
				6 Haemolysis								Haemolysis, jaundice		Sugar+		
				7								B.S.—82 mg./100 ml.		Sugar+		
				10 15	7·25	3·80	3·45	9	Neg.	0·64	14	No jaundice		Sugar—nil	150	BAL eight hourly, total 34 amps.
18	F 30	Moderate	2 dr.	1 2	6·14	3·86	2·28	6	Neg.	0·1	15			Alb. Sugar—trace	250	BAL four hourly
				3										Sugar—nil		
19	F 28	Moderate	1 dr.	1 2	5·92	3·1	2·82	8	Positive biphasic	1·3	13	B.S.—92 mg./100 ml. Jaundice—mild	0·45%—0		230	BAL six hourly Patient left hospital on risk bond apparently well
20	M 32	Mild	1 dr.	1	6·18	3·20	2·98	9	Neg.	0·2	18				190	

N.B.—Bluish stool in bed on the 2nd day of ingestion of copper sulphate in Case 17.

In many instances, the amount of copper sulfate ingested could only be approximated; the severe clinical symptoms were associated with 0.5 to 4.5 ounces. Transfusions and BAL (British Anti-Lewisite) appeared to be a successful treatment, and no fatalities occurred. The symptoms of copper sulfate poisoning are described as:

> Mild cases (6) were those who complained of a metallic taste in the mouth, slight pain and tenderness over the epigastric region and sometimes nausea. Moderate cases (9) had in addition to the above, retrosternal burning sensation, vomiting, tachycardia, low blood pressure, tender liver, scanty urine and albumin and sometimes blood. In severe cases, (5) all the above signs and symptoms were present but they were more acute. These patients complained of severe tenderness, persistent tachycardia, falling blood pressure, bluish or greenish loose stools often with blood, urine with blood, albumin and sometimes sugar. Soon jaundice developed, and some of the patients became restless and apathetic with a toxic look or became stuporose and developed muscular weakness. In this series no death was recorded.

Chuttani et al. (131) reported that 33.6% of all cases (238) of poisoning admitted to the Irwin Hospital, New Delhi, 1961, were due to acute copper sulfate poisoning. The 48 subjects actually studied ranged in age from 14 to 60 years; two thirds were male, one-third female. The amounts of copper sulfate ingested were generally unknown, but probably varied from 1 g to 4 ounces. Seven deaths occurred among the 48 patients studied; early deaths (within 24 hours of ingestion) were due to shock, whereas later deaths appeared to be due to hepatic or renal complications or both. The levels of serum total copper, serum ionic copper and whole blood copper were found to be much higher in these patients than in normal subjects. A significant correlation was noted between whole blood copper levels and the severity of toxic manifestations.

A report by Mital et al. (453) in 1966 noted that "the use of copper sulfate as a suicidal agent is suprisingly common in the lower strata of people at Agra" (India). Fifty cases of acute copper sulfate poisoning hospitalized in Agra were studied in detail. The symptoms were those already described by Chowdhury (128). Tables II and III show the amounts of copper sulfate ingested and the more important complications encountered:

TABLE III

Showing amount of copper sulphate ingested

Quantity of copper sulphate ingested	Number of Cases
Upto ½ oz.	16
½ oz to 1 oz.	18
More than 1 oz.	5
Quantity not known	11

TABLE IV

Complications encountered in acute copper sulphate poisoning

Complication	Number of cases
Haemolytic episodes	19
Clinical juandice	9
Neurological symptoms	7
Renal complications	6
Cardiovascular symptoms	5
Deaths	3

The most common complication observed was hemolysis; reticulocyte counts increased following ingestion of the sulfate and hematocrit values fell, sometimes as much as 16% within a week. Increased excretion of urinary urobilinogen and hemoglobinuria was frequent.

The following table shows the incidence of hemolytic reactions among various cases reported by other investigators as well as of those in the present study:

TABLE V

Incidence of haemolytic reactions in acute copper sulphate poisoning

Author	Year	Total No. of cases	Number of cases with haemolysis	Percent
Chowdhury et al	1961	20	10	50
Gupta et al	1962	150	33	13
Wahal et al	1963	50	10	20
Present series	1964	50	19	38

Singh and Singh (606) reported in 1968 the biochemical changes in blood occurring in acute copper sulfate poisoning, with special emphasis on the study of copper in serum and whole blood and the serum transaminases. Forty patients were included in the study. Serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were determined in all (SGOT) and in 6 cases (SGPT). The blood chemical changes are shown in the following table:

TABLE 6—SHOWING BLOOD BIOCHEMICAL CHANGES

Case No. and name	Duration after ingestion of copper sulphate in days (approx.)	Serum copper (μg. per cent)		Whole blood copper (μg. per cent)	SGOT level (units/ml.)	SGPT level (units/ml.)	Haemolysis in serum	Icterus in serum	Serum bilirubin (mg. per cent)	Blood urea (mg. per cent)	Remarks (clinical toxicity)
		Total	Direct								
1	2	3 A	3 B	4	5	6	7	8	9	10	11
1 P	3	—	—	—	—	—	—	—	0·6	38	Moderate
	4	—	—	480	84·6	—	No	Yes	—	—	
2 SU	2	—	—	—	—	—	—	—	0·4	38	Moderate
	10	—	—	265	45	—	No	No	—	—	
	14	—	—	—	—	—	—	—	0·4	—	
	16	—	—	180	—	—	No	No	—	—	
3 S	2	—	—	—	250	—	Yes	Yes	1·4	40	Severe
	4	—	—	—	180	—	Yes	Yes	—	—	
	7	—	—	303	200	—	Yes	No	—	—	
	8	—	—	—	—	—	No	No	0·2	—	
	10	—	—	180	100	—	No	No	—	—	
4 SK	2	—	—	—	95	—	—	—	—	—	Mild
	5	—	—	340	—	—	—	—	—	—	
5 JK	27/24	—	—	380	220	—	Yes	No	—	37	Moderate
	5	—	—	280	143	—	No	No	—	—	
6 MC	1	—	—	320	144	—	No	No	0·2	—	Mild
7 BP	4½	—	—	292	96	—	No	No	0·2	—	Mild
8 KL	2	—	—	210	33	—	No	No	—	30	Mild
9 BS	15/24	—	—	—	—	—	—	—	1·5	—	Severe
	39/24	—	—	436	212	—	Yes	Yes	—	—	
	5½	—	—	—	—	—	—	—	1·2	—	
10 DC	20/24	—	—	174	106	—	No	No	0·4	—	Mild
11 ML	1½	—	—	220	112	—	No	No	0·3	—	Mild
12 AK	1	—	—	—	—	—	No	No	0·6	—	Mild
	2	—	—	300	112	—	No	No	0·6	38	
13 D	17/24	—	—	274	275	—	Yes	No	—	—	Moderate
	4	—	—	—	148	—	No	No	0·4	—	
14 CL	26/24	—	—	360	120	—	No	No	0·2	—	Mild
15 DR	2	—	—	527	240	—	Yes	No	—	32	Moderate
	6	—	—	350	130	—	No	No	—	—	
16 RK	4	—	—	380	85	—	No	No	2·0	—	Mild
17 BB	11/24	269	30·4	—	146	—	No	No	0·2	33	Mild
18 SD	1	—	—	—	264	—	Yes	Yes	0·8	41	Severe
	2	266·6	—	—	288	—	Yes	Yes	—	—	
	5	277·5	—	—	180	—	No	Yes	1·2	—	
19 BL	2	105	30·9	—	220	—	No	No	—	—	Mild
20 MR	1	—	—	—	378	—	Yes	Yes	—	70	Severe
	2	—	—	—	—	—	No	Yes	2·0	—	
	5	—	—	—	143	—	No	Yes	—	—	
	6	190	95	—	220	—	No	No	0·2	—	
	8	220	0	—	110	—	No	No	—	—	
	10	173	6·6	—	44	—	No	No	—	—	
	13	248	9·5	—	36	—	No	No	—	—	
21 JK	1	99	4·5	550·2	120	—	Yes	No	0·2	—	Mild
22 NM	8/24	142	10·0	307·0	150	—	No	No	—	—	Mild
23 Rag	3	114	41·2	—	154	—	No	Yes	—	47	Moderate
	6	116	0	282	80	—	No	No	—	—	
	24	116	13·0	133	165·6	—	No	No	0·4	—	

TABLE 6—(*Contd.*)

Case No. and name	Duration after ingestion of copper sulphate in days (approx.)	Serum copper (μg. per cent)		Whole blood copper (μg. per cent)	SGOT level (units/ml.)	SGPT level (units/ml.)	Haemolysis in serum	Icterus in serum	Serum bilirubin (mg. per cent)	Blood urea (mg. per cent)	Remarks (clinical toxicity)
		Total	Direct								
1	2	3 A	3 B	4	5	6	7	8	9	10	11
24 PB	1	131	33	342	120	—	No	No	0·4	23	Mild
25 SD	21/24	127	27	647	121	—	Yes	Yes	1·75	37	Moderate
	2½	109·5	20·5	208	132	—	No	Yes	—	—	
	4	132·5	220	—	110	—	No	No	—	—	
26 SD	29/24	113·0	6·5	888	248·4	—	No	Yes	—	—	Moderate
	2½	210·5	—	—	72·0	—	No	No	—	—	
27 GD	18/24	79	0	200	77	—	No	No	—	33	Mild
28 YR	13½/24	—	—	709	88	—	No	No	—	—	Mild
	2	—	—	371	88	—	No	No	—	—	
	5	95	21	257	80	—	No	No	0·25	37	
29 RK	35/24	211	0	800	165	—	No	No	0·2	—	Mild
30 SD	7/24	242·2	61	284	132	—	Yes	No	—	—	Mild
	4	134·5	8·1	—	121	—	No	No	0·2	—	
31 VD	3½/24	444	150	449	165	—	Yes(±)	Yes(±)	—	—	Moderate
	8	165	35	169	93·15	—	No	No	0·2	—	
32 NK	5/24	413·5	298	483	150	—	No	No	—	—	Severe
	1	—	—	—	—	—	—	—	0·4	40	
	63/24	178	64	166	429	—	No	Yes	—	—	
	10	—	—	—	130	—	No	No	—	37	
33 RS	3/24	848	557	1047	154	—	No	No	—	—	Severe
	25½/24	139·9	0	176	154	—	No	Yes	—	47	
	3½/24	70·0	0	216	146	—	No	No	0·2	—	
34 CD	25/24.	199	15	3240	143	—	No	Yes	—	—	Expired (severe intravascular haemolysis)
	50/24	—	—	—	270	—	Yes	Yes	—	30	
35 JS	1	—	—	—	120	—	Yes	No	—	35	Expired (hepatorenal failure)
	1½	—	—	310	190	—	Yes	Yes	—	73	
	2	—	—	—	250	—	Yes	Yes	—	380	
	4	177	—	—	310	—	Yes	Yes	—	318	
	10	240	—	500	540	—	Yes	Yes	30	262	
	12	129·8	—	—	238	224	Yes	Yes	—	—	
36 GR	11/24	977·6	277	277·3	364	187	Yes	No	—	—	Expired (severe intravascular haemolysis)
37 MR	3	205·2	43	603·7	128	66	Yes	No	—	—	
38 BL	1	410	150	2150	220	429	Yes	Yes	—	—	Severe
	2½	—	—	309·3	400	440	Yes	Yes	—	—	
	4	131·5	—	280	280	440	No	Yes	—	—	
	6	200	43·1	113·2	308	429	No	Yes	—	—	
	10	172·3	74·2	175·3	145	364	No	Yes	—	—	
	11	57·0	0	167·2	198	428	Yes	No	1·2	—	
39 Zi.	16/24	—	—	—	88	132	No	No	—	—	Mild
	40/24	123	5	100	116	139	Yes	No	—	—	
40 KL	2	302	—	1200	264	228	Yes	No	—	—	Expired (haemolysis)

The authors suggest that "the main brunt of copper toxicity is borne by erythrocytes, the liver and kidneys, in descending sequence". It is also suggested that conventional liver function tests are, in their opinion, of very little importance, either in the diagnosis or prognosis of copper sulfate poisoning.

Papadoyanakis et al. (507) reported in 1969 a case of suicide in Belfast, Ireland by the ingestion of 250 g (more than half a pound) of copper sulfate. The 36 year-old-man survived 11 days under intensive hospital treatment. Autopsy showed extensive intestinal ulceration with one ulcer perforated; some liver and kidney cytological abnormalities were observed. Death was most likely caused by the intestinal perforation and subsequent peritonitis. Jaundice, which has frequently been associated with copper poisoning, appeared within a day following the hospitalization of this case; initially probably caused by intense hemolysis, but later attributable to hepatocellular damage. Ashraf (21) described in 1970 the results of a study of 118 cases at Lucknow, India of acute copper sulfate poisoning for biochemical hepatic derangements; 35 of these cases developed jaundice and tests indicated hepatocellular dysfunction.

Recent indications of unexpected copper intoxication due to hemodialysis - induced hypercupremia were reported by Klein et al. (377) in 1972. The use of copper heating coils in dialysis equipment was apparently responsible, and copper tubing has been generally eliminated from the equipment, though some systems still contain copper or brass parts which serve as a potential source of copper ion. Three cases of copper intoxication are described which resulted from prolonged dialysis; in addition to the usual findings in copper poisoning, the authors observed pancreatitis and myoglobinemia. In two of the patients studied, chronic and acute orchitis were observed. This was the first such observation, as far as the authors knew, of orchitis being a feature of copper intoxication. Physicians using dialysis should be aware of the symptoms, myalgia, abdominal pain, diarrhea, acidosis, pancreatitis, methemoglobin formation and hemolysis which indicate acute copper intoxication. A similar report appeared in 1971 by Blomfield et al. (73).

Semple et al. (588) described in 1960 an instance of moderate to severe gastroenteritis among workers in a Liverpool, England, factory. Investigation revealed copper poisoning as the cause; water for tea was heated in a geyser (British term for a water heater, utilizing injected steam for quick heating) which was lined with copper. Deposits of copper sulfate encrusted the boiler lid and opening for the float control arm. Presumably some copper sulfate had contaminated the water from which some of the tea drunk by the affected workers was brewed. Several other similar cases were noted. It was suggested that instructions for periodic cleaning of copper geysers accompany the equipment, together with warning of the hazards of copper sulfate contamination.

An unusual case report of copper poisoning (not copper salt) in a 15-month-old infant was given by Salmon and Wright (563). The clinical appearance of the infant upon hospitalization was that of pink disease (acrodynia) with prostration, misery, red extremities, hypotonia, photophobia and peripheral edema. Tests indicated raised serum copper levels and abnormal transferases values; treatment for copper poisoning was initiated. The infants' family had moved three months earlier (before its hospitalization) into a new house equipped with an all-copper hot water system; hot water was used for cooking and for beverages and was shown to have a high copper content. The infant's illness was presumably due to chronic ingestion of copper over the 3-month period. The authors believed this to be the first such case reported.

Pande and Gupta (506) reported in 1969 a case of thrombocytopenia apparently due to administration of small (2 mg/day) doses of copper sulfate in the treatment of leucoderma. It was presumed to have resulted from an acquired sensitivity to copper sulfate and the treatment was stopped. The evidence to substantiate the presumption was not available as the patient refused testing with copper sulfate after recovering.

A rare case of hemolytic anemia was described by Fairbanks (192). A 22-year-old woman accidentally ingested an unknown amount of saturated copper sulfate solution. Nausea and diarrhea over several days time led to her hospitalization. Treatment, primarily with BAL, for several days alleviated the symptoms of anemia. Studies have shown that copper sulfate produces hemolysis by: inhibiting glucose-6-phosphate dehydrogenase competitively; penetrating the intact erythrocyte and inhibiting glycolysis; accelerating oxidation of nicotinamide-adenine dinucleotide phosphate; denaturing hemoglobin; and oxidizing glutathione.

II. Short-Term Studies

No appropriate information found.

III. Long-Term Studies

No appropriate information found.

IV. Special Studies

Emesis

The use of copper sulfate as an emetic has been widely recommended in the immediate treatment of poisoning by ingestion, as was reported by Karlson and Noren (347) in 1965.

Although emesis is readily induced by copper sulfate, Holtzman and Haslam (300) determined serum copper and ceruloplasmin in 6 young children given 250 mg $CuSO_4$ as an emetic and compared the results with 6 children given ipecac for emesis. Emesis from $CuSO_4$ was rapid (2-5 minutes), but despite this, some copper was absorbed; two of the 6 children receiving $CuSO_4$ had marked elevation of serum ceruloplasmin. The authors suggested that in view of hazards associated with copper sulfate, its use as an emetic appears questionable.

Decker et al. (159) in 1972 observed that the high potential for systemic absorption of toxic amounts of copper sulfate, especially when emesis does not occur, raises questions regarding the safety of the process. Various studies have shown that significant concentrations of administered copper have been found in liver, kidney and plasma; copper sulfate is both hepatotoxic and nephrotoxic.

Adult male albino Water Reed strain rats, weighing 275-350 g were given by stomach tube a solution of $CuSO_4$ containing radioactive Cu^{64} with an activity of 100 microcuries. The dose was comparable to that used as an emetic in young children. At 1, 3, 6 and 24 hour intervals, the animals were sacrificed and various tissue samples taken for the determination of radioactivity by a gamma ray spectrometer. The bulk of the administered copper passed through the gastrointestinal tract and was excreted in the feces. Relatively large concentrations of copper appeared in liver and kidney; heart, lung, spleen, and plasma showed intermediate copper deposition; femur, erythrocytes, skeletal muscle and skin contained minimal amounts. The following table summarizes the findings:

TABLE 7

CONCENTRATIONS OF ADMINISTERED COPPER IN TISSUES OF RATS GIVEN PO CUPRIC SULFATE[a]

Tissue	Hours after copper administration			
	1	3	6	24
Skeletal muscle	10 ± 0.0	20 ± 0.0	10 ± 0.0	40 ± 10
Skin	30 ± 10	50 ± 20	30 ± 10	70 ± 40
Femur	0	30 ± 0.0	30 ± 20	90 ± 10
Erythrocytes[b]	10 ± 0.0	20 ± 10	30 ± 10	120 ± 10
Depot fat (pooled from 3 rats)	90	170	10	0
Heart	20 ± 0.0	50 ± 10	60 ± 10	230 ± 10
Spleen	10 ± 0.0	50 ± 10	50 ± 10	260 ± 60
Lung	170 ± 140	2400 ± 3000	60 ± 10	350 ± 170
Plasma[b]	40 ± 10	60 ± 20	100 ± 10	480 ± 110
Liver	350 ± 140	430 ± 200	370 ± 150	1100 ± 180
Kidney	410 ± 20	850 ± 160	1230 ± 200	2700 ± 900

[a] ng copper per g wet weight, mean of 3 animals ± SD.
[b] ng/ml.

Since liver and kidney are primary target organs for many poisons, it was concluded that copper sulfate, which tends to further poison these organs, could be "extremely hazardous when used as an emetic in poison ingestion".

Nutrition

The use of copper sulfate as a feed additive for the promotion of growth in pigs is a generally accepted practice; some disagreement regarding the level of such supplementation, however, was noted by Brande and Ryder (84) in a report on feeding studies using form levels of copper sulfate given to pigs. The four diets were supplemented by copper sulfate providing 0, 150, 200 or 250 mg Cu/kg diet. Nine to 11-week-old pigs were grouped and placed on one of the diets and in each instance were weighed weekly; upon reaching 87.5 kg live weight, the animals were slaughtered and carcass measurements taken. Gram samples of inner and outer subcutaneous fat were removed and melting points determined. Liver samples were taken for estimates of copper content.

The improvements in growth rate due to copper supplementation expressed as percentage increases over controls were 3.2, 4.4 and 6.1 with 150, 200 and 250 mg Cu/kg diet respectively. Feed/gain ratios amounted to 3.4, 3.8 and 5.3%. Copper storage in the liver increased considerably with the dietary intake of copper. The effect of copper supplementation on the melting point of subcutaneous fat parallels the findings of Thompson et al. (648) with regard to the desaturation of fatty acids by copper; melting points of subcutaneous fats were significantly lower when copper was present in the diet (more unsaturated?).

The authors concluded "that the best performance and the highest increase in profitability were obtained with copper sulfate supplementation at the rate of 250 mg Cu/kg diet".

Elliot and Bowland (184) reported on efforts to determine if the rat responded to supplementary dietary copper in a manner similar to the pig, chiefly for cost considerations in the use of rat versus pig. Weanling Sprague-Dawley rats were given diets with and without 250 ppm supplemental copper. Weight gains and food conversion were not improved by the supplemental copper. The accumulation of copper in the liver was subject to wide variability, precluding conclusions regarding similarity between rat and pig. The composition of lipids in the rat carcass was not influenced by copper in the diet, contrary to the lipid changes found in the pig. It appeared that the response of the rat to dietary, supplemental copper was different from that of the pig, and thus "the rat cannot serve as a suitable pilot species in which to study the effects of high levels of supplemental copper on fat metabolism in the pig".

Allergy

Barranco (46) reported in 1972 an allergic reaction to copper in an

intrauterine contraceptive device (IUCD) inserted in a 26-year-old woman several weeks before the onset of a pruritic dematitis. Various treatments failed to relieve the condition which during a months duration developed into an eczematous state with significant papulo-urticarial component. A review of the patients' history revealed the insertion of the IUCD; removal of the device resulted in a complete clearing of the dermatitis. Patch tests revealed a positive reaction to 5% copper sulfate solution.

Copper Disorders and Disease

Copper as a trace metal and as an essential element in human nutrition can be associated with numerous metabolic disorders related to disease. Davies (156), in Chapter III of his text, discusses the clinical significance of copper, and the following listing of disorders causing change in blood copper level is included here for its general interest:

Disorders causing change in blood copper level
(Adelstein and Vallee, 1962)

Disorders of human metabolism associated with hypocupraemia

I. Inability to synthesize ceruloplasmin
   a. Conditioned copper deficiency
   b. Failure to absorb copper
      (1) Sprue
      (2) Coeliac disease
   c. Inability to synthesize other copper-carrying proteins
      (1) Newborn
      (2) Hepatolenticular degeneration
      (3) Kwashiorkor
      (4) Idiopathic hypoproteinaemia
II. Excessive loss or destruction of ceruloplasmin
   a. Loss through excretion
      (1) Nephrotic syndrome
      (2) Protein-losing enteropathy
   b. Accelerated catabolism

Disorders of human metabolism associated with hypercupraemia

Pregnancy
Viral and bacterial infection
Myocardial infarction
Lupus erythematosus
Haemochromatosis
Biliary cirrhosis
Hodgkin's disease
Leukaemia
Malignant disease
Aplastic anaemias
Thyrotoxicosis
Schizophrenia
Alcoholism

The biochemical effects of copper deficiency are mainly a reduction of copper-containing enzymes or sometimes an increase in enzymes in which the copper ion acts as an inhibitor.

A few recent studies are included but not abstracted here, demonstrating the relationship of copper and a variety of diseases: epilepsy, Brunia, 1972 (92); leprosy, Khaire and Magar, 1972 (359); Wilson's disease, Sternlieb and Scheinberg, 1972 (621); "torsion dystromy", Herishanu and Loewinger, 1972, (288); Menkes's kinky hair syndrome, Danks et al., 1972 (152); and Hodgkin's disease, Hrgovcic et al., 1973 (304).

Biochemical Aspects

I. Breakdown

No information available

II. Absorption - Distribution

A study of the effects of dose and duration of administration of copper sulfate on the concentration of copper in rat tissues was reported by Lal and Sourkes (410) in 1971. Male Sprague-Dawley rats with an initial weight of 50 g were injected intraperitoneally with 0.625, 1.25, 2.5 or 3.75 mg Cu/kg body weight daily as $CuSO_4$ solution in 0.9% NaCl. Controls received only the NaCl. The animals were sacrificed at 24 hours, 1, 2, 6, 12 or 18 weeks and the copper concentration in 13 tissues was estimated. In all, 225 rats were used in the experiment.

At the higher dose levels, 2.5 and 3.75 mg/kg, growth was consistently depressed; all animals treated beyond 10 weeks with 3.75 mg/kg had adhesions and megacolon. With all but the lowest dose, liver weight was significantly increased. Incremental changes in liver Cu concentrations were generally more striking at the higher doses and linear up to about 12 weeks, after which copper accumulated at the same rate at all dose levels.

Only a small amount of the injected copper was retained by the kidney; significant increases in renal copper concentrations after a single injection were observed only with highest two doses. In general, significant, through quantitatively small, increments of copper concentrations in bone, spleen, lung, brain, testis, skin and hair, and muscle were seen only at the higher doses. Dosage and duration of loading with copper determine the degree of copper deposition in tissues, and varies with the individual tissue.

Lal and Sourkes (409) also reported in the same year a related study of the distribution of copper in the liver, in detail. The animals used, and the dosages were the same as in the foregoing report. At the higher doses (2.5 and 3.75 mg/kg significant decreases in liver weight, paralleling body weight losses, were observed. Effects of duration and dose were variable among the rats treated, but generally, the concentration of copper in the liver increased with time and dose. Hepatic copper concentrations between 30 and 100 micrograms/g liver are somewhat higher in the mitochondrial fraction than in the nuclear fraction: beyond 100 micrograms Cu/g liver, the mitochondrial fraction levels off at about 175, the nuclear fraction increases progressively without a sign of a plateau. The supernatant (from the centrifuged liver homogenate) portion of the cells is affected by the dose of $CuSO_4$ and at the highest dose, by time; concentration is higher at lower doses and lower as time of treatment increases. Generally, the storage of copper in the microsomal fraction is very small compared with the other fractions. The authors concluded the report with a lengthy discussion of copper cytotoxicity.

III. Metabolism and Excretion

The handling of copper in the liver, the mechanism of its excretion into bile, and the nature of biliary copper were subjects of studies reported by Terao and Owen (643). Radioactive copper, Cu^{67} as $CuCl_2$, was injected intravenously into adult male Sprague-Dawley rats at levels of from 30 to 100 microcuries and the animals sacrificed at 30 minutes, or 1, 6, 24 and 48 hours after injection. Livers were removed, homogenated and centrifuged; the supernate was subjected to chromatography and the fractions measured for radioactivity. Fractional proteins were determined spectrophotometrically. Bile was obtained from another group of animals, with cannulated bile ducts, before injection with Cu^{67}, and at various intervals thereafter up to a week following injection. Bile collected was centrifuged and chromatograms made.

The chromatograms from liver supernates made between 15 minutes and 48 hours after injecting Cu^{67} presented three peaks, the prominences of which shifted with time, as shown in the following figure; the distribution of radioactivity with time is shown in the table:

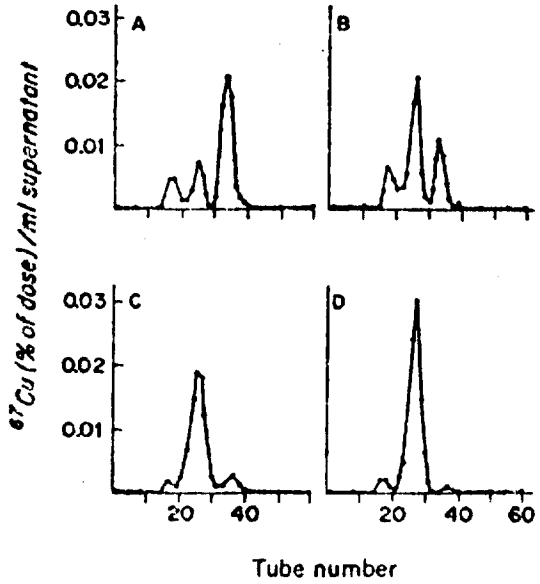

FIG. 1. Radioactivity in fractions (4 ml/tube) from Sephadex G-100 chromatography of liver supernatant fraction from normal rat at various times after intravenous injection of carrier-free ^{67}Cu. *A*: 30 min. *B*: 1 hr. *C*: 6 hr. *D*: 24 hr.

TABLE 1. *Distribution of ^{67}Cu in rat liver supernate at various time intervals after ^{67}Cu administration*

Hours After ^{67}Cu	No. of Animals	% of Dose In Supernate (From 1 g Liver)	% of Total Radioactivity		
			Peak 1	*Peak 2*	*Peak 3*
.5	5	1.26 ± 0.11	21.0 ± 8.2	37.6 ± 11.8	41.5 ± 15.4
1	5	1.38 ± 0.14	19.8 ± 5.8	49.6 ± 3.7	30.6 ± 7.3
6	5	1.56 ± 0.04	4.7 ± 0.9	85.2 ± 4.6	10.1 ± 4.9
24	5	1.50 ± 0.16	5.1 ± 0.9	92.8 ± 1.2	2.2 ± 1.0
48	6	0.91 ± 0.25	4.7 ± 2.1	93.4 ± 3.9	1.9 ± 1.9

Values are means ± SD.

Measurements of the oxidase activity of the three peaks suggested that ceruloplasmin, characterized by its oxidase activity, coincided with peak 1. Estimates of molecular weights showed peak 1 to be about 150,000 (similar to reported values for ceruloplasmin; peak 2 was about 31,000 and peak 3, 11,000.

Chromatograms of bile fractions collected between 6 and 12 hours after Cu^{67} injection showed 2 major peaks, with occasionally a small peak corresponding to peak 2 of the liver supernate. The following figure and table show the radioactive fractions of the bile and the distribution of the radioactivity between peaks 1 and 2 with time:

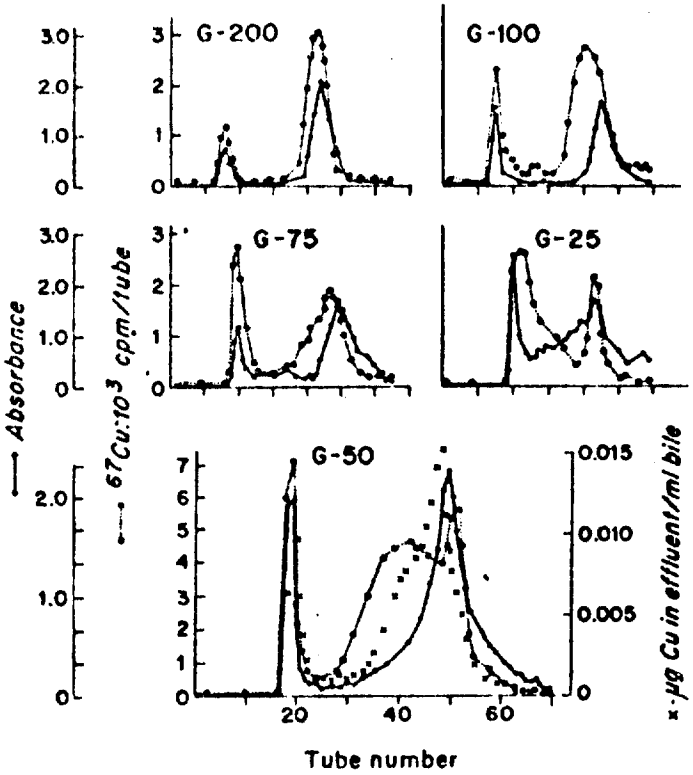

FIG. 2 Radioactive fractions (4 ml/tube) from gel filtration through Sephadex columns of different fraction ranges of normal rat bile collected between 6 and 12 hr after intravenous injection of carrier-free ^{67}Cu.

TABLE 2. *Distribution of radioactivity in bile between peak 1 and peak 2 (Sephadex G-50) at various times after intravenous administration of ^{67}Cu*

Hours	% of Radioactivity	
	Peak 1	*Peak 2*
0–1	7.6	92.4
0–1	6.3	93.7
0–1	9.0	91.0
0–1	8.8	91.2
0–1.5	8.9	91.1
0–1.5	14.7	85.3
6–12	11.7	88.3
6–12	16.7	83.3
6–18	15.3	84.7
6–18	25.7	74.3
11–19	23.8	76.2
24–28	22.7	77.3
30–45	20.6	79.4
30–45	21.3	78.7
30–45	26.9	73.1
30–45	33.5	66.5
30–45	35.3	64.7
6–7 days	16.1	83.9

The determination of molecular weights of the components in bile comprising the 2 chromatographic fractions or peaks gave values of greater than 800,000 for peak 1, and about 5000 for peak 2. Neither peak could be matched with any hepatic component.

The copper content of human saliva and gastric juice in normal human subjects and in patients with Wilson's disease was determined in studies by Gollan et al. (242) in 1971. It was noted that bilary and unabsorbed dietary copper do not account for all the fecal copper excreted. Samples of human saliva and gastric juice were collected and analyzed, as were samples of serum and bile. The copper concentrations in normal subjects are shown in the following table:

TABLE 3

COPPER CONCENTRATION IN HUMAN SERUM, SALIVA, GASTRIC JUICE AND GALL BLADDER BILE

Sample	No. of Persons	μg Copper/100 ml		
		Mean	S.D.	Range
Serum	20	111	16.7	86–152
Gall bladder bile	20	354	155	130–715
Saliva	18	29	11.7	8–55
Gastric juice	46	39	19.0	10–85

Patients with Wilson's disease, a genetic disorder characterized by an undefined, abnormal copper metabolism which results in copper deposition in tissues, and dysfunction of hepatic and nervous systems, were similarly tested for salivary and gastric copper concentrations. The values found were not significantly different from those of normal subjects.

IV. Effects on Enzymes and Other Biochemical Parameters

The role of dietary copper in the metabolism of hemoglobin was the subject of a study by Evans and Abraham (190) in 1973. Periodic monitoring of the levels of liver iron, liver copper and serum ceruloplasmin as related to the development and remission of anemia during a state of copper depletion and subsequent repletion in rats was employed in the study. Hooded Norway male rats, 21 to 25 days of age, were fed either low copper (less than 1 ppm) or control diets (35 ppm) with copper as $CuCO_3$. After 42 days of depletion, the copper-deficient animals were divided into 2 groups, one of which continued on the low copper diet and the other was given the control diet of 35 ppm copper. The rats were sacrificed at weekly intervals from day 0 to 84 and on days 44 and 47; 6 replicates comprised each group at each interval. Hemoglobin, hematocrit, plasma ceruloplasmin oxidase activity, liver iron and copper were determined. Copper-deficient diets reduced growth rate, changed the hair color and resulted in a 15% mortality; hemoglobin and hematocrit values were reduced at day 7, the hemoglobin value falling to 50% of control value in 4 weeks time and continuing low throughout the experiment. In two weeks, the ceruloplasmin oxidase activity of the blood in copper-deficient rats was lowered to 5% of control values; liver copper content showed reduction at day 7, reaching 50% of control values at 2 weeks. Iron accumulated rapidly in the liver of copper depleted animals, increasing to 65% above control values at 42 days and 140% above controls at 84 days.

When copper was fed to the copper-depleted animals, hemoglobin and hematocrit values suddenly increased, reaching control values by day 70. Ceruloplasmin oxidase activity of the plasma increased 25-fold in 2 days and reached control values within 5 days of repletion; between days 56-77 the activity increased over and above the levels of those animals which had received copper throughout the experiment. Increase in liver copper content occurred slowly, requiring 2 weeks for a return to normal values; liver iron content decreased by 30% within 2 days, reaching normal values by day 5.

The changes observed in liver iron concentrations together with the corresponding changes in serum ceruloplasmin oxidase activity during copper depletion and repletion suggested that the role of copper in hemoglobin metabolism is in the mobilization of absorbed iron to the site of hemoglobin synthesis.

Thompson et al. (648) in 1973 reported that supplementing the diet of pigs with 250 ppm copper (sulfate) resulted in a significantly greater oleic/stearic acid ratio of outer subcutaneous fat and perirenal fat than in controls given no supplemental copper. The two fatty acids, stearic and oleic constitute a major portion of porcine adipose tissue; the present study was done to determine if copper alters the activity of fatty acyl desaturase enzyme in liver and adipose tissue, thus changing the fatty acid composition of the fat.

Twenty-four crossbred barrows were divided into two groups: group A received a basic growing and finishing diet; group B received the same diet supplemented with copper sulfate to provide 250 ppm supplemental copper. The animals were slaughtered when reaching 100 kg weight, and subcutaneous fat samples were taken, as were samples of perirenal fat, for fatty acid analyses. Supplemental copper in the diet increased palmitoleic acid content and decreased stearic acid content in outer subcutaneous fat; perirenal fat in copper-supplemented diets showed increased oleic and decreased stearic acid levels. Other fatty acids were unchanged. Supplemental copper influenced the ratios of the monounsaturated fatty acids to their saturated fatty acid precursors. Liver samples were analyzed for stearic acid desaturase activity, and showed that supplemental copper significantly increased the activity in the liver.

V. Drug Interaction

No information available

VI. Consumer Exposure Information

Only copper gluconate has been approved for addition to foods for nutritional purposes and is limited to 50 mg/kg food (7 mg copper/kg).

The following table was compiled from data submitted by user firms. Food consumption values for each food category were derived from the Market Research Corporation of America (MRCA) data on the frequency of eating, and from the USDA data on mean portion size of foods in each category. The food consumption values thus derived were coupled with the usage level data obtained in the surveys to calculate the daily intake of the substance.

Table 14, Part A - Potential Daily Intakes of NAS Appendix A Substances (Group I, II), Per Food Category, Based on Food Consumption by Eaters Only.

Food Category	Substance	Age	Potential Daily Intake, mg. Average	High A	High B	Very High
Soft Candy	Copper gluconate	0-5 mo.	.252000	.423000	.280000	.470000
	Copper gluconate	6-11 mo.	.337500	.765000	.375000	.850000
	Copper gluconate	12-23 mo.	.333000	.720000	.370000	.800000
	Copper gluconate	2-65 yr.	.513000	1.111500	.570000	1.235000
Chewing Gum	Copper gluconate	6-11 mo.	.037680	.065940	.037680	.065940
	Copper gluconate	12-23 mo.	.028260	.061230	.028260	.061230
	Copper gluconate	2-65 yr.	.032970	.070650	.032970	.070650
Formulas	Copper gluconate	0-5 mo.	1.531020	2.480640	3.223200	5.222400
	Copper gluconate	6-11 mo.	1.109980	2.316860	2.336800	4.877600
	Copper gluconate	12-23 mo.	1.363060	2.826060	2.869600	5.949600

COPPER SALTS

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Hepatic Derangements (Biochemical) in Acute Copper Sulphate Poisoning

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Acute copper sulphate poisoning is one of the commonest poisonings. During a period of 4·5 years no less than 118 cases of acute copper sulphate poisoning were admitted in G.M. Association Hospital, Lucknow, a figure which could not be surpassed by another poison.

In this study an effort has been made to elucidate the pathologic physiology. Simple clinical presentations were noted. Biochemical evaluations made were correlated with these.

Scanty literature is available on the subject. Choudhry and Paul (1961) reported 10 cases of jaundice due to acute copper sulphate poisoning. Their findings in these cases were: (1) Serum bilirubin varying from 0·5 to 2·6 mg. per cent. (2) diminution of total protein varying from 5·8 to 6·14 g. per cent. One case showed 7·2 g. per cent initially but came down to 6 g. per cent in 9 days. The above case showed a tendency to the reversal of albumin globulin ratio, (3) serum alkaline phosphatase varying from 11 to 25 K.A. units and (4) thymol turbidity range between 6 and 16 units.

Material and Method

Cases of alleged copper sulphate poisoning admitted in G.M. Association Hospital, Lucknow, were selected for the study. Material obtained by stomach wash was subjected to qualitative analysis for $Cu++$ and SO_4. In the event of positive test the case was taken to be that of acute $CuSO_4$ poisoning. Detailed history was obtained and a thorough general and systemic examination was undertaken.

Regular assessment of hepatic function was made by determining (1) van den Bergh reaction, (2) serum bilirubin, (3) serum albumin, (4) serum globulin, (5) serum alkaline phosphatase and (6) glutamic oxalacetic transaminase.

Observation

Serum bilirubin levels—The values ranged from 2·8 to 10·6 mg. per cent. Lower figures were seen in mild cases of poisoning whereas higher readings were encountered in severe cases.

Van den Bergh test—In all these cases it was biphasic positive.

Qualitative test of urinary urobilinogen—Twenty-four out of these 35 cases gave positive qualitative test for urinary urobilinogen.

Determination of bile salts in urine—Hay's test was positive in all these 35 jaundiced cases.

Serum protein level—In the majority (31 out of 35 jaundiced cases) serum protein level was normal (6·5 to 8·0 g. per cent). In the remaining four cases it was as follows:

Total protein g. %	Serum globulin g %	Serum albumin g. %
6·0	1·8	4·2
5·7	1·9	3·8
5·5	1·4	4·1
5·1	1·5	3·6

Serum alkaline phosphatase—In all these 35 jaundiced cases serum alkaline phosphatase was raised. Raised values were: Mild rise 14 to 17 K.A. units in 9 cases. Moderate rise 18 to 21 K.A. units in 19 cases. Severe rise 22 to 25 K.A. units in 7 cases.

Thymol turbidity and cephalin cholesterol flocculation test—In all these cases the values were normal.

Serum glutamic oxalacetic transminase level—This was determined in 14 cases. None of these showed normal value. The findings were:

35 to 100 units ...	... 6 cases
101 to 200 units ...	... 4 cases
301 to 400 units ...	... 2 cases
401 to 1000 units ...	... 2 cases

Discussion

Normal serum contains less than 1 mg. per cent of bilirubin. Thirty-five cases (29·6 per cent) of this series of 118 developed hyperbilirubinaemia (jaundice) ranging from 2·8 to 10·6 mg. per cent Lower figures were seen in mild cases of poisoning whereas higher readings were encountered in severe ones. Chowdhry and Paul (*loc. cit.*) have reported 10 cases of jaundice due to acute copper sulphate poisoning. The range of serum bilirubin was 0·5 to 2·6 mg. per cent. It is not possible to pinpoint the exact defect concerned to produce jaundice in these cases. The disturbance may be at the level of uptake, transport, or conjugation of bile.

In all these cases van den Bergh test was biphasic positive. This pointed towards hepatocellular damage.

Twenty-four out of these 35 jaundiced cases gave positive qualitative test for urobilinogen. An increase of urobilin in urine is found when hepatocellular function is inadequate to re-excrete all the bile pigment absorbed from the intestines. It is thus a sensitive index of hepatocellular dysfunction. Chowdhry and Paul (*loc. cit.*) while reporting 10 cases of jaundice due to acute copper sulphate poisoning have not mentioned about urinary urobilinogen finding.

In the great majority (31 out of 35 jaundiced cases), serum protein (both albumin and globulin fractions) level was normal. In the cases of acute jaundice this finding was not unexpected. Protein changes are slow to develop and do not immediately reflect acute liver damage. Even complete cessation of albumin production would result in only 25 per cent decrease in blood levels after 8 days (Martin and Neuberger, 1957). Thus, patients with severe acute damage may have normal serum albumin level. Four patients of the series presented with low serum protein levels ranging from 5·1 to 6·0 g. per cent. These also presented with anaemia and other deficiencies. Thus this hypoproteinaemia is in all probability, nutritional in origin.

Serum alkaline phosphatase was high in all the (35) jaundiced cases. It ranged from 14 to 25 K.A. units. Chowdhry and Paul (*loc. cit.*) have mentioned serum alkaline phosphatase to vary from 11 to 25 K.A. units in seven of the jaundiced cases. In hepatocellular jaundice, raised levels may be observed but they are usually less than 30 K.A. units.

Seroflocculation tests (thymol turbidity and cephalin cholesterol flocculation in all these 35 jaundiced cases of acute copper sulphate poisoning were normal. Abnormal results reflect inflammatory activity.

Serum glutamic oxalacetic transaminase (SGOT) level was determined in 14 cases. Values were high and were found to range from 35 to 1000. SGOT is present in large quantities in the liver. Serum level of the enzyme increases whenever these tissues are acutely destroyed presumably due to release of intracellular enzyme into the blood stream from damaged cells. Very high values are found in hepatocellular necrosis. (Normal is 5 to 17 units per cent).

Summary

118 cases of acute copper sulphate poisoning were studied for biochemical hepatic derangements. Thirty-five of these developed jaundice. Serum bilirubin levels varied from 2·8 to 10·6 mg. In all these van den Bergh test was biphasic (positive). This pointed towards hepatocellular damage. Twenty-four out of these 35 jaundiced cases gave positive qualitative test for urobilinogen. This was a sensitive index of hepatocellular dysfunction. In the great majority (31 out of 35 jaundiced cases) serum protein (both albumin and globulin fractions) levels were normal. Serum alkaline phosphatase was high in all these (35) jaundiced cases. It ranged from 14 to 25 K.A. units. Seroflocculation tests (thymol turbidity, cephalin cholesterol flocculation) in all these 35 cases of acute copper sulphate poisoning were normal. SGOT level was determined in 14 cases. Values in all these were high ranging from 35 to 1000.

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Eczematous Dermatitis Caused by Internal Exposure to Copper

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Allergic reactions to copper are rare and have taken the form of eczematous allergic contact dermatitis. This is the first instance of eczematous dermatitis to copper in which the site of exposure was "internal" rather than epicutaneous.

A review of the literature would indicate that allergic reactions to copper are rare and to date have taken the form of eczematous allergic contact dermatitis.[1-4] The following case of eczematous dermatitis to a copper-containing intrauterine contraceptive device (IUCD) acts as a reminder of the allergenic potential of copper. To our knowledge, it is the first instance of eczematous dermatitis to copper in which the apparent eliciting dose of copper was "internal" rather than epicutaneous.

Report of a Case

A 26-year-old woman was in her usual state of good health except for moderately severe hay fever, until one month prior to her initial office visit. At that time, she developed a pruritic dermatitis involving the arms. When first seen, there was a diffuse, erythematous, maculopapular eruption of the arms and scattered areas involving the trunk; excoriations were present. Initially, the diagnosis of atopic dermatitis or allergic contact dermatitis was made. Careful questioning failed to uncover any suspicious topical or systemic allergens. Her initial treatment consisted of 0.1% betamethasone valerate cream applied at least four times daily.

One week later, she was markedly worse with areas of subacute dermatitis involving most cutaneous surfaces except for the face, palms, and soles. Treatment with diphenhydramine hydrochloride, 50 mg four times a day, was started, and she was given 1 ml of a mixture of betamethasone acetate with betamethasone sodium phosphate, intramuscularly. For a short time, some improvement was noted, but ten days later a marked flare occurred. She was started on a regimen of diazepam, 5 mg four times a day for anxiety and prednisone, 30 mg every other day for ten days. Nine days later or approximately one month from when she was first seen and while she was still on a regimen of prednisone (30 mg every other day), diphenhydramine hydrochloride, diazepam, and betamethasone valerate cream and was avoiding the use of topical allergens, another severe flare developed which was generalized in nature. This flare was now not only eczematous but also contained a significant papulo-urticarial component. At that time, her history was reviewed, but again was normal except that she recalled that two weeks before the onset of dermatitis she had an IUCD inserted. A call to her gynecologist revealed that the IUCD contained copper. The copper-containing IUCD was promptly removed. Approximately four days later the dermatitis had markedly subsided, and patch testing was performed. At that time, she had had no systemic corticosteroids for seven days.

Closed patch testing was done with a 5% solution of copper sulfate and a current routine chemical patch testing tray. The metals in the testing tray are listed in the Table. All patch tests were negative except for a 4+ reaction to 5% copper sulfate.

The dermatitis began to fade 48 hours after the copper-containing IUCD was removed, and two weeks later her condition was completely cleared. She has had no treatment for four months following the removal of the copper-containing IUCD and has continued to remain free of all skin diseases.

Review of Literature

Allergic Contact Dermatitis to Copper.—Only six cases of allergic contact dermatitis to copper have been reported. In 1952, Morris[1] reported three cases of allergic contact dermatitis to copper involving industrial exposure to brass. Brass is an alloy containing two parts of copper and one of zinc with trace amounts of other elements. The three patients had positive patch tests to copper only. Bazex and Parant[2] reported a case of allergic contact dermatitis to copper in a worker at a chemical products warehouse who transported sacks of copper sulfate. Patch test revealed a 4+ reaction to copper sulfate only. Van Der Meer[3] reported a case of allergic contact dermatitis to copper in a telephone wireman. He was found to be allergic to pure copper only. In the final and most recent case, copper was found to be the cause of allergic contact dermatitis to various pieces of jewelry.[4] Patch testing with aqueous copper sulfate and other metals resulted in a 4+ positive reaction to copper only.

Gaul[5,6] has shown that copper may corrode in the presence of sweat, producing a primary irritant reaction to rings and metal spectacles. Often a greenish discoloration is produced at the site of the reaction and should suggest the diagnosis.

Eczematous Dermatitis Following Internal Exposure to Metals.—Apparently this subject has received little attention. In a current general textbook of dermatology[7] and one on contact dermatitis,[8] the subject of eczematous dermatitis following internal exposure to metals is not mentioned.

Dental Amalgams and Metal Dentures.—In 1966, Foussereau and Laugier[9] reviewed 12 cases of eczematous dermatitis from mercury present in dental amalgams. They also reported three cases of eczematous dermatitis following the use of metal dentures. In two of the cases

Metals Used for Testing

Metal	Vehicle
Copper sulfate, 5%	Aqueous
Nickel sulfate, 3%	Petrolatum
Potassium dichromate, 0.25%	Petrolatum
Cobalt sulfate, 2.3%	Petrolatum
Mercuric chloride, 5%	Petrolatum

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the denture contained chromium and nickel. Only one patient was patch tested and was strongly positive to nickel and chromium. In the third case, the dermatitis cleared after removal of a gold dental crown. The patient's patch tests to gold powder and various salts of gold were positive.

In 1970, Brendlinger and Tarsitano[10] reported a case of severe generalized eczematous dermatitis due to sensitivity to a chrome-cobalt removable partial denture. Removal of the metal denture resulted in prompt clearing of the dermatitis. A flare occurred immediately after reinsertion of the denture. The patient's patch test to pure samples of nickel and chrome was positive.

In the most recent case report, Elgart and Higdon[11] describe a case of allergic contact stomatitis to a gold dental crown in a patient who also had a flare of dermatitis at previous sites of contact dermatitis to jewelry. The patient's patch test to gold chloride was positive, but those to mercury, chromium, copper, or silver were negative.

Metal Surgical Implants.—Forster and Dickey[12] describe an interesting case of eczematous dermatitis of the eyelid and adjacent skin after a gold-ball orbital implant was inserted. The patient's patch tests to gold sodium thiosulfate, gold leaf, and the gold-ball implant were positive, but not to pure silver, tin, and copper leaf which are the other components of the gold-ball. Symptoms promptly subsided following removal of the implant.

Foussereau and Laugier[9] reported four cases of generalized or widespread eczematous dermatitis following insertion of surgical metal implants. All four cases involved bone fractures repaired with metal screws or plates or both. Two cases were unequivocal in that the dermatitis developed shortly after insertion of the implant, and the implants were known to contain a high percent of nickel. The patients' patch tests to nickel were positive but those to other metals were negative; and complete cures resulted only after removal of the implants. The other two cases were suggestive only. In one the metal implant was never removed yet the patient's condition cleared with treatment, and patch tests to nickel, chromium, cobalt, and molybdenum were negative. In the other case the patient was patch test positive to nickel, but when the nickel-containing plate was removed the dermatitis did not clear and treatment was still necessary.

McKenzie et al[13] describe a case of chronic urticaria following insertion of a cobalt-chromium alloy (Vitallium) nail for a femoral fracture. The patient's patch and scratch tests were positive to nickel but not to other components of the nail (chromium, cobalt, molybdenum, iron, carbon, silicon, and manganese). Following removal of the nail there was prompt and permanent resolution of the urticaria.

All metal alloy implants which are used for orthopedic correction contain nickel (8% to 18%), chromium, and often molybdenum or cobalt.[9]

Comment

The copper-containing IUCD was recently devised to overcome some of the undesirable side-effects of the older IUCD, such as metrorrhagia, pain, and expulsion.[14] Metallic copper was added to the device because of its anticonceptional properties. Other components of this type device are polyethylene and barium.[14]

A copper-containing IUCD was the type used in the present case, and the resulting dermatitis most likely represents an example of "internal" or allergic contact-type dermatitis. In such cases a systemically administered drug elicits an eczematous dermatitis in a patient previously sensitized by external contact with the drug or a chemically related substance.[8] The eliciting exposure may be ingested, inhaled, injected, or absorbed through a mucous membrane and transported by the lymphatics and hematogenous routes to the skin.[15] This concept might be broadened to include sensitization to metals by external contact followed by elicitation of a generalized or widespread dermatitis after internal exposure to the metal. The present case was one of apparent external sensitization to copper with the internal exposure occurring within the intrauterine cavity.

The history of insertion of a copper-containing IUCD two weeks prior to the onset of her dermatitis, the fruitlessness of all attempts at treatment, clearing of the dermatitis following removal of the device, and a positive patch test to copper, but not to other metals or other routine patch testing chemicals, confirms the fact that the copper portion of the IUCD was the cause of the widespread dermatitis seen in this case.

It is likely that with increasing use of the copper-containing IUCD that other cases of copper-induced eczematous dermatitis may occur. It is hoped that this very puzzling and fascinating case will raise the index of suspicion.

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Potential Hepatotoxicity of Copper in Recurrent Hemodialysis

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Tap water acting on copper plumbing contributes up to 1 ppm of copper to dialysis fluid in Australian hemodialysis units. Patients dialyzed against 0.4 ppm of copper show uptake of copper by both plasma and red blood cells during hemodialysis. In experimental dialysis of dogs against 0.4 and 1 ppm of copper, the initial uptake is mainly by the plasma, but copper concentration occurs in the red blood cells and in the liver. The possibility that, in patients on long-term hemodialysis, copper accumulation in the liver might eventually lead to hepatitis and hemolysis is discussed in relation to chronic copper poisoning of sheep and to Wilson's disease.

Acute copper poisoning has been shown to be the cause of severe hemolysis and gastrointestinal disturbances following isolated hemodialysis treatments.[1,2] Retrospective investigation has established a correlation between the hemolytic crises and the formation of acidic water from partially exhausted deionizers which acted on copper components of the artificial kidney machine, resulting in high, and at times lethal, copper concentrations.

The purpose of the present study is to draw attention to the added possibility that copper from relatively low dialysis-fluid copper concentrations derived from untreated tap water, may insidiously accumulate in the liver and eventually present clinically as hepatitis.

In chronic copper poisoning of sheep,[3-6] and in Wilson's disease,[7-10] copper concentrates in the liver without apparent ill-effect until the binding sites are saturated. Then the toxicity is manifested as a necrotic hepatitis or cirrhosis and as a hemolytic crisis due to red blood cell absorption of excessive circulating copper.

Although chronic copper intoxication is virtually unknown in man, hemodialysis supplies an unusual situation in which the blood is subjected to recurrent dialysis against large volumes of fluid over prolonged periods. During hemodialysis, ionic copper in low concentration has been shown to have a one-way flow from the dialysis fluid into the plasma because of protein-binding of the copper.[11] Evidence is presented in this study to show that with slightly higher copper concentrations (within the range encountered in Australian dialysis units), copper transfers from the plasma to the red blood cells and to the liver.

Methods

Samples of dialysis fluid and of tap water used in its dilution were examined from hemodialysis units throughout Australia. One unit used distilled water but no units used deionizers.

The uptake of copper by patients undergoing hemodialysis was assessed in three different units: (1) in four patients dialyzed by a twin-coil machine (Kolff) against a dialysis fluid copper of 15μg/100 ml; (2) in four patients dialyzed by a single-coil of a twin-coil machine (Kolff), with two patients per machine, and a dialysis fluid copper of 40μg/100 ml; and (3) in one patient dialyzed by another dialyzer (Kiil) against a dialysis fluid copper of 40μg/100 ml. The dialyzer was supplied by fluid from a plastic tank with the use of a monitor (Watson Marlow). In each of these three units the established source of the copper in the dialysis fluid was the copper plumbing of the tap water, with the membranes (Cupraphan) providing a possible addi-

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tional contribution in the Kiil dialyzer. No copper-containing parts were detectable in the dialysis machines or delivery systems. Arterial blood was collected before dialysis and samples of blood entering ("arterial") and leaving ("venous return") the dialyzer were collected one hour after commencement and at eight hours, just prior to the end of the dialysis. Simultaneously, dialysis fluid samples from main storage tanks and from the compartment adjacent to the dialysis membranes were also collected.

Liver biopsy specimens were collected from 4 patients and posttransplant 24-hour urine specimens from 12 patients in the unit with dialysis fluid copper of 15μg/100 ml.

Sixteen dogs were also dialyzed with an artificial kidney machine (Travenol-Kolff type) using either cellulose or Cuprophan tubing. Each dog was dialyzed for two hours only and either the dialysis fluid copper level was that of the tap water level of around 40μg/100 ml or copper sulfate solution was added to raise the concentration to approximately 100μg/100 ml (1 ppm). A total of 12 to 24 liters of dialysis fluid was used during the two-hour period. Arterial blood was taken before dialysis and arterial and venous-return samples after ten minutes and again after two hours of dialysis. Two to two and one half liters of 0.9% saline were run through the tubing before commencing dialysis. When indicated this was followed by 500 ml of a degraded gelatin plasma expander, special intravenous gelatin solution (Haemaccel), which was discarded after passing through the tubing. The cellulose tubing coils of the Kolff dialyzer were finally primed with blood from a donor dog prior to dialysis, but the Cuprophan tubing coils, being of lesser capacity, were not. Samples of liver obtained by open biopsy were taken one week prior to dialysis (to avoid bleeding after heparinization) and again at the end of the two-hour dialysis.

The concentration of free ionic copper in tap water, dialysis fluid, plasma, and urine and total copper of red blood cells was measured by the atomic absorption method of Blomfield and MacMahon.[7] Liver copper was estimated by drying liver segments to constant weight over phosphorus pentoxide in a vacuum desiccator, ashing the dried samples in platinum crucibles overnight in a muffle furnace at 450 C, dissolving the ash in copper-free 4N hydrochloric acid, and measuring the copper concentration with an atomic absorption spectrophotometer (Perkin-Elmer).

Liver sections were stained for copper by the method of Mallory and Parker.[11]

Results

Survey of Australian Dialysis Fluid Copper Levels.—Dialysis fluid from most Australian hemodialysis units had a copper concentration lower than 25μg/100 ml, but several had values in the 25μg/100 ml to 100μg/100 ml range. The level in the dialysis fluid closely resembled the level of tap water after the tap had been running for five minutes. With the higher copper levels, tap water values tended to be at least 30μg/100 ml to 40μg/100 ml higher when the tap was first turned on. These results demonstrated that the major source of copper in dialysis fluid was tap water and that this copper was largely derived from copper plumbing. With one machine the dialysis-fluid copper concentration rose by 40μg/100 ml after passing through a copper heat exchanger.

Hemodialysis of Patients.—*Copper Uptake by the Blood.*—1. With patients dialyzed against a copper level of 15μg/100 ml, the venous-return plasma free-copper levels were a little higher than those in the arterial plasma, but there was no increase in red blood cell levels (Fig 1*a*).

2. With a dialysis fluid copper of 40μg/100 ml, there was uptake by both plasma and red blood cells as shown by increased venous-return concentrations, and, after one hour of dialysis, arterial copper levels of both plasma and red blood cells had risen above the predialysis values (Fig 1*b*).

3. With a single patient dialyzed by Kiil dialysis, using Cuprophan membranes, against a dialysis fluid copper of 40μg/100 ml, there was appreciable uptake of copper by both plasma and red blood cells (Fig 1*c*).

Liver Copper.—Liver biopsy specimens for copper measurement have only been available from four patients. Two of these patients had little or no hemodialysis and normal liver copper concentrations of 1.0 and 1.8 mg/100 gm of dry tissue. One patient, after four months of hemodialysis against dialysis fluid with a copper concentration of 15μg/100 ml, had a normal liver copper value of 2.4 mg/100 gm, and one other patient after six months in the same unit showed possible evidence of copper accumulation with a liver copper concentration of 19.6 mg/100 gm. This latter figure is above the normal range of liver copper reported by most workers[10,11,13,15] (Table). The higher normal ranges found by Levi et al[8] were from children, and the discrepancy in results between the two sample sizes suggests that the yellow color of the copper-diethyldithiocarbamate complex is not sufficiently intense or specific to be reliable with low copper concentrations.

Posttransplant Urine Copper.—Twenty-four hour urine specimens were collected within two weeks of kidney transplantation from 12 patients in a unit with a dialysis-fluid copper concentration of 15μg/100 ml. Four of these patients, who had received prior dialysis with copper-free peritoneal dialysis fluids only, had a urine copper concentration range of 2.5μg/100 ml to 8.0μg/100 ml (mean, 5.6μg/100 ml), and a 24-hour urine copper output of 70μg/day to 130μg/day (mean, 110μg/day). The other eight patients, who had been treated by hemodialysis before receiving the renal transplant, had a urine copper concentration range of 2.3μg/100 ml to 11.7μg/100 ml (mean, 6.8μg/100 ml) and 24-hour urine copper output of 45μg/day to 275μg/day (mean, 145μg/

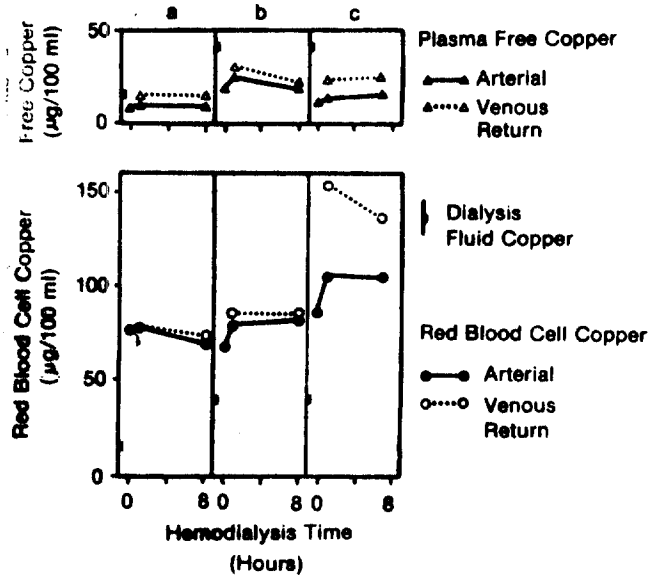

Fig 1.—Uptake of copper by plasma and red blood cells of patients during hemodialysis. (a) Mean results of four patients on twin-coil Kolff dialyzers. (b) Mean results of four patients on single-coil Kolff dialyzers. (c) Results of one patient on a Kiil dialyzer.

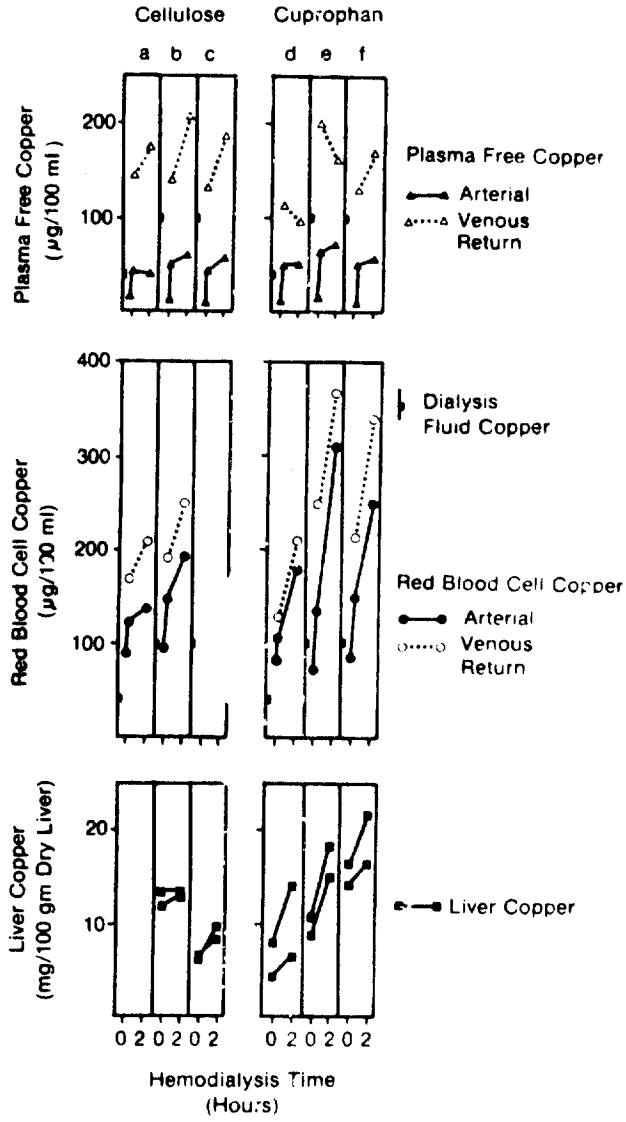

Fig 2.—Uptake of copper by plasma, red blood cells, and liver during hemodialysis of dogs on single-coil Kolff dialyzers. Predialysis gelatin solution primes were used in (c) and (f). Red blood cell and plasma results of (a) and (b) are means of four dogs and (c) to (f) of two dogs.

day). The normal ranges in our laboratory are 1μg/100 ml to 4μ/100 ml (mean, 2.7μg/100 ml) and 10μg/day to 87μg/day (mean, 20μg/day).

Hemodialysis of Dogs.—*Copper Uptake by the Blood.*—Dogs were dialyzed using either cellulose (Fig 2*a* to *c*) or Cuprophan (Fig 2*d* to *f*) dialysis tubing and using the tap water copper concentration of 40μg/100 ml (Fig 2*a* and *d*) or the supplemented copper level of 100μg/100 ml (Fig 2*b*, *c*, *e*, and *f*). All tubing was rinsed with 0.9% saline, and on occasion this was followed by gelatin solution (Fig 2*c* and *f*) to clear copper which was bound to the membranes during the predialysis soaking of the coils.

In all cases the venous-return plasma free-copper levels were considerably higher than the arterial levels. The arterial plasma free-copper concentrations had risen significantly after ten minutes, but, although the elevation was sustained, there was little additional increase during the remainder of the two hours (Fig 2). The copper values of the gelatin solution run through the coil rose from nil to a mean level of 225μg/100 ml, but this preliminary clearing of copper bound to the dialysis membranes failed to prevent the early arterial

Normal Values for Liver Copper

Author	Year	Method: Sample Preparation	Method: Copper Estimation	Dry Weight of Liver Sample	Copper (mg/100 gm of Dry Liver): Mean	SD	SE	Range
Butt et al [14]	1954	Drying, 140 C, 16 hr Ashing, 500 C, 24 hr	Emission spectrograph		2.76		0.10	
Levi et al [9]	1967	Ashing, 243 C	? Diethyldithiocarbamate	2-10 mg 10-30 mg	8.07 3.56			1.5-19.5 0.87-11.2
Smallwood et al [15]	1968	Freeze-drying; wet-ashing in nitric: perchloric acid, 9:1	Neutron activation		3.53		1.20	1.5-5.5
Fell et al [16]	1968	? Drying in vacuo; wet-ashing in nitric acid	Neutron activation		2.55	1.15		0.92-4.68
Sternlieb and Scheinberg [10]	1968	Wet-ashing in sulphuric and perchloric acids	Dicyclohexanone-oxalyldihydrazone		3.15	0.68		2.0-4.5
Cumings [17]	1968	?	Atomic absorption		2.76 (2 SD)	1.78	0.26	
Volini et al [18]	1968	Drying, 120 C, 16 hr; preliminary digestion with nitric acid and heat; ashing, 500-550 C 24-hr	Atomic absorption		5.78		0.87	

copper rise.

The red blood cell copper concentrations also increased during the passage of the blood through the dialysis tubing although not to the same degree as the plasma. However, after an initial rise during the first ten minutes, the arterial red blood cell copper values continued to increase progressively over the two-hour period (Fig 2).

Liver Copper Uptake.—Accumulation of copper in the liver was found in nine of the ten liver biopsy specimens in which comparisons were made of the copper concentrations of samples collected one week before dialysis with a further sample collected immediately after the two-hour dialysis period (Fig 2). The predialysis liver copper concentrations of the dogs tended to be higher than the human normal range, but in this study each dog acted as its own control. Goresky et al[19] have reported liver copper concentrations in dogs which ranged from 18.10 to 57.80 mg/100 gm of dry liver, with a mean and standard deviation (SD) of 33.64 ± 16.11 mg/100 gm. Whether the higher copper levels found in dogs' livers are due to a species difference or to environmental accumulation is not known.

Liver Histopathology.—Despite increases in liver copper ranging up to 7.8 mg/100 gm of dry liver, no copper could be detected in the postdialysis liver biopsy sections by histochemical staining.

Dialysis Fluid Copper Loss.—After two hours of hemodialysis, simultaneous samples of dialysis fluid were collected from the main storage tank and from the separate dialysis compartment adjacent to the membranes. With the 1-ppm fluid there was a mean decrease in copper concentration of 15%. The actual copper loss was probably greater than this as some copper contamination occurs within the machine.[11] At the lower copper concentration of 0.4 ppm, the mean loss or gain was nil, presumably because copper contamination equaled blood uptake.

Comparisons of Cellulose and Cuprophan Tubing.—In vitro studies with dialysis tubing showed that Cuprophan membrane not only contained copper (500μg/100 × 40 cm/sheet), but it bound copper more readily than cellulose tubing. When 10 × 10 cm pieces of Cuprophan or cellulose tubing were immersed in 10 ml of aqueous copper sulfate solution with a copper concentration of 100μg/100 ml, at 37 C for ten minutes, the Cuprophan membrane bound 90% of the copper compared with 40% by the cellulose. The original or the bound copper was not significantly released by 0.9% saline, by 10% formaldehyde solution, or by the dextrans (Rheomacrodex, Macrodex, or Intradex), but it was removed by 3% acetic acid, 5% albumin, or by the degraded gelatin polymer.

Comment

Hepatitis is not an uncommon problem in hemodialysis units, and the viral nature of an outbreak is often evident by a high incidence of the disease among staff and close relatives. However, in some units hepatitis has been confined to patients and the possibility that liver dysfunction could be due to hepatotoxic agents such as azathioprine, halothane, or other known hepatotoxic chemicals or insecticides has been considered.[20-22] Long-term accumulation of copper in the liver has known hepatotoxic consequences in animals and man and has previously been suggested as a potential hazard in hemodialysis.[11]

The strong affinity of the liver for copper has been demonstrated by isotope studies in normal man.[7] Within two hours of the intravenous administration of radioactive copper (^{64}Cu

and ^{67}Cu) compounds only 4% remains in the plasma, and a marked localization in the liver can be demonstrated by whole-body profile scanning or by scintiscanning of the abdomen. Some 60% to 80% of the injected dose is still present in the liver after 24 hours.

In our series of dogs, despite continual uptake of copper by the plasma, the arterial plasma free-copper initially rose, then levelled off, forming an inverted L pattern (Γ) (Fig 2). The arterial red blood cell copper concentration, however, rose progressively, tending towards the shape of a stroke (/). The increase of liver copper would, therefore, appear to have been derived mainly from the plasma rather than the red blood cells.

The present investigation of the hemodialysis of patients and of dogs against concentrations of copper up to 1 ppm has shown that copper is taken up by the plasma, the red blood cells, and the liver. One part per million is 1 mg of copper per liter of dialysis fluid, and patients on long-term maintenance hemodialysis are subjected to 200 to 500 liters of dialysis fluid two to three times per week. The total body copper of a normal adult is 75 to 150 mg, and the normal daily oral intake is 2 to 5 mg.[7] When the copper concentration is high in the dialysis fluid and the dialyses are carried on over a long-term period, it is probable that copper accumulation in the liver would occur and that eventually hepatitis and hemolysis might result. The danger levels in terms of copper concentration and time are not known, but some indication of the degree of risk may be obtained by analogy with studies of chronic copper poisoning in sheep and of patients with Wilson's disease.

Chronic copper poisoning of animals, particularly of sheep, has been extensively studied over the past 40 years. Sheep given copper orally in the range 125 to 500 mg per day usually die within one to eight months.[8-10] Copper accumulates in the liver in a clinically benign manner until the sudden onset of an acute hemolytic crisis and severe liver dysfunction, from which the animal usually dies within a few days. The approach of a hemolytic crisis can be predicted biochemically three to eight weeks before the appearance of clinical symptoms by moderate rises in the levels of serum glutamic oxaloacetic transaminase (SGOT) and lactic dehydrogenase (LDH). At the time of the hemolytic crisis there is a sudden massive increase in the levels of SGOT and LDH, and of the more specific liver enzymes, serum glutamic pyruvic transaminase (SGPT) and arginase, and the clearance of sulfobromophthalein (BSP) is abruptly reduced. Blood copper levels are normal to within a few days of hemolysis, when enormous increases in red blood cell and plasma copper occur. With the crisis the hematocrit level drops sharply, hemoglobin is found in plasma and urine, methemoglobinemia may give the blood and liver a brown color, red blood cell glutathione is reduced to very low levels, and a profound hyperbilirubinemia follows. Liver copper concentration shows a 10- to 30-fold increase above normal, the amount of copper retained by the liver being of the order of 100 to 300 mg.

The terminal phase in sheep of intravascular hemolysis and hepatic toxicity resembles acute copper poisoning seen in human subjects,[11,12] in which the predominant clinical features are a marked hemolysis occurring 6 to 24 hours after exposure to copper and associated jaundice, methemoglobinemia, hypoglycemia, vomiting, diarrhea, abdominal pain and liver tenderness. The similarity between chronic copper poisoning of animals and the hemolytic episode with related hepatic involvement sometimes seen in Wilson's disease has been emphasized by McIntyre et al.[13] From the normal daily dietary intake of copper, a child with Wilson's disease may accumulate sufficient copper in the liver to present as clinical hepatitis by the age of [illegible] years.[7] In presymptomatic Wilson's disease, an increase of SGOT has been found to correlate well with abnormal liver histology and increased liver-copper concentrations.[7]

A direct analogy cannot, of course, be drawn between dialyzed patients and patients with Wilson's disease who have a defect of copper metabolism or with sheep, which are believed to be particularly susceptible to copper poisoning. However, from the evidence presented, we feel that chronic copper accumulation must be regarded as a possible cause of hepatitis and hemolysis in maintenance hemodialysis.

Soft water, where deionizing is not usually felt to be necessary, is the water most likely to dissolve significant amounts of copper. Measurement of tap water and dialysis-fluid copper concentrations should give an indication of the possibility of excessive copper loading. Estimation of plasma free-copper and red blood cell copper in arterial and venous-return samples taken at the end of the dialysis should give some idea of the degree of copper uptake by the patient. It may be noted here that, even with excessive liver copper accumulation, the predialysis plasma free-copper and red blood cell copper are likely to be normal and that measurement of plasma ceruloplasmin has no value as it is

unaffected by copper uptake.

Postdialysis urine copper estimations may give some indication of excessive copper uptake but have limited value in patients with impaired renal function. Posttransplant urine copper outputs may give an indication of high liver copper accumulation, although this aspect requires further investigation. All of the posttransplant patients in the present series had urine copper levels above our normal ranges. However, they were all receiving prednisone (1.5 mg/kg/day), and a recent report by Henkin et al[24] has shown that administration of 50 mg of prednisone for five days to normal volunteers caused a more than twofold increase of urine copper excretion. Wiesel[25] also found an increased urinary copper excretion on administration of glucogenic corticosteroids to normal subjects. In the present study liver copper and posttransplant urine copper tended to be slightly higher in dialyzed than in nondialyzed patients, which suggests some degree of copper accumulation, but probably insufficient to cause serious toxicity.

Excessive copper stored in the liver may be bound so that it does not react to copper stains,[8,10] but an accumulation sufficient to cause an acute hepatic reaction would probably give a positive stain. A retrospective survey of liver biopsy and autopsy sections of dialyzed patients by copper staining could reveal the presence of copper as a primary or contributory cause of hepatic dysfunction in some dialysi patients, and, as a prospective study the more accurate chemical assessment of liver copper levels coul prove an invaluable indicator of cop per accumulation in patients under going hemodialysis.

J.H. Stewart, MB, ChB, of Sydney Hospita Sydney, Australia, and E. Haywood, MB, of t Royal Perth Hospital, Perth, Australia, assist with hemodialysis. Mr. K. Curtis and the Depar ment of Pathology, Royal Children's Hospit Melbourne assisted with hemodialysis of dogs. I Bale, MB, and Mr. H.J. Mitchell of the Institut of Pathology, Royal Alexandra Hospital for Chil dren, Sydney, performed the liver histopatho logical examinations. The special intravenous gelatin solution used in the studies was donated as Haemaccel by Hoechst Australia Ltd.

Nonproprietary and Trade Names of Drug

Azathioprine–*Imuran*.

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Copper levels in diets for growing pigs

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SUMMARY

Results of a co-ordinated trial carried out at twenty-one centres indicated that the performance of growing pigs (20–90 kg) can be improved by supplementation of their diet with copper sulphate.

Three levels of copper supplementation were tested, 150, 200 and 250 mg Cu/kg diet. Though significant improvements were recorded on all three levels, the best performance and the highest increase in profitability were obtained with 250 mg Cu/kg diet.

With increasing dietary intake of copper, the copper content of the pigs' livers increased markedly.

The melting-point of subcutaneous fat was lower in carcasses of pigs that were given supplementary copper sulphate in their diet.

INTRODUCTION

The use of copper sulphate as a feed additive for promoting the performance of pigs is now generally accepted (see reviews by Braude (1965), Wallace (1967) and Meyer & Kröger (1973)). The level of supplementation is still a matter of controversy: 250 mg Cu/kg diet is the level recommended by Braude and his co-workers and convincing evidence exists that this is to be preferred to 125 mg/kg (see Braude, 1965). Until recently there has been very little evidence about the effects of levels within the 125–250 mg/kg range. Results of a recent experiment (Braude *et al.* 1970) suggested that the optimum level of supplementation may be in the 170–210 mg/kg range.

The aim of this experiment was to study the average effects of feeding copper sulphate to growing pigs at levels supplying 0, 150, 200 and 250 mg Cu/kg diet.

METHODS

Four treatments were used; a cereal-fishmeal diet was supplemented with 0, 150, 200 or 250 mg Cu/kg diet respectively. These are subsequently referred to as diets 1–4.

At each of the twenty-one centres 9- to 11-week-old pigs were allocated to the four experimental treatments at random, except that initial weight, sex and litter distribution were equalized as far as possible. Pigs receiving the same treatment were housed together in one pen, care being taken that the four pens used had, as near as possible, the same environment. The minimum number of pigs/pen was six, the actual number being at the discretion of each centre. The number of pigs/pen, the average initial weight of the pigs, their breed and the type of housing are given for each centre in Table 1.

All the diets were compounded at a central depot from which they were distributed to all participating centres. When the pigs in each pen reached an average of 54·4 kg live weight, their diets were changed to 'finishing' diets with lower amounts of the protein supplement, adjusted mineral and vitamin content, but unchanged level of copper sulphate. The composition of the 'standard' and 'finishing' experimental mixtures is given in Table 2.

Several random samples of the experimental mixtures were taken throughout the experimental period and analysed for main chemical constituents. Table 3 gives the average values for the 'standard' and 'finishing' diets. Checks were also made for the Cu content of the diets. The average values were 6, 147, 194 and 240 mg Cu/kg for diets 1–4 respectively.

The pigs were weighed once weekly. They were given feed wet, twice daily in amounts related to live weight and according to a scale, the details of which are the same as in previous co-ordinated experiments (e.f. Braude, Lyon & Rowell, 1965).

Table 1. *Number of pigs per treatment, average initial weight, breed of pigs and type of housing at each centre*

Centre*	No. pigs/ treatment	Av. initial wt. (kg)	Breed	Housing
Beecham	8	18·5	Thornber hybrid	Danish fattening house
Bibby's	9	19·6	Camborough	Inlet and outlet piggery
BOCM	6	17·5	L.W. × L.	Kennelled pens with no separate dunging area
Chadacre	9	23·9	L. × L.W.	Controlled environment, slatted dunging passage
Cheshire	6	18·5	L.W.	Modified Harper Adams
Compton	8	21·9	L.W., L.W. × L.	Danish
Cumberland	6	29·0	L.W.	Open plan six-pen fattening house
E. Sussex	6	22·3	L.W.	Converted Danish
Greenmount	6	19·8	L.; L.W.	Danish type piggery
Harper Adams	10	22·2	L.W., L. × L.W.	Harper Adams bacon house
Herts	6	18·3	L.W.; (L.W. × B.S.) × L.W.	Danish
Hillsborough	6	19·7	L. × (L.W. × L.)	Danish
Lancashire	8	18·9	L.W.	Danish
NIRD	6	18·8	L.W.	Danish
N. of Scotland	7	15·8	L. × (L.W. × L.)	Danish
Reading	10	22·5	Camborough	Danish
Terrington	7	22·8	L.W.	Insulated roof with extractor fans and lateral feeding passages
Toddington	11	20·0	L.W.	Danish
Trawscoed	6	22·3	L.W.	Controlled environment Pears Ballard centre slatted dunging passage
W. of Scotland	6	19·1	L.W.	Scandinavian
Yorks W.R.	6	22·8	L., L.W.; L.W. × L.	Intensive controlled environment

* Full names and addresses of the participating centres are given at the end of the paper.
L., Landrace; L.W., Large White; B.S. British Saddleback.

Table 2. *Composition of the diets*

	'Standard' up to 54·4 kg live weight	'Finishing' from 54·4 kg to slaughter
Barley meal (%)	57	57
Wheat, ground (%)	20	20
Fine miller's offals (%)	15	17·5
White fish meal (%)	7	3·5
Limestone (%)	1	1·5
Salt (%)	—	0·5
Vitamin A (i.u./ton)	4 million	2 million
Vitamin D (i.u./ton)	1 million	0·5 million
Zn supplement, mg Zn/kg	100	100

Granulated fine crystal copper sulphate ($CuSO_4.5H_2O$) was used to supply 0, 150, 200 or 250 mg Cu in the four 'standard' and 'finishing' diets respectively.

Table 3. *Average chemical analysis of experimental diets*

	'Standard' diet	'Finishing' diet
Moisture	10·4	10·2
Cr. protein	15·2	13·4
Oil	2·2	2·0
Cr. fibre	5·1	5·6
Ash	4·7	5·0

The ratio of water to the meal was 2½:1 (w/w). The water was added just before feeding.

As soon as possible after a pig exceeded 87·5 kg live weight at a weekly weighing, it was despatched for slaughter to one of the co-operating slaughter-houses. On the day of despatch the pig was weighed at a specific time, and a similar slaughter routine was followed at each of the slaughter-houses. Carcass measurements were taken according to the routine described in detail by Buck, Harrington & Johnson (1962).

Following slaughter 1 g samples were removed from the inner and outer subcutaneous fat, over the centre of the L. dorsi muscle, and the lipids extracted and purified by the method of Folch, Lees & Sloane-Stanley (1957). A sample of the fat was placed in melting-point tubes. The ends of the tubes were sealed by heating and they were kept in a refrigerator overnight. The tubes were then placed in a water bath, which was heated until the fat became completely clear. The temperature at which this occurred was taken as the melting point.

A sample of the liver, a 1 cm cube to the left of the gall-bladder, was taken for estimation of Cu content in liver.

RESULTS AND DISCUSSION

The experiment was analysed statistically as a randomized block design using the pen means as variates, with blocks corresponding to centres and the treatment sum of squares was partitioned into the three components: presence or absence of copper in the diet and the linear and quadratic effects of copper over the three levels 150, 200 and 250 ppm of copper. Records of the pen means for each centre have been lodged at the National Institute for Research in Dairying and may be obtained on request.

Diet means and their standard errors are given in Table 4. The growth rate and the feed/gain ratio for the three periods of growth (up to 54·4 kg, from 54·4 kg to slaughter, and overall) together with the width and depth of eye muscle at slaughter were significantly improved when the diets which had copper added were used. Significant linear effects were obtained for the growth rate and feed/gain ratio for the periods, up to 54·4 kg and overall. The melting-points of both inner and outer subcutaneous fat were significantly lower when copper was present in the feed. For the variables dressing percentage, carcass length, shoulder depth, shoulder fat, mid-back fat, loin fat and fat over eye muscle, treatment differences were not significant.

The data for the amount of copper in the liver were transformed by taking logarithms before calculating the pen means and the analysis was weighted by the number of pigs in each pen. There was a highly significant increase in the amount of

Table 4. *Treatment means and their standard errors*

	Copper sulphate supplement				S.E. of treatment means	Significance level †	
	None	150 ppm Cu	200 ppm Cu	250 ppm Cu		Cu v. no Cu	Linear effects of Cu
Growth rate (kg/day)							
Start to 54·4 kg live weight	0·496	0·510	0·520	0·532	0·0047	***	**
54·4 kg live weight to slaughter	0·688	0·714	0·716	0·721	0·0082	**	
Start to slaughter	0·573	0·591	0·598	0·607	0·0042	***	**
Feed conversion							
Start to 54·4 kg live weight	3·12	3·01	2·98	2·92	0·022	***	**
54·4 kg live weight to slaughter	3·83	3·70	3·73	3·68	0·037	**	
Start to slaughter	3·46	3·34	3·33	3·28	0·022	***	*
Dressing percentage	75·6	76·0	76·1	75·9	0·17		
Carcass length (mm)	810·3	809·1	809·5	807·6	1·54		
Shoulder depth (mm)	317·1	317·1	318·8	319·4	0·99		
Shoulder fat (mm)	45·1	45·4	45·6	45·2	0·36		
Mid-back fat, minimum (mm)	21·3	21·7	22·0	21·9	0·26		
Loin fat, maximum (mm)	29·7	30·0	30·6	30·6	0·38		
Loin fat, minimum (mm)	23·9	23·8	24·5	24·2	0·33		
Eye muscle							
Width A (mm)	76·3	76·6	77·5	77·9	0·43	*	
Depth B (mm)	46·8	47·7	47·7	48·4	0·39	*	
Fat over eye muscle							
C (mm)	20·7	21·1	20·7	20·7	0·28		
K (mm)	25·6	26·1	25·5	25·3	0·35		
J (mm)	5·6	5·8	5·6	5·7	0·18		
Melting point of fat							
Outer	45·47	44·45	44·24	44·08	0·324	**	
Inner	47·52	46·72	46·81	46·78	0·263	*	
Copper level in liver‡							
Transformed data (ln x)	3·63	4·40	4·97	5·66	0·17	***	**
Original unit: (ppm dry wt)	37·63	81·68	143·80	286·57			

† All treatment comparisons for which level is not indicated were not significant ($P > 0·05$).
‡ weighted analysis.
* $0·01 < P < 0·05$; ** $0·001 < P < 0·01$; *** $P < 0·001$.

Table 5. *Pairwise comparisons of treatment differences in growth rate (GR) and feed/gain ratio (FGR)*

	Treatments					
	1 *v.* 2	1 *v.* 3	1 *v.* 4	2 *v.* 3	2 *v.* 4	3 *v.* 4
GR: start to 54·4 kg	*	***	***	NS	**	NS
54·4 kg to slaughter	*	*	**	NS	NS	NS
start to slaughter	**	***	***	NS	**	NS
FGH: start to 54·4 kg	**	***	***	NS	**	NS
54·4 kg to slaughter	*	*	**	NS	NS	NS
start to slaughter	***	***	***	NS	*	NS

NS, $P > 0.05$; * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$

copper present in the liver for those treatments with copper in the diet and a highly significant linear effect of the amount of copper present.

In Table 5 the effects of the treatments on growth rate and the feed/gain ratio are examined in more detail. It can be concluded that the biggest effect occurred during the period up to 54·4 kg live weight, and that the differences in response to 150 or 200 mg Cu/kg and 200 or 250 mg Cu/kg diet were not significant while the difference between 150 and 250 mg Cu/kg was significant.

The improvements in growth rate due to supplementation with copper sulphate expressed as percentage increases over the control pigs were 3·2, 4·4 and 6·1 with 150, 200 and 250 mg Cu/kg diet respectively. The corresponding improvements in the feed/gain ratio amounted to 3·4, 3·8 and 5·3% respectively. The values obtained for the diet containing 250 mg Cu/kg can be compared with a weighted average from 83 tests of 8·1% for daily gain and 5·4% for feed/gain ratio calculated by Braude (1965). With present costs of feed and of the copper supplement it can be roughly calculated that the financial benefit arising from copper supplementation at 250 mg Cu/kg diet was about 50p per pig.

The amount of copper stored in the liver increased considerably with increasing dietary intake of copper. The average value for the 250 mg Cu/kg diet was 286·6 mg/kg dry matter. This is considerably lower than the weighted average for the same intake of copper as assessed from published data, namely 578·4 mg/kg dry matter (Braude, 1965). No explanation can be offered for this difference.

The effect of copper supplementation on melting point of subcutaneous fat confirms the finding by Moore *et al.* (1969), but in this experiment the effect was very much smaller.

The results of this large-scale field trial indicate that the best performance and the highest increase in profitability were obtained with copper sulphate supplementation at the rate of 250 mg Cu/kg diet.

This test was conducted as Co-ordinated Experiment No. 15 organized on behalf of the Agricultural Research Council, and the following co-operated in it:

D. B. Bellis, British Oil & Cake Mills Ltd., Risborough Road, Stoke Mandeville, Bucks (BOCM)

J. C. Bowman, Department of Agriculture, University of Reading, Reading, Berks (Reading)

M. Boyd, Greenmount Agricultural and Horticultural College, Antrim, Northern Ireland (Greenmount)

J. R. Cowper, Toddington Manor Research Farms Ltd., Toddington, Dunstable, Beds (Toddington)

C. J. England, Cheshire College of Agriculture, Reaseheath, Nantwich, Cheshire (Cheshire)

W. M. R. Evans, Trawscoed Experimental Husbandry Farm, Trawscoed, Aberystwyth, Cardiganshire (Trawscoed)

L. C. G. Gilling, Askham Bryan College of Agriculture and Horticulture, Askham Bryan, York (Yorks W.R.)

R. Kenney, Harper Adams Agricultural College, Newport, Shropshire (Harper Adams)

E. V. Knight, A.R.C. Institute for Research on Animal Diseases, Compton, near Newbury, Berks (Compton)

A. J. Mudd, Beecham Research Laboratories, Walton Oaks, Dorking Road, Tadworth, Surrey (Beecham)

N. J. D. Nickalls, Plumpton Agricultural College, near Lewes (E. Sussex)

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...lker, Agricultural Research Institute of ...orthern Ireland, Hillsborough, Co. Down (Hillsborough)

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Serum Copper Levels and Epilepsy

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INTRODUCTION

Elevated serum copper levels in epileptic patients have been reported by several authors (Plum and Hansen 1960, Canelas *et al.* 1964, Brunia 1966, Merlo and Fernandez Martin 1969). Serum copper levels are increased in a multitude of diseases (see for a review Scheinberg and Sternlieb 1960). Although this phenomenon may be a general reaction to stress its significance is in fact not known. Merlo and Fernandez Martin (1969) found, however, a relation between increased serum copper concentration and the presence of focal lesions in epilepsy. This finding could point to a more direct relation between at least certains forms of epilepsy and copper metabolism. At the time Merlo and Fernandez Martin (1969) published their results on 58 epileptic patients we had just finished a similar study on 174 patients. The results of this first part of our investigation are reported briefly here.

MATERIAL AND METHODS

94 epileptic children admitted to the "Dr Hans Berger Kliniek" at Breda and 80 adults in the epilepsy centre "Kempenhaeghe" at Heeze took part in the investigation. All patients had at least once "specific disturbances" in their EEG, namely spike-wave discharges. The nature and frequency of seizures were diverse; they were classified by the International Classification of Gastaut *et al.* (1969). The control groups consisted of healthy school children (n = 107) and blood donors (n = 141). Controls and patients were matched for age and sex since both factors influence the copper and ceruloplasmin content of serum (Buyze unpublished observations). The anamnesis of the controls mentioned no serious illness for at least one year before blood was collected. Anti-epileptic medication used by the patients

Key words: *epilepsy—copper metabolism, seizure—serum copper levels—international classification.*

consisted of one or more of the following drugs: phenobarbital, diphenylhydantoin, primidone, ethosuximide, methosuximide, acetazolamide, amphetamine, dextramphetamine and caffein.

In patients with behaviour disorders the benzodiazepines librium and valium were administered as additional neuroleptic agents. Blood was collected after an overnight fast under the usual precautions against copper contamination. Copper levels were determined by the method of Rice (1960), ceruloplasmin by the method of Van den Hamer and Buyze (1961) (see also van den Hamer and van der Heyden 1963).

RESULTS

In the first 80 patients (50 children and 30 adults) the concentration of ceruloplasmin and copper in the serum were closely related. Therefore we did not measure the serum level of ceruloplasmin in subsequent persons, and are presenting only the serum levels of copper here.

Serum level of copper in patients and controls (Table I)

In every age group, the concentration of copper in the serum was significantly higher in the patients than in the controls ($p < 0.01$).

TABLE I

age	sex	patients	controls	p (Student t-test)
8–12	♀ + ♂	n = 46 x = 155.2 s.d. = 26.5	50 124.0 21.9	$p < 0.01$
12–17	♀ + ♂	n = 48 x = 146.6 s.d. = 30.5	57 106.4 18.9	$p < 0.01$
17–40	♂	n = 36 x = 132.8 s.d. = 23.5	59 96.1 21.9	$p < 0.01$
17–40	♀	n = 44 x = 148.8 s.d. = 23.9	82 107.3 16.4	$p < 0.01$

Table I: Difference of serum copper levels in epileptic patients and in matched control groups n: number of patients, x: mean serum copper level, expressed in microgram/100 ml, s.d. standard deviation.

Serum levels of copper, age and sex (Table I)

There was no difference between males and females in the age group 8–12 and 12–17 year, so boys and girls are combined in Table I. In the age group 17–40, females had an unchanged mean serum level of copper, whereas the men had a lower mean level than patients aged 8–12 yr. In the controls, too, the mean serum copper concentration fell with age in the men, not in the women.

Serum level of copper and type of seizure (Table II)

The size of the groups was too small to allow them to be divided by age and sex, so the control groups were matched for these variables. The patients had again higher values than controls. There was no systematic difference between the groups of patients according to the form of the seizures. The highest levels were found in patients with convulsions, whether primarily or secondarily generalized (IC and IIB). The difference was not systematic, however, since patients with both non-convulsive and convulsive primarily generalized seizures (IIA + IIB, Table II) had the same mean serum level of copper as patients with partial seizures with elementary and complex symptomatology (IA and IB, Table II). Patients with primarily generalized non-convulsive seizures (IIA, Table II) also had high serum levels of copper, partly attributable to age, since many of them were children.

TABLE II

	generalized seizures		*partial seizures*		
	elementary and complex symptomatology IA + IB	*secondarily generalized IC*	*non convulsive seizures IIA*	*convulsive seizures IIB*	*both IIA + IIB*
n	35	56	26	29	28
x	138.2	152.4	146.7	151.1	136.9
s.d.	28.9	32.1	34.0	30.9	19.6
p against weighted control group	$p < 0.02$	$p < 0.02$	$p < 0.02$	$p < 0.02$	$p < 0.02$

Table II: Mean serum copper levels of patients with different kinds of epilepsy. Patients of both sexes and different age groups are taken together.

DISCUSSION

Our study confirms previous reports of a higher serum level of copper in patients with epilepsy than in healthy control persons matched for age and sex. The matching is necessary since both factors are related to the serum level both in patients and in controls. The reason for the difference between patients and controls is not clear. The three possibilities to be discussed are that the high level of copper in the serum is a nonspecific effect of stress of muscular activity, that it is caused medication, or that it is related to the illness itself.

Concentration of copper in the serum and stress or muscular work. A nonspecific stress cannot be ruled out as yet and may be responsible for the observed increase. The possibility that the elevated serum levels are caused by the muscle movements during the seizures does not seem very probable. Costa Furu (1961) reported a de-

crease in serum concentration of copper after excercise. We ourselves could not find any change in the serum concentration of copper after exhausting physical excercise in normal men (Buyze, to be published). Furthermore, if the elevated serum copper concentration in patients with epilepsy were related to muscular activity during convulsions, one would expect to find a systematic difference between patients with generalized convulsions and other forms of epilepsy, and this was not the case (Table II).

Concentration of copper in the serum and medication

Diphenylhydantoin may influence the metabolism of copper. Peters *et al.* (1966) reported increased urinary excretion of copper in patients intoxicated on diphenylhydantoin. On the other hand, Cantu and Schwab (1966) found no increase in the serum concentration of copper in healthy persons given diphenylhydantoin, though the content of ceruloplasmin did rise. Merlo and Fernandez Martin (1969) thought the increased serum concentration of copper in their 58 patients might be due to diphenylhydantoin. There was no evidance in our patients of such a relation. The patients who did not receive diphenylhydantoin (IIA in Table II) had as high a serum level of copper as the patients in the other groups, who often were treated with diphenylhydantoin. However, it was not possible to match the groups of patients for age and sex, so our material does not give decisive evidence on the possible relation of diphenylhydantoin to elevated level of copper in the serum. The ethosuximide medication used most often for patients in group IIA does not seem to alter the serum level of copper (Buyze and Brunia, unpublished observations).

Concentration of copper in the serum and age and sex

Since the level of copper in the serum is related to age and sex (Buyze unpublished data), healthy controls need to be matched, and this has not been done in previous series. That the serum level falls after the age of 12 yr, and continues to fall with age in men seems not to have been reported previously.

Concentration of copper in the serum and epilepsy

Although we were not able to confirm the finding of Merlo and Fernandez Martin (1969) that an elevated serum level of copper is related particularly to focal epilepsy (see Table II), the notion that copper metabolism may have some relation to epilepsy has some support. The level of copper in the spinal fluid seems to be lower in patients than in normal controls (Canelas *et al.* 1964, Marques de Assis 1966, Buyze and Brunia, unpublished observations), and the level decreases further after grand mal convulsions (Canelas *et al.* 1964, Marques de Assis 1966). Furthermore we have some indication that there is a deficit of copper in certain areas of the brain in persons with epilepsy (Buyze and Brunia, unpublished data). Although the evidance is still scanty, the question of some relation of copper metabolism and epilepsy seems worth pursuing further, and we feel it desirable to attract the attention of others to this problem while continuing our own investigations.

SUMMARY

Serum copper levels of 94 epileptic children and 80 adult patients were determined. The children were divided in two age groups, 8–12 and 12–17 years. The adult patients (17–40 years) were divided according to sex. Serum copper levels of the different patient groups were significantly higher than in normals. Patients were also classified by the International Classification of Gastaut *et al.* All types of epilepsy showed the elevated serum copper levels. Three possible explanations are discussed, viz. a nonspecific effect of stress or muscular activity during the seizures, a medication effect and a possible relation to the illness itself. There seems to be some support for the latter hypothesis.

RESUME

Les niveaux sériques de cuivre ont été déterminés chez 94 enfants et 80 adultes. Les enfants ont été divisés selon leur âge, en 2 groupes: l'un âgé de 8 à 12 ans, l'autre de 12 à 17 ans. Les adultes, âgés de 17 à 40 ans, ont été groupés par sexe.

Les niveaux sériques de cuivre des malades des différents groupes étaient significativement plus élevés que la normale.

Les malades ont également été classés selon la classification internationale de Gastaut *et al.* L'élévation du taux sérique de cuivre, se voyait dans tous les types d'épilepsie. Trois explications possibles sont discutées, cette élévation pouvant résulter soit du stress ou de l'activité musculaire durant les crises, soit d'un effet dû aux médicaments, soit de la maladie elle-même, cette dernière hypothèse paraissant la plus probable.

(C. A. Tassinari, Marseille)

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COPPER SULPHATE POISONING*

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Statistical study.—All cases of poisoning admitted in the Medical College Hospitals of Lucknow, Agra and Kanpur and Balrampur Hospital, Lucknow in 1957 and 1958 (2 calendar years) were analysed.

Out of a total of 729 cases of poisoning, 134 or 19·3% were due to Copper sulphate.

TABLE I

Showing the various types of poisoning in 729 cases

Types of poisoning	Total No.	Percentage	Deaths	Percentage
Copper sulphate	134	19·3%	16	12%
Alcohol	119	16·3%	1	·83%
Opium	96	14·1%	13	13·6%
Datura	85	12·64%	7	8·3%
Kerosene oil	70	9·6%	5	7·1%
Acid	31	4·2%	6	19·3%
Food poisoning	30	4·2%	3	10%
Bhang	16	2·2%	Nil	0%
Rat-killing poison	12	1·64%	,,	0%
Barbiturates	11	1·2%	1	9·39%
Lint. Camphor	7	·96%	1	14·3%
Arsenic	6	·82%	2	33%
Tobacco	4	·56%	Nil	0%
Pot. permanganate	4	·56%	,,	0%
Glass powder	5	·69%	,,	0%
D.D.T.	4	·56%	,,	0%
Corrosives	2	·22%	,,	0%
Castor oil	1	·14%	,,	,,
Petrol	1	·14%	,,	,,
Chloroform	1	·14%	,,	,,
Jamalgota	1	·14%	,,	,,
Turpentine oil	1	·14%	,,	,,
Cyanide	1	·14%	1	100%
Lint A.B.C.	1	·14%	Nil	0%
Undetermined	66	9·2%	6	9·99%

It will be seen for the above tabular statement that copper sulphate poisoning topped the list with nearly 20% of the total number.

As the literature available on this subject is meagre, we made a study of the natural history, course and progress of copper-sulphate-poisoning cases. The following tests were done in addition to routine clinical and pathological investigations: (1) Urine for bile-salt pigments and urobilinogen; (2) repeated liver function tests; (3) reticulocyte count every

*Specially contributed to the ANTISEPTIC

alternate day; (4) blood-urea estimation; (5) S.G.O.T. (6) liver biopsy where possible.

Case study.—In a detailed study of 36 patients, the following observations were made. The poison was taken in the f of '*Tootia*.' The dose being from a few grains to 1 tola. three cases a solution of the salt was taken.

Purposes of taking copper sulphate:—(1) Accident (2) suicidal; (3) medicinal; (4) homicidal; (5) experiment (6) undetermined or (7) alleged accidental (suicidal).

Copper sulphate was used most often as a suicidal a although some accident is usually alleged to be the cause intake of the drug.

Of the 36 cases initially investigated, 12 were over 20 ye of age and 24 below 20; 19 were Hindus and 17 Muslims; were males and 11 were females.

The mental and social condition of the cases was fou to be half-literate working class families.

Presenting symptoms.—(1) Vomiting; (2) pain in the abd men: within a few minutes 100%; (3) hæmatemesis: 4 case (4) four patients were brought semiconscious within a few hou of ingesting the poison; (5) jaundice apparent: in six cas during the stay in the Hospital in these cases.

(*i*) Serum bilirubin level varied from 2·2 to 8·0 mg.%; (i van den Berg: direct 4, indirect 2; (*iii*) all showed evidence o moderate parenchymatous damage; (*iv*) alkaline phosphata was within normal limits.

Urine:—Albumin was present in 20 cases; bile pigment and salts in 6 cases; R.B.C. present in 10 cases and *S.G.O.T.* level done in three cases showed a level between 50 and 6 units.

The four cases that ended fatally were semiconscious befor death and died within four hours of admission due to peripher circulatory failure. One died on the fourth day due to hepato renal failure.

Treatment:—(1) Stomach wash with Sodium ferrocyanide (2) treatment of shock; (3) treatment of hæmatemesis; (4) B.A.L and (5) treatment of hæmolysis.

Discussion.—Copper has been used as an emetic in doses o 5 to 10 grains and for anæmia 1/50 g.

Today we know that traces of copper are essential for the formation of hæmoglobin. In Wilson's syndrome there is gros alteration of copper metabolism. The occasional use of thi drug by quacks and for self-experimentation (as found by us) only goes to prove that beliefs die hard.

It will not be out of place to give a summary of the results of animal experiment in mammals. I.V. injection of copper slows down locomotion which becomes, clumsy, weak and then there is complete paralysis of spontaneous movements.

There is a fall of B.P., and also weakness of heart muscle. This is followed by bloody diarrhœa (copper being excreted more by the intestines) and death. There is evidence of hæmolysis of blood; on *p.m.*, fatty degeneration of the liver, kidney and heart, and ecchymosis of the kidney are the chief lesions.

There is no vomiting.

In human beings we say that if the dose is small the patient does not usually suffer from systemic effects; larger doses cause hæmolysis of the blood and act as hepato- and renal toxic agents.

Follow-up :—About 20 cases so far have been followed-up for 1 to 2 years and there has been no evidence of any complications developing.

Summary.—(1) Copper sulphate is the commonest form of poisoning in Uttar Pradesh. (2) It is commoner in Muslims and in illiterates, the younger age group being more prone. (3) Though reported often as accidental the great majority of them appear to be suicidal. (4) Hæmolysis appears only occasionally when the dose is excessive. (5) A moderate damage to the liver occurs in severe cases following the ingestion of large doses. (6) Kidney lesions are present—toxic nephritis. (7) Reticulocyte count is not altered in most cases.

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ACUTE COPPER SULPHATE POISONING

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Cases of acute copper sulphate poisoning often turn up in general hospitals for treatment. The patients usually ingest easily procurable copper sulphate for suicidal purpose, although they rarely succeed in their attempts. Copper sulphate is taken in the form of a lump, powder, or paste with other materials, or sometimes as a drink. The exact quantity ingested can rarely be ascertained, as the patient's relatives either do not know its exact weight or would not like to disclose it for obvious reasons. The signs and symptoms of poisoning vary from minimal to severe degrees and these are described in standard text books of forensic medicine where a reference is made to jaundice (Modi, 1959) in some of the cases.

In the present investigation attention is drawn to the serum copper level, jaundice, haemolysis and transient glycosuria without hyperglycaemia in some of the cases of acute copper sulphate poisoning. Liver function tests were carried out and during investigations we often noticed haemolysis in the samples of blood collected for the above purpose, particularly in severe cases of poisoning. Haemolysis, haemoglobinuria and port-wine urine had also been seen in this series, and it is of interest to note that Goldberg *et al*

(1956) referred to the less recognised haemolytic anaemia in copper sulphate poisoning. The cause of jaundice has been discussed and a brief reference has also been made to the treatment given, including administration of BAL and exchange transfusion.

Materials and Methods

Twenty cases of acute copper sulphate poisoning were admitted mostly soon after the ingestion of the substance and samples of blood were taken for investigation as early as possible on the same day or soon after. When necessary, further samples were taken for follow-up. Erythrocyte fragility tests and urine examinations were also carried out. Blood sugar was estimated when necessary, using the standard procedure. Liver function tests and serum copper estimations were done according to the following procedures.

Serum proteins were estimated by biuret method of Wolfson *et al* (1948) using Weichselbaum's (1946) biuret reagent. The colour was measured in a photo-colorimeter at 530 mμ. This method was first standardised against the standard Kjeldahl distillation method. The fractionation of proteins into albumin and globulin was carried out by Howe's (1921) procedure using 22·2 per cent sodium sulphate.

Thymol turbidity test—Maclagan's (1944) method was followed and the turbidity was measured in a photo-colorimeter at 650 mμ. The unit calculated was based on the chemical standard of Shank and Hoagland (1946).

Serum alkaline phosphatase—The methods of King and Armstrong (1934) and King *et al* (1937, 1942) in which disodium phenyl phosphate was hydrolysed with liberation of phenol and formation of sodium phosphate, were followed. Phenol was estimated photocolorimetrically at 650 mμ.

Van den Bergh test—Serum was treated with diazotised sulphanilic acid. Bilirubin present reacted to form coloured compounds, e.g., azorubin and azobilirubin. The reaction consisted of two parts, the direct and indirect reactions, the first being the qualitative one. From the diazotised solution the proteins were precipitated with saturated ammonium sulphate and ethyl alcohol following the procedure of King and Coxon (1950) and the reading of the resultant coloured solution was measured photocolorimetrically at 520 mμ. The results were calculated from the curve prepared in the same way from the known amounts of bilirubin. It is to be noted that the room temperature at which the reaction was carried out was much higher in our place, which probably affected the serum bilirubin values, particularly in summer.

Serum copper level was estimated from blood serum following the method of Cartwright *et al* (1945). This method is based on the fact that approximately 75 per cent of copper contained in serum is present in filtrates prepared by precipitation of the serum with trichloracetic acid. Three warm extractions of the trichloracetic acid precipitate remove approximately 97 per cent of the copper. Based on the above, a triple warm extraction of a trichloracetic acid precipitate of the serum was followed by photocolorimetric determination of copper with sodium diethyldithiocarbamate.

Observations

Twenty cases of acute copper sulphate poisoning were investigated over a period of years of which 6 were mild, 9 moderate and 5 severe. Mild cases were those who complained of a metallic taste in the mouth, slight pain and tenderness over the epigastric region and sometimes nausea. Moderate cases had in addition to the above, retrosternal burning sensation, vomiting, tachycardia, low blood pressure, tender liver, scanty urine with albumin and sometimes blood. In severe cases, all the above signs and symptoms were present but they were more acute. These patients complained of severe tenderness, persistent tachycardia, falling blood pressure, bluish or greenish loose stools often with blood, urine with blood, albumin and sometimes sugar. Soon jaundice developed, and some of the patients became restless and apathetic with a toxic look or became stuporose and developed muscular weakness. In this series no death was recorded (Table 1).

It was observed that acute poisoning was slightly more common in women than in men (12 : 8) and the age of the sixteen patients varied between ten and thirty years. The quantity of copper sulphate ingested was rather a guess, as it could not be ascertained from most of the patients or their relatives. It might have been anything between a few grains to four and a half ounces. In order to solve this problem, thirteen estimations of copper in serum were made in the later cases in this series. Their range was between 132 μg. and 510 μg. per cent. The estimated values of the normal controls (5 estimations) were between 125 and 130 μg. per cent. From the serum copper estimations, an idea of copper absorption was made. A reddish tinge noticed in many of the blood samples was confused for increased bilirubin but this was actually due to increased copper.

TABLE I—SHOWING DETAILS OF THE CASES AND INVESTIGATIONS INCLUDING SERUM COPPER LEVEL. ALL THE CASES WERE CURED

Case No.	Sex, age (yrs.)	Clinical types	Quantity of copper sulphate (approx.)	Days of ingestion	Liver function tests: Total protein (g./100 ml.)	Albumin (g./100 ml.)	Globulin (g./100 ml.)	Thymol turbidity (units)	van den Bergh: Direct	van den Bergh: Indirect (mg.%)	Alkaline phosphatase (K-A units)	Haemolysis, jaundice. Blood sugar (B.S.)	Erythrocyte fragility	Urine	Serum copper level (μg. %)	Treatment with BAL (250 mg./amp.)
1	F 25	Mild	Small lump	3	6·8	4·6	2·2	3	Neg.	0·2	12					
2	M 22	Moderate	Small lump	3	6·1	3·0	3·1	12	Delayed positive	1·2	16	Jaundice				
3	F 25	Severe	Paste of a lump with mustard oil	2	Serum haemolysed							Haemolysis, jaundice		Alb. RBC		BAL four hourly
4	F 47	Moderate	Small lump	2 4	6·4	3·1	3·3	16	Neg.	0·2	12		0·54%—0	Alb. RBC		BAL six hourly
5	M 40	Moderate	2 tea-spoon-fuls	3 4	Haemolysis							Jaundice, haemolysis				BAL six hourly
				5	7·2	3·5	3·7	10	Immediate positive	2·6	11			Haematuria		
				9								No jaundice		,,		
				14	6·0	3·2	2·8	9	Neg.	0·05	11					
6	F 25	Moderate	1 table spoonful	2	Serum haemolysed							Haemolysis, jaundice				BAL 2 amp. six hourly
7	F 35	Severe	½ oz.	1 6	Serum haemolysed							Haemolysis, jaundice		Alb. RBC Portwine Sugar ++ Few RBC		
				8 12	5·8	2·9	2·9	11	Neg.	0·4	21	No jaundice	0·54%—0	Alb. sugar +		BAL four hourly
8	M 23	Moderate	20 gr. ?	2 5 7								Jaundice No jaundice				
9	M 20	Moderate	Small lump	2	5·84	3·1	2·74	10	Neg.	0·3	20					
					Serum slightly haemolysed. Tinge of copper							Haemolysis				
10	F 14	Mild	10 gr.	3 4	Haemolysis—mild				Delayed positive	Icteric index—12		Haemolysis		Alb.		BAL four hourly
11	F 19	Severe	160 gr.	2 3	6·14	3·25	2·89	12				Haemolysis	0·54%—0	Alb. RBC Sugar—trace	510	BAL four hourly Blood six bottles of 280 c.c. each —exchange transfusion
					Haemolysis							,,				
				6					Delayed positive	2·4		,, B.S.—118 mg./100 ml.		Port-wine urine (0·14 mcg. ca in 12 mg. dried residue from centri-fuged deposit		

12	F 19	Severe	Drink of copper sulph.	1 3	5·86	2·7 Serum haemolysed	3·15	16	Delayed positive	1·2	25	Haemolysis, jaundice		260 (average of three normal —130)	BAL four hourly
13	M 14	Moderate	Two lumps	2	5·04	3·5	2·44	6	Neg.	0·2	16		All samples haemolysed	132 (normal control 125)	BAL six amps.
14	M 14	Mild	Small lump	2	6·62	3·95	2·67	4	Neg.	0·05	14			200 (normal control 130)	
15	F 20	Mild	15 gr. ?	1	6·24	3·8	2·44	6	Neg.	0·1	13	Haemolysis slight	0·45%—0	158	
16	M 20	Mild	10 gr. ?	2 3	6·56 6·24	3·4 3·3	3·16 2·94	10 10	Neg. Neg.	0·2 0·2	16 18			240 200	
17	F 30	Severe	4½ oz.	1 2 4 6 7 10 15	7·65 7·38 7·25	4·14 3·65 Haemolysis 3·80	3·51 3·73 3·45	6 10 9	Neg. Immediate positive Neg.	0·08 1·6 0·64	14 15 14	Haemolysis, jaundice B.S.—82 mg./100 ml. No jaundice	Alb. RBC sugar + Sugar+ Sugar+ Sugar—nil	280 260 150	BAL six hourly BAL eight hourly, total 34 amps.
18	F 30	Moderate	2 dr.	1 2 3	6·14	3·86	2·28	6	Neg.	0·1	15		Alb. Sugar—trace Sugar—nil	250	BAL four hourly
19	F 28	Moderate	1 dr.	1 2	5·92	3·1	2·82	8	Positive biphasic	1·3	13	B.S.—92 mg./100 ml. Jaundice—mild	0·45%—0	230	BAL six hourly Patient left hospital on risk bond apparently well
20	M 32	Mild	1 dr.	1	6·18	3·20	2·98	9	Neg.	0·2	18			190	

N.B.—Bluish stool in bed on the 2nd day of ingestion of copper sulphate in Case 17.

Liver function tests were carried out mostly within 3 days in all the cases except in three where the tests could not be performed on account of marked haemolysis of the blood samples drawn for the above study. Haemolysis in varying degrees was also noticed in ten samples out of fourteen of the moderate and severe cases. If the blood samples were kept for a longer time progressive haemolysis was seen in many of the samples, though the fragility of the erythrocytes, except in severe cases, was, not much altered when suspended in different strengths of saline as is done in routine fragility tests. In three severe cases it went upto 0·54 per cent saline, and in another all the samples were haemolysed.

Jaundice was present in ten cases. van den Bergh test was negative only in ten cases, delayed positive in four, transient immediate positive in two cases and biphasic in one, but in the above eleven severer cases, negative tests only were seen in five, whereas it was delayed positive in three, immediate positive in two and biphasic in one. The range of serum bilirubin was between 0·05 and 2·6 mg. per cent, the lower figures being mostly confined to milder cases and in severe cases it was between 0·2 and 2·6 mg. per cent except in one. Out of the eleven severer cases where the liver function tests could be carried out, nine cases showed a diminution of total protein which was between 5·8 and 6·14 g./100 ml. and one showed 7·2 g./100 ml. initially, but it came down to 6 g./100 ml. in nine days. The above cases showed a tendency to reversal of albumin/globulin ratio. Thymol turbidity units increased to 6-16 units in the above cases. The serum alkaline phosphatase was between 11 and 25 King-Armstrong units and it was between 15 and 25 K-A units in seven of the severer cases. Albumin and red blood cells were present in urine in most and sugar in five of the severer cases. In two cases of glycosuria, the blood sugar was within normal limits. Port-wine urine was present in two severe cases where blood samples were also found haemolysed during the most acute phase and one of these cases was saved by BAL therapy and exchange transfusion.

Discussion

A correlation of the signs and symptoms of poisoning with the amount of copper sulphate ingested appeared to us necessary but as the information about the quantity ingested was inadequate or unreliable, we had to take recourse to serum copper estimation in the later cases of our series. Thus, Case 11 gave the ingested dose as 160 gr. but her serum copper was 510 μg. per cent whereas in Case 17 the ingested amount was stated to be 4½ oz. but her serum copper was 280 μg. per cent only. This discrepancy can, however, be explained by difference in absorption from the intestines. Other local factors may play some part as well. Wyllie (1957) reported copper poisoning symptoms with as little as 5·3 - 32 mg. of copper which was precipitated by ingestion of alcohol in an empty stomach. From our control study of the few cases (five determinations), it appeared that the normal serum copper level was rather on the higher side in local inhabitants, being 125 to 130 μg. per cent. In some of our cases (thirteen determinations in ten cases) we found the value varying from 132 to 510 μg. per cent. In three severe cases it was between 260 and 510 μg. per cent, where varying degrees of haemolysis and jaundice were also observed. It appears that the haemolytic reaction in cases with serum copper level above 260 μg. per cent contributed to the development of jaundice. Further, when the blood samples and centrifuged urine samples of these cases were kept for sometime, bluish deposits could be observed sticking to the sides of the test tubes, which on chemical analysis indicated the presence of copper derivatives.

The nature of jaundice that we have referred to in our cases is ill understood (Goldberg *et al*, 1956). When a sample of blood was kept for investigation for liver function tests, varying degrees of haemolysis were soon observed in many of the severer cases, but erythrocyte fragility tests with different strengths of saline did not reveal significantly increased fragility in all such cases. Hence the blood samples were examined as quickly as possible. In some of the severest cases, blood samples drawn for the above study were found already haemolysed, partly or completely, and naturally our attention was drawn to the haemolytic anaemia and haemolytic jaundice in such cases. The direct van den Bergh reaction indicated that there were more delayed positive cases in the severer group than in the milder cases of poisoning and only in one case it was biphasic. In two severer cases it was immediate positive for a short period suggesting transient obstruction to bile flow. Serum bilirubin in one case went up to 2·6 mg. per cent. According to Hawk *et al* (1954), in normal plasma serum bilirubin value lies between 0·1 and 0·25 mg./100 ml., and low values may be seen in secondary anaemia. King (1951), however, points out that there is no generally accepted range of normal values. We often noted that the serum bilirubin values were much lower than what we expected, but this unexpected feature was not uniformly seen throughout the year. The above discrepancy has been explained

by Nath *et al* (1960) to be due to higher temperature at which diazotization is carried out. They have shown that at a higher temperature diazotization is hampered wholly or partly and this accounts for low values for bilirubin. Thus, it seems probable that serum bilirubin figures might have been much higher if the temperature of the reaction was controlled.

It was also observed in moderate and severe cases that there was a tendency to diminution of total proteins with reversal of albumin/globulin ratio, the values of total proteins being 5·8-6·4 g./100 ml., as against normal values 6·3-8·2 g./100 ml. (Bayliss, 1954). From all available information, it appears that cases of liver disease were not included in the present series and it may possibly be inferred that acute copper sulphate poisoning produces hepatocellular dysfunction leading to the changes stated above. This view was further strengthened by the finding of higher values of thymol turbidity units in this series—the normal range being 0 to 4 units (Bayliss, 1954). Serum alkaline phosphatase was also higher in the more severe cases though it did not exceed 25 K-A units—the normal being 3 to 13 K-A units per 100 ml. (Bayliss, 1954). Thus, the hyperbilirubinaemia in the more severe cases may be interpreted in the light of the above findings to be partly due to hepatocellular damage and/or temporary obstruction to bile flow. We may mention here that clinically, the liver was found to be tender on palpation in the severe cases under report. Modi (1959) recorded soft and fatty liver in fatal cases. These findings suggest involvement of the liver in acute poisoning and the liver function tests in our series also supported this view. The delayed positive van den Bergh test, progressive haemolysis of the blood samples on keeping, increased fragility in some and haemolytic reaction along with port-wine urine of haemoglobinuria in others suggest that jaundice may also partly be due to haemolytic reaction from elevated serum copper level which often imparts reddish tinge to the serum. Intravascular haemolysis may occur in severe copper sulphate poisoning. How far the copper level and the liver are involved in this reaction is difficult to surmise from the present study. Goldberg *et al* (1956) have shown that copper in low concentration causes agglutination and/or haemolysis when incubated with chicken, rabbit or human erythrocytes suspended in saline. They found that copper fragility studies with human erythrocytes suspended with 0·9 per cent saline indicated that (1) normal human erythrocytes begin to haemolyse at 75 μg. per cent of copper concentration, and (2) agglutination begins at 83 μg. per cent (75 to 100 μg.). Copper, however, failed to do so even at a higher concentration when erythrocytes were suspended in normal serum. In a total of 150 determinations of plasma copper in 105 normal individuals, Cartwright *et al* (1948) found the range to be between 116·2 and 121·0 μg. per cent, and the mean ± standard error of the mean was 118·6 ± 1·2 μg. per cent. A few determinations of serum copper level in normal individuals in our series gave a higher figure of 125 to 130 μg. per cent. It appears that copper even at such a high concentration will not lead to haemolysis possibly due to the erythrocytes being suspended in normal serum. But in poisoning when the serum copper level is much elevated and liver functions are deranged, haemolytic reactions may set in. Goldberg *et al* (*loc. cit.*) also produced haemolytic anaemia in the chicken by administering copper. It is suggested that the erythrocytes are destroyed in the liver after exposure to excessive copper concentration in that organ. Alternatively, they suggest that there is an increased concentration of copper in red blood cells leading to increased susceptibility to their destruction, as shown also by other studies on copper metabolism (Gubler *et al*, 1953; Bush *et al*, 1955). Albiston *et al* (1940) in drawing attention to the importance of haemolysis in copper poisoining reported enzootic jaundice in Australian sheep characterised by haemolytic anaemia and increased liver copper. They also suggested a mechanism of sudden mobilisation of copper from liver to plasma causing intravascular haemolysis, whereas Goldberg *et al* (*loc. cit.*) have drawn attention to erythrophagocytosis in liver.

We also observed glycosuria in severe acute copper sulphate poisoning which is probably due to liver damage or from lower threshold from renal tubular changes, as in two cases blood sugar recorded during glycosuria was 118 mg. and 82 mg. per 100 ml. respectively. This liver damage causing plasma protein changes, albuminuria and temporary glycosuria without hyperglycaemia in acute copper sulphate poisoning, reminds one of its similarity to hepatolenticular degeneration (Sherlock, 1958) associated with increased tissue copper and to Debre-de Toni-Fanconi syndrome (Sherlock, 1958), in both of which aminoaciduria may be associated with liver damage and glycosuria with normal glucose tolerance curve and renal tubular changes. Lastly, BAL therapy tried in our cases seems to be of definite value in the treatment of severe cases of poisoning and exchange transfusion along with BAL saved the life of one of our severest cases with marked intravascular haemolysis and persistent haemoglobinuria.

Summary

Twenty cases of acute copper sulphate poisoning of varying degrees of severity were studied. The majority of patients were young women who ingested copper sulphate for suicidal purpose. The maximum serum copper level in the observed series was 510 μg. per cent in cases of poisoning and was over 250 μg. per cent in cases associated with haemolytic reaction. The range in five cases of normal controls was between 125 and 130 μg. per cent. In severe cases of poisoning bluish copper derivatives could be seen separating out from blood and urine samples. Three of the severer cases had increased fragility of the erythrocytes ranging from 0 to 0·54 per cent saline and in another case all the samples were haemolysed. Clinically, jaundice was observed in ten cases in five of which varying degrees of progressive haemolysis were encountered in blood samples kept for liver function tests and elevated serum bilirubin was seen in seven cases where it could be estimated and in the remaining three, liver function tests could not be performed due to marked haemolysis. Of these cases three had delayed positive, two indirect and one biphasic van den Bergh reaction. The serum bilirubin values were between 0·2 and 2·6 mg. per cent in ten of the eleven severer cases. The probable reason for the low values of serum bilirubin even in clinically jaundiced patients has been discussed. The cause of jaundice appeared to be hepatocellular or haemolytic and sometimes obstructive damage. Liver function tests revealed in nine moderate and severe cases diminution of total protein to 5·8 - 6·14 g./100 ml, with a tendency to reversal of albumin/globulin ratio. In these cases thymol turbidity was elevated to 6-16 units and serum alkaline phosphatase to 13-25 K.A. units (where it could be performed), suggesting hepatocellular damage. Transient immediate positive van den Bergh reaction was seen in two cases which indicated temporary obstruction to the bile flow. The delayed positive van den Bergh reaction in four cases, progressive haemolysis of the blood samples, increased fragility of the erythrocytes, haemolysis and haemoglobinuria in some of the above cases suggest that jaundice is at least partly due to haemolytic reaction in severe cases of acute copper sulphate poisoning. Transient glycosuria was observed in five of the acute cases and in two of them blood sugar was normal which suggests lowering of renal threshhold due to renal tubular changes. BAL therapy appeared useful in the severer cases and exchange transfusion along with BAL saved the life of one of the severest cases of our series.

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Acute Copper Sulfate Poisoning*

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Acute copper sulfate poisoning is common in Delhi and is implicated in about one-third of the cases of poisoning encountered in the Irwin Hospital, New Delhi (Table I). Copper sulfate is a cheap, readily available substance used mostly in whitewashing and the leather industry. It is quite commonly employed as a suicidal poison among people in the lower income group in India. Occasionally, it is the cause of accidental poisoning in children [1].

An attempt was made to determine the incidence of suicide with this poison in other parts of India since there is a general impression that it is not a common agent in areas culturally and ethnologically different from this metropolitan area. Data from various hospitals were analysed. Although the data have limitations, they do give some idea of the prevalence of copper sulfate poisoning in other parts of the country. The maximum incidence (200 to 300 cases per 10,000 hospital cases) was found in Delhi and Uttar Pradesh. The next greatest incidences occurred in West Bengal, Rajasthan, Madhya Pradesh and East Punjab. Prevalence was lowest in Kashmir, Gujarat, Maharashtra and the southern states. It was reported that copper sulfate is not used for this purpose in Assam, Bihar and Himachal Pradesh. The reasons for such a varying prevalence of this form of poisoning in different parts of the country are not clear.

Copper sulfate causes a metallic taste in the mouth, a burning sensation in the epigastrium, eructations and repeated vomiting of greenish material. In severe cases diarrhoea, shock, smoky urine, oliguria, anuria, uraemia, an enlarged tender liver and jaundice may occur [2-4].

Acute poisoning with copper sulfate has not been studied intensively. In an earlier series [3] it was reported that in the past the mortalities were numerous, but since the introduction of corticosteroid therapy the prognosis has improved somewhat.

MATERIAL AND METHODS

The present study comprises fifty-three subjects, forty-eight who sought admission to this hospital and five from whom autopsy material was made available after they had died of acute copper sulfate poisoning in other hospitals in the city. Patients admitted to the paediatric unit were not included.

For each patient admitted to the hospital a detailed form was used to record the clinical features and to maintain regular progress notes. Although efforts were made to determine the quantity of copper sulfate ingested, the information provided often was not reliable because those who had attempted suicide hesitated to divulge the exact quantities they had taken.

In each patient vomit was tested for the presence of copper sulfate, using ammonium hydroxide [5]; the urine was examined for various abnormal constituents, especially urobilinogen and haemoglobin, and blood was tested for haemoglobin, red blood cell count, total and differential leukocyte counts, reticulocyte count, blood urea, serum bilirubin, zinc sulfate turbidity, alkaline phosphatase, serum glutamic oxalacetic transaminase (SGOT) and prothrombin time. The direct-reacting (ionic) fraction of plasma copper, total serum copper and whole blood copper were estimated by methods described by Gubler et al. [6]. A fractional test meal was given to twenty patients and a barium meal study carried out in sixteen. A liver biopsy was performed in thirty-three patients and a kidney biopsy, using the Vim-Silverman needle, was performed in four. Autopsy material was available in nine cases.

COMMENTS

The age range was fourteen to sixty years (average twenty-three and six-tenths years). The majority (71 per cent) of patients were between sixteen and twenty-five years of age which is significant statistically as only 61.5 per cent of the total attendance in the casualty department was in this age group. (Table II.) Two-thirds (thirty-two) of the patients were male and one-third (sixteen) were female.

Quantity of Copper Sulfate Ingested. Reliable

* From the Departments of Medicine and Pathology, Maulana Azad Medical College, New Delhi, India. Manuscript received December 22, 1964.

TABLE I
INCIDENCE OF VARIOUS CASES OF POISONING IN IRWIN HOSPITAL IN 1961*

Nature of Poisoning	Percentage
Copper sulfate	33.6
Dhatura	23.5
Barbiturate	9.2
Opium	5.0
Caustic (chlorine, ammonia)	6.3
Kerosene oil	3.7
Rat poison (barium carbonate)	3.3
Acids and alkalies	5.9
Naphthalein and dettol	2.5
Arsenic, mercury	2.5
Miscellaneous	4.5

* Total number of cases = 238.

TABLE II
AGE DISTRIBUTION OF COPPER SULFATE POISONING

	1-15	16-25	26-35	36-45	46-55	55+
No. of cases	3	34	8	2	1	Nil
Percentage of cases	6.5	70.7	16.7	4.2	2.1	Nil
Percentage of casualties	30.8	31.5	19.3	8.6	5.2	4.6

TABLE III
QUANTITY OF COPPER SULFATE INGESTED AND ITS RELATION TO THE LEVELS OF SERUM AND WHOLE BLOOD

Quantity Ingested (gm.)	Copper Level (µg./100 ml.) Serum	Whole Blood
1-5	264.6 ± 181.2	383.4 ± 124.9
6-15	332.4 ± 112.4	519.0 ± 134.3
16-30	346.2 ± 280.2	684.0 ± 513.2
30+	239.5 ± 78.1	605.5 ± 146.3
Normal values	151.6 ± 75.0	217.0 ± 52.0

TABLE IV
INCIDENCE OF VARIOUS CLINICAL FEATURES

Clinical Features	No. of Cases	% of Cases
Metallic taste, nausea, vomiting and burning in epigastrium	48	100
Diarrhoea	14	29.1
Jaundice	11	23.0
Haemoglobinuria and/or haematuria	14	29.1
Anuria	13	27.0
Oliguria	5	10.3
Hypotension	4	8.3
Coma	4	8.3
Melaena	1	2.1

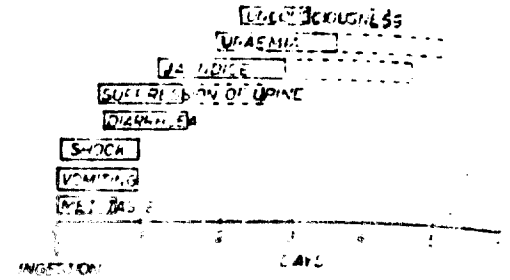

FIG. 1. Diagramatic representation of the order of appearance of various manifestations after the [illegible] of copper sulfate.

information regarding the quantities of copper sulfate ingested was not available. According to statements obtained from the patients, the amount varied from 1 gm. to 4 ounces. The poison, in the form of crystals or powder, was swallowed with water. No correlation between the amount of copper sulfate ingested and the level of serum copper was found, although there appeared to be some correlation between the amount ingested and the whole blood copper values. (Table III.)

Clinical Features. The symptoms presented by the patients are given in Table IV.

Gastrointestinal Symptoms. Metallic taste, nausea, burning in the epigastrium and repeated vomiting of greenish material were the chief symptoms. (Fig. 1.) The episodes of vomiting ranged from two to ten. In the first few episodes the vomit was greenish blue due to the presence of copper sulfate. The vomiting usually subsided with stomach washout, although nausea and anorexia persisted. Diarrhoea occurred in fourteen patients, usually appearing on the first or second day and lasting for a maximum of 24 hours. On the average, four or five loose stools were passed. Only one patient had frank melaena.

The cause of the gastrointestinal symptoms is presumed to be injury of the mucosa by copper sulfate. The fractional test meal given to twenty patients on the third or fourth day showed the presence of blood in all patients and decreased free and total acidity in ten. Barium meal studies performed in sixteen patients during the second week of the hospital course did not demonstrate any roentgenologic evidence of injury; however, erosions of gastric mucosa, if there were any, could have healed by this time. The gastric mucosa from patients who had died was stained green and showed superficial and deep erosions

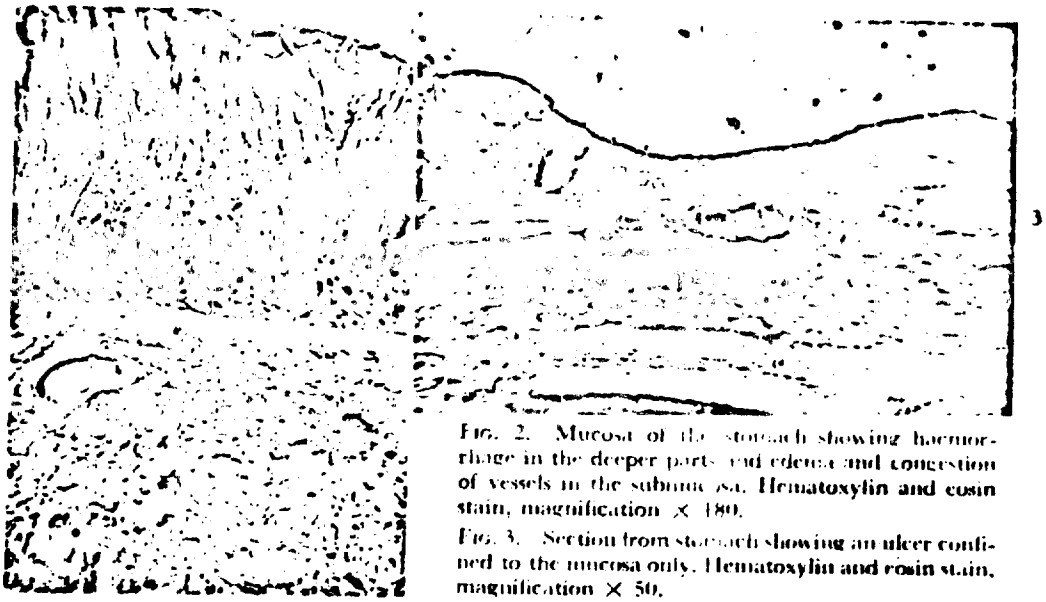

FIG. 2. Mucosa of the stomach showing haemorrhage in the deeper parts and edema and congestion of vessels in the submucosa. Hematoxylin and eosin stain, magnification × 180.

FIG. 3. Section from stomach showing an ulcer confined to the mucosa only. Hematoxylin and eosin stain, magnification × 50.

The small intestine showed areas of haemorrhage. Microscopic examination of the stomach disclosed haemorrhage in the mucosa as well as edema and congestion of the vessels in the submucosa (Fig. 2); mucosal ulcers were also observed. (Fig. 3.)

Jaundice. Jaundice appeared in eleven patients (23 per cent). It invariably occurred on the second or third day. In those who died the jaundice had deepened gradually until the time of death, but in the other patients it lasted for only two to five days. These patients can be divided into two groups. In the first group consisting of five patients jaundice was deep, the liver was enlarged and tender, the average serum bilirubin was 11.2 ± 8.9 mg. per 100 ml. and the results of liver function tests showed gross derangement. (Table v.) The liver biopsy in these patients showed centrilobular necrosis (Fig. 4) and biliary stasis. In the one patient in this group who died, examination of the liver at autopsy revealed severe histologic changes. (Fig. 5.)

In the second group (Table v) consisting of six patients jaundice was mild, the liver was either not palpable or just palpable, and nontender. The results of liver function tests were within the normal range (Table v) but the reticulocyte count was slightly increased and the urobilinogen was markedly increased or haemoglobinuria was present. In these patients the biopsy specimen of liver was normal histologically. In one patient in this group who died, the liver on microscopic examination showed dilatation of the sinusoids and central veins. In the first group the predominant cause of the jaundice appeared to be toxic liver injury, whereas in the second group it was intravascular haemolysis.

There was a third group of eight patients in whom jaundice was not present and the serum bilirubin was within normal limits but the urobilinogen was increased markedly and the reticulocyte count was increased (average 3.5 ± 1.3 per cent). Biopsy specimens of liver in these

TABLE V
ANALYSIS OF CASES OF JAUNDICE

Studies	Group I (5 cases)	Group II (6 cases)
Liver	Enlarged 2-3 cm., tender	Not palpable or just palpable, nontender
Serum bilirubin (av., mg./100 ml.)	11.2 ± 8.9	3.03 ± 0.98
Zinc sulphate turbidity (av., units)	6.6 ± 1.2	2.8 ± 0.97
Prothrombin time (av., sec.)	35.25 ± 4.56	18.2 ± 2.
S.G.O.T. (av., units)	252.4 ± 142	144 ± 80
Urobilinogen	Normal	Increased
Bile salts, pigments in urine	Present	Absent
Liver histology	Centrilobular necrosis, biliary stasis	Normal or focal areas of necrosis in some patients

FIG. 4. Liver showing centrilobular necrosis. Hematoxylin and eosin stain, magnification × 180.

FIG. 5. High power view of liver tissue showing centrilobular biliary stasis. Hematoxylin and eosin stain, magnification × 600.

patients showed no pathologic changes. The haemolytic reaction may have been so mild that it did not give rise to an increase in serum bilirubin.

Several workers [2,3,7-9] have demonstrated, in experimental or clinical studies, haemolysis of red blood cells caused by copper salts. It has also been observed that injury of the liver parenchyma can be produced by giving injections of copper sulfate to rats [10].

Haemoglobinuria and Haematuria. The urine became smoky or red-coloured on the second or third day in fourteen patients. Examination revealed the presence of red blood cells (six patients) and haemoglobin (eight patients). Both manifestations usually cleared by the fourth or fifth day. Eight patients also had jaundice and in all fourteen suppression of urine developed with a fatal outcome in three.

Suppression of Urine. Significant oliguria was observed in five patients and anuria occurred in thirteen. Anuria appeared within 24 to 48 hours after the ingestion of copper sulfate and lasted from 12 to 56 hours (average 23.7 ± 7.9 hours) in patients who survived. The blood urea in-

FIG. 6. Kidney showing dilated and congested glomerular capillaries. Hematoxylin and eosin stain, magnification × 180.

FIG. 7. Proximal convoluted tubules showing the lining cells in various stages of necrosis and desquamation into the lumen. Hematoxylin and eosin stain, magnification × 600.

FIG. 8. Kidney showing haemoglobin casts. Hematoxylin and eosin stain, magnification × 180.

creased in seven patients and ranged from 47 to 395 mg. per 100 ml. (average 118.1 ± 122.1 mg. per 100 ml.). Before the onset of anuria and even subsequently the blood pressure remained normal. Therefore hypotension could not have been the cause of the suppression of urine. Biopsy specimens of kidney tissue showed swelling or necrosis of the tubular cells in two patients. In the four patients in the group with suppression of urine who died, the kid-

neys on gross examination were swollen and congested. On histologic examination there was congestion of glomeruli (Fig. 6), necrosis and denudation of tubular cells (Fig. 7), and in some instances haemoglobin casts were present. (Fig. 8.) The presence of haemoglobin casts suggests that the kidney lesion could have been due to excessive haemolysis. Vogel [9], after giving intraperitoneal injections of copper thymin complex to mice, found lesions of the renal tubules which were filled with cellular debris. Many of the basement membranes were ruptured and erythrocytes were present in the tubules. There were no haemoglobin casts but there was deposition of copper in degenerated tubules. It is possible that renal damage could have been due to a direct action of copper.

Hypotension. Hypotension occurred in four patients, on the first day except in one patient. The exact cause of shock is not known. It could have been due to oligaemia resulting from excessive vomiting and diarrhoea. Plasma volume studies were not carried out in these patients, but the vomiting and diarrhoea were not of a degree severe enough as ordinarily results in hypotension. There is experimental evidence [7] to support the view that copper sulfate has a cardiotoxic action in animals and this could have been the cause of hypotension in these patients. Shock was of bad prognostic significance as three patients of the four died.

Coma. Coma occurred in four patients, developing on the second day or later. Probably it was due to uraemia resulting from renal damage. The blood urea values in these patients were high (mean 118 mg. per 100 ml.). Two patients recovered and the other two died. It seems unlikely that copper sulfate has a direct toxic effect on the brain as those patients in whom uraemia did not develop remained conscious even though serum copper levels were high. Wolff and Tenn [11], after administering intraperitoneal injections of copper to albino rats daily for 256 consecutive days, and not find brain lesions or increases in the copper content of brain tissue. None of our patients showed any evidence of extrapyramidal lesions, suggesting that acute copper sulfate poisoning does not injure basal ganglia as it does in a chronic condition such as hepatolenticular degeneration.

Deaths. Of the forty-eight patients studied, seven died (14.6 per cent). Deaths occurring within 24 hours after ingestion were due to shock, whereas deaths occurring at a later stage

TABLE VI
AVERAGE SERUM AND WHOLE BLOOD COPPER

Cases	Copper (μg./100 ml.)	
	Total Serum	Whole Blood
Normal	151.6 ± 75	217 ± 52
Poison		
Mild*	294.2 ± 126.7	287.0 ± 126.8
Severe†	333.4 ± 214.6	798.0 ± 396.4
Total	310.7 ± 180.5	727.2 ± 600.04

* Gastrointestinal symptoms only.

† Jaundice, renal manifestations or shock in addition to gastrointestinal symptoms.

TABLE VII
SERUM IONIC COPPER

Cases	Serum Ionic Copper (μg./100 ml.)
Normal	21.8 ± 20
Poison	
Estimation within 12 hours of ingestion	257.20 ± 144.2
Estimation after 12 hours of ingestion	23.4 ± 12.4
Total	140.03 ± 155.6

appeared to be due to hepatic or renal complications or both.

Copper Estimation. Serum total copper was estimated in forty-one patients, whole blood copper in twenty and direct reacting (ionic) copper in fourteen. (Tables VI and VII.) Although the serum total copper levels were increased in these patients with copper sulfate poisoning, no correlation could be established between the levels of serum copper and the severity of symptoms. However, a significant relationship was found between the levels of whole blood copper and the severity of the disease, as the values of whole blood copper in mild cases were 287 ± 126.8 μg. per 100 ml. and in severe cases 798 ± 396.4 μg. per 100 ml. (Table VI.)

The values for serum ionic copper were about seven times higher in patients with poisoning than in normal subjects. (Table VII.) The levels of serum ionic copper were much higher (257.2 ± 144.2 μg. per 100 ml.) when determined within 12 hours after ingestion of the poison than the values obtained (23.4 ± 12.4 μg. per 100 ml.) when the tests were performed at a later time. Furthermore, the initially high levels of

rum ionic copper were considerably lower when tests were repeated on subsequent days. From these observations it may be concluded that after ingestion of copper sulfate, copper is absorbed quickly from the gut and starts to move almost immediately at a rapid rate into the red blood cells where it achieves a very high concentration.

Gubler et al. [6], after administering copper to mice, observed an increase in direct-reacting (ionic) copper which quickly reverted to normal values in 4 hours. These investigators also observed an increase in red blood cell copper starting 10 minutes after the administration of copper. This is probably the reason why there is no correlation between the serum copper levels and the severity of symptoms, whereas there is a distinct correlation between the levels of whole blood copper and the severity of various manifestations. It is suggested that to assess the prognosis of patients with acute copper sulfate poisoning the blood copper should be estimated. Goldberg et al. [8] demonstrated haemolysis of the red blood cells and suggested that an increased concentration of copper in erythrocytes may make them vulnerable to haemolysis. The majority of the patients in this series with high levels of whole blood copper showed evidence of haemolysis.

SUMMARY

Acute copper sulfate poisoning constituted 33.6 per cent of all cases of poisoning admitted to the Irwin Hospital, New Delhi, in 1961. The highest incidence was found in the hospitals of Delhi and Uttar Pradesh.

Forty-eight cases of acute copper sulfate poisoning were studied, with special reference to clinical features and for evidence of biochemical and histopathologic injury. An autopsy was performed in nine fatal cases. The levels of serum total copper, serum ionic copper and whole blood copper were found to be much higher in these patients than in normal subjects. A significant correlation was found between the levels of whole blood copper and the severity of manifestations.

There were superficial or deep ulcerations of gastric and intestinal mucosa. Histologic examination of the liver revealed dilatation of central veins, varying degrees of liver cell necrosis and bile thrombi. In the kidneys there was congestion of glomeruli, swelling or necrosis of tubular cells, and in some cases haemoglobin casts were observed.

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ARTICLES

MENKES'S KINKY HAIR SYNDROME

An Inherited Defect in Copper Absorption With Widespread Effects

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ABSTRACT. Seven new cases of Menkes's kinky hair syndrome are described from five families. These patients were seen in a period of 3 years in Melbourne and the frequency of the disease is estimated to be 1 in 35,000 live births. Seven other affected males were present in these families and each pedigree was compatible with X-linked inheritance.

Hypothermia was noted to be an important clinical feature which has escaped previous attention. Widespread arterial tortuosity and variation in the lumen of arteries has been demonstrated by arteriography and by microscopic examination at necropsy. Fragmentation of the internal elastic lamina is seen in the arterial walls with thickening of the intima in the most severely affected vessels. This process can lead to complete obliteration of major arteries. Involvement of cerebral arteries is the probable cause of the gliosis and cystic degeneration seen in the brain. Metachromasia was observed in fibroblasts cultured from patients and from heterozygous females.

Low levels of serum copper and ceruloplasmin were found in all patients studied and a defect in the intestinal absorption of copper has been demonstrated. This appears to be the basic defect in the disease and copper deficiency provides an adequate explanation of all the features of the disease. Parenteral administration of copper may prove therapeutically effective.

These findings suggest many new lines of research on copper metabolism and trace metal deficiency. *Pediatrics*, 50:188, 1972, KINKY HAIR, MENKE'S SYNDROME, ARTERIAL DEGENERATION, ELASTIC FIBERS, COPPER DEFICIENCY, COPPER ABSORPTION.

IN 1962 Menkes, *et al.*[1] described a syndrome characterised by slow growth, progressive cerebral degeneration with death before 3 years, pili torti, and X-linked recessive inheritance. Since that time cases have been reported from only five other families.[2-6] Consequently, the literature gives the impression that this disease is extremely rare. This cannot be true because seven cases from five families have been seen at the Royal Children's Hospital, Melbourne, in the past 3 years. Family histories revealed six other probable cases in earlier generations of these families, and a cousin of one patient has recently been diagnosed in Sydney.

This paper describes these seven cases and their families, indicates the possible reasons for under-diagnosis, and discusses the main clinical features. A defect in copper metabolism is described and copper deficiency is postulated to be the cause of all the features of the disease, including newly described abnormalities of the elastic fibers of arteries.

CASE REPORTS

Case 1

J.B., male, was delivered uneventfully at term after labor was induced by rupture of the membranes. He weighed 3.24 kg (50th percentile for gestational age) and was stated to have progressed satisfactorily until he presented at the age of 2 months with convulsions. The convulsions were easily controlled, but he remained lethargic and floppy, and hypothermic (33-35°C) for the first week in hospital. He then improved gradually and became able to maintain his temperature later,

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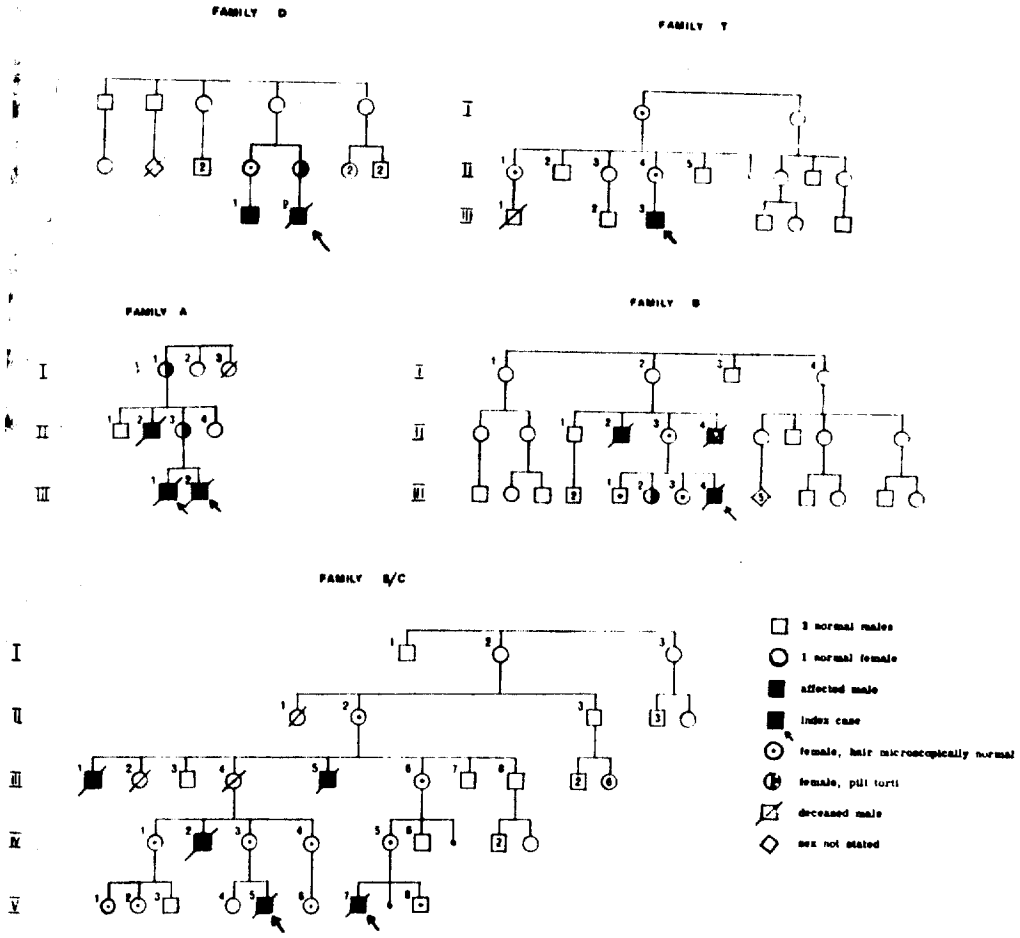

FIG. 1. Pedigrees of the five families described. Details regarding some family members appear in the text.

but mental retardation, mild generalized muscular hypertonia, and a lack of spontaneous movement became apparent.

His hair appeared sparse but was really broken short leaving a palpable stubble, especially in occipital and temporal regions. On the vertex hair was about 1 cm long, rather tangled, lusless, and almost colorless. The scalp was scaly d mild seborrhea was present on the face. Micropically the hair showed pili torti.

During the remaining 6 months of his life he deloped no new skills, and the lack of movement d mild hypertonia persisted. Drowsiness and yoclonic jerking increased. Episodes of hypothermia occurred spontaneously during the winter onths, and his temperature became very unstable uring respiratory infections. He died during a sere attack of measles, at the age of 8 months.

Growth had been normal. His weight was close to the 50th percentile throughout, and his height was at the 5th percentile at 2 months, and at the 25th percentile when he died. Head circumference was above average at 2 months, but at the 2nd percentile by 8 months.

Radiologically, Wormian bones were apparent in the lambdoid suture at 2 months. Metaphyseal changes (see below) developed in the femur at 4 months, and in the ribs and radii at 5 months. At 8 months, striking changes developed in the upper metaphyses of the humeri.

Peripheral blood examinations, serum electrolyte and acid-base studies, serum calcium, blood glucose, thyroid function studies and serum cholesterol were normal. Amino acid chromatography of urine and serum revealed no consistent abnormalities. Electroencephalography showed diffuse abnormality with multi-focal sharp waves. Cerebrospinal fluid was normal at 2 months.

Muscle biopsy performed at 5 months was normal to light and electron microscopy and contained

FIG. 2. Typical appearance of face and hair (J.T. at age 5 months).

1.7% glycogen (normal 0 to 2%). Spectroscopy revealed normal muscle cytochrome peaks (Prof. F. Gibson). Peripheral leucocyte culture showed a normal male karyotype.

Necropsy revealed a small brain weighing 454 gm and showing atrophy of the cerebral gyri and cerebellar folia with cystic degeneration of the grey matter of the frontal lobes. The lateral and third ventricles were moderately dilated. Microscopically there was extreme neuronal loss and gliosis together with degeneration and cyst formation in both grey and white matter. There was a mild accumulation of lymphocytes and larger mononuclear cells in the meninges and some of the mononuclear cells contained hemosiderin.

The cerebral arteries were extremely dilated, the thickness of the walls was irregular, and small outpouchings were visible. The internal elastic lamina in the larger vessels was irregular, but there was no significant intimal hyperplasia.

Systemic arteries showed splitting, irregularity, fragmentation and focal loss of the internal elastic lamina, associated with extreme hyperplasia of the overlying intimal layer. These changes were particularly noted in the mesenteric vessels, but could be seen in the aorta, the carotid, splenic, renal and coronary arteries. The changes were most noticeable at points of branching.

The scalp hair showed the typical rotational twists of pili torti. Sections of the scalp revealed many hair follicles which extended deep into the dermis. These were small and the hair shafts developing within them were slender and apparently brittle since most had broken off below the skin level in the sectioning process. Several hair follicles contained no hair shafts and were filled with masses of keratin.

Bone sections stained with hematoxylin and eosin appeared normal, but under polarized light the cortical bone of the rib showed a pronounced woven pattern instead of the usual lamellar pattern.

Case 2

D.S. was delivered uneventfully at 36 weeks' gestation. The baby weighed 2.15 kg (20th percentile for gestational age) and was lethargic from birth. Intragastric tube feeding and careful temperature maintenance were required for 3 weeks. He was admitted at 5 weeks because of extreme lethargy, refusal of feedings, hypothermia, and circulatory collapse. He gradually improved with intravenous fluids, antibiotics and warming, but severe motor and intellectual retardation was apparent. Convulsions developed at 5 months and an electroencephalogram showed diffuse multifocal epileptic activity.

The diagnosis was made at 6 months when the facies and hair abnormality were recognized. Microscopic examination of the hair confirmed the diagnosis. The scalp was scaly.

Over the next 8 months he deteriorated slowly in mental state and physical health with episodes of urinary tract infection, respiratory infection and hypothermia. He died rather abruptly at 14 months of age, without a clinically obvious final cause.

Radiological changes were seen in the skull, ribs, and femoral metaphyses. Blood examinations, serum electrolytes, serum calcium, blood sugar, tests of adrenal function, and urinary amino acid excretion were normal. Thyroid function studies were not performed. Muscle cytochromes were normal on spectroscopy (Prof. F. Gibson).

Necropsy revealed widespread atrophy and cystic changes in the brain and a moderate-sized left subdural hematoma. Changes in the arterial walls were widespread throughout the cerebral and visceral vessels examined. Hair and rib ends showed changes similar to those seen in J.B. The liver copper content was found to be 14.7 μg per gm dry weight.

Case 3

B.C. was born at 36 weeks' gestation after normal pregnancy. He was a cousin of D. He weighed 2.97 kg (80th percentile for gestational age), but fed poorly. Repeated hypothermia and vomiting led to admission at 6 weeks of age. He had unusual facies and severe mental retardation.

noted, ...d his hair was abnormal. The diagnosis was no... made until after his death when D.S. was seen a... reviews of photographs showed the characteristi... facies and hair. During the 18 months that he ...urvived, extreme mental retardation, myoclonic ...ilepsy, hypothermia, and recurrent infections ...e the main features. A scaly skin rash waxed ...d waned. He died in a private nursing home ...he age of 20 months and no autopsy was perfor...d.

Marked cupping of the anterior ends of the ribs was described in a chest x-ray at the age of 7 months, but limb films were not taken.

Case 4

S.A. was born at 36 weeks' gestation and weighed 2.41 kg (40th percentile for gestational age). He fed poorly and gained weight slowly. Jaundice was unusually severe, but did not require treatment. He was referred because of an enlarging head and one very brief convulsion. A large subdural hematoma was aspirated repeatedly over the next 2 weeks, but neurological abnormality persisted. Skeletal x-rays showed no fractures, but physical maltreatment was suspected. Unfortunately, these x-rays were subsequently destroyed.

Severe mental retardation, repeated respiratory infections, episodes of hypothermia, and seborrheic dermatitis persisted. The hair was described as sparse, but pili torti was not recognized until 15 months of age. He died suddenly at home before further investigations were arranged.

Necropsy by the City Coroner's pathologist revealed cerebral gliosis with areas of cystic degeneration, but no further details are known.

Case 5

M.A., the younger brother of S.A., was born at 36 weeks' gestation and weighed 2.27 kg (30th percentile for gestational age). Jaundice developed at 36 hours of age, required an exchange transfusion, and persisted for 10 days. Diarrhea due to intolerance of all monosaccharides developed, and persisted for 4 weeks. His temperature was unstable for 3 weeks.

...e had a moderate amount of fine hair at birth ... microscopic examination of several samples ...ed no abnormality. He was discharged home ... 0 weeks of age. At 14 weeks he was growing ..., and was smiling and active. However, at 18 ...ks he had ceased to smile and he looked dull ... abnormal. Some short new hairs showed pili ... After this he deteriorated steadily, and hypo...mia, repeated infections, and myoclonic epi...sy developed. Seborrheic dermatitis was a per...ent problem. Ascorbic acid (100 mg daily) was ...en throughout his life and trials of riboflavin (60 ... daily), nicotinamide (500 mg daily), pyridoxine (50 mg daily), and sodium glutamate (3 gm daily) were given without effect upon the course of the disease. He died of bronchopneumonia at 10 months of age.

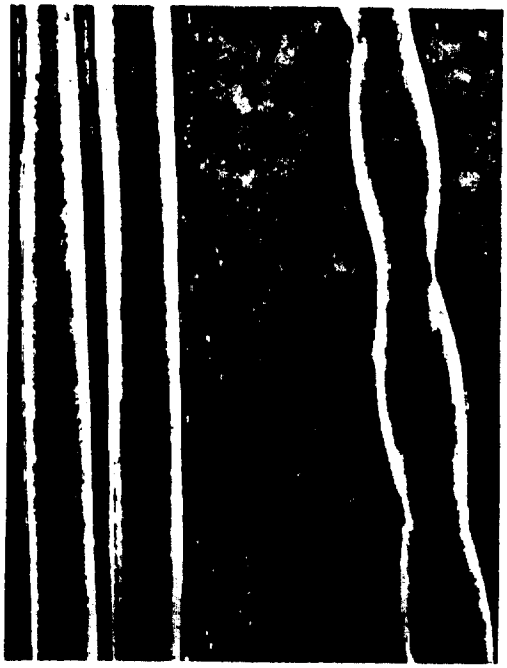

FIG. 3. Hair photographed with phase contrast illumination to show pili torti.

Blood examinations, serum electrolyte estimations, thyroid function studies, and urinary amino acid chromatograms were within normal limits. Serial x-rays of the limbs and chest showed no metaphyseal abnormalities. Unfortunately no films were taken of the skull.

Necropsy revealed a moderately atrophic, firm brain weighing 610 gm (normal 852 gm). Vessels around the base were prominent and irregular in

FIG. 4. Spurs on the proximal femoral metaphyses (P.D. at 4 months of age).

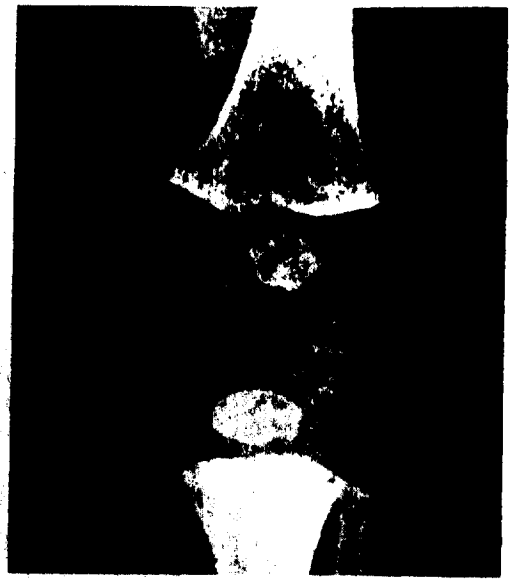

FIG. 5. Metaphyseal spurs on the lower end of the femur (P.D. at 5 months).

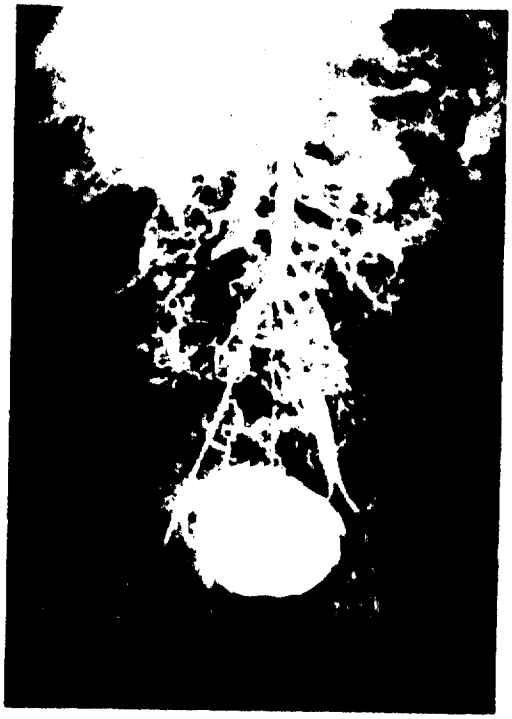

FIG. 6. Aortogram showing widespread arterial changes. The right renal artery is narrowed proximally and dilated and tortuous distally. The left common iliac artery is narrowed. (J.T. at 4 months of age.)

diameter with apparent aneurysmal dilatations. Mi croscopically the brain showed widespread glios associated with neuronal loss.

The cerebral arteries were thin walled, tort and dilated. Elastic tissue, however, appe within normal limits. Systemic arteries showed ilar changes to those observed in the pre cases. Tortuous arteries were seen in the left l limb on postmortem angiography (Fig. 8).

Case 6

J.T. was born at 36 weeks' gestation weighed 2.24 kg (30th percentile for gesta age). He grew well and weighed 3.55 kg weeks, when he was admitted because of hypo mia (28.9°C). He was mildly edematous an facial appearance and hair suggested Menkes's drome. Hair was cut from behind the ears wh was longest and appeared normal, but the sparse hair from the vertex showed pili torti.

His subsequent developmental failure, recu hypothermia, lethargy, myoclonic jerking, and s ceptibility to infection have been typical of t syndrome. Hair abnormality became more obvio macroscopically and microscopically. At the ti of reporting he was 11 months old.

A few Wormian bones were apparent in the s gittal suture posteriorly at 6 weeks. Changes in t rib ends and proximal femora were seen at months and have persisted. Other bones have a peared normal.

Retinal photographs showed no abnormality the vessels. Thyroid function studies were norma Studies of serum and urinary amino acids reveale transient lysinuria without hyperlysinemia, whic resolved spontaneously. Serum copper was 15 µ per 100 ml (by atomic absorption) and serum cop per oxidase was 0.035 O.D. units (by spectr photometry). The liver copper content was 15.4 µ per gm dry weight. Intravenous administration o ^{64}copper revealed normal clearance and reappea ance (presumably as ceruloplasmin). Oral ^{64}copp was poorly absorbed. Transient clinical and bi chemical improvement followed the dose of intr venous ^{64}copper (80 µg), but oral copper thera caused no change in the serum copper. Analysis hair keratin revealed a ninefold increase in fr sulphydryl groups (Dr. J. M. Gillespie). These r sults have been reported in more detail.[3]

Fibroblastic cells cultured from a skin biop showed granules which stained metachromatical with toluidine blue in primary culture, but the granules were not seen in serial subcultures.

Case 7

P.D. was born at 34 weeks' gestation a weighed 1.93 kg (40th percentile for age). He w admitted at the age of 14 weeks because of refus of feedings, vomiting and twitching. He was l thargic, inactive, pale and dehydrated, and seve seborrheic dermatitis covered the scalp which w

devoid of hair. Intravenous rehydration and treatment with antibiotics were followed by some improvement. *E. coli* septicemia was demonstrated. His body temperature and convulsions were difficult to control.

The diagnosis was made by the radiologist who noted Wormian bones in the sagittal and lambdoid sutures, spurs on the proximal and distal femoral metaphyses, and flaring of the anterior rib ends. The features, the mild hypertonia, the lack of movement, and the mental dullness were typical. Short stubble was palpable on the scalp, but there was no hair long enough to examine microscopically. Nonetheless, sufficient features were present to allow confident diagnosis, and arteriographic findings provided further support (see below).

Thyroid function studies and amino acid chromatography of urine were normal. Fibroblastic cells grown from a skin biopsy showed granules which stained metachromatically with toluidine blue in the primary culture, but not in subcultures. Serum copper was 25 μg per 100 ml and serum copper oxidase 0.056 O.D. units.

Aortography revealed the widespread and remarkable elongation and tortuosity of arteries throughout the systemic circulation. The left profunda femoris artery was completely occluded.

His general condition deteriorated, hypothermia became more constant, and he died at the age of 6 months. By this time sufficient hair had grown to allow recognition of pili torti.

Necropsy revealed the typical changes in the brain, and in the cerebral and visceral arteries. The occlusion of the left profunda femoris artery was confirmed. Liver and brain copper content were 10.8 and 4.2 μg per gm dry weight respectively.

PEDIGREES (Figure 1)

Family B (Index case J.B., III.4)

III.2 Female, born 1958. Brittle hair and dry scaly skin noted when younger; pili torti present at 10 years.

II.3 Mother of J.B., born 1932. Had brittle hair as a child. Skin was dry and atrophic with some hyperkeratotic patches in 1969, but hair was microscopically normal. Hypothyroidism diagnosed in 1970.

II.2 Male, born 1930, died at 9 months, after showing slow mental development. "Scurvy, subdural hematomas, and abnormal hair" were described in the records of Royal Alexandra Hospital for Children, Sydney.

II.4 Male, born 1937, died at 30 months. Stated to have abnormal hair and severe mental retardation. No hospital records traced.

Family A (Index cases S.A., III.1 and M.A., III.2)

I.1 and II.3—Pili torti in a few hairs, but hair macroscopically normal.

FIG. 7. Abnormal tortuosity of the arteries in the lower limbs of J.T. at 4 months of age.

II.2—Male, born 1948, died at 6 months of pneumonia—no necropsy. Periods of hypothermia occurred during a previous attack of bronchitis. Scalp always scaly, very little hair. Motor development severely retarded.

Family S/C (Index cases D.S., V.5 and B.C., V.7)

II.2 Alert 82-year-old informant who stated that all the affected males looked just the same, had

FIG. 8. Abnormal tortuosity of the arteries in the lower limbs of M.A. at necropsy.

similar hair and followed the same course.

III.1 Male, born 1915, died at 13 months. Never sat. Hair abnormal.

III.2 Female, born 1916, died of pneumonia at 13 months. Development and hair were normal.

III.5 Male, born 1921, died at 27 months. Never sat up. Hair abnormal.

IV.2 Male, born 1948, died at 5 months of pneumonia. Parents were told he would have had brain damage if he had lived. Hair abnormal.

V.4 Female, adopted at 2 weeks of age and cannot be traced.

Family T (Index case J.T., III.3)

III.2 Male, born 1969, with multiple malformations and mental retardation; died at 18 months. Hair not examined microscopically, but appeared normal to observers who were familiar with other cases of Menkes's syndrome. Brain changes at necropsy were not suggestive of this condition and the cerebral arteries appeared normal.

Family D (Index case P.D., III.2)

III.1 Male, born July 1971. Presented to Royal Alexandra Hospital for Children, Sydney, at 3 months with mental retardation, convulsions, pili torti, and hypothermia. Copper deficiency, defective copper absorption and excessive free sulphydryl groups in hair keratin have been demonstrated.[7]

DISCUSSION

Experience in Melbourne has shown that Menkes's syndrome is not the rare curiosity that the literature would suggest, but that it is a condition which should be remembered among the causes of severe mental retardation, unexplained hypothermia, or septicemia in young male babies. The facial appearance, the hair changes, the radiological features, and a seborrheic rash provide helpful clues to the diagnosis. Correct diagnosis has always been important for prognostication and genetic counselling, but early diagnosis is especially necessary now that treatment by parenteral copper seems a possibility (see below).

Clinical Features

Mental retardation has been severe, and progressive deterioration has developed at an early stage, in all the present patients, and in all those previously described. Smiling and some degree of head control seem to comprise the maximum achievements in this disease, and only one of these patients developed to this stage before deterioration began. Muscle tone has usually been increased, but the limbs may be flaccid when the baby is very ill. Severe spasticity with postural deformity is not usual, but has been described.[8] Spontaneous movement is very limited. Drowsiness and lethargy are the presenting symptoms in most of the cases and delayed development was no

FIG. 9. Section of the brain meninges. Meningeal arteries are abnormally wide and irregular in outline and their walls vary in thickness. (Original magnification ×15.)

parent to the parents, or even to the doctors, at this stage.

Hypothermia led to suspicion of septicemia, meningitis, or hypothyroidism in five patients. Although susceptibility to infection is certainly a feature of the disease, hypothermia and instability of temperature control proved to be striking and persistent problems in these patients and was also described in the affected males in the earlier generations of Families B and S/C. It is surprising that this important feature has attracted so little attention in previous reports.

Convulsions were the reason for presentation in three patients, and occurred in all babies. Myoclonic jerking movements were not frequent and were best controlled by diazepam or nitrazepam.

Growth retardation was given great prominence in the original report[1] but has been less evident in the subsequent reports and absent in some.[6] Six of our seven patients were born prematurely, but all were between the 10th and 90th percentiles for birth weight when gestation is taken into account.[8] Growth then proceeded at a normal rate and showed some catch-up as expected in prematurity so that all reached the 3rd to 25th percentiles for mature babies by the age of 6 to 9 months. Six of our seven patients, and three of the six previously reported cases for which gestational age was stated, were born before 38 weeks' gestation.

All our patients had a striking *facial resemblance* to one another and to the previous published photographs (Fig. 2). Although the hair changes contribute to the characteristic facies, it is recognizable even when there is no hair (as in P.D.). Pallor and lack of expressive movement are important components, but the pudgy appearance of the cheeks and the rather horizontal and twisted eyebrows also play a part.

Many samples of *hair* have been studied from five of the seven affected males described. Pili torti has been the only abnormality found in any of these samples. The characteristic appearance is shown in Figure 3. The apparent narrowing at the site of the single twist may have been mistaken for monilethrix in some previous reports. Trichorrhexis nodosa was shown in the original report,[1] but has not been illustrated in other cases.

It is clear from our observations of M.A. and J.T. that pili torti is not necessarily

present at birth, and one other report had also implied that the primary hair is normal.[4] This point is of considerable diagnostic importance in a newborn male who may have Menkes's syndrome.

The *skeletal changes* developed in a fairly definite sequence in the x-rays which were available for five of our seven patients. Wormian bones were present in the posterior part of the sagittal suture as early as 6 weeks of age and persisted up to 5 months. Later skull films were not available. In two babies (J.B. and P.D.) with very pronounced changes, the lambdoid sutures were also involved. The remaining bone changes consisted of widening of the metaphyses of certain bones with formation of lateral spurs which fractured or fragmented in some babies. These changes developed progressively after about 2 months and then resolved in the latter part of the first year, leaving a wavy irregularity of the metaphyseal growth plate in some bones. The ribs and femora (upper and lower ends) were most often involved in our patients, and similar experience is recorded by others.[4] One patient (J.B.) also developed changes in the upper end of the humeri and the lower end of the radii. Flaring of the anterior ends of the ribs with some cupping occurred in four patients appearing as 2, 3, 5, and 5 months. Sharp medial spurs were seen at the proximal end of the femora (Fig. 4) in four patients, and medial and lateral spurs occurred on the distal femoral metaphyses in three babies (Fig. 5). One patient (M.A.) developed no bony changes at all. Bone maturation proceeded normally.

Wesenberg, *et al.*[4] described most of these changes in the cases they reported and also noted subperiosteal new bone formation. This feature was not observed in any patient in this series, but osteoporosis became apparent after the age of 6 months in three of the five patients.

The bone changes are easily confused with those seen in maltreated babies and the tendency for subdural hematomas to occur in Menkes's syndrome adds to the risk of confusion of the two entities. No difficulty should be encountered in correct diagnosis if the other features are remembered. There is some resemblance to scurvy, also, and it is very interesting that the one child who showed no metaphyseal changes (M.A.) was treated with ascorbic acid (10 mg daily) throughout his life. Very similar changes have been described in nutritional copper deficiency.[9,10] Microscopically, the bones sectioned in J.B., D.S., and M.A. showed no evidence of scurvy, but the persistence of woven bone in the cortex of the ribs suggested an error in bone modelling.

Pathology

ARTERIAL ABNORMALITIES: Tortuosity and elongation of cerebral arteries was demonstrated in J.T. and P.B. and resembled findings reported previously.[3,4] Aortography showed that the arterial abnormalities were not confined to the brain, but occurred in the limbs and viscera with variable distribution (Fig. 6 and 7). Irregularity of the lumen of arteries was also demonstrated, with marked narrowing of the right renal and left common iliac arteries in J.T. (Fig. 6), and occlusion of the left profunda femoris artery in P.D.

These arteriographic findings in J.T. and P.D. led to a review of the microscopic appearance of vessels present in the tissues available from the previously necropsied patients (J.B., D.S., and M.A.). Changes in the internal elastic lamina and intima of many arteries were found, but the distribution of these lesions was very variable. The subsequent death of P.D. allowed study of a wider range of vessels and correlation of the microscopic and arteriographic findings. The correlation was found to be excellent and the occlusion of the left profunda femoris artery was confirmed.

Microscopically, distortion of arteries and variation in the thickness of their walls was seen (Fig. 9). The elastic tissue in systemic arteries, and to a lesser extent in pulmonary arteries, showed extensive beading, fragmentation and splitting, with overlying intimal hyperplasia in which irregular elastic

fibres developed (Fig. 10 and 11). The histochemistry of the mature elastin appeared normal, but the damaged elastin showed irregular intensity of staining, as might be expected if the chemical structure of elastic tissue were abnormal.

BRAIN PATHOLOGY: The changes found in the brain have been discussed at great length in previous publications[1-3] and the observations in the present series confirm the existence of diffuse neuronal damage, gliosis, and cystic degeneration in the most severely damaged areas. The changes observed could be the end result of vascular insufficiency.

Genetic Features

PATTERN OF INHERITANCE: Four of the five families provide further support for the X-linked inheritance of this condition. The fifth family contributes no information because there is just the one affected male, and there are few unaffected males in the previous generations. The abnormal gene can be traced through four generations in the S/C Family and through three generations in Families B and A (Fig. 1).

FREQUENCY: All seven cases described were born in Victoria during a period of 4 years. The total births in Victoria during that period were approximately 265,000 and about 80% of babies with serious chronic disease in Victoria are referred to this Hospital. The frequency of the disease is therefore approximately 1 in 35,000 live births. (The 95% confidence limits for the estimate are 1 in 17,000 and 1 in 110,000 live births.)

It is probable that this condition has often passed unrecognized, as happened in J.C. and nearly happened in S.A. Pediatricians generally take very little interest in the hair of young babies, probably because many babies have very little hair until about 3 months of age. The primary fetal hair is apparently unaffected in Menkes's syndrome, and it seems possible that some babies die of septicemia before the secondary hair grows.

HETEROZYGOTE MANIFESTATIONS: There were only two females in these families who had symptoms which may be relevant. The 11-year-old sister of J.B. was described as having brittle hair and rather dry skin when younger, and a mild degree of pili torti was found in some hairs. Her mother described similar brittleness of hair in her own childhood, and had rather atrophic skin with patches of hyperkeratosis. Her hair showed no pili torti and she was subsequently found to be hypothyroid.

FIG. 10. Section of a large artery. Disruption, fragmentation, and reduplication of the internal elastic lamina and thickening of the intima are present. (Original magnification ×130.)

Hair from nine definite heterozygotes, and from 10 other females who could be heterozygotes, was examined microscopically. Pili torti was found in four of these females—in the sister of J.B. (mentioned above), and in the mother and maternal grandmother of M.A. and S.A., and in the mother of P.B. Pili torti has not been encountered among hairs from more than 100 normal individuals examined in another study, nor among about 50 individuals with

FIG. 11. Section of a large artery. Disruption, fragmentation, and reduplication of the internal elastic lamina and thickening of the intima are apparent. (Original magnification ×430.)

a variety of other ectodermal diseases.[11] Consequently, it seems probable that the finding of pili torti in a female relative of a boy with Menkes's syndrome can be regarded as evidence of heterozygosity. Unfortunately, the presence of normal hair cannot be used to exclude heterozygosity.

Skin biopsies from four known heterozygotes have been cultured and the fibroblastic cells from all four have shown metachromasia in primary culture, but not in subcultures. This test may prove of value in detecting heterozygous females among the close relatives of affected males, but further evaluation is needed before any such claim could be made. The disappearance of metachromasia in subcultures in patients and heterozygotes is unusual and may be related to the high copper content of fetal calf serum.

Serum copper and copper oxidase levels were normal in five heterozygotes tested. Direct measurement of intestinal transport of copper may be necessary to reveal any abnormality in heterozygotes.

Pathogenesis

Previous authors have suggested that this disease is the result of an inborn error of metabolism[1,12] and have implied that the brain damage is probably the direct result of the metabolic disturbance. The disturbance of glutamate metabolism found in the original case[1] has not been found consistently, and none of our cases showed abnormal serum levels of glutamic acid. A molecular abnormality of cytochrome oxidase has been suggested.[13] Absorption spectra of muscle cytochromes were normal in J.B. and D.S. (Prof. F. Gibson, A.N.U.) and electron microscopy of muscle revealed normal mitochondria.

Arterial elongation and tortuosity had been noted previously, but little pathogenetic significance had been attributed to these changes.[3,4] The arteriographic and microscopic evidence of vascular narrowing and occlusion in the present cases make it possible that the brain damage is the result of widespread cerebral vascular insufficiency, and the changes seen in the brain are not inconsistent with this suggestion. The arterial abnormalities were obvious at 3 months of age in both J.T. and P.D. The microscopic changes described in the arteries, the changes in hair and bones, and the finding of metachromasia in cultured fibroblastic cells all suggest that Menkes's syndrome may be a "heritable disorder of connective tissue."[14] Since the earliest change in the arterial wall appears in the internal elastic membrane, a search for a chemical process or compound involved in this structure and in hair was initiated.

Investigations of copper metabolism in these patients was undertaken because of the similarity of the changes in the arterial walls to those described in copper deficient animals[15,16] and because copper deficient sheep were known to produce abnormal wool.[17,18] The finding of very low serum

copper and copper oxidase levels in J.T. and P.D. encouraged more detailed studies of copper handling in J.T. (the only surviving Melbourne patient), in the surviving cousin P.D. living in Sydney and in two other patients discovered in Sydney (in collaboration with Dr. B. Turner, and Miss J. Blomfield of the Children's Medical Research Foundation, and Dr. J. Walker Smith of the Royal Alexandra Hospital for Children) and chemical analysis of hair keratin (Dr. J. M. Gillespie, C.S.I.R.O., Melbourne). The detailed results of these studies have been published elsewhere.[7]

In summary, these studies demonstrated defective intestinal absorption of copper, but normal conversion of intravenously administered copper into ceruloplasmin. Severe copper deficiency was found in all patients studied with very low levels of copper in serum, liver, and brain (but normal levels in red blood cells). Prolonged oral administration of copper caused no significant clinical or biochemical change. Hair keratin contained free sulphydryl groups in great excess as does the wool of copper deficient sheep.[18]

It is therefore postulated that the basic biochemical defect in Menkes's syndrome involves the intestinal absorption of copper, and that all of the features of the disease are the direct or indirect result of copper deficiency. Copper is known to be required for the formation of the disulphide bonds between the polypeptide chains of wool keratin in sheep,[18] and for the formation of cross links between lysine residues in elastin and probably in collagen.[16] Copper deficiency could therefore explain the hair and vascular abnormalities. It seems probable that the gross degenerative changes seen in the brain are the consequence of ischemia due to arterial abnormalities. Ease of rupture of abnormal arteries may account for the frequent finding of intracranial hemorrhage.[4] Cytochrome oxidase contains copper in its heme prosthetic group, and deficient function of this vital enzyme may explain the hypothermia seen in this disease, and may contribute to the neurological problems for there is extensive evidence that cytochrome oxidase deficiency is important in the neurological disorder in copper deficiency in sheep and in other species.[19] It is, however, interesting to note that very little seems to have been written about the state of the cerebral arteries in these copper deficient animals.

Bone changes similar to those seen in Menkes's syndrome have been described in copper deficient babies[3,10] and the resemblance to scurvy has been attributed to the copper requirement of ascorbic acid oxidase.[10] The bone changes seen in experimental copper deficiency are said to resemble scurvy.[19] Osteoporosis is prominent in these animals and became pronounced in the older babies reported here.

In the articles written about nutritional copper deficiency in babies, the main emphasis has been upon the development of anemia and neutropenia.[3] Neither of these effects was seen in these patients and the red cell copper levels were normal. This suggests that the red cells bind copper more avidly than other tissues and supports those workers who claim that other aspects of the nutritional deficiency contribute to the production of anemia. Anemia is one of the more variable effects of induced copper deficiency in animals.[19] Detailed studies of patients with Menkes's syndrome may help to define the role of copper in erythropoeisis.

These findings raise the possibility of treatment by parenteral administration of copper, or even by giving very large doses orally since some absorption occurs. Research will be needed to discover which copper compounds can be used most safely. Very early diagnosis will be necessary if structural brain damage is to be prevented. It may even be necessary to start treatment before birth, because the early onset of symptoms suggests that these babies lack the large store of copper present in the normal newborn liver.[20] There may be a defect in the placental transport of copper. However, the normal copper stores are known to accumulate only during the last weeks of

pregnancy,[20] and it is possible that premature birth, which was common in this series, predisposes to early development of copper deficiency. The one full-term baby in the series (J.B.) developed symptoms later than the other six patients. Studies of newborn male siblings of known cases are needed, and these studies may need to start *in utero*.

These findings also open avenues of investigation of the normal mechanism of copper absorption, and suggest the need to look at copper metabolism in other heritable disorders of connective tissue, and to look for diseases due to defective absorption of other trace elements.

SUMMARY

The recognition of seven patients with Menkes's kinky hair syndrome born into five families during a 3-year period in Melbourne has indicated that this disease is not rare and has suggested that babies with this disease may sometimes die undiagnosed. Hypothermia is an important clue to the diagnosis. The arterial tortuosity previously observed in cerebral vessels has been demonstrated to occur throughout the body and microscopic studies have shown fragmentation and disintegration of the internal elastic lamina of arteries along with an irregular thickening of the intima. These changes appear to provide the explanation of the arteriographic findings and for the brain damage. Copper deficiency and defective intestinal absorption of copper have been demonstrated and the latter is postulated as the basic biochemical defect. All the features of the disease appear to be the direct or indirect consequence of copper deficiency. Treatment of this serious disease may soon become possible.

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EDITOR'S NOTE: See also p. 181.

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Systemic Absorption of Copper after Oral Administration of Radioactive Copper Sulfate Emetic in Rats[1,2]

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Systemic Absorption of Copper after Oral Administration of Radioactive Copper Sulfate Emetic in Rats. DECKER, WALTER J., GOLDSMITH, WILLIAM A., MILLS, ROY C., and BANEZ, RAPHAEL J. (1972). *Toxicol. Appl. Pharmacol.* 21, 331–334. Copper sulfate ($CuSO_4$) has been advocated as a rapidly acting emetic for use in the immediate treatment of poisoning by ingestion. There is, however, a high potential for systemic absorption of toxic amounts of copper, especially when emesis does not occur. To test this possibility, a dose of $CuSO_4$ (2.5 mg in 0.25 ml H_2O, equivalent to the recommended human emetic dose of 250 mg) tagged with copper tracer ($^{64}CuSO_4$) was administered to rats, a species that cannot vomit. Rats were sacrificed 1, 3, 6 and 24 hr after copper administration, and blood and other tissues were counted to determine copper content. Significant concentrations of administered copper were found in kidney, liver and plasma. These concentrations increased with time from 0.41, 0.35 and 0.4 μg/g at 1 hr to 2.7, 1.1 and 0.5 μg/g at 24 hr in the respective tissues. These results militate against the use of copper sulfate as an emetic agent.

Copper sulfate has been used as a po emetic agent for many yr (Decker, 1971) and was recently widely recommended because it appeared to induce emesis in a greater % of patients more rapidly than syrup of ipecac (Karlsson and Noren, 1965). Recent reports have questioned its safety (Holtzman and Haslam, 1968) as well as its efficacy (Cashman and Shirkey, 1970). Copper has been shown to be both nephrotoxic (Vogel, 1960) and hepatotoxic (Bal and Haldar, 1961).

The present study was undertaken to determine if po administration of cupric sulfate following use of the recommended emetic dose would result in significant systemic absorption of copper when emesis does not occur and, if so, whether absorbed copper would concentrate in organs intimately involved in the metabolism and excretion of poisons.

METHODS

A solution of cupric sulfate was made from 156 mg $CuSO_4 \cdot 5H_2O$ in 10 ml of deionized distilled water. Before diluting to that volume, 4 mCi of carrier-free $^{64}CuSO_4$ was

[1] Presented at the Tenth Annual Meeting, Society of Toxicology, Washington, D.C., March 7–11, 1971.

[2] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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added. The 0.25-ml aliquot of this solution given to each rat (a species that cannot vomit) contained 1 mg cupric ion at an activity of 100 μCi. The dose of cupric sulfate recommended as an emetic (Karlsson and Noren, 1965) is 250 mg (containing 100 mg cupric ion); this dose was scaled down by a factor of 0.01, an approximate ratio of rat weight to young child weight.

Unanesthetized unfasted adult male albino Walter Reed strain rats, 275–350 g, were given the cupric sulfate solution by stomach tube. At 1, 3, 6 and 24 hr intervals, 3 rats were sacrificed by chloroforming and immediately dissected. Whole intact organs and specimens of depot fat, skeletal muscle and skin were weighed. Heart blood was drawn into a heparinized plastic syringe. After centrifugation, the buffy coat was discarded and the volumes of erythrocytes and plasma were recorded. All specimens were placed in glass test tubes and radioactivity was determined by an automatic gamma ray spectrometer (NaI-Tl crystal). Using a programmable calculator, net counts, corrected for activity at the time of po administration, were used to calculate the amounts of copper present in each tissue by the radioisotope dilution technique. Means and SD were determined with a second calculator program.

RESULTS

The brain and eye did not take up measurable amounts of copper; the concentrations of administered copper in other tissues are shown in Table 1. The femur, erythrocytes, skeletal muscle and skin contained minimal amounts. The heart, lung, plasma and spleen were intermediate in behavior, while the liver and kidney took up relatively high amounts. Depot fat was the only tissue examined which showed a definite decrease in concentration with time. The bulk of the administered copper passed through the gastrointestinal tract and was excreted in the feces.

TABLE 1

CONCENTRATIONS OF ADMINISTERED COPPER IN TISSUES OF RATS GIVEN PO CUPRIC SULFATE[a]

Tissue	Hours after copper administration			
	1	3	6	24
Skeletal muscle	10 ± 0.0	20 ± 0.0	10 ± 0.0	40 ± 10
Skin	30 ± 10	50 ± 20	30 ± 10	70 ± 40
Femur	0	30 ± 0.0	30 ± 20	90 ± 10
Erythrocytes[b]	10 ± 0.0	20 ± 10	30 ± 10	120 ± 10
Depot fat (pooled from 3 rats)	90	170	10	0
Heart	20 ± 0.0	50 ± 10	60 ± 10	230 ± 10
Spleen	10 ± 0.0	50 ± 10	50 ± 10	260 ± 60
Lung	170 ± 140	2400 ± 3000	60 ± 10	350 ± 170
Plasma[b]	40 ± 10	60 ± 20	100 ± 10	480 ± 110
Liver	350 ± 140	430 ± 200	370 ± 150	1100 ± 180
Kidney	410 ± 20	850 ± 160	1230 ± 200	2700 ± 900

[a] ng copper per g wet weight, mean of 3 animals ± SD.
[b] ng/ml.

DISCUSSION

The presence of relatively large amounts of administered copper in liver and kidney, primary target organs for many poisons, is most disturbing. Soman *et al.* (1970) showed that normal liver contained approximately 3 μg/g and normal kidney approximately 5 μg/g of copper. Data obtained in the current study indicate possible respective increases of 40 and 60% copper concentration 24 hr after administration of the recommended emetic dose when emesis does not occur. Although the rats did not show any toxic effect at the dose level employed, the results presented here strongly indicate that the significant extra burden of copper placed upon these organs, vital to the metabolism and excretion of poisons, could quite conceivably add to the effect of hepatotoxic and nephrotoxic poisons.

Even when induction of emesis by copper sulfate was successful, about 30% of the administered copper, according to Holtzman and Haslam (1968), could not be accounted for in the vomitus; children had marked elevation of serum copper, on the order of 20–100% of preemetic serum copper concentrations.

Pathologic findings in acute copper poisoning include corrosive lesions on mucous membranes of the gastrointestinal tract and parenchymatous degeneration of the liver and kidney. Blood acquires a dark color due to formation of hematin. Jaundice, oliguria, albuminuria and hematuria may appear. Abdominal pain, tetanic spasms, delirium, paralysis and collapse may result (Gonzalez *et al.*, 1954).

Acute copper poisoning can occur when surprisingly little copper has been ingested. In 1 well-documented situation (Semple *et al.*, 1960), 18 adult workers collapsed with severe abdominal pain, vomiting, diarrhea, headache and dizziness 5–10 min after drinking tea made from water heated in a corroded copper-lined boiler. These manifestations are compatible with those described in the preceding paragraph. Analysis of water taken from the boiler revealed a dissolved copper concentration of 44 mg/l. It would appear that these workers had ingested considerably less than 44 mg of copper on their tea break. This observation is particularly significant in view of the fact that the recommended dose of copper is 100 mg for children 1.5 yr and older, with a repeat dose if emesis fails (Karlsson and Noren, 1965).

Although the most commonly encountered adverse effect of copper sulfate emetic is colicky abdominal pain (Karlsson and Noren, 1965), a definite potential for more serious sequelae is always present. Since this agent is corrosive to the gastrointestinal tract, its use following ingestion of corrosive compounds such as ferrous sulfate and salicylates would appear to be very dangerous. Similarly, it is quite possible that hemolysis produced by an ingested poison could be greatly increased (Holtzman and Haslam, 1968; Manzler and Schreiner, 1970). The same may be said when compounds causing convulsive seizures or paralysis are involved.

The foregoing considerations lead to the conclusion that copper sulfate could be extremely hazardous when used as an emetic in poison ingestion.

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RESPONSE OF RATS TO DIETS CONTAINING 250 PPM SUPPLEMENTAL COPPER: GROWTH, FOOD CONVERSION, LIVER COPPER ACCUMULATION, AND FAT COMPOSITION

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ABSTRACT

Thirty-two weanling rats were used to study the effects on food intake, average daily gain, food conversion, accumulation of copper in the liver, and fatty acid composition of body fat when 250 ppm supplemental copper was added to a barley–fish meal or barley–soybean meal diet. Rats were killed at either 4 or 8 weeks following the start of the experiment. Supplemental dietary copper did not influence food intake, reduced daily gain ($P < 0.10$ for 0–4 weeks only), depressed food conversion ($P < 0.05$ for 0–4 weeks only), increased liver copper concentration ($P < 0.05$), and did not alter the composition of body fat in comparison with control animals receiving either of the nonsupplemented diets. Based on these results the rat does not appear to be a suitable pilot species for investigating metabolic responses in pigs to high level copper supplementation of diets.

RESUME

Trent-deux de rats au sevrage ont été utilises pour étudier les effets de l'addition de 250 ppm de cuivre dans la moulée sur le taux de consommation de la moulée, le gain journalier moyen et le nombre de gm d'aliments/gm de gain. Les deux moulées étaient composées d'orge-farine de poisson et orge-farine de soya. Les rats ont été abattus à 4 ou 8 semaines après le début de l'expérience. L'addition de cuivre n'a pas affecté le taux de consommation de la moulée, diminué le gain journalier moyen ($P < 0.10$ pour 0–4 semaines seulement). Cette addition a abaissé le taux de conversion ($P < 0.05$ pour 0–4 semaines seulement) augmenté la teneur en cuivre du foie ($P < 0.05$) et n'a pas changé la composition du gras en comparaison avec les témoins qui ont été nourris avec une ou l'autre des moulées ne contenant pas de cuivre additionel. D'après ces résultats le rat ne semble pas être l'espèce animale pouvant servir dans l'étude des réactions metaboliques chez le porc à la suite de l'ingestion d'une niveau éléve de cuivre additionel.

INTRODUCTION

It was suggested by Taylor and Thomke (1964) that high levels of dietary copper might influence the absorption of dietary fat components, influence the mobilization from or deposition of fatty acids in depot fat, or that copper stored in the liver might interfere with endogenous fat metabolism, and thereby cause an increase in the proportions of unsaturated fatty acids in porcine depot fat.

In the course of experiments designed to study the effects of dietary copper on depot fat composition in pigs (Drouliscos et al. 1970; Elliot and Bowland 1968, 1970) it became of interest to attempt to define more specifically the site of action of copper with respect to fat metabolism. If such studies were to be carried out, cost considerations would make the rat a more desirable experimental animal than the pig. This paper reports on the results of an experiment to determine if the rat responded to supplemental dietary copper in a manner similar to the pig.

MATERIALS AND METHODS

Thirty-two 3-week-old weanling Sprague-Dawley (University of Alberta strain) rats were assigned at random to each of four treatment groups with four males and four females per group. The rats were individually housed in 24 × 20 × 18-cm stainless steel cages and fed barley–fish meal or barley–soybean meal diets with and without 250 ppm supplemental copper. The basal diets were the same as those fed in a concurrent study (Elliot and Bowland 1970). The fish meal-supplemented diet

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containing 90.0% barley, 7.6% herring meal with iodized salt, calcium, phosphorus, zinc, and vitamin supplements. On an "as fed" basis this diet averaged 2648 kcal digestible energy (DE)/kg, 13.8% crude protein, and 4 ppm Cu prior to copper supplementation. The soybean meal-supplemented diet contained 82.0% barley, 15.0% soybean meal, the same mineral and vitamin supplements as in the fish meal diet, and averaged 2520 kcal DE/kg, 14.2% crude protein, and 7 ppm Cu in the basal diet (Drouliscos et al. 1970). Water was available free choice at all times. Rats were individually weighed and their feed consumption recorded weekly.

Two males and two females from each group were killed at 7 and 11 weeks of age (4 or 8 weeks after initiation of the experiment). The rats were killed by stunning and decapitation and were hung by their tails to bleed for approximately 5 min. Feet, skin, and tails were removed and the carcasses eviscerated in such a way as to leave a large proportion of the internal body fat with the carcass. The carcasses were then placed in plastic bags, the bags flushed with N_2, sealed, placed in a container with dry ice to facilitate rapid freezing, and stored, until analysis, in a freezer thermostatically controlled at −40 C.

At time of analysis the frozen carcasses were chopped into small pieces that were ground in an electric meat grinder (Grind-o-matic, Model No. 772; Rival Mfg. Co., Kansas City, Missouri). The resulting material was freeze-dried and then further ground with the aid of a mortar and pestle. Lipids were extracted from a representative sample using methods previously described by Elliot and Bowland (1968).

Copper level of the liver of each rat was determined using a Techtron atomic absorption spectrophotometer model AA-4 following the method of Allan (1961).

The data for daily food intake, daily gain, food conversion, total saturated fatty acids, total unsaturated fatty acids, and liver copper levels were analyzed statistically as a 2 × 2 × 2 factorial design including two protein sources (fish meal and soybean meal), two sexes, and two supplementary copper levels (0 and 250 ppm).

RESULTS AND DISCUSSION

Average Daily Food Consumption, Daily Gain, and Food Conversion

Rats receiving the barley–soybean meal diet tended to consume more feed in both periods (0–4 weeks and 4–8 weeks on experiment) than did those receiving the barley–fish meal diet; however, this increase was significant ($P < 0.05$) only for the overall period. Dietary protein source did not significantly affect either average daily gain or food conversion (Table 1). These results are in general agreement with those reported earlier for pigs fed diets formulated on the same basis (Elliot and Bowland 1968, 1970).

Male rats consumed more food, gained significantly ($P < 0.01$) faster, and were more efficient at converting food to gain than were females. Females in the second period required significantly ($P < 0.01$) more food per gram gain than did males (Table 1). These results agree with those for pigs as regards food intake and rate of gain, but with pigs, food conversion is usually more efficient in females (Elliot and Bowland 1968, 1970).

Rats receiving 250 ppm supplemental copper did not consume any more food than those receiving the basal diet. They tended to gain at a slower rate and were less efficient at converting food to gain than were those receiving the basal diet

Table 1. Effects of protein source, sex, and supplemental copper on average daily food consumption, average daily gain, and food conversion in rats 4 and 8 weeks post-weaning

Period (weeks):	1–4			1–8		
	ADF g	ADG g	FC g	ADF g	ADG g	FC g
Protein source						
FM†	13.1	3.43	4.17	16.5 *a*	3.73	4.57
SM	13.9	3.93	3.67	18.4 *b*	3.89	4.84
Sex						
Male	13.8	4.02 *a*	3.80	18.3	4.52 *a*	4.06 *a*
Female	13.2	3.35 *b*	4.04	16.6	3.10 *b*	5.35 *b*
Copper						
0	13.3	3.97	3.38 *a*	17.4	3.87	4.59
+	13.7	3.39	4.46 *b*	17.5	3.75	4.82

†FM, fish meal; SM, soybean meal.
ab Within comparison groupings, means carrying different letters are significantly ($P < 0.05$) different.

(Table 1). However differences in average daily gain and food conversion were significant only for the 0–4 week period ($P < 0.10$ and $P < 0.05$ respectively). Beames and Lloyd (1965) and Beames (1969) reported that supplemental copper increased body weight gain of rats in some diets and not in others, but copper resulted in improved food conversion in both studies. However, pigs receiving supplemental copper at this level grow faster on the average and are more efficient at converting food to gain in comparison with control animals (Braude 1965; Beames 1969).

From these results it would appear that insofar as gain and food conversion are concerned, the rat does not respond to high level copper supplementation of the diet in the same way as the pig.

Accumulation of Copper in the Liver

Supplemental copper resulted, on the average, in markedly increased levels of copper in the liver (Table 2). Accumulation of copper in the liver of the rats in this experiment was subject to wide variability. Beames (1969) reported only slight increases in liver copper concentration in rats receiving diets containing

Table 2. Effects of protein source, sex, and supplemental copper on the accumulation of copper in the liver of rats 4 and 8 weeks post-weaning

	Liver Cu, ppm	
	4 Weeks	8 Weeks
Protein source		
FM	75.3±110.3†	104.5±206.5
SM	41.4±74.4	66.6±111.5
Sex		
Males	43.6±99.8	143.5±217.0
Females	67.9±83.5	27.6±33.6
Copper		
0	22.9±50.5	7.8±6.0
+	99.6±114.8	163.2±205.1

†Standard deviation from the mean.

250 ppm of supplemental copper compared with control rats. Underwood (1962) noted that the rat maintains a low liver copper level in spite of a relatively high absorption of copper from the diet. Pigs fed identical diets to those used in the present study displayed increased liver copper levels (Drouliscos et al. 1970). Rats receiving the fish meal diet supplemented with copper accumulated more copper in their livers than did those receiving the soybean meal diet. This observation is in agreement with the findings of Drouliscos et al. (1970) for accumulation of copper in the liver of pigs receiving the same diets. However, due to the wide variability in the data and the observations of others (Beames 1969 and Underwood 1962) no conclusion can be drawn with respect to the similarity between rats and pigs in terms of liver copper accumulation in the presence of supplemental dietary copper.

Fatty Acid Composition of Body Fat

On the average, the fatty acid composition of fat extracted from carcasses of rats fed the nonsupplemented diets was similar to that reported by Hilditch (1956) and Bezard and Bugaut (1969). To facilitate presentation, the sums of the saturated fatty acids (SFA = C 14.0 + 16.0 + 18.0 + 20.0) and the sum of the unsaturated fatty acids (UFA = C 16.1 + 18.1 + 18.2 + 18.3 + 20.4) were obtained. Protein source did not significantly affect the proportions of SFA and UFA in the fat extracted from rat carcasses at either 4 or 8 weeks post-weaning (Table 3).

At 4 weeks post-weaning, male and female rats displayed similar fatty acid composition. However, at 8 weeks post-weaning, females had significantly ($P < 0.01$) lower proportions of SFA and significantly ($P < 0.01$) greater proportions of UFA (Table 3).

The composition of lipids from rat carcasses was unaffected by the addition of 250 ppm supplemental copper to the diet. This observation is in direct contradiction to the observations in pigs fed the same diets (Drouliscos et al. 1970). Pigs receiving supplemental copper displayed increased proportions of UFA and decreased proportions of SFA in their depot fat, an observation confirmed in other reports (Elliot and Bowland 1968, 1970).

Based on the foregoing observations and discussion, it appears that the rat responds to supplemental dietary copper in a manner different from the pig.

Table 3. Effects of protein source, sex, and supplemental copper on the fatty acid composition of fat extracted from carcasses of rats 4 and 8 weeks post-weaning

	4 Weeks		8 Weeks	
	SFA†	UFA	SFA	UFA
Protein source				
FM‡	40.5	56.4	37.2	60.4
SM	39.4	57.2	36.3	60.0
Sex				
Males	40.1	56.6	37.7 *a*	59.0 *a*
Females	39.8	56.9	35.8 *b*	61.4 *b*
Copper				
0	39.5	57.3	36.6	60.0
+	40.5	56.2	36.9	60.5

†SFA, sum of saturated fatty acids 14:0 + 16:0 + 18:0 + 20:0; UFA, sum of unsaturated fatty acids 16:1 + 18:1 + 18:2 + 18:3 + 20:4.
‡FM, fish meal; SM, soybean meal.
ab Within each season groupings, means carrying different letters are significantly ($P < 0.01$) different.

Possibly the difference between species is purely quantitative. Since with 250 ppm supplemental dietary copper the rat (Table 2) stores less copper in its liver than the pig (Drouliscos et al. 1970), the rat may have insufficient copper in its system to affect fat metabolism. Regardless of explanation, the rat cannot serve as a suitable pilot species in which to study the effects of high levels of supplemental copper on fat metabolism in the pig.

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Anemia, Iron Storage and Ceruloplasmin in Copper Nutrition in the Growing Rat[1,2,3]

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ABSTRACT Several studies have shown that copper-deficient animals accumulate iron as anemia persists, indicating the unavailability of absorbed iron for hemoglobin formation. Recently, it was suggested that ceruloplasmin by its ferroxidase activity is the regulating agent that mobilizes the iron from cells to plasma. This experiment was conducted to examine in vivo this suggestion by the periodic monitoring of the levels of liver iron and copper and hemoglobin in relation to ceruloplasmin oxidase activity during a state of copper depletion and subsequent repletion. A total of 230 weanling rats were divided into two groups and fed either a low copper (<1 ppm) or a control diet (35 ppm copper as $CuCO_3$). Both diets contained 135 ppm iron as Fe_2O_3. After 42 days half the rats on the low copper diet were fed the control diet and continued for another 42 days of repletion. Animals were killed at weekly intervals for 84 days and on days 44 and 47. Hemoglobin, hematocrit, plasma ceruloplasmin oxidase activity, liver iron and liver copper were different ($P < 0.05$) from the control values starting at day 7 of depletion. Ceruloplasmin activity was reduced to 5% of the control by day 14 of depletion, and an accumulation of iron in the liver amounted to 65% more than the control values by the end of the 42-day depletion period. Hemoglobin and hematocrit values were reduced to 50% of the control values during this period. With copper repletion the marked increase in ceruloplasmin oxidase activity (25-fold in 2 days) corresponded with the rapid removal of iron (30% decrease in 2 days) and the accumulation of copper in the liver. Further increases in the ceruloplasmin activity in the repleted animals were tied with the corresponding removal of iron from the liver during repletion. These changes were followed by a steady increase in hemoglobin and hematocrit values equaling the control values by days 70 and 63 of repletion, respectively. These results are compatible with the suggestion that copper exerts its influence in the metabolism of hemoglobin through ceruloplasmin by mobilizing the absorbed iron, bound to transferrin, from the cells to the plasma. J. Nutr. *103:* 196–201, 1973.

INDEXING KEY WORDS copper depletion–repletion · ceruloplasmin-ferroxidase · anemia · liver iron

The importance of the role of copper in the metabolism of hemoglobin became evident when Hart et al. (1) showed that anemia of rats fed a whole milk diet, supplemented with iron, could be cured by feeding copper. Anemia observed in copper-deficient rats and swine was shown to be hypochromic and microcytic with normoblastic hyperplasia of the bone marrow, hypoferremia and increased iron-binding capacity of the plasma (2–4). These changes were the same as those of an iron deficiency state, and the morphological and biochemical similarities between these two deficiency states suggested that copper deficiency anemia was a result of a defect of iron metabolism (5). Lee et al. (6) have shown that there was no abnormality in the heme biosynthetic pathway in copper-deficient swine and further indicated a defect in the

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[2] Preliminary report of these results was presented at the Federation of American Societies for Experimental Biology, Chicago, IL, 1971, Federation Proc. 30: 491 (abstr.).

[3] Assistance with the statistical analyses was provided by Dr. J. R. Tarr.

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metabolism of iron. A step toward understanding the biological role of copper in the metabolism of hemoglobin was made by Osaki et al. (7), when they proposed that ceruloplasmin, by virtue of its ferroxidase activity, could enhance the incorporation of absorbed iron into apotransferrin before it is transported to the site of hemoglobin synthesis. This proposal was validated by experimental evidence recently be Lee et al. (8) and Roeser et al. (9). These workers have observed that the defect in the transfer of iron from cells to plasma caused by copper deficiency could be corrected by the intravenous administration of ceruloplasmin. Further they have observed that the administration of ceruloplasmin to copper-deficient swine with normal iron stores caused an increase in plasma iron which was proportional to the log of the ceruloplasmin dose.

Previous studies in this area were restricted to the direct administration of ceruloplasmin to copper-deficient animals and observing its consequent effects on iron metabolism. This study was conducted to examine the role of dietary copper in the metabolism of hemoglobin by periodic monitoring of the levels of liver iron, liver copper and serum ceruloplasmin as related to the development and remission of anemia during a state of copper depletion and subsequent repletion.

MATERIALS AND METHODS

Hooded Norway male rats (230) 21 to 25 days of age were fed either a low copper (<1 ppm) or a control diet of 35 ppm copper as $CuCO_3$. Both diets contained 135 ppm iron as Fe_2O_3. The composition of the diet is shown in table 1. Rats were kept in stainless steel cages in rooms controlled for temperature and humidity. The diets and deionized water were given ad libitum. At the end of a 42-day depletion period the copper-deficient rats were divided into two groups. One continued on the low copper diet while the other group was fed the control diet containing 35 ppm copper. Rats were killed at weekly intervals from zero to 84 days and on days 44 and 47. There were six replicates in each group at each time interval. Hemoglobin, hematocrit, plasma ceruloplasmin oxidase activity, liver iron and liver copper were determined.

TABLE 1

Composition of diet

Ingredient	%
Milk, dry fat-free	55.0
Starch	17.0
Sucrose	16.5
Nonnutritive bulk	2.0
Corn oil [1]	5.0
Vitamin mixture [2]	2.0
Mineral mixture [3]	2.5

[1] Santoquin (Monsanto Co., St. Louis, Mo.) added to corn oil to make 0.01% in the diet. [2] Vitamins (Nutritional Biochemicals, Cleveland, Ohio) per 100 g of diet: (in IU) (supplied by fish liver oil purified for each vitamin activity solely) vitamin A, 1800; vitamin D, 200; (in mg) alpha tocopherol, 10.0; inositol, 10.0; choline chloride, 1.0; riboflavin, 2.0; menadione, 4.5; *p*-aminobenzoic acid, 10.0; niacin, 9.0; pyridoxine hydrochloride, 2.0; thiamin, 2.0; calcium pantothenate, 9.5; biotin, 0.04; folic acid, 0.15; and vitamin B_{12}, 0.02 mg. [3] Mineral mixture supplied the following per 100 g of diet: (in mg) manganese, 4 as $MnCO_3$; zinc, 5 as $ZnCO_3$; cobalt, 0.5 as $CoCO_3$; molybdenum, 0.06 as $Na_2MoO_4 \cdot 2H_2O$; iodine, 0.2 as KI; fluoride, 0.001 as NaF; selenium, 0.01 as H_2SeO_3; iron, 13.5 as Fe_2O_3; copper (control diet, 3.5 as $CuCO_3$). The mineral powder by analysis provided potassium, 30%; sodium, 10%; and magnesium, 50.

Hemoglobin was determined by the method of Cohen and Smith (10); and hematocrit, by the method of McGovern et al. (11). Ceruloplasmin was assayed spectrophotometrically by the method of Scheinberg et al. (12) as modified by Rice (13), and the values were expressed as international units of enzyme activity. Liver copper and iron were determined using atomic absorption spectrometry according to the method of Berman (14) after wet ashing the dried tissue with perchloric and nitric acids. Least significant differences (LSD) were computed for comparing diet and time-within-diet means (15).

RESULTS

Rats fed the copper-deficient diet showed a reduced growth rate (fig. 1), change in hair color and suffered 15% mortality. Copper deficiency resulted in a reduction ($P < 0.05$) in the hemoglobin and hematocrit values of blood starting at day 7 of the experiment. The hemoglobin values were lowered to 50% of the control in 4 weeks time and these lowered values were maintained throughout the course of the experiment (figs. 2 and 3). The ceruloplasmin oxidase activity of the blood in the copper-deficient animals was lowered to 5% of the control values in 2 weeks of copper depletion (fig. 4). Liver copper content of the

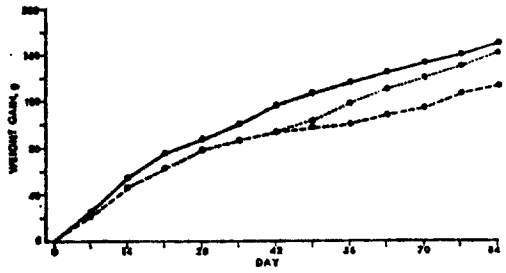

Fig. 1 Weight gain of copper-fed (———), copper-depleted (– – – –), and copper-repleted (. . . . .) rats. Each point represents the mean of the six rats assayed. The zero day controls had an average weight of 61 ± 1.4 g (SE). The pooled standard errors from analyses of variance for body weight for each diet group were 8, 7 and 5 g for copper-fed, copper-depleted and copper-repleted rats, respectively.

copper-depleted animals showed a reduction ($P < 0.05$) starting at day 7 on the low copper diet. The ceruloplasmin oxidase activity was lowered to 5% of the control values in 2 weeks, whereas the reduction in liver copper was much slower and lowered only to 50% of the control values during the same period (figs. 4 and 5). Iron accumulated in the livers of copper-depleted rats at first very rapidly and then at a slower rate showing a quadratic response with time, reaching a 65% increase over the control values by the end of 42 days and 140% increase by day 84 (fig. 6).

When copper was fed to the copper-depleted animals hemoglobin and hematocrit values showed a sudden increase and reached the control values by about day

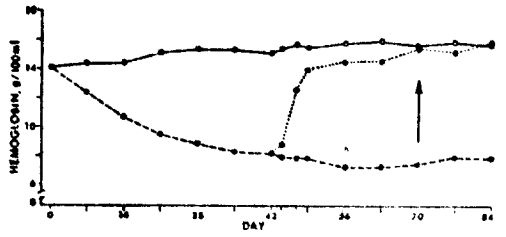

Fig. 2 Change in hemoglobin associated with dietary copper. Recovery of copper-repleted rats indicated by arrow. (Copper-fed ———, copper-depleted – – – –, and copper-repleted . . . . .) The least significant difference was 1.26 ($P < 0.05$). The pooled standard errors from analyses of variance were 0.45, for each of the three groups. Each point represents the mean of the six rats per diet assayed.

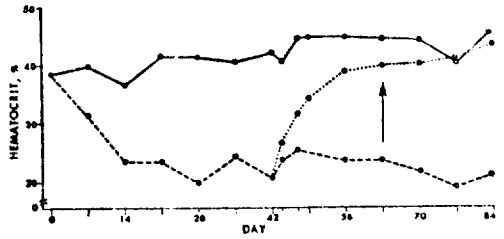

Fig. 3 Change in hematocrit associated with dietary copper. Recovery of copper-repleted rats indicated by arrow. (Copper-fed ———, copper-depleted – – – –, and copper-repleted . . . . .) The least significant difference was 5.0 ($P < 0.05$). The pooled standard errors from analyses of variance were 1.8 for each of the three groups. Each point represents the mean of the six rats per diet assayed.

70, increasing in a quadratic manner with time (figs. 2 and 3). Ceruloplasmin oxidase activity of the plasma increased 25-fold in 2 days on repletion and reached the control values within 5 days of repletion (fig. 4). An increase in liver copper with time was slower than that of ceruloplasmin and equalled the control values only after 2 weeks. Further, the ceruloplasmin oxidase activity of the copper-repleted animals showed an increase ($P < 0.05$) over and above the activity of the copper-fed animals from day 56 to 77 (fig. 4). This change was noteworthy because it was reflected in a corresponding decrease in liver iron values during repletion. There was a 30% decrease in liver iron content during the first 2 days of repletion, and a further lowering to the control values by day 5. The liver iron values were lowered ($P < 0.05$) below the control values from days 49 to 70, after which they were increased to equal the control values showing a cubic response with time (fig. 6).

DISCUSSION

As observed in previous studies, copper deficiency resulted in microcytic and hypochromic anemia which is characteristic of iron deficiency. But iron accumulated progressively in the liver. Thus copper deficiency did not alter substantially the absorption of iron in copper-depleted animals. Elvehjem and Sherman (16) have also shown that in the absence of copper inorganic iron was readily assimilated and stored in the liver and spleen of rats. Simi-

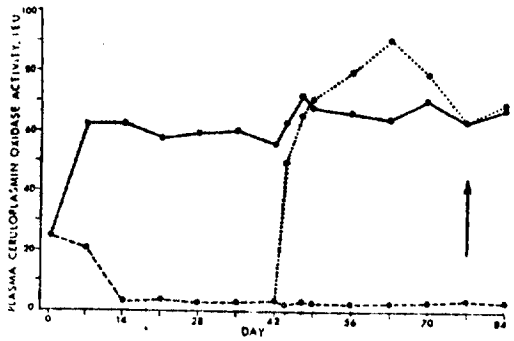

Fig. 4 Change in plasma ceruloplasmin oxidase activity associated with dietary copper. Recovery of copper-repleted rats indicated by an arrow, Abraham and Evans (29). (Copper-fed ———, copper-depleted – – – –, and copper-repleted .....) The least significant difference was 7.8 ($P < 0.05$). The pooled standard errors from analyses of variance were 2.8, 0.7, and 2.8 for the copper-fed, copper-depleted and copper-repleted groups respectively. Each point represents the mean of the six rats per diet assayed.

lar accumulation of iron has been observed in rats (17, 18), rabbits (19) and ruminants (20). The slower rate of accumulation of iron after sudden initial pile-up might be a consequence of the decrease in iron absorption resulting from the large storage of iron in the animal. Thus copper deficiency can have a secondary effect on the absorption of iron mediated through something akin to the mucosal block.

It is generally believed that the iron entering the blood stream from the intestine is mostly in the ferrous form (21). Recent studies using ultraviolet difference spectroscopy have confirmed that a strong complex of iron-transferrin is formed only when the iron is in the trivalent state (22). According to Osaki et al. (7) the copper-protein, ceruloplasmin, has a biological role here and it is the ceruloplasmin by virtue of its ferroxidase activity which oxidizes the ferrous iron into ferric iron before incorporation into transferrin (23). Thus in the absence of ceruloplasmin this oxidation step is hindered, and the incorporation of absorbed iron into the carrier-protein apotransferrin for delivery into the site of hemoglobin synthesis is consequently slowed down.

Absorbed iron is stored in the storage depots, mainly in the liver. In this study during the first week of depletion when

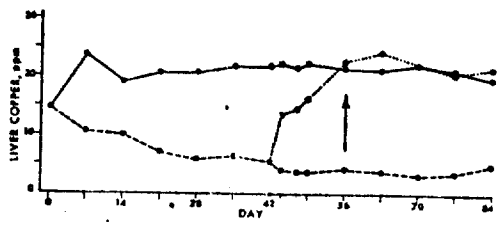

Fig. 5 Change in liver copper associated with dietary copper. Recovery of copper-repleted rats indicated by arrow. (Copper-fed ———, copper-depleted – – – –, and copper-repleted .....) The least significant difference was 3.2 ($P < 0.05$). The pooled standard errors from analyses of variance were 1.2 for each of the three groups. Each point represents the mean of the six rats per diet assayed.

there was a sudden decrease in ceruloplasmin oxidase activity, a corresponding increase in liver iron accumulation was observed. Thus as more iron accumulated in the liver indicating its unavailability for hemoglobin synthesis, hemoglobin and hematocrit values were observed to undergo a slow but steady decrease.

A much slower rate of reduction of liver copper as compared to the ceruloplasmin oxidase activity noted here is a physiological attempt to preserve the sparsely available copper to the animal. Similar changes were observed in previous studies (24). Copper is excreted through ceruloplasmin and the first notable symptom of the copper deficiency was the drastic reduction in the ceruloplasmin oxidase activity within 5

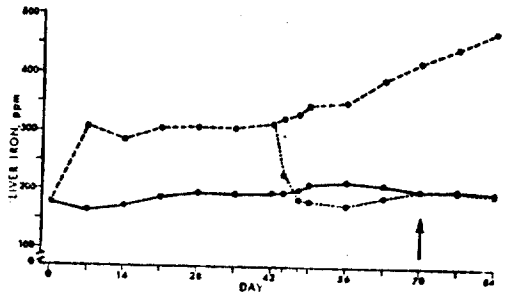

Fig. 6 Change in liver iron associated with dietary copper. Recovery of copper-repleted rats indicated by arrow. Copper-fed ———, copper-depleted – – – –, and copper-repleted .....) The least significant difference was 15.8 ($P < 0.05$) and the pooled standard errors from analyses of variance were 5.7 for each of the three groups. Each point represents the mean of the six rats per diet assayed.

days of copper depletion. Copper functions as the cofactor for many vitally important enzymes such as cytochrome oxidase, and severe copper deficiency can impose a great strain on the overall economy of the sparsely available copper, thus necessitating preferential distribution with a fair sense of preservation and priorities. During repletion the pattern of recovery of liver copper did not follow that of ceruloplasmin (figs. 4 and 5). There was a 25-fold increase in the ceruloplasmin oxidase activity during 2 days of repletion. Ceruloplasmin is one of the copper–proteins by which copper is transported to the various tissues, and the extraordinary demand for copper at the various tissue sites resulting from severe copper deficiency, might have prompted the newly absorbed copper to exist in the transportable ceruloplasmin-linked form rather than to be stored in the liver (25). A normal buildup in liver copper was accomplished only after the hematologic values almost returned during repletion to those of the copper-fed rats.

Marston and Allen (18) have observed a significant reduction in plasma iron in copper-deficient rats and reported an increase after injection of copper to these animals. Similar results were observed in copper-deficient swine (8). Ragan et al. (24) have demonstrated that administration of homologous ceruloplasmin to copper-deficient swine with adequate iron stores resulted in a rapid, marked and substantial increase in plasma iron concentrations. Roeser et al. (9) have observed that in copper-deficient swine the administration of ceruloplasmin resulted in an increase of serum iron at a rate proportional to the log of the ceruloplasmin dose. In this study, when copper was fed to the copper-depleted rats with large iron stores, the marked increase in ceruloplasmin (25-fold in 2 days) was reflected in a 30% decrease in liver iron during the same period, indicating a rapid mobilization of stored iron. Further the increase in ceruloplasmin oxidase activity over the control values during repletion corresponded with the lowering in liver iron concentrations and thus provided additional evidence for the proposed role of ceruloplasmin as the agent responsible for the mobilization of stored iron. These fluctuating changes in liver iron stores and serum ceruloplasmin oxidase activity were followed by steady but slower increases in hemoglobin and hematocrit values, when more and more iron was progressively released and made available along with newly absorbed iron for the formation of hemoglobin. As the iron is transported by transferrin through the blood stream these changes might have resulted in an increase in the serum iron concentrations in repleted animals as compared to copper-depleted animals. But the extremely low serum iron levels and the not too infrequent hemolysis in the copper-depleted animals made the accuracy of serum iron values questionable and are not reported here.

It is not clear why the ceruloplasmin values rose above the control values during repletion. One possible reason might be the increased absorption of copper in the copper-repleted rats as a result of the increased demand for copper for all the recovery processes in progress. It has been shown that the administration of copper can cause an induction of the synthesis of ceruloplasmin in rats (26). The source of iron used in the diet was ferric oxide, which was considered as a poor source of iron (27). But the copper-fed animals grew normally (fig. 1) and showed no signs of anemia while the copper-depleted animals accumulated substantial quantities of iron, indicating adequate absorption of iron from the diet.

There are several objections to the above hypothesis regarding the physiological role of ceruloplasmin in mobilizing the iron for the formation of hemoglobin. Thus, in Wilson's disease, where serum ceruloplasmin oxidase activity is very low or virtually absent, no defect in hemoglobin metabolism is observed. A possible explanation of this anomaly was recently furnished by Topham and Frieden (28), who have purified a nonceruloplasmic ferroxidase (ferroxidase II) from human serum. Ferroxidase showed no *p*-phenylene diamine oxidase activity, the property upon which the estimation of ceruloplasmin oxidase activity is generally based. They observed further that in Wilson's disease ferroxidase II activity in serum has a higher percentage of the total serum ferroxidase activity. Again, if absorbed iron is transported by

transferrin it is not clear how this iron finds its way to liver and other storage sites in the absence of ceruloplasmin. But it is possible that iron is not transported to the storage sites through transferrin, and ceruloplasmin may not have a role in this process.

The observed changes in liver iron concentrations as linked to the corresponding changes in serum ceruloplasmin oxidase activity during copper-depletion and subsequent repletion are compatible with the suggestion that the role of copper in hemoglobin metabolism is in the mobilization of absorbed iron to the site of hemoglobin synthesis.

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Copper Sulfate-Induced Hemolytic Anemia

Inhibition of Glucose-6-Phosphate Dehydrogenase and Other Possible Etiologic Mechanisms

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MANY substances have been found to cause hemolytic anemia in persons whose erythrocytes are deficient in glucose-6-phosphate dehydrogenase (G6PD). However, the basis for the hemolysis observed with heavy metals has not been elucidated. In 1965, the author treated a patient who experienced severe acute hemolytic anemia after accidental ingestion of a saturated solution of copper sulfate. At that time, only one previous report [1] of copper sulfate-induced hemolytic anemia in man could be found. Subsequently, two papers [2,3] have documented several cases of copper sulfate-induced hemolytic anemia. It also has become apparent that this disorder has been repeatedly recorded in India. The case observed by the author is briefly recorded and investigations of possible mechanisms are described.

Report of a Case

A 22-year-old white woman inadvertently ingested an unknown amount of saturated copper sulfate solution. She vomited promptly and repeatedly. Diarrhea developed on the same day. Two days later she continued to have nausea and diarrhea, and she noticed for the first time that her urine was black and her stools were dark. She was hospitalized.

There was no medical history of anemia or icterus. The family history was also negative in this respect. Her ancestry was northern European.

On physical examination the patient was found to be pale, anxious, and restless. A slight icterus of skin and sclerae was observed. There was diffuse abdominal tenderness, and bowel sounds were slightly hyperactive. There was a scant, sanguineous, uterine discharge. Digital rectal examination disclosed soft, rust-colored stools.

On admission the hematocrit value was 29.5% and the leukocyte count was 13,300/cu mm. Erythrocytes displayed slight anisocytosis. The differential cell count was normal, and platelets were adequate on smear. Coagulation time, prothrombin time, serum bilirubin, blood urea nitrogen (BUN), carbon dioxide content, and serum electrolyte values were within normal limits. The urine was the color of black coffee. It contained 5 to 7 leukocytes and 17 to 20 erythrocytes per high power field. It was strongly positive for protein and for hemoglobin by the benzidine test. It was negative for bile. Examination for urobilinogen was inconclusive because of the dark color. The stool was also strongly positive for occult blood. The results of the following studies were all within normal limits: Coombs' antiglobulin test, presumptive Ham's test for acid hemolysis, and a screening test [4] for deficiency of erythrocyte G6PD. Hematocrit value, plasma hemoglobin level, and urinary copper excretion were followed serially (Table 1).

Treatment was with bland liquid diet, antacids, and dimercaprol (BAL) in oil, 100 to 150 mg intramuscularly every four hours for two days. Low-grade fever was present for four days. Gastrointestinal bleeding and hemoglobinuria subsided by the third hospital day and did not recur. She was dismissed on the sixth day. When she was reexamined six months later, no further episodes of jaundice, anemia, or passage of dark urine were described; the hematocrit value was 40%; and the leukocyte total and differential cell counts were normal. The urine was free of hemoglobin and was normal except for 20 leukocytes per high power field. Repeated Ham's and G6PD tests were negative. The plasma hemoglobin concentration was 4.2 mg/100 ml (normal), and urinary copper excretion was 25μg/24 hr (normal).

Materials and Methods

The G6PD activity was assayed by a modifica-

Table 1.—Laboratory Data in a Case of Copper Sulfate-Induced Acute Hemolytic Anemia

Date	Hematocrit Value (%)	Plasma Hemoglobin (mg/100 ml)	Urinary Copper * (μg/24 hr)
5-28-65	30	390.0	...
5-29-65	27	120.0	628
5-30-65	23	9.0	135
5-31-65	22	7.0	268
6-1-65	20	...	...
6-2-65	20	...	...
6-3-65	20	...	...
12-12-65	40	4.2	25

* Normal value, up to 30μg/24 hr.

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tion[4] of the method of Kornberg and Horecker.[5]

Activity of the pentose phosphate shunt pathway was studied in intact, metabolically active, normal erythrocytes by supravital tetrazolium stain (Fairbanks, V. F.: unpublished data). This technique provides semiquantitative estimation of glycolytic activity by this pathway in intact erythrocytes. For studies of enzyme inhibition, erythrocytes were incubated with the substrate in the absence of copper sulfate and in the presence of copper sulfate in concentrations ranging from 10^{-5} to 10^{-3}M.

Erythrocyte reduced glutathione (GSH) was assayed by the method of Beutler and co-workers.[6] The rate of oxidation of GSH was measured in a system containing GSH (80 mg/100 ml) and copper sulfate (10^{-3}M) buffered at pH 7.0. Also studied was the effect of copper sulfate on the stability of erythrocyte GSH. For this purpose, 1 ml of whole blood (anticoagulated with acid-citrate-dextrose solution) was incubated for two hours at 37 C with and without copper sulfate (10^{-3}M). The GSH content was assayed before and after completion of incubation. Tubes were agitated at the end of one hour. For comparison of effect on GSH stability, this study was done on the blood of five normal and of four G6PD-deficient patients and a GSH stability test[7] was performed on the same samples.

The rate of in vitro oxidation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) was followed at 340 mμ at room temperature in the presence and in the absence of copper sulfate (10^{-3}M) in an ultraviolet spectrophotometer with a recorder.

Normal human erythrocyte G6PD was purified by a modification of the method of Kirkman.[8] The partially purified G6PD had a specific activity 1,000 times that of crude hemolysate and was devoid of 6-phosphogluconate dehydrogenase activity. The Michaelis-Menten constants determined for this material were slightly lower than those found for normal human erythrocyte G6PD by Kirkman but agreed closely with the values obtained by Waller and co-workers[9] and by Marks and co-workers.[10] It is believed that these differences are due to the type of buffer used and its pH. Inhibition of activity of this preparation by 10^{-3}M copper sulfate was determined independently for various concentrations of glucose-6-phosphate and for various concentrations of NADPH in accordance with conventional methods for the study of enzyme inhibition.[11]

Results

The effect of copper sulfate on oxidation of NADPH is shown in Fig 1. Copper sulfate enhanced NADPH oxidation almost ninefold over that occurring nonenzymatically in vitro.

The inhibition of glycolysis in intact erythrocytes is demonstrated in Fig 2. In concentrations as low as 10^{-5}M, copper sulfate caused profound inhibition of the pentose phosphate glycolytic pathway. (Plasma copper concentrations of 10^{-5}M are attainable in acute copper intoxication.)

Studies with purified normal human erythrocyte G6PD indicate that copper sulfate is an effective inhibitor of G6PD and that inhibition is of the competitive type for both glucose-6-phosphate (Fig 3) and for NADPH, since maximal velocity (1/V intercept) is identical in the presence or absence of copper sulfate.

In a solution of copper sulfate (10^{-3}M) and GSH (80 mg/100 ml), GSH is completely oxidized in 30 minutes. Oxidation of erythrocyte GSH also occurs, although more slowly, when G6PD-deficient erythrocytes are incubated with copper sulfate (Table 2). Normal erythrocytes, on the other hand, are able to maintain normal GSH values at this concentration of cupric ion.

Comment

Hemolytic anemia due to the ingestion of copper sulfate has long been recognized in sheep and in rabbits, and it may have been responsible for the hepatic hemosiderosis observed by Mallory in copper-fed rabbits.[12,13] Acute hemolytic anemia has also been recorded occasionally early in the course of Wilson's disease.[14] On the other hand, hemolytic anemia in man has rarely been reported as occurring after ingestion of copper sulfate despite the exposure of large populations to low concentrations of copper sulfate as a color preservative for canned vegetables.[15] It is likely, therefore, that the hemolytic effect of copper is dose-dependent, as has been observed for many other hemolytic substances.[16] At the time the patient described herein was observed, the single previous reported[1] case of hemolytic

anemia due to copper sulfate ingestion in the United States was that of a Negro man who had ingested 20 gm of copper sulfate in a single dose.

Studies to be reported elsewhere have shown that copper sulfate, mercuric chloride, saline extracts of the beans of *Vicia faba*, dihydroxy vitamin K_1, N-ethylmaleimide, and p-hydroxymercuribenzoate inhibit glycolysis via the pentose phosphate pathway in the intact human erythrocyte. On the other hand, primaquine, sulfonamides, nitrofurantoin, 1-acetylphenylhydrazine, and other agents do not inhibit this pathway of glycolysis in the intact cell during a two-hour incubation period. It is probable that many of the latter agents exert their deleterious effect on the erythrocyte membrane[17] or accelerate the intraerythrocytic generation of hydrogen peroxide.[18,19] It is unfortunate that, in the cases of copper sulfate-induced hemolytic anemia previously reported, studies of erythrocyte enzymes were not made. However, the geographic and ethnic distributions of these cases, together with the data here reported, suggest that in some cases the proclivity of the erythrocytes to hemolyze on exposure to copper sulfate may have been evidence of deficiency of G6PD.

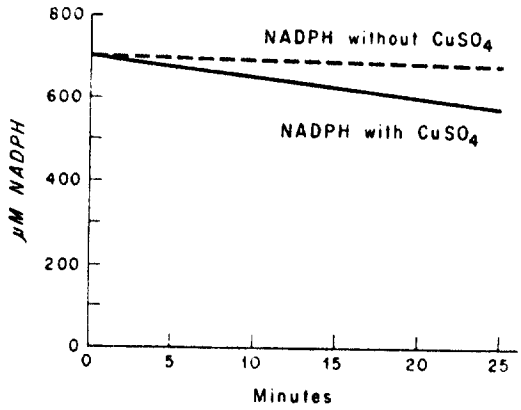
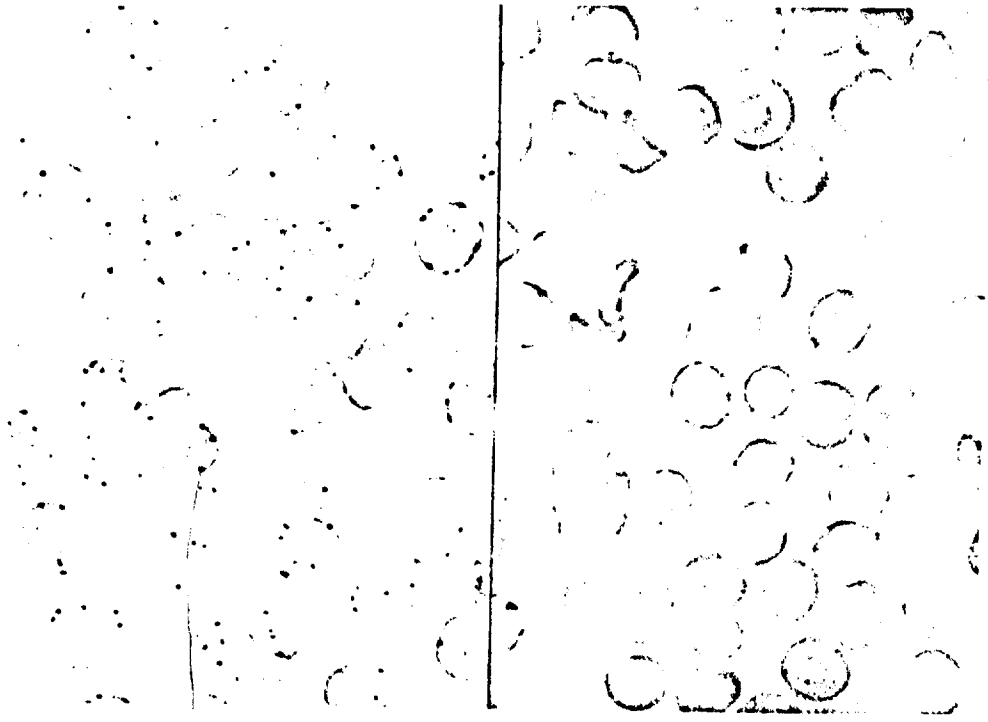


Fig 1.—Effect of 10^{-3}M copper sulfate on oxidation of NADPH in vitro at 37 C, shown as micromoles of NADPH oxidized plotted against time in minutes.

There is a close similarity between

Fig 2.—Tetrazolium (MTT) supravital stain for G6PD in normal human erythrocytes (× 3,000). Left, Incubated in the absence of copper sulfate, pH 7.0, 37 C. Monoformazan granules are abundant in every cell. Right, Incubated in the presence of 10^{-4}M, copper sulfate, pH 7.0, 37 C. Few monoformazan granules are seen. Small refractile intracellular particles represent Heinz bodies.



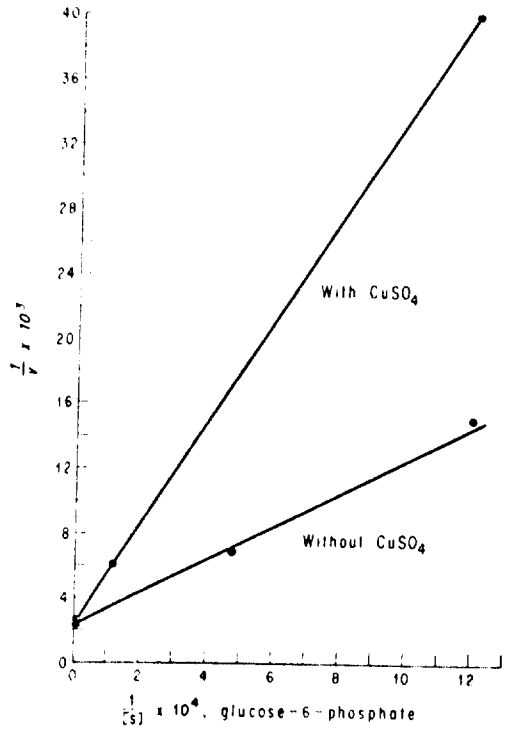


Fig 3.—Inhibition of purified normal human erythrocyte G6PD by copper sulfate (10^{-3}M), shown as double-reciprocal plot of reaction velocity against substrate concentration.

Table 2.—Stability of Glutathione in Normal and in G6PD-Deficient Erythrocytes

Source of Blood	No. Samples Tested	Glutathione (mg/100 ml of Erythrocytes) * Unincubated	Incubated With Acetylphenylhydrazine, 3.3×10^{-2}M	Incubated With Copper Sulfate, 10^{-3}M
Normal (White)	5	52.5	60.8	62.1
G6PD-deficient (1 Filipino, 3 Negro)	4	60	7.1	31.8

* Values given represent mean value for each group.

primaquine-induced and copper sulfate-induced hemolytic diseases with respect to the latent interval (approximately two to three days) between time of drug administration and onset of manifestations of hemolysis. This suggests that the difference observed between the effects of these agents on the intact erythrocyte is not merely due to the time required for penetration of the cell by the drug. Thus, copper sulfate rapidly penetrates the cell, inhibits glycolysis, oxidizes GSH, and denatures hemoglobin (Heinz bodies are demonstrable), whereas the metabolic effects of primaquine appear to be more limited.

Some caution may be justified in interpretation of results of the tetrazolium-G6PD stain and its inhibition by copper sulfate. This procedure is dependent on anaerobic glycolysis (Embden-Meyerhof pathway) for endogenous production of adenosine triphosphate and glucose-ti-phosphate. Inhibition of any of several enzymes could, theoretically, be responsible for the results observed. The method is quite sensitive, however, for detection of glycolytic inhibition nonspecifically, and, in the absence of inhibitors, appears to be specific for G6PD because no other naturally occurring disorder of erythrocyte metabolism has been found to give abnormal results by this technique. However, precise localization of the metabolic defect requires studies with purified enzymes, as here reported. These investigations indicate that copper adversely affects the erythrocyte in many ways, any of which might of itself contribute to hemolysis. Besides the inhibition of G6PD and denaturation of hemoglobin, effects on critical erythrocyte components other than those here documented may also be exhibited by copper.

A comparison of the results of this investigation with those previously reported [20-22] suggests that copper sulfate may be useful in studies of the effects of hemolytic drugs for the following reasons:

1. Copper is the only agent thus far shown both to penetrate the erythrocyte membrane and to cause competitive inhibition of G6PD.

2. Previous studies of other G6PD inhibitors have not demonstrated nonenzymatic enhancement of the rate of oxidation of NADPH as here reported for copper.

3. In contrast to the effect of N-ethylmaleimide, which preferentially oxidizes

GSH, copper sulfate inhibits G6PD at concentrations which do not affect GSH concentration in the normal cell.

On the basis of the competitive inhibition of G6PD herein documented, it is tempting to speculate that cupric ion competes with substrate at the active site of the G6PD molecule, and that sulfhydryl groups may therefore play an important role at this locus. This possibility is supported by the observation that other sulfhydryl inhibitors (N-ethylmaleimide, *p*-hydroxymercuribenzoate, sodium iodoacetate, and mercuric chloride) are also inhibitors of human erythrocyte G6PD.

Summary

A case is reported of acute hemolytic anemia and gastrointestinal hemorrhage due to accidental ingestion of copper sulfate. Investigations of the mechanism of hemolysis show that copper sufate (1) inhibits purified glucose-6-phosphate dehydrogenase competitively, (2) penetrates the intact erythrocyte and inhibits glycolysis, (3) accelerates the oxidation of NADPH in vitro, (4) denatures hemoglobin (Heinz body formation), and (5) oxidizes glutathione. Each of these phenomena probably contributes to the hemolytic effect of copper sulfate. It is suggested that persons with erythrocyte glucose-6-phosphate dehydrogenase deficiency may be more susceptible than normal persons to the hemolytic effect of copper sulfate.

The medical staff of the Bess Kaiser Hospital, Portland, Ore, permitted the publication of the clinical and laboratory details of this case.

Generic and Trade Names of Drugs

Dimercaprol—*BAL*.

Nitrofurantoin—*Furadantin*.

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NOTE

COPPER CONTENT OF HUMAN ALIMENTARY SECRETIONS*

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BILE IS THE MAJOR EXCRETORY VEHICLE for copper in man, yet the amount of copper in the feces is more than can be accounted for by the unabsorbed dietary copper and copper excreted in bile (*1*). It has been suggested that a portion of the copper found in the feces is secreted by some unspecified mechanism directly through the intestinal wall (*2, 3*).

In order to determine whether intestinal secretions other than bile could serve as significant excretory pathways for this metal, a study was made of the copper content of human saliva and gastric juice. Few such determinations for normal subjects have been reported (*4, 5, 6*). None has previously been reported for patients with Wilson's disease, a genetic disorder characterised by an undefined disturbance in copper metabolism which leads to increased tissue copper levels with hepatic and nervous system dysfunction.

Copper was determined colorimetrically using the oxalydihydrazide-acetaldehyde complex (*7*) in samples which had been wet-ashed with 2 M HCl in 10% w/v trichloracetic acid (*8*). Samples of human serum and gall bladder bile were used to standardize and test the precision of the method. Precautions were taken to minimize metal contamination by using analytical grade reagents, demineralised water and acid-washed glassware for the determinations, and samples were collected and stored using plastic tubes, syringes and containers. Particulate matter was removed from saliva and gastric juice by centrifugation at 2,600 G for 20 min. Gastric juice free of swallowed saliva or nasopharyngeal secretions was aspirated from fasting subjects. Samples contaminated with blood or bile were excluded. Gall bladder bile was collected by needle puncture and aspiration of the gall bladder at laparotomy in six patients who had had normal liver function tests and who were found to have a normal liver and biliary tract at operation. Further bile samples were taken from fourteen patients who were undergoing cholecystectomy for symptomatic biliary tract disease, but who had been shown by preoperative cholecystography to have functioning gall bladders.

Analytical precision was determined by the analysis of ten aliquots of normal human serum sample. The value obtained was 105.8 ± 4.8 (±S.D.) μg copper/

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TABLE 1

COPPER CONCENTRATION IN HUMAN SERUM, SALIVA, GASTRIC JUICE AND GALL BLADDER BILE

Sample	No. of Persons	μg Copper/100 ml		
		Mean	S.D.	Range
Serum	20	111	16.7	86–152
Gall bladder bile	20	354	155	130–715
Saliva	18	29	11.7	8–55
Gastric juice	46	39	19.9	10–85

100 ml. The copper concentrations found by the analysis of normal serum and bile samples are shown in Table 1. No significant difference in values for bile was found between the patients with symptoms of biliary disease and those with a normal biliary tract. The values obtained are consistent with those reported by previous workers for serum (*9–13*) and for gall bladder bile (*14–16*).

The values for the copper content of saliva and gastric juice are reported in Table 1 for normal subjects and in Table 2 for patients with Wilson's disease. Human saliva and gastric juice contained much smaller amounts of copper than did bile. However, when considered on the basis of their daily volume (saliva 1–2 l, gastric juice 2–4 l) (*17*) an amount of 1–2 mg would be expected to pass to the bowel lumen each day. Together with pancreatic juice, these secretions do represent a significant pool of luminal copper.

The amount of copper found to be present in the saliva and gastric juice of the patients with Wilson's disease did not differ significantly from normal values ($P > 0.05$).

TABLE 2

COPPER CONCENTRATION IN SALIVA AND GASTRIC JUICE OF PATIENTS WITH WILSON'S DISEASE

Sample	No. of Patients	μg Copper/100 ml	
		Mean	Range
Saliva	5	27	13–38
Gastric juice	4	34	22–57

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Bulletin
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Public Health Service
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COPPER CONTAINERS FOR FOOD AND DRINK

Richard Hall, Pharm. D.

Copper poisoning from copper pots and pans has long been feared by housewives, not entirely without cause. Nearly two centuries ago, an article appearing in the Medical Transactions of the College of Physicians in London described several cases of copper poisoning, including that of a young woman who ingested 3 to 4 ounces of pickles heavily impregnated with copper after which she "slaked her thirst with vinegar" and died following ten days of incessant vomiting. Some interesting cases which have been reported during recent years are presented below.

CASE REPORTS OF COPPER POISONING

1. Nine cases of copper intoxication with vomiting and diarrhea were caused by ingestion of apples boiled in a copper vessel at a school lunch kitchen.

2. An outbreak of nausea and vomiting occurred at a military base in the United States. It was caused by the use of a communion decanter whose worn silver plating left the copper base exposed. After a one hour contact with the worn vessel, grape juice used in the church service was found to have a pH of 3.1 and a copper content of 830-840 p.p.m.

3. Ten of fifteen persons attending a party exhibited weakness, abdominal cramps, dizziness, and headache after drinking a cocktail containing lemon juice, rye whiskey, sugar, and water. The cocktail had been chilled for two hours before serving in cocktail shakers whose inner plating appeared worn. The amounts ingested varied from one-half to three glasses which, on the basis of a subsequent test batch, represented ingestion of 5 to 32 mg. of copper per person.

4. Fifteen of thirty-seven persons at a boat club luncheon exhibited nausea, vomiting, cramps, or diarrhea after drinking a punch containing

carbonated soft drinks, club soda, vodka, and slices of lime and orange. The punch was being served from a three tiered fountain punch bowl made of chrome plated copper. A recirculating pump is used to force the liquid to the top tier of this fountain. It then drops through multiple holes to the second tier creating a falls effect. Glasses are filled by holding them under the four spouts to the bottom tier. The chrome of this fountain was eroded away in both the top and bottom tiers, and the punch was found to contain 50 p.p.m. of copper.

5. Several instances of nausea and vomiting have been reported from ingestion of carbonated beverages from post-mix vending machines. When the check valves on these machines become defective, they allow carbon dioxide gas under pressure to leak into the water supply lines. If the water lines are copper, rather than plastic or stainless steel, the carbonated water dissolves some of the copper. Water from such machines has been found to contain 35 to 260 p.p.m. of copper.

6. In England, copper hot-water boilers (geysers) have been employed to heat water for tea. In one instance, twenty workmen exhibited nausea, vomiting, and diarrhea after drinking their morning tea. It was prepared with water which had been standing in a gas-heated geyser for 48 hours. Inspection of the geyser showed corrosion and over-burning of the gas jets producing excessive sulfur containing fumes which formed copper sulfate on the copper lid of the geyser over the condensation bowl. A sample of tea taken from the brew pan contained 30 p.p.m. of copper even though more water had been added to the initial infusion. In another instance, eighteen workers exhibited moderate to severe gastroenteritis after drinking tea prepared from water heated in a similar geyser. This geyser had a blue-grey deposit of copper sulfate encrusted on the lid and float control arm. Water from it showed a brownish tinge due to suspended particles of matter which were found to be copper sulfate. The water was very slightly acidic (pH 6.7) and contained a total copper content in the suspension of 44 p.p.m.

Copper has been extensively mined throughout the world and used by mankind for thousands of years. Many of the copper mines of Europe have been worked since before the Roman Empire. Workings of copper in the oldest known copper mine associated with the smelting of this metal have been traced back to 4300 B.C. and archeological research indicates that the use of copper probably began about 5000 B.C. Moses who lived about 1200 B.C. recommended that copper vessels should be kept absolutely clean. Herodotus in the fifth century B.C. in writing of his travels in

Egypt noted that the Egyptians "drink from cups of bronze and rinse them out every day, and not some only do this, but all." Thus copper containers for food and drink have been used since long before Christ, and are still in use today. Copper kettles are used in the canning industry and in the processing of evaporated milk. In many European countries, copper cookware is still commonly used by many housewives.

There appears to be no unanimity of opinion regarding the acute and chronic toxicity of copper and its salts. It is apparent that the acute oral toxicity will be more dependent upon the amount of copper or its soluble salts retained in the stomach than on the amounts actually ingested. Because of their irritant effects, even small amounts of soluble copper salts such as copper sulfate will cause vomiting in humans, and are indeed administered therapeutically for this purpose.

Copper sulfate intoxication causes a metallic taste in the mouth, a burning sensation in the epigastrium, eructations and repeated vomiting of greenish material. In severe cases, bloody diarrhea, shock, smoky urine, oliguria, anuria, uremia, enlarged tender liver, jaundice, coma, and death may occur.

In India, copper sulfate is a cheap, readily available substance which is quite commonly employed as a suicidal poison among people in the lower income group. A study of 53 adult patients suffering from acute copper sulfate poisoning was made in New Delhi, India in 1964. Most of the patients were 16 to 25 years of age, and 12 of these 53 cases of copper intoxication were fatal. A study of these attempted suicides was made by Dr. Chuttani and his associates. They found there was no correlation between the amount of copper sulfate ingested and the level of serum copper. However, there appeared to be some correlation between the amount ingested and the whole blood copper values (Table 1).

Table 1

Quantity Ingested	Copper Level in Micrograms/100 ml	
	Serum	Whole Blood
1 - 5 grams	264.6 ± 181.2	383.4 ± 124.9
6 -15 grams	332.4 ± 112.4	519.0 ± 134.3
16 -30 grams	346.2 ± 280.2	684.0 ± 513.2
Over 30 grams	239.5 ± 78.1	605.5 ± 146.3
Normal Values	151.6 ± 75.0	217.0 ± 52.0

Statements obtained from the patients indicated that the amounts ingested varied from 1 to over 100 grams. Of the twelve fatal cases, seven died apparently either from shock and hypotension during the first 24 hours or from subsequent hepatic or renal damage with coma and uremia. The clinical course of the other five fatalities was not given. Autopsy reports showed superficial to deep ulcerations of the gastric and intestinal mucosa, liver cell necrosis, bile thrombi, and renal damage with congestion of the glomeruli and swelling or necrosis of the tubular cells.

In 1896, a medical journal reported that human volunteers had consumed 20 to 30 mg. of copper daily in coppered preserves without any effect. Hoffman, in a Textbook of Inorganic Chemistry published in 1918, states that an adult can tolerate daily ingestion of 100 mg. of metallic copper without harm. A dose of 250 to 500 mg. of copper is stated to be the largest dose which can be taken unnoticed in food. Copper sulfate has been used in oral doses of 30 to 100 mg. as an astringent, and 250 to 500 mg. in a 1% solution as an emetic, although smaller amounts may also produce vomiting. Vomiting is produced by similar doses of other soluble copper salts, but metallic copper, copper oxide, and copper sulfide which are all practically insoluble in water are reported to be harmless. One author took daily for 8 months a dose of 240 mg. of copper oxide, and another took a single oral dose of 15 grams of finely divided metallic copper without apparent harm. However, aspiration of metallic copper dust has produced fatalities.

Chronic toxicity studies in animals showed that rats receiving ad libitum 500 p.p.m. of copper in their diet exhibited retarded growth, while 4000 p.p.m. caused starvation and death. Copper poisoning in animals leads to severe degenerative changes of the liver, kidneys, and spleen. Sheep having access to salt licks containing 5 to 9% (50,000 to 90,000 p.p.m.) of $CuSO_4$ over a 25 to 86 day period, and each ingesting an amount estimated to be not more than 50 grams, showed sudden onset of anorexia, hemolytic anemia, icterus, and hemoblobinuria followed by death in a day or two. Livers showed concentrations as high as 1.47 mg. Cu per gram compared to a normal maximum of 0.04 mg./gm. Coppersmiths have reportedly absorbed so much copper that they exhibit green stains of the teeth, green tint of the hair, beard, and complexion, and "their bones in the churchyard are green". However, their morbidity and mortality was no greater than that of other industrial workers.

In the United States, copper utensils have been largely replaced by ones made of aluminum and stainless steel, and the copper pots of the eighteenth century have been relegated to the antique dealers. However,

copper is still employed in the manufacture of some kitchen utensils because of its high thermal conductivity which provides an even distribution of heat. Many pots and kettles are in use which have copper on the bottom of stainless steel so that the copper does not come in contact with the food. With pots and pans made exclusively of copper and copper alloys, including bronze, and in copper containers having worn or defective tin coatings, direct contact does occur.

In the presence of moist air, copper tarnishes (corrodes) rapidly, becoming coated with either a brown oxide or a green basic carbonate (verdigris). Organic acids such as acetic, citric, malic, oleic, and tartaric acids which are present in certain fruits, jellies, jams, candies, and citrus juices, will react with the basic oxides and carbonates of tarnished copper utensils to form soluble copper salts which can produce symptoms of toxicity. The amount of soluble copper salts formed by these foods is dependent upon their degree of acidity, the length of contact, and to some degree on the amount of air present and the temperature. Even highly polished copper vessels can add small traces of copper to foods, but unless the foods are acidic, the amount of copper added will be negligible and should cause no harm. Therefore, it has been suggested that if copper pots are to be used for cooking or storing foods, they should be adequately tinned or kept brightly polished, a process in which our grandmothers took great pride, but which many of their descendants find too time-consuming.

Although any water will take up traces of copper if allowed to stand long enough in contact with it, significant amounts of copper are not introduced into water from copper tubing until the pH falls below 5.6 (Table 2). At pH values of 5.0 and below, the amount of copper dissolved is increased. In the presence of CO_2, even higher concentrations of copper are dissolved into the acidic carbonated solution than are found with acid buffer solutions alone. With but few exceptions, the drinking water in the United States has pH values well above 5.0, with the usual limits for untreated natural waters being 5.5 to 8.5. In those few instances where it is lower, the acidity is likely to be caused by dissolved tannic acid from heavily forested areas. Tannic acid will form insoluble salts with copper, iron, and other metals.

In one experiment, it was found that there is no appreciable difference in the amount of copper dissolved by water from old versus new copper tubing. In water, a protective layer consisting mainly of insoluble cuprous oxide is formed on copper. After formation of the protective layer, the copper content in either cold or warm waters flowing through copper tubing does

not usually exceed 0.3 p.p.m. (0.1 to 0.3 mg./liter). The use of copper and brass pipes and fittings to carry drinking water appears to be perfectly safe, except in those few instances where the water is strongly acidic or contains a strong dissolved gas content.

Table 2

EFFECT OF pH ON COPPER TUBING

Buffer pH	Time of Exposure	Copper (p.p.m.)	CO_2 Bubbled Thru
5.60	18 hours	3.5	yes
5.00	2 hours	25.0	yes
4.75	20 hours	500.0	yes
4.00	2 hours	400.0	yes
3.50-3.90	24 hours	166.0	no
3.60	21 hours	600.0	yes
3.35	24 hours	200.0	no

Cases of poisoning have been reported from ingestion of certain foods and beverages which were processed or stored in contact with copper. Distilled liquors have been contaminated with copper dissolved from the copper worm piping of the condenser by the action of volatile organic acids such as acetic and citric. Acid foods including jellies, jams, and candies have been contaminated by being cooked in copper vessels. Plum jam, for example, is often cooked in copper vessels without causing any problems, but when the product is left too long in the vessels considerable quantities of copper compounds can be formed with the organic acids in the presence of atmospheric oxygen.

The French were reportedly the first to place on the market preserved food which was "greened" by addition of copper. This greening (coppering, verdissage, Fr.) of canned foods such as peas, beans, cucumbers, etc., also became popular in Austria, Germany, Italy, Switzerland, and England.

Canned fresh peas which contained 150 to 200 mg. of copper per kg. of food (150-200 p.p.m.) were reported to be perfectly edible from a standpoint of taste and absence of toxic symptoms. The amount of copper necessary to produce the desired amount of color is about 50 mg. Cu/Kg. of food (125 mg. copper sulfate/Kg.). The copper forms a firm compound with the chlorophyl of green vegetables which preserves their color. No effects were observed from prolonged consumption of greened foods corresponding to a daily intake of 12 to 15 mg. of copper.

German and Rumanian food laws in the early 1900's limited the amount of copper which could be added to foods at 55 and 66 mg./kg. of food.

In Bucharest in 1924, a grocer was fined for selling peas and pickles adulterated with copper sulfate to the extent of 91 mg. Cu/Kg. peas and 73 mg. Cu/Kg pickles. In the United States, the Food and Drug Administration has limited the amount of copper gluconate which can be added for nutritional purposes to 50 mg. per kg. of food (7 mg. copper/kg.). However, at the present time, no copper salts are approved for use as food coloring additives under the provisions of the U. S. Food, Drug, and Cosmetic Act.

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'Torsion Dystony' and Abnormal Copper Metabolism

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Abstract. A patient suffering from torsion dystony presenting abnormal copper metabolism is reported. Low ceruloplasmin concentration was found in other members of his family. The problem of the causal relationship between torsion dystony and abnormal copper metabolism is discussed.

Key Words
Torsion dystonia
Ceruloplasmin
Wilson's disease

Introduction

The torsion dystony syndrome was described in the past by some authors in connection with liver involvement and lenticular lesions.

This clinical and pathological condition was called 'dystonia lenticularis' 4–8]. It is the aim of this paper to report a patient who developed the classical symptomatology of pure torsion dystony associated with abnormal copper metabolism.

Case Report

A 29-year-old man, a Jew of Iranian origin, was repeatedly admitted in 1969 and 1970 to the neurological department because of involuntary movements of the neck, limbs and body of 14 years' duration.

At the age of 14 the patient started to suffer from some writing disturbances which did not change the next few years. In 1961 the patient noticed involuntary movements of the right hand when he was tired or excited: extension and fanning of the fingers, abduction of the thumb, and a very fine tremor. Later on, contractions of the muscles of the abdomen appeared which spread to the neck and back. When he was supine there were involuntary contractions of the abdominal muscles only. In 1969 when walking, he twisted his head to the right, turned it to the left

and bent his body to the right because of contractions of the muscles of the neck and mainly of the right side. These contractions caused him pains in his back and neck. When writing, sudden involuntary movements of the hand occurred, especially when the hand got tired. In 1969 he had some interviews with a psychiatrist, but since his condition deteriorated, he was admitted to the neurological department.

The birth and childhood were uneventual. His parents are first, degree cousins (the pedigree of his family is attached). His father is suffering from essential tremor. The ceruloplasmin concentration was found to be low in his parents and in the brother examined: 7 mg% in his father, 12 mg% in his mother and 15 mg% in his young brother. The patient is working as a social worker in an institution for young delinquents.

On admission, the general physical examination revealed a man in a relatively good nutritional state. The blood pressure was 130/80 the pulse rate 80 min; a split first heart sound was found and the liver and spleen were not palpable. No jaundice or pallor was found.

The neurological examination revealed a well-oriented man with a good mentation. The cranial nerves were normal. The speech was normal. The Kayser-Fleischer's ring was not found, on repeated slit lamp examinations done by three senior ophthalmologists. All modalities of sensation were normal. The crude force in the four limbs was normal and the deep tendon relexes were equally elicited, except for the triceps reflexes. No pyramidal signs were found. Torticollis to the right was seen (fig. 1), as well as scoliosis of the thoraco-lumbar spine (fig. 2), with the convexity to the left side and tortipelvis to the right. His gait was straight, but he alternately flexed his body to the right side and then straightened it again, rotating his head to the left and bending it to the right, in a rather slow way. No tremor or any other cerebellar signs were found; the Romberg test was negative.

The laboratory tests disclosed normal ESR, 14 g% hemoglobin, 110,000 thrombocytes, 6,200 white blood cells, 40 mg% serum urea and 98 mg% blood sugar. The liver function tests were within normal limits on several occasions. The SGOT and SGPT were normal, except for one determination when SGOT showed a value of 71 U. The urinalysis was normal and an increased concentration of aminoacids was found in the urine. The CSF examination did not reveal any pathological findings. The EEG, brain Tc^{99} scan, echoencephalogram were normal. The plain X-ray of the skull and chest were normal, X-ray of the cervical spine revealed spondylarthrotic changes of the C_5–C_7 vertebras.

Serum ceruloplasmin was constantly found below 20 mg%, between 4.5 and 1.5 mg%. The cupriuria was 220 μg/24 h. Radioactive copper-67 studies revealed a rapid liver utpake, but the liver copper-67 content increased in the next three days (fig. 3), instead of the normal decrease seen during this time. The thigh muscle copper-67 uptake was low and did not change subsequently.

The patient received a maintenance dose of 60 mg of Diazepam daily, without any benefit. Therapeutic trials with L-dopa also did not help. Now the patient is receiving D-penicillamine 250 mg, 6 tablets daily, a low copper diet and potassium sulfide, 20 mg tid. During the first 1½ months of treatment the cupriuria increased to 13.2 mg/24 h, and afterwards decreased progressively, reaching a value of 600 μg/24 h. No clinical improvement was observed meanwhile.

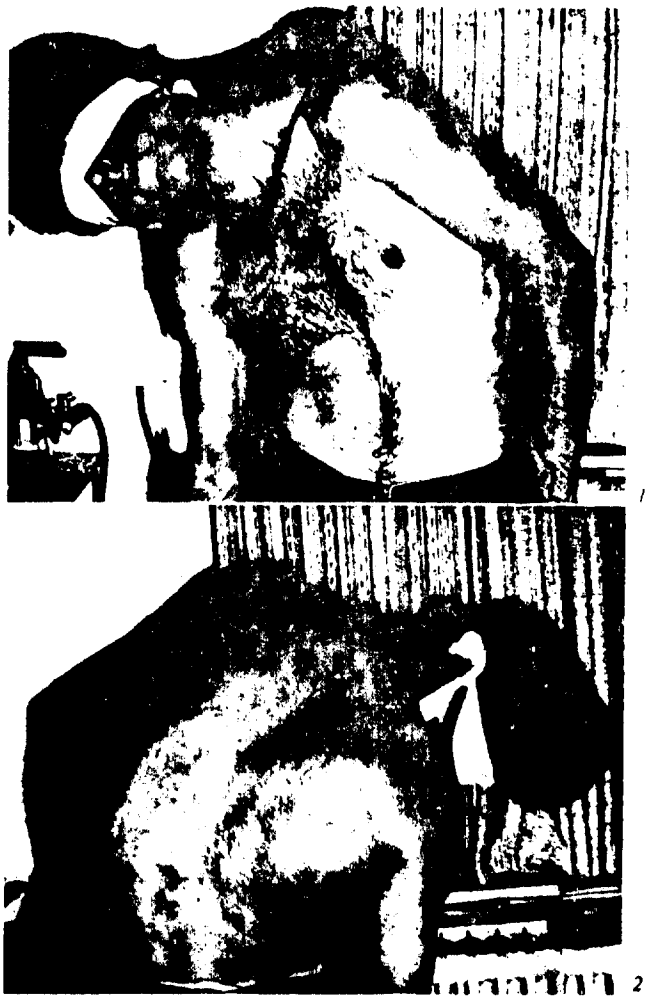

Fig. 1 and 2

Discussion

The case described showed a combination of two different pathological entities: clinically, the patient behaved as a classical torsion dystony, and biochemically he presented a clear-cut copper metabolism disorder. This association of conditions raises the question of a common etiology for both.

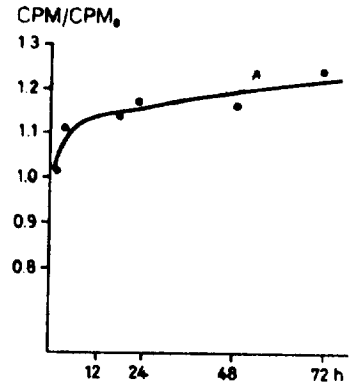

Fig. 3

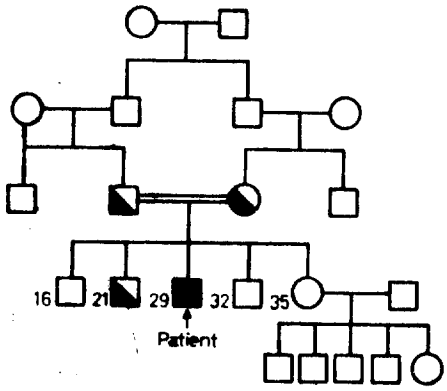

Fig. 4. Pedigree of the family. Double-line signifies consanguineous marriage. Numbers indicate age in years.

Since 1918, there are several cases in the literature in which the involvement of the lenticular nuclei and liver were described in patients suffering from dystonic features. In 1918, TOMALLA [7] described a young boy suffering from liver disease with spasmodic torsion movements in his right arm and neck, rigidity, stiffness of the right leg and severe dysarthria. The pathological findings were those of Wilson's dis-

ease, and Tomalla named this condition 'dystonia lenticularis'. Two years later SPILLER [5] described a patient suffering from severe universal spastic contractions, athetoid movements, mental deterioration and severe dysarthria. The pathological examination revealed central nervous system findings of Wilson's disease. In the same year TAYLOR [6] reported two siblings: one of them had suffered since the age of 7 from progressive spasmodic movements of the limbs and presented a gait severly disturbed by torsion movements. The other sibling suffered from widespread choreiform movements, mental failure, disturbances in speech and facial contortion. He suggested that the first case suffered from pure torsion dystony and the second one probably from other extrapyramidal condition. He thought that there was no sharp difference between various extrapyramidal conditions. TAYLOR suggested a striatum lesion as the pathological basis for these conditions, associated probably with alterations in the liver and concluded that the most suitable term for the torsion dystony as symptom is dystonia lenticularis'. WIMMER in 1929 [8] described a new case of torsion dystony presenting pathological findings of Wilson's disease. He mentioned two similar cases reported by others which presented Kayser-Fleischer rings. RODRIGUEZ-ARIAS *et al.* [4] in the same year reported a patient with severe dystonia associated with sialorrea, speech disturbances, Kayser-Fleischer's rings and disturbed liver function tests. ZADOR [9] and ASCHER [1] pointed out the fundamental differences between Wilson's disease and torsion dystony, and showed that dystonia in Wilson's disease is always accompanied by mental changes and rigidity. Recently, ZEMAN and WHITLOCK [10] in their discussion on symptomatic dystonias wrote: 'We have never encountered difficulties in distinguishing the two diseases.' In their opinion, Wilson's disease should not longer be mentioned in the differential diagnosis of dystonia.

Our patient, however, presented clear-cut progressive signs of dystonia musculorum without any mental changes, speech disturbances or pigmentary deposits into the iris, and from a clinical point of view no other diagnosis could be considered. However, determinations of the ceruloplasmin in the serum revealed consistent and constant pathological levels and, therefore, the diagnosis of torsion dystony was questionable. Studies with radiocopper showed rapid, normal uptake in the liver and prolonged retention of the isotope there with some increased uptake three days after copper administration and no significant muscle uptake. O'REILLY *et al.* [3] and BECKNER *et al.* [2] showed that Wilsonian hetero-

zygotes do not show significant muscle uptake of radiocopper and present a normal initial rate of liver uptake, but delayed and incomplete net release of the isotope from the liver. The urine copper in our patient was found to be 220 μg/24 h – at least twice the normal value.

All these biochemical findings point to disturbed copper metabolism in our patient, similar to that found in Wilsonian patients.

It is interesting to note that, in spite of long-term and very severe neurological signs, Kayser-Fleischer's ring, which is always seen in Wilson's disease, was not found in the patient. We are of the opinion that there exists a causal relationship between the abnormal copper metabolism and dystonia, and we must consider the possibility that we are dealing with a special form of Wilson's disease – the dystonic type. This dystonia might be due to the inborn error of copper metabolism.

In light of the laboratory findings in our case, it seems to us that in each case of torsion dystony an examination for copper abnormality is necessary.

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ELEVATION OF SERUM COPPER FOLLOWING COPPER SULFATE AS AN EMETIC

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ABSTRACT. Copper sulfate in a dose equivalent to 100 mg of copper was administered orally to six young children who came to the outpatient department because of ingestion of substances in toxic amounts. As a control, six other children presenting with the same complaint received 10 to 15 ml of syrup of ipecac. Although vomiting occurred within 5 minutes after administering the copper sulfate, significant elevations of serum copper occurred in each child. Only one of the children receiving ipecac had any rise in serum copper concentration.

Because of hazards associated with copper sulfate, its use as an emetic appears questionable. *Pediatrics*, 42:189, 1968. CERULOPLASMIN, COPPER, EMETICS, IPECAC, POISONING.

The effectiveness of copper sulfate ($CuSO_4$) as an emetic in intoxications in children was recently reported[1,2] and circulated widely to pediatricians.[1] Although capable of inducing emesis in a greater percentage of patients and more rapidly than ipecac, $CuSO_4$ administered orally may be hazardous.[3] In this study children with acute ingestions were given $CuSO_4$. The absorption and effects of copper were assessed. Karlsson and Noren's[1] regimen was employed.

METHODS

Three groups of children were studied. Pertinent clinical data is shown in Table I.

Group I

Four children presenting to the outpatient department with a history of recent ingestion of potentially toxic substances were admitted to the Clinical Research Unit following administration of 250 mg of $CuSO_4$ in 5 ml of water by mouth. This dose, containing 100 mg of copper, was given after the children drank 75 to 200 ml of juice. Had it not been for this study, only one child, G.L., would have been admitted. The duration of the hospital stay for each child was 24 hours, except for G.L. who remained for 48 hours. Routine hemogram and blood for plasma hemoglobin and liver function studies were obtained on each of these four children prior to receiving $CuSO_4$ and again 24 and 48 hours later. Serum copper and ceruloplasmin concentrations were determined before administration of the $CuSO_4$ and 1 to 3, 6 to 8, 10 to 12, 20 to 24, and 42 to 48 hours later.

Group II

Two other children who had ingested potentially toxic substances also received 250 mg of $CuSO_4$ as an emetic. These children presented after the studies on Group I were completed. They were not admitted to the hospital. Blood for serum copper and ceruloplasmin concentrations was obtained before and 1 and 8 to 12 hours after the administration of $CuSO_4$.

Group III

Six children presenting to the outpatient department because of recent ingestions re-

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TABLE I

CLINICAL DATA ON CHILDREN RECEIVING $CuSO_4$ OR IPECAC AS EMETICS FOLLOWING INGESTION OF POTENTIALLY TOXIC SUBSTANCES

Name	*Age (mo)*	*Ingested Substance*	*Minutes After Emetic Given Before Emesis*	*Initial Serum Copper (μg/100 ml)*	*Initial Serum Ceruloplasmin (mg/100 ml)*
Group I (250 mg $CuSO_4$ given at 0 time)					
R.H.	18	aspirin	2	128	31
H.G.	20	aspirin	2	94	[illegible]
J.D.	40	Tussionex*	5	128	[illegible]
G.L.	22	aspirin	2	173	68
Group II (250 mg $CuSO_4$ given at 0 time)					
B.T.	28	aspirin	3	238	73
J.H.	36	aspirin	3	168	54
Group III (10 to 15 ml of Syrup of Ipecac given at 0 time)					
Y.K.	18	Pro-Duosterone†	14	210	78
H.W.	30	aspirin	17	165	73
K.E.	36	aspirin	12	94	43
J.B.	42	aspirin	7	115	61
La.W.	36	Vistaril‡	10	140	73
Li.W.	18	Vistaril	5	105	73

* Dihydrocodeinone, phenyltoloxamine.
† Ethisterone, ethinyl estradiol.
‡ Hydroxyzine.

ceived 10 to 15 ml of syrup of ipecac as an emetic. Serum copper and ceruloplasmin concentrations were determined at the same intervals as for Group II.

Specimens

Specimens were obtained in specially washed° glassware rinsed with deionized, distilled water. Plasma hemoglobin was determined by the Crosby and Furth method.[4] Total serum copper was measured by the method of Gubler, *et al.*[5] Fourteen determinations of five samples were reproducible to within 6%. This method was also used for determinations of copper in vomitus and stools. Stools were first homogenized in a Waring blender. Known amounts of $CuSO_4$ were added to aliquots of vomitus and feces. Recoveries of 90 to 110% were obtained. Urine copper was determined by atomic absorption spectrophotometry and ceruloplasmin was determined by its oxidase activity.[6] By the latter method, 15 determinations on 4 samples were reproducible to within 3%. Urine amino acids were determined by high voltage electrophoresis[7] on aliquots equivalent to 0.03 mg creatinine.

° Pyroneg, The Diversey Corp., Chicago, Illinois 60606.

RESULTS

As shown in Table I, the $CuSO_4$ was rapidly effective as an emetic. The vomitus was green in color and between 75 and 180 ml in volume. Guaiac on the vomitus was negative in each case. In three children in Group I, where all the vomitus was collected and analyzed for copper, only 54 to 67% of the 100 mg (by assay) of copper administered was recovered. In the one child in whom fecal copper was analyzed

G.L.) only 2% of the administered copper was found in stools passed spontaneously and following Fleet's enema up to 48 hours after administration of the copper. The total recovery of copper in feces and vomitus in this child was 69%. Stools on all four children in Group I remained guaiac negative, and there was no diarrhea in the children in this group.

It thus appeared that, despite rapid emesis, some copper was absorbed. Additional evidence for this is shown in Figure 1. Each of the six children receiving $CuSO_4$ as an emetic had a significant elevation of serum copper within the first 6 hours following administration of $CuSO_4$. In two children, B.T. and G.L., the maximum serum copper values exceeded the upper limit found in normal children by this method.[8] In B.T. the 0 time value was also above the normal range. Only one of the six children receiving ipecac had any elevation of serum copper.

Two of the six patients had marked elevations of serum ceruloplasmin after receiving $CuSO_4$ (Fig. 2). Smaller rises were seen in two other children receiving $CuSO_4$ and in two of the six children who received ipecac.

In two of the three children in Group I where complete urine collections were obtained, the 24-hour excretion of copper significantly exceeded the normal value of 58.5 ± 24.6 µg/24 hours (Table II).[9]

One of the patients, G.L., with a significant cupruria had trace proteinuria with a generalized amino aciduria in the urine specimen obtained 12 to 18 and 18 to 24 hours after copper was given. Although asymptomatic, this child had a serum salicylate of 53 mg/100 ml 23 hours after admission. A clean-catch urine obtained 40 hours after admission had 3+ protein and 20 to 40 round cells per spun high power field. The culture of this specimen was negative. Urinalysis of this patient 2 and 12 days later, including culture, was completely normal. No amino aciduria was found in the 18 to 24 hour specimen from H.G. or J.D. Urinalysis on R.H., H.G., and J.D. between 24 to 48 hours revealed no abnormality.

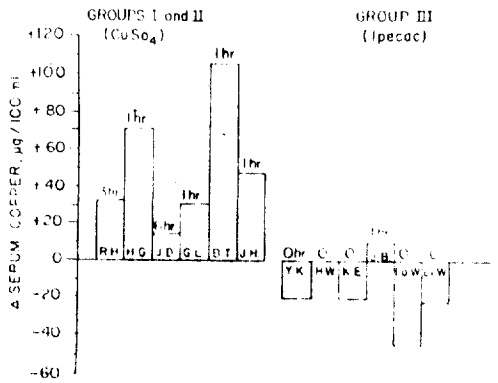

FIG. 1. Change in serum copper concentration following emetics. The time of the maximum value (hours after emetic given) is indicated above the bar for each patient.

The amount of copper found in plasma and urine accounted for less than 1% of the administered copper. It seemed possible that the copper unaccounted for could be sequestered in the liver or erythrocytes. Because of the occurrence of hemolysis and hepatic damage in copper intoxication, indications of such complications were sought in the patients in Group I.

There was no evidence of hemolysis as measured by bilirubin, reticulocyte count, or plasma hemoglobin up to 48 hours. Slight declines in hematocrit were compatible with the volume of blood drawn. Plasma hemoglobin followed no consistent pattern. The variations were attributed to

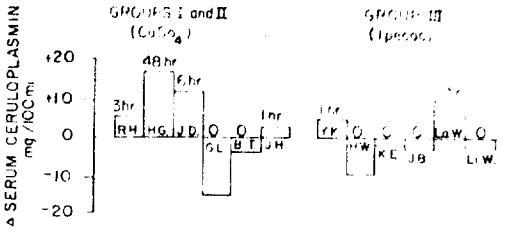

FIG. 2. Change in serum ceruloplasmin concentration following emetics. The time of the maximum value (hours after emetic given) is indicated above the bar for each patient.

TABLE II

URINARY COPPER EXCRETION FOLLOWING ORAL ADMINISTRATION OF $CuSO_4$

Patient	*Time Following Administration of $CuSO_4$ (hr)*	*Urine Volume (ml)*	*Total Urine Copper (μg)*
R.H.	0–6	53	56.2
	6–12	26	21.1
Total	12–18	66	20.5
	18–24	16	6.9
		161	104.7
H.G.	0–6	—	—
	6–12	97	12.7
Total	12–18	270	15.8
	18–24	355	14.8
		722	43.3
G.L.	0–6	105	81.0
	6–12	130	19.2
	12–18	190	19.8
	18–24	263	26.4
Total		688	96.4

hemolysis resulting from difficulties in obtaining individual specimens.

In the three patients where liver chemistries were obtained elevations of 7 to 13 units of SGOT and 3 to 13 units of SGPT compared to the specimens obtained just before $CuSO_4$ was given were found at 24 to 48 hours. These are of only doubtful significance. There was no change in bilirubin, alkaline phosphatase, cephalin flocculation, thymol turbidity or albumin, and globulin concentration.

COMMENT

The most striking findings in copper intoxication are jaundice secondary to hemolytic anemia and liver damage, oliguria, hypotension, coma, and death.[5,6] None of the children reported here who received $CuSO_4$ as an emetic showed any toxicity definitely attributable to copper. The amino aciduria and celluria in the child with elevated serum salicylate levels could be attributed to the ingested salicylate.[9,10] The rise in transaminases after administration of the copper was of only doubtful significance.

Most disconcerting, however, was our inability to account for about 30% of the administered copper in the vomitus. This together with the elevations of serum copper in each child, leaves little doubt that significant amounts of copper were absorbed. Since copper can cause renal damage,[5,6] it might well potentiate the nephrotoxicity of drugs like salicylates[13] and other agents frequently taken in toxic amounts. Similar potentiation due to its hepatotoxic and hemolytic effects might be expected as well. Since copper also corrodes the gastrointestinal tract—undoubtedly a source of its emetic action—its use as an emetic seems questionable following ingestions of corrosive substances like salicylate and ferrous sulfate.

Two of the patients receiving $CuSO_4$ (H.G. and J.D.) had marked elevations of serum ceruloplasmin concentrations (Fig. 2). It appears reasonable that a rise in body copper in these children stimulated ceruloplasmin synthesis. However, in view of the failure to observe a similar response in the other children receiving $CuSO_4$, the significance of the rise in ceruloplasmin in these two is difficult to evaluate. In adults receiving 20 mg of copper intravenously as allycuprothiocarbamide, no elevation of ceruloplasmin was seen in the next 24 hours.[14] On a per weight basis, however, this is a much smaller dose than the amount absorbed (i.e., unaccounted for in the vomitus and stools) in our patients in Groups I and II.

One of us recently recorded a striking elevation of ceruloplasmin in a child poisoned by repeated application of $CuSO_4$ to burned skin, also suggesting that copper might stimulate ceruloplasmin synthesis.[4] Furthermore, the administration of copper to hypoceruloplasminemic, copper deficient infants results in an elevation of serum ceruloplasmin concentration within 24 hours.[15]

The two children showing the most pronounced rise in serum ceruloplasmin concentration following copper had the lowest initial ceruloplasmin concentrations. It is possible that, in those children with high

initial values, no further stimulation of ceruloplasmin synthesis was possible. The ability of salicylate to chelate copper[16] may account for the decreased ceruloplasmin in G.L. She was the only patient in this study in whom an elevated serum salicylate concentration was observed.

The dangers of ipecac as an emetic result largely from the inadvertent use of the extract rather than the syrup.[17] Fluid extract of ipecac is no longer included in the United States Pharmacopeia. In the experience of the Children's Medical Center Outpatient Department, syrup of ipecac serves as an effective and safe emetic.

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SIGNIFICANCE OF SERUM COPPER LEVELS IN ADULT PATIENTS WITH HODGKIN'S DISEASE

MARTIN HRGOVCIC, M.D., DMSc,* CARL F. TESSMER, MD,† FORREST B. THOMAS, MS,† LILLIAN M. FULLER, MD,‡ JESS F. GAMBLE, MD,* AND C. C. SHULLENBERGER, MD*

A study of 896 serum copper levels (SCL) in 191 adult patients with Hodgkin's disease has demonstrated a clinically useful relationship between SCL and the disease activity. Elevated SCL in the active disease decreases as disease activity is reduced in response to therapy, with normal SCL during remission and elevated SCL in relapse; the latter often precedes clinical signs. These relations of SCL do not seem to be present in the limited number of lymphocytic predominance cases observed. A relation between SCL and extent of disease is shown by marked elevation in SCL in generalized in contrast to localized disease and is more pronounced in patients with systemic symptoms. A control group of 35 patients with prostatic carcinoma treated with radiotherapy showed no relationship of SCL to disease or therapy. The findings in this study of serum copper confirm its clinical significance in the evaluation of Hodgkin's disease activity.

THE RELIABILITY AND CLINICAL SIGNIFICANCE of laboratory tests which have been reported for the evaluation of Hodgkin's disease is controversial.[8,10,13,15,26]

All are essentially nonspecific in character, and similar test results are encountered in a wide range of conditions and disease states, particularly inflammatory and infectious conditions.

Pagliardi and Giangrandi[17] and Jensen et al.[8] reported high correlation between serum copper levels (SCL) and disease activity. Our studies extended the previous observations on the clinical significance of SCL determinations in patients with lymphoma and leukemia, particularly Hodgkin's disease.[4-6] An increasing number of reports supporting these observations has been noted.[5,7,16,23,27-30,32]

This report is a review of the total 6-year experience of serum copper observations in adult patients with Hodgkin's disease with particular reference to the relationship with the clinical state of the disease, and to significant deviations from this relationship including interfering substances or clinical conditions. Some consideration of histologic subclassifications, stage of disease, and other factors involved has been included.

CLINICAL MATERIAL

This study is based on 896 observations of serum copper levles in 191 adult patients with Hodgkin's disease, treated and followed in the Department of Medicine (Hematology Section), Department of Developmental Therapeutics, and Department of Radiotherapy of The University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

All adult patients (65 female and 126 male) with tissue diagnosis of Hodgkin's disease, on whom one or more SCL determinations have been made during the period from February 1966 to June 1972, are included in this study. Copper values when a patient is known to be

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The authors wish to acknowledge the assistance and support of Dr. H. Hussey, Department of Radiotherapy, in the provision and organization of control cases, the Department of Developmental Therapeutics, Dr. P. S. Ong, and Dr. W. O. Russell, Head, Department of Anatomic Pathology. Special thanks must be given to Dr. Grant Taylor, Dean, The University of Texas Graduate School of Biomedical Sciences at Houston, Division of Continuing Education, and to Dr. D. M. Mumford, Department of Pediatrics, for their support in this and other studies of copper metabolism. The able technical assistance was provided by Tom Goka, Peter Chang, Hubert Daniel, Pearl Gervais, Donna Mitcham, Kenneth Jurden, and Catherine Johns of the Department of Antaomic Pathology.

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pregnant or receiving estrogens have been excluded, in keeping with the recognized relation between estrogens and elevated SCL..[2,12,20,22]

Clinical and laboratory investigations were performed as indicated to determine the extent of disease. Thirty-nine patients, clinically Stage I and II, were subjected to a staging laparotomy.

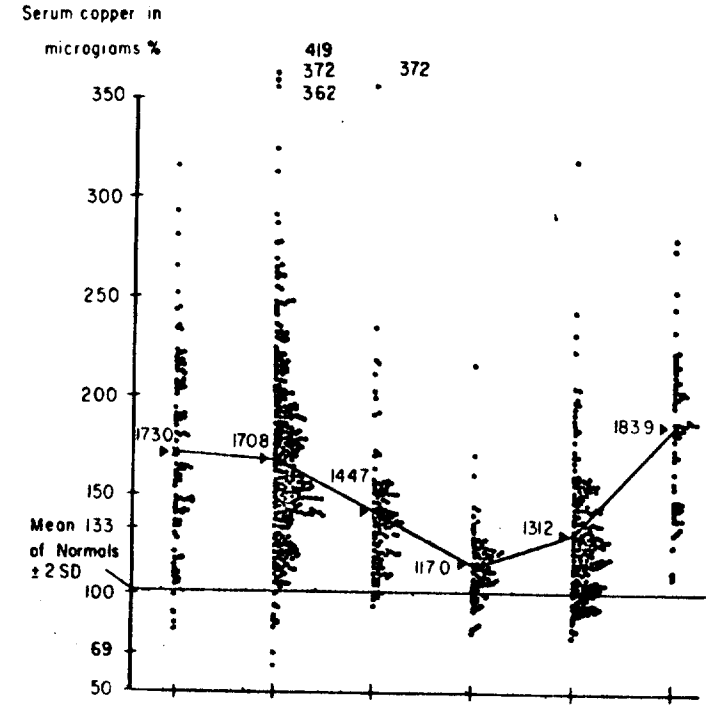
Tissue examination findings were reported according to the Lukes and Butler classifications.[14] Nodular sclerosis (100 patients) and mixed cellularity (72 patients) were the two largest groups. Ten patients were classified as lymphocytic depletion and nine as lymphocytic predominance. The Rye classification was used to stage the extent of disease.[19]

As a control group, 33 patients with carcinoma of the prostate were studied throughout the interval of radiotherapy providing a solid tumor group having no known relation to SCL, and a group undergoing radiotherapy to exposure levels equal to or exceeding those of the Hodgkin's disease patients.

METHOD

Serum copper levels were determined in the early part of this study by the colorimetric method of Jensen et al.[9] as modified by Hrgovcic et al[6] More recently, SCL have been determined by an atomic absorption technique utilizing direct dilution.[27]

The normal adult values utilized in this study are those determined previously in this laboratory,[6] a mean value of 101 μg/100 ml with a standard deviation of ± 16 μg/100 ml, the normal range thus being determined as 69–133 μg/100 ml. This is in general conformity with the values reported in the literature.[9,11,17,21,31]



Clinical Category	1	2	3	4	5	6
Abbreviation	Pre Rx	Rx WED	Rx Dund	Rx NED	No Rx NED	Relapse
Number of Samples	93.0	321.0	112.0	89.0	218.0	63.0
Number of Patients	80.0	72.0	42.0	21.0	50.0	44.0
Standard Dev.	48.0	47.5	37.6	18.5	31.6	36.2

FIG. 1. All SCL are plotted in the clinical category to which assigned (see text). Represented by the mean SCL of each category, it may be seen that increased SCLs in the active phase of disease decrease with response to treatment and rise with relapse.

The SCL was determined in this patient group at varying times during their clinical course, at intervals ranging from every other day to every 6 months. The number of SCL for each patient ranges from a single observation from each of 87 cases to as many as 34 from one individual taken over a 4-year period.

Six clinical categories of patient status of disease activity were established as follows:

	Abbreviation
1. Pretreatment	PreRx
2. Under treatment with evidence of disease	Rx WED
3. Under treatment, activity of disease undetermined	Rx, D. Und.
4. Under treatment, no evidence of disease	Rx NED
5. No current treatment, no evidence of disease	No Rx, NED
6. Relapse, or recurrent disease, with or without maintenance treatment	Relapse

The appropriate clinical category is assigned from the medical record, utilizing only the date of each copper value, without reference to the SCL.

All SCL values are plotted according to the assigned clinical category. The same method is applied to correlate SCL with histologic classifications, stages, and symptoms. Sixty-six patients presented values in two or more clinical categories during the period of observation.

Samples in the control group (carcinoma of prostate) were taken twice weekly during the course of radiotherapy which was administered as 200 rads daily for 5 days of the week, a total of 5,000 rads to the pelvic field (12 × 12 cm) continued to 7,000 rads in a smaller field (8 × 8 cm).

OBSERVATIONS

All SCL in 191 adult patients with Hodgkin's disease are presented in graphic form in Fig. 1 according to clinical category, including number of samples and number of patients in each category. The basic overall relation of SCL to disease activity is represented by the mean SCL of each category. Elevated SCLs in the active phase of the disease gradually decrease with sequential reduction of

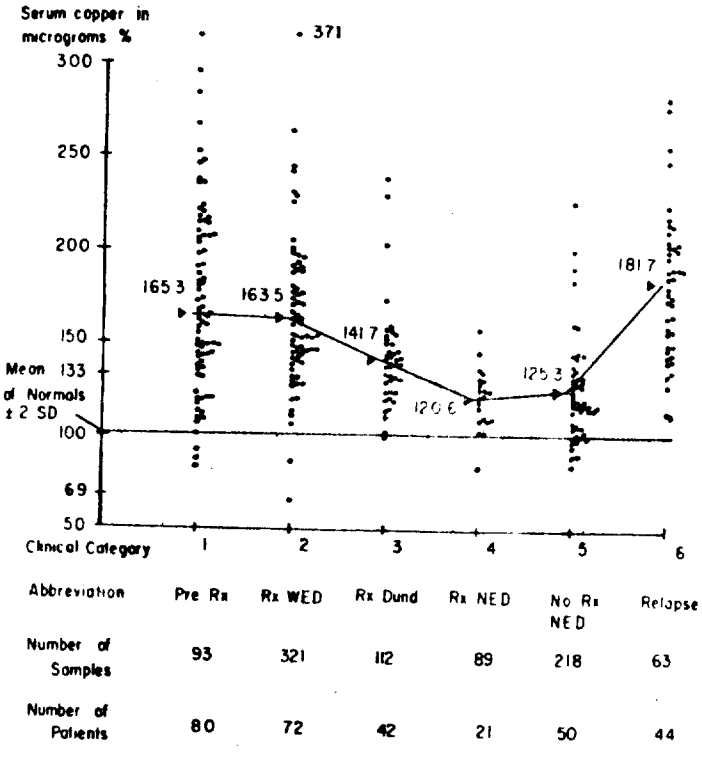


FIG. 2. Each value in this plot represents the mean of all SCLs in a given clinical category from one patient. This is presented to avoid possible distortion of the overall mean by a large number of values from single patient. The pattern, however, is essentially similar to that in Fig. 1.

the disease activity as response to therapy, with normal SCL during remission, and an elevation of the SCL in relapse.

Since a number of patients has contributed more than one value to each of the clinical categories, and the mean may be unduly influenced by the multiple values of single patients, the mean values of all SCL from each patient in a given clinical category have been plotted in Fig. 2. The same sequence of the basic relationship of SCL to the disease activity is noted.

A comparison of Figs. 1 and 2 indicates an essentially similar pattern with no significant distortion by multiple values of single cases. These figures also serve to point out wide variations of SCL in certain categories, and groups seemingly inconsistent with the hypothesis that serum copper is mirroring the activity of the disease process. Details of these aspects are presented in the discussion.

The mean values of SCL in each of the initial histologic classifications of Hodgkin's disease are presented according to clinical category in Fig. 3. Again, similar basic relation of SCL with disease activity is noted in three histologic subgroups (nodular sclerosing, mixed cellularity, and lymphocytic depletion). SCLs of patients with lymphocytic predominance type appear to show no elevation. The limitations of these data will be alluded to in the discussion.

A review of the relations of SCL (mean values) to clinical category by stage of disease (Fig. 4)shows, in general, the same sequence noted in previous figures, i.e., reflection of disease activity by SCL. In addition, a significant difference in SCL between localized and generalized Hodgkin's disease is noted, being consistently higher in stages representing more extensive disease. Patients with systemic symptoms show SCL consistently higher than those without systemic symptoms.

CASE REPORT

The disease course with pertinent data of a 20-year-old Caucasian man with Hodgkin's disease, nodular sclerosing type, is presented in Fig. 5. Patient was symptom-free and underwent staging procedures which failed to reveal any abnormality, thus establishing this case as Stage IA. The only abnormal biochemical test was an elevated SCL of 205 μg/100 ml. Clinical remission obtained by irradiation (XRT) was of short duration. Six weeks after completion of radiotherapy, the patient re-

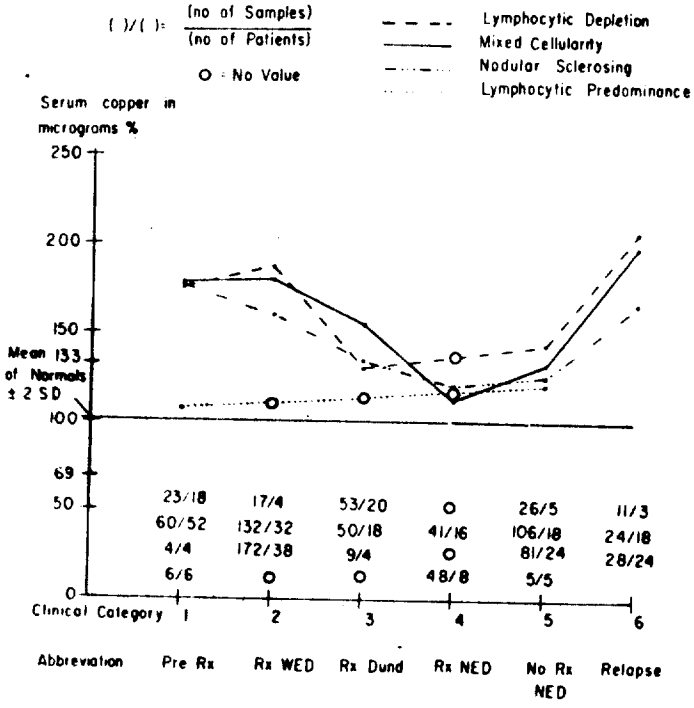


FIG. 3. The mean values of SCL in the four histologic classifications of Hodgkin's disease according to assigned category shows generally similar relation to the clinical evidence of disease activity, i.e., high SCL in the active phase of the disease, falling with response to treatment, and rising with relapse. The exceptions are SCL of patients with lymphocytic predominance type which appear to show no elevations.

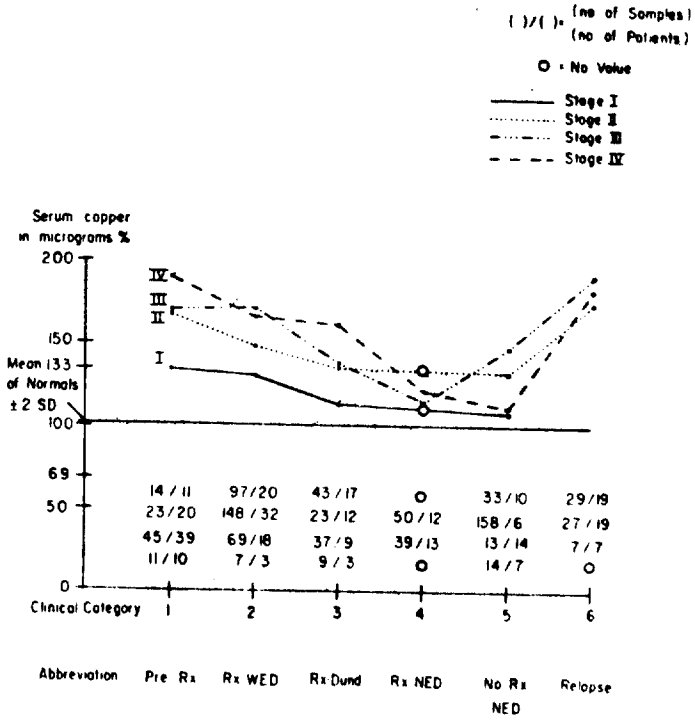


FIG. 4. The mean values of SCL in each stage (Rye classification)[8] show generally similar relation to the clinical evidence of disease. In addition, there is a relationship between SCL and extent of disease ("tumor volume"), showing higher serum copper values in stages representing more extensive disease, particularly in pretreatment period.

turned with systemic symptoms, and liver and bone marrow involvement with Hodgkin's disease. Remission reinduction was obtained by six courses of MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone) and maintained with actinomycin D. This case is an example of SCL serving as an index of the status of disease activity which changed four times during a 6-month period. It will be noted that the serum copper values in this case fall in all clinical categories. Each phase of disease activity was accompanied by significant change in the SCL.

The variability of SCL in patients with extended periods of observation in a clinical category has been analyzed in 40 patients in complete remission (categories 4 and 5). These patients have had two or more SCLs over a period of 2 months or longer while remaining in a single category. Thirty-two of these patients without any evidence of disease activity or complications showed no significant variation in SCL in the same individual. The remaining eight patients from this group had elevated SCL. These will be presented in the discussion with other inconsistent cases.

SCL in 33 patients with carcinoma of the prostate undergoing radiotherapy were found to be within the normal range of values. The mean SCL for each of the 8 weeks of therapy varied from 107.6 to 115.6 μg/100 ml, and did not differ significantly from pretreatment values (Table 1).

DISCUSSION

Hodgkin's disease presents many manifestations, and after apparently effective therapy,

TABLE 1. Serum Copper Levels in 33 Patients with Prostatic Carcinoma during 8 Weeks of Radiotherapy

	PreRx	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week	Total
No. of samples	4	59	65	63	64	64	56	43	9	426
Mean	113.5	115.6	112.8	114.7	113.7	110.9	109.8	112.2	107.6	112.8
Standard deviation	11.30	22.24	19.98	19.83	19.08	18.87	15.02	15.44	16.14	13.7
No. of patients	3	33	33	33	33	33	30	26	7	33

No. 76061		
SYMPTOMS		
Absent	Fever Chills	Absent
CLINICAL FINDINGS		
Right axill mass 15x10 cm Chest x-ray I V P Lymphangiogram Bone marrow biopsy (ALL NEGATIVE)	Liver 8cm bcm Spleen 1cm Liver and Bone marrow biopsy positive for Hodgkin's disease	Negative Bone marrow reboopsy negative for Hodgkin's disease

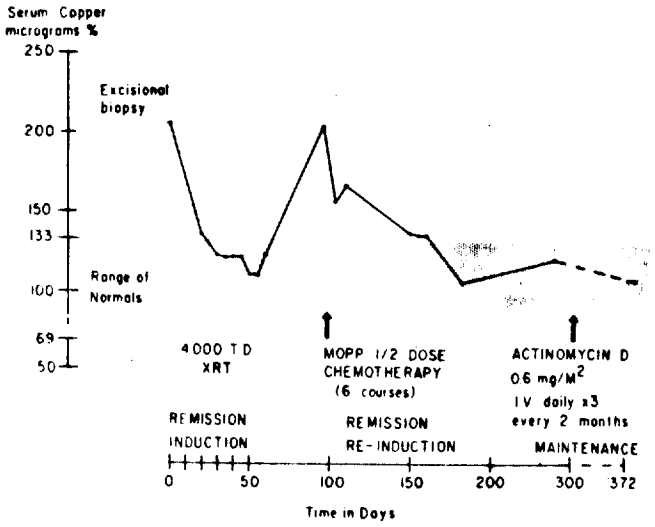


FIG. 5. Sequential SCLs in same patient with pertinent data reflecting status of disease activity and effectiveness of various forms of therapy used.

may reappear many years later.[24,25] Elevated SCL may well be the first indication of recurrent disease.

The general relationships of the mean SCL in Figs. 1 and 2 show the active phase of disease characterized by increased SCL and the inactive phase by SCL within the normal range. There is progressive reduction with response to therapy, coinciding with reduced disease activity, and an elevation in relapse, with recurrent activity.

The wide spread of the individual values of SCL in each category, as indicated by the scatter plots in Figs. 1 and 2, is probably a reflection of many factors. These, in our opinion, include degree of disease activity and its extent, clinical behavior, presence or absence of systemic symptoms, histologic type, effect of medication and response to therapy, individual pattern of SCL and differences between individuals, infections and impending relapse without manifest clinical findings.

Clinical category 2 consists of those patients with active disease undergoing treatment, including those entering this category from 1 (untreated) and 6 (relapse). Analysis of patients in category 2 disclosed three general patterns: 1. persistent disease activity without response to therapy and with persistently high SCL values; 2. partial response to therapy with gradual decrease in SCL accompanying improvement, and 3. complete response to therapy characterized by rapid fall in the SCL preceding clinical remission. The details of this category, which largely concern responses to therapy, are the subject of a separate report.

Analysis of individual cases of Hodgkin's disease in this clinical material (Fig. 5) confirms the close correlation between the changes in the serum copper level and the disease activity process noted in the collective presentations by clinical categories (Figs. 1 and 2).

There are certain general observations to be made regarding the relationships of SCL to

disease activity based on the cumulative experience and observations in this area, in many instances illustrated in detail in prior reports.[4–6] A rapid fall of serum copper from abnormal high levels to the normal range, followed by persistence of a normal level over many months, indicates that response has been good. A slow fall in SCL does not necessarily exclude complete response, particularly if it is followed by persistence of a normal SCL. In some cases, a temporary increase in SCL was noted soon after therapy was initiated, after which there was a fall with treatment response. The decrease in SCL during therapy without reaching the normal range at any time suggests a residual active disease. The only exception to the general relationships of SCL and disease activity that seems to be clearly identified is the fall of SCL which occurs at times as a terminal event in active disease. A persistently elevated or steadily rising SCL is a reflection of continued presence of disease activity and is related to an ultimately fatal prognosis, in the absence of a known alternative cause. An increase of SCL in patients apparently in remission indicates the presence of occult or recurrent disease and the need for further investigations (if otherwise unexplained).

The identification of interfering substances or conditions is of utmost importance to avoid misinterpretation of SCL. Pregnancy,[12] estrogen therapy,[20] and contraceptive drugs[2,22] are the most frequent causes of SCL elevations, and when in doubt, patients must always be questioned precisely about these causative factors.

A number of other conditions reported to influence SCL has been adequately summarized in comprehensive reviews.[1,5,12,21,31] The presence of infections (in incubation period or manifest) must always be taken into account when interpreting the copper level in Hodgkin's disease.

It is of value to examine in detail what appear in this series to be exceptions to the general pattern.

Fifteen patients in complete clinical remission (categories 4 and 4), Fig. 2, presented with high SCL. Of these seven were in prerelapse, as determined by subsequent clinical course. An additional five patients showed infection. The remaining three (20%) are without explanation of the hypercupremia in either history or clinical findings. Twenty-five patients in category 3 (R), disease activity undertermined) presented with hypercupremia. This is admittedly a difficult group to analyze, inasmuch as clinical findings themselves are inconclusive. Of the 25, there was adequate explanation for the condition in 11 (7 prerelapse and 4 with manifest infection), with a remaining 14 (56%) without adequate explanation. Incomplete control of disease and occult infection are possibilities, particularly in this category.

Considering the SCL in untreated patients (category 1), 17 of 80 patients were within the normal range (Fig. 2). Of these 17, 8 were considered to have localized disease (Stage I); 6 of the patients had lymphocytic predominance type. Since there is substantial evidence of a difference in SCL in localized (borderline SCL) vs. generalized (high SCL) disease (Fig. 4), we believe that in 8 of the 17 patients the apparently inconsistent values in the pretreatment period represent a low volume of disease tissue, or a characteristic of the particular histologic subgroup of Hodgkin's disease, or both. The remainder of this group (nine patients) are without explanation for what we consider an inconsistent pattern of SCL.

In evaluating other apparently inconsistent values, in this instance normal SCL in relapse, 4 of 44 patients were identified. One had SCL of 133 μg/100 ml and 129 μg/100 ml which dropped to 86 μg/100 ml accompanying remission reinduction. Two were in the terminal phase of disease. The reason for a rapid fall of SCL as a terminal event[28] observed in some cases is not known and may be a reflection of many factors, particularly liver failure and compromised immune mechanisms. The occurrence of a "relative" change even within normal ranges will be mentioned below. One case is without apparent explanation.

Serum copper shows a significant relation to the disease activity and therapy as indicated by the six clinical categories in the nodular sclerosing, mixed cellularity, and lymphocytic depletion types (Fig. 3). Similar values for these histologic subgroups occur in the pretreatment period, although somewhat different in relapse, which reflects the differences in clinical behavior of the histologic types (lowest values in nodular sclerosing, highest in lymphocytic depletion).

The lymphocytic predominance group seems to show no significant variations with SCL regardless of the disease activity. Due to a limited number of patients in this group (six patients) and since five of these six patients had localized Hodgkin's disease, Stage IA, it is not possible at the present time to interpret

the lack of correlation in this group, which could be related either to histologic type (lymphocytic predominance) or to small tumor volume (localized disease) or a combination of both. It is perhaps possible that the biopsy procedure itself has reduced the volume of disease tissue sufficiently to influence SCL.

Considering SCL with respect to stage of disease, as shown in Fig. 4, there is an obvious relation of SCL in the pretreatment period to stage, as well as the general response to therapy, and in relapse. In Stage I, 8 of 10 untreated patients had normal SCL (average 115 μg/100 ml) and have been in clinical remission since completion of radiotherapy. Two remaining patients with localized disease and marked hypercupremia relapsed in a short period of time after completion of radiotherapy (one of these cases is depicted in Fig. 5). This observation suggests that marked hypercupremia in localized disease may represent either occult foci of disseminated Hodgkin's disease or subsequent adverse course of disease.

In the control group of 33 patients with carcinoma of prostate, it may be seen that the general pattern is that of minor variation within the normal range. There are no significant serum copper alterations in this particular form of solid tumor, or as a result of radiotherapy delivered to a substantial pelvic tissue mass.

Function of serum copper and ceruloplasmin, the major serum copper-containing protein, in metabolic processes is not well understood.[1,12,18,21,31] Of great interest are current studies emphasizing the role of ceruloplasmin in iron oxidase activity.[3] The characterization of the elevated copper proteins, other than ceruloplasmin, may provide some insight into this index of Hodgkin's disease activity, including its source, degree of specificity, and true relation to the disease process, which is not yet apparent.

It is of interest to note that in approximately 6,000 sera from 2,000 cancer hospital patients in whom SCL and SMA 12/60 values were determined, it was clearly established that SCL was essentially an independent value not reflected in any of the other biochemical determinations. A relatively small degree of correlation was found only between SCL and alkaline phosphatase and lactic dehydrogenase, with correlation coefficients 0.24 and 0.20, considered in this context to have relatively little specific value.[6]

Activity of Hodgkin's disease, in our opinion, is much better reflected in serum copper levels (if properly interpreted) than in any other biomedical index, and as such, SCL can be of substantial assistance in the management of patients with Hodgkin's disease as a significant part of the entire clinical picture.

SUMMARY

This study of 896 serum copper observations from 191 adult patients with Hodgkin's disease has shown a relationship of serum copper levels (SCL) with activity of the disease. High SCL are seen in the active phase of disease, with reduced levels in decreased disease activity and normal values in inactive phases. SCL rise to abnormal levels in relapse, often preceding clinical signs.

These relations of SCL to disease activity are noted in three histologic types of Hodgkin's disease (nodular sclerosing, mixed cellularity, and lymphocytic depletion) and do not seem to be present in the limited number of lymphocytic predominance cases observed.

Data in this study point to a relation between SCL and extent of disease, as shown by marked elevation in SCL in generalized as compared to localized disease. Patients with systemic symptoms have higher SCL compared to those without symptoms.

A control group of 33 patients with carcinoma of the prostate treated with radiotherapy showed no relationship of SCL to disease or therapy.

There is variation of individual values from the general pattern, and evaluation of those which appear to be inconsistent often present clinical findings which provide a satisfactory basis for the particular SCL. The data in this study provide a sound basis for the utilization of SCL in the evaluation of Hodgkin's disease activity.

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Ipecacuanha and Copper Sulphate as Emetics in Intoxications in Children

by BENGT KARLSSON and LARS NORÉN

Intoxications in children are with very few exceptions oral. Trials to remove the poison through gastric lavage or induced vomiting are the most common procedures in handling such cases.

Induced vomiting is often considered as a more preferable way of treatment in pediatric practice than is gastric lavage. Ipecacuanha seems to be the most widely used emetic [7, 15]. It cannot be taken for granted, however, that ipecacuanha is superior to other emetics. The emetic effect of ipecacuanha is partly based on resorption. Other agents, e.g. copper sulphate, act only by local mucosal irritation [4, 6], and have to some extent been successfully used [10].

The purpose of this study was to compare copper sulphate and ipecacuanha regarding how often and how fast the two emetics induce vomiting and also to compare the effect of larger and smaller dose of the agents.

Material

In the study were included 257 children with intoxication or suspected intoxication, treated at the Department of Pediatrics, Karolinska Sjukhuset, Stockholm, during January through August, 1963. To induce vomiting copper sulphate was given to 132 children and ipecacuanha to 125 children. The age distribution was about the same in the two groups (Fig. 1). A considerable number of patients were below the age of one year (0–12 months).

The ingested agents were the same in the two groups dominating was tobacco (Fig. 2).

Methods

Copper sulphate was used as follows:

Preparation. Powder preparations with 0.25 g and 0.15 g, dissolved in 20 ml of water immediately before use.

Dosage. Patients below the age of 1½ years: 0.15 g. Patients above the age of 1½ years: 0.25 g.

Technique. 1. Administration of 100–200 ml of juice. 2. Administration of copper sulphate. 3. If negative result: Alt. (*a*) Repeated dose after 15–20 min. Alt. (*b*) Gastric lavage (see 4 below). 4. At the latest 15 min after 3*a* – if no vomiting occurs – aspiration of the gastric content and gastric lavage with 1,000–1,500 ml of physiologic saline containing 0.15 g of potassiumferrocyanide.

Ipecacuanha was used as follows:

Preparation. Syrup with the following preparation [7]: Extr. fluid. ipecac. 14 g, Glycerin 10 g, Syr. Sacchari ad 130 g.

Dosage. Patients below the age of 1½ years: 10 ml, Patients above the age of 1½ years: 15 ml.

Technique. 1. Administration of 100–200

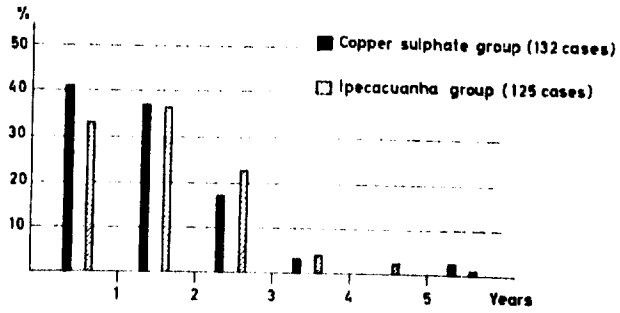

Fig. 1. Age distribution.

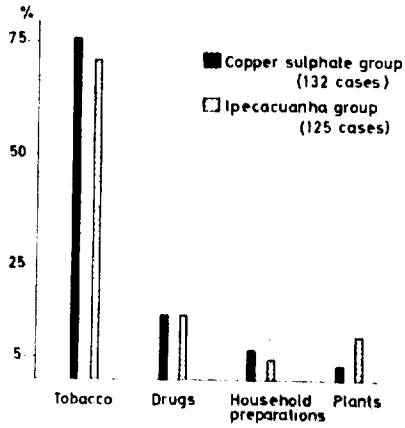

Fig. 2. Ingested agents.

ml of juice. 2. Administration of syrup of ipecacuanha. 3. If negative result: Alt. (*a*) Repeated dose after 15–30 min. Alt. (*b*) Gastric lavage (see 4 below). 4. At the latest 15 min after 3*a*—if no vomiting occurs aspiration of the gastric content and gastr lavage with 1,000–1,500 cc of physiolo saline.

When needed, the treatment was suppl mented with universal or special antidot antagonists or cathartics. Emetic was n given to semiconscious or unconscio children, nor to children who had ingest petroleum products or when a corrosi lesion was suspected.

Results

A single dose of copper sulphate had considerably better effect than a sing dose of ipecacuanha. In the copper sulphate group 81 % of the patients vomite while only 55 % vomited in the ipeca cuanha group (Fig. 3). The difference statistically significant (p less than 0.1 %

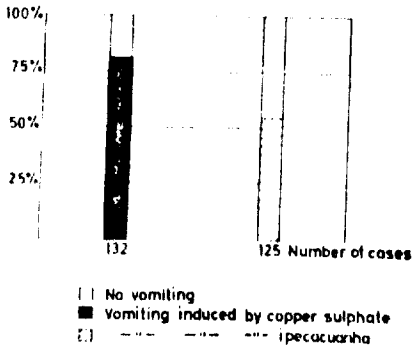

Fig. 3. Effect of single dose of emetic.

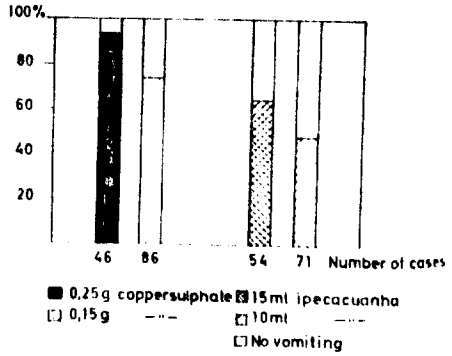

Fig. 4. Effect of large and small dose of emetic

TABLE 1. *Number of vomitings after single dose of emetic.*

No. of vomitings	Copper sulphate		Ipecacuanha	
	No. of patients	(%)	No. of patients	(%)
1	73	68.2	21	30.4
2	30	28.1	25	36.3
3	4	3.7	18	26.1
4	0	0.0	4	5.8
5	0	0.0	1	1.4
Total	107	100.0	69	100.0

When no vomiting occurred after administration of emetic, it was not always possible to repeat the dose. Because of an unpleasant "after-taste" of copper sulphate the children often refused to take another dose. Repeated dose of the syrup of ipecacuanha was more easily accepted.

A comparison between large and small dose of emetic shows that a large dose of copper sulphate was effective in 95 % of the cases. The smaller dose was effective in 75 % of the cases. The difference is statistically significant (p less than 1 %).

A certain but not statistically significant difference also occurs between large and small dose of ipecacuanha, but vomitings were constantly induced less frequently with this emetic (Fig. 4).

To our knowledge there are as yet no studies, which clarify if one, two or several vomitings are required to remove a sufficient amount of ingested material from the stomach.

In the group of patients, who vomited after administration of a single dose of copper sulphate, 68 % vomited only once, 28 % twice and 4 % three times.

Ipecacuanha considerably more often induced repeated vomitings. In 30 % of the cases vomiting occurred once, in 36 % twice, in 26 % three times and in 7 % more than three times (Table 1).

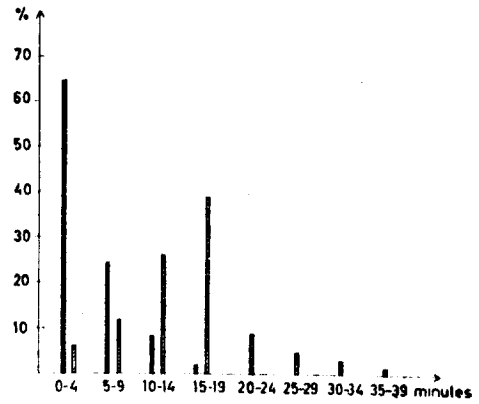

Fig. 5. Delay between administration of emetic and onset of vomitings in 107 children who vomited after a single dose of copper sulphate (full line); 69 children who vomited after a single dose of ipecacuanha (hatched line).

The value of a rapid effect of an emetic is obvious. In the copper sulphate group 65 % of the children vomited within 4 minutes and 98 % within 14 minutes after administration of the emetic. In the ipecacuanha group 50 % of the patients vomited after a latent period of 15 minutes or more (Fig. 5).

Undesired effects of the emetics can be suspected in two of our cases. One child in the copper sulphate group complained of colicky pain in the abdomen during 1–2 hours after administration of emetic. These symptoms were possibly caused by copper sulphate. More pronounced reactions were noted in one child in the ipecacuanha group. A nine-month old girl, who had ingested part of a cigarette, was given 10 ml of ipecacuanha. After repeated dose of ipecacuanha the girl vomited one hour after the first dose. The vomiting contained some tobacco. The girl then continued to vomit for 6 hours and during 12 hours had a tachycardia up to 170/min. Those symptoms may have been caused by nicotine or ipecacuanha, probably by both.

Comments

The study clearly shows that a single dose of copper sulphate induced vomiting in a significantly higher number of cases than a single dose of ipecacuanha. The vomitings occurred more rapidly after administration of copper sulphate than ipecacuanha. Copper sulphate provoked fewer vomitings and a shorter period of nausea.

The study gives no information of how effectively induced vomiting removes ingested poison from the stomach. Completely reliable procedures to study this important question have so far hardly been presented. Studies by Harstad *et al.* [8] showed that gastric lavage is of a very limited value.

Aspiration of the gastric content instead of lavage was considered more sufficient. Arnold *et al.* [1] found that vomiting induced by ipecacuanha was in several respects more effective than gastric lavage. Their conclusions were based upon experimental studies on animals. Goldstein *et al.* [5] performed an x-ray study of the effect of ipecacuanha-induced vomitings in children. The preliminary results were disappointing.

So far there is no general agreement about the most effective way of emptying the stomach in intoxicated children.

In our study it was noted that gastric lavage performed after induced vomiting could still remove considerable amounts of ingested poison, as well as the reverse conditions also occurred.

If induced emesis is preferred as treatment of poisoning, our study indicates that copper sulphate has in many ways advantages over ipecacuanha. Factors which limit the use of ipecacuanha are:

The ipecacuanha induces vomiting pa through central effect [4, 6]. This ef is counter-acted by antihistamines, w are not uncommonly ingested in case intoxication. To a certain extent sim toxic effects of ipecacuanha and nicot are reasons why the largest group of toxications and suspected intoxications children in Sweden – ingestion of toba – should not be given this emetic. T effect of copper sulphate is not ba upon resorption and can be used wh histamines as well as tobacco have be ingested.

Risk for intoxication occurs with the u of all types of emetics. Several severe t toxications have been reported after th use of ipecacuanha as emetic [2, 9, 13, 14 Copper sulphate poisoning may also occu even if this agent has not been reporte to give serious intoxications when use as emetic. Against copper sulphate there is a specific antidote, potassium ferrocya nide, which combines with copper sulphat to an unsoluble, nonirritating salt.

At present we consider that the fol lowing method to induce vomiting ha the widest range of use, the most rapid effect and has the highest incidence of positive results:

1. Administration of 100–200 ml of juice or other appropriate fluid.
2. Administration of 0.25 g copper sulp hate in 1 % solution.
3. If no vomiting occurs within 15 minute 0.15 g potassium ferrocyanide dissolve to a 1 % solution is given. Then the stomach content is aspirated and gastric lavage is eventually performed

Summary

A comparison of the emetic effect of ipecacuanha and copper sulphate is made in 257 children with intoxication or suspected intoxication. Copper sulphate was given to 132 children and ipecacuanha to 125 children. A single dose of copper sulphate induced vomiting in a considerably higher number of cases than ipecacuanha. Vomitings were induced more rapidly by copper sulphate than by ipecacuanha. Copper sulphate gave fewer vomitings and a shorter period of nausea than ipecacuanha. A method to induce emesis is suggested.

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Copper In Leprosy Blood Plasma Concentration And Urinary Excretion

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Copper (free, bound and total) and ceruloplasmin levels have been determined in fasting blood plasma of normal individuals and leprosy patients. Plasma concentration of these constituents was higher in non-treated leprosy patients than in normal persons. There was an increase in copper excretion through urine in all patients. The urinary copper excretion was higher in lepromatous than in non-lepromatous type. Results indicate that the above values for the copper components in leprosy patients of all types decreased significantly after DDS treatment, thereby showing a tendency to attain normal values.

Introduction

The present investigation was undertaken to find out the concentration of copper components of blood plasma and also the effect of DDS administration on the copper components in leprosy patients. For the sake of comparison, copper components from normal healthy men and women were also studied.

Material and Methods

The leprosy patients under investigation were classified into four different types as—(1) Lepromatous, (2) Tuberculoid (major), (3) Tuberculoid (minor), and (4) Maculo-anaesthetic.

Fasting venous blood samples were withdrawn in test tubes containing heparin as anticoagulant. Heparinized blood was centrifuged and plasma separated and kept in freeze at 4°C. Plasma copper was estimated by the method of Gubler **et al** (1952). Ceruloplasmin in plasma was estimated by the method of Ravin (1961). Twenty-four hour urine samples were collected under toluene and stored at 5°C till taken for copper estimations. Copper in urine was estimated by the method of Green and Eden (1940). Control observations were made on apparently healthy normals from the same socio-economic conditions and corresponding age groups. All the reagents were either of A.R. or E. Merck grade and glass distilled water was used for the investigations.

Results

Copper (free, bound and total), ceruloplasmin in plasma and urinary copper : Table I shows the values for the direct reacting copper i.e. free copper, bound copper total copper, ceruloplasmin levels in plasma, and copper in urine in untreated leprosy

cases of different types. There was a significant increase found in the plasma levels of all the three forms of copper (i.e. free, bound and total) as well as plasma ceruloplasmin in all types of leprosy patients ($P < 0.001$). The increase in the concentration of plasma copper and ceruloplasmin was much higher in lepromatous leprosy cases than that in non-lepromatous type. The urinary excretion of total copper was elevated significantly ($P < 0.001$) in all leprosy patients in proportion to the severity and mycobacterium leprae infection in leprosy patients.

Table II gives the values for plasma concentration of free, bound and total copper and ceruloplasmin and copper excretion through urine in leprosy patients after the treatment with DDS for about five years. Table III gives the values for total copper and ceruloplasmin in plasma and urinary copper of leprosy patients after the DDS treatment for about ten years. It can be seen from Tables II and III that the plasma concentration of copper, ceruloplasmin and urinary copper were decreased in the Leprosy patients after the treatment with DDS.

Discussion

Holmberg and Laurell (1954) observed that using paraphenylene diamine as substrate, ceruloplasmin acted as an oxidase. Ravin (1956) proposed a test for the estimation of oxidase activity using paraphenylene diamine dihydrochloride as substrate. Cumings (1961) reported a good correlation between the copper content and the oxidase activity of the sera. Data presented in the Tables I, II and III also indicate a fairly good correlation between the plasma copper and the ceruloplasmin (i.e. paraphenylene diamine oxidase activity).

In leprosy, the ceruloplasmin and total copper in plasma increased significantly in proportion to the severity of the M. leprae infection. There was an increase in the values of free copper in non-treated leprosy patients as compared to those of normal ones. Acute and chronic disorders of viral and bacterial etiology are associated with serum copper elevations (Cartwright et al 1946). The serum copper was elevated in certain pathological conditions, such as cirrhosis of liver (Chopra and Balasubrahmanyan 1958), subacute and chronic infections, lipochromic anaemia and in hepatitis. A considerable increase in the urinary excretion of copper occurred in leprosy. This is in agreement with the increase of urinary copper as reported in Wilson's disease (Huntington 1951), and nephrotic syndrome (Cartwright et al 1954). The level of copper in plasma is closely associated with alterations in plasma proteins. Copper is transported as an α_2-globulin complex, ceruloplasmin in blood. Dhople and Magar (1963) reported decreased levels of serum proteins in leprosy. Copper may be splitting from the protein complex and an increase in free copper occurred. M. leprae bacteria brought about the degeneration of muscle and the chronic infection which possibly then caused the elevation of plasma copper. Breindstrup (1953) had reported an increase in serum copper in acute and chronic infection.

Paikar and Magar (1967) observed an increase in the activities of lysosomal enzymes in leprosy tissue, which bring about bacterial lysis (Dingle and Allen 1962).

Table I. Distribution of copper, ceruloplasmin in plasma and urinary copper in normal and untreated leprosy patients

Type of patients	No. of samples	Plasma copper µg/100 ml			Plasma ceruloplasmin mg/100 ml	Urinary excretion of copper µg/24 h
		Free	Bound	Total		
Lepromatous	24	33·42 ± 9·56	148·00 ± 24·25	181·42 ± 33·61	74·12 ± 22·22	366·22 ± 197·20
Tuberculoid major	26	29·58 ± 10·42	129·65 ± 16·34	158·23 ± 21·37	69·15 ± 19·83	231 46 ± 109·10
Tuberculoid minor	25	18·59 ± 9·88	131·75 ± 16·74	150·34 ± 23·71	65·00 ± 19·23	195·17 ± 90·15
Maculoanaesthetic	24	17·12 ± 8·56	120·16 ± 14·67	137·28 ± 18·14	57·87 ± 20·12	151·72 ± 70·92
Normal	12	4·00 ± 1·00	110·52 ± 14·50	114·52 ± 16·52	34·00 ± 5·12	12·00 ± 5·96

All values indicate Mean ± S.D.

Table II. Distribution of copper, ceruloplasmin in plasma and urinary copper in leprosy patients treated with DDS for the period of about five years

Type of patients	No. of samples	Plasma copper, µg/100 ml			Plasma ceruloplasmin µg/100 ml	Urinary copper µg/24 h
		Free	Bound	Total		
Lepromatous	22	19·66 ± 7·68	125·74 ± 9·20	146·39 ± 13·77	60·25 ± 13·36	101·22 ± 36·41
Tuberculoid major	23	13·11 ± 5·50	129·64 ± 11·23	134·61 ± 14·84	53·40 ± 11·56	68·59 ± 24·12
Tuberculoid minor	21	12·04 ± 5·52	116·41 ± 12·41	128·86 ± 13·67	55·16 ± 13·38	62·96 ± 23·94
Maculoanaesthetic	18	8·95 ± 2·12	112·69 ± 12·36	121·98 ± 11·27	39·20 ± 7·82	51·68 ± 19·52
Normal	12	4·00 ± 1·00	110·52 ± 14·50	114·52 ± 16·52	34·00 ± 5·12	12·00 ± 5·96

All values indicate Mean ± S.D.

These enzymes act on the host cell with their lytic action and the bacteria bring about the destruction of tissues to a certain extent, and thus remove copper from the tissues. This ultimately results in the elevation of plasma copper and ceruloplasmin. Some of the excess copper in the body fluids is excreted through urine.

Table III. Copper, ceruloplasmin of plasma and urinary copper in leprosy patient treated with DDS for about ten years

Type of patients	No. of samples	Plasma copper μg/100 ml	Plasma ceruloplasmin mg/100 ml	Urinary copper μg/24 h
Lepromatous	20	129·36 ± 11·86	48·33 ± 9·75	49·02 ± 20·56
Tuberculoid major	16	122·51 ± 12·26	44·35 ± 8·25	36·97 ± 19·38
Tuberculoid minor	22	123·70 ± 11·37	39·16 ± 7·95	38·89 ± 17·41
Maculoanaesthetic	17	119·65 ± 11·56	37·52 ± 7·12	29·19 ± 15·13
Normal	12	114·52 ± 16·52	34·00 ± 5·12	12·00 ± 5·96

All values indicate Mean ± S.D.

With the administration of DDS (Depsone) to the leprosy patients, there is a suppression of the infection due to **M. leprae** and also the degeneration of muscle tissues. The values for copper components therefore tend to lean towards those found in normal, following the DDS treatment.

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Acute Copper Intoxication

A Hazard of Hemodialysis

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Clinical and laboratory finding from three patients with hemodialysis-induced copper intoxication included pancreatitis and myoglobinemia in addition to previously observed feature of this syndrome (gastrointestinal symptoms, leukocytosis, metabolic acidosis, and hemolysis). Copper concentration was measured in samples of serum, erythrocytes, whole blood, and dialysate. In all instances there was a significant hypercupremia following the inciting dialysis. A demonstrated erythrocyte to serum copper concentration gradient documents the ability of the erythrocyte to concentrate copper. It is important to measure the copper concentration in whole blood or erythrocytes in suspected instances of copper intoxication. The possible mechanisms of copper entry into the dialysate include oxidation of metallic copper to cupric ion or contamination of tap water from copper pipes. Copper ion significantly alters erythrocyte metabolism.

Acute copper intoxication and hemolytic anemia have occurred after the ingestion of copper-containing compounds,[1,2] or following irrigation of wounds with copper sulfate.[3] Lyle had postulated that hemodialysis with the use of water with a high copper concentration could cause copper intoxication with attendant hemolysis by yet another route.[4] Recently, acute copper intoxication with hemolytic anemia has been recognized as a complication of hemodialysis.[5,6] Oxidative release by heat and acid of copper in dialysis heating coils was proposed as the mechanism through which copper entered the dialysis fluid. These reports prompted physicians and equipment manufacturers to eliminate copper tubing from dialysis machines and to monitor the pH and conductivity of water used for dialysis.

Since few copper determinations were made during the acute illness in the reported cases, a direct relationship could not be established between the serum copper level and the presence of hemolysis. Matter et al measured the serum copper in only one of the three cases reported and no difference was found in serum copper concentrations before and after dialysis.[5] Ivanovich et al reported single determinations of serum copper concentration in each of three patients. Two patients had concentrations of 418μg/100 ml and 747μg/100 ml (normal < 140μg/100 ml), respectively, yet only one of these patients had hemolysis. Another patient with a serum copper concentration of 155μg/100 ml had severe hemolysis. They were thus unable to make a positive correlation between the level of serum copper and the presence of hemolysis. Both investigators reported measurements of copper in the dialysis water prior to and after its contact with the copper heating coil on the dialysis machine. A marked rise in copper concentration of the water after contact with the heating coil was demonstrated.[5,7]

We present and discuss the clinical and laboratory features of acute copper intoxication as observed in three patients undergoing repetitive hemodialysis.

Materials and Methods

Hemodialysis System.—Within a three-week period in 1968 a characteristic syndrome developed in three patients during hemodialysis. All patients were dialyzed with a tank delivery system (Sweden

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Freezer) in which water is premixed with acetate-buffered dialysis chemicals prior to passage of dialysate mixture through a copper heating coil. The dialysate used for our first two patients was made from deionized water. Patient 3 was hemodialyzed against tap water mixed with a batch of chemicals different from that used for patients 1 and 2.

Patient and System Monitoring.—All routine hematological and biochemical analyses were performed in the clinical laboratories of the Durham Veterans Administration (VA) Hospital. Blood, urine, sputum, and dialysate were cultured in the Clinical Microbiology Laboratory. Serum, dialysate fluid, and returns from peritoneal dialysis were tested for methanol[9] and formaldehyde,[9] reagents used to clean the dialysate mixing tank. Levels of sodium, potassium, chloride, and osmolality were measured on dialysate samples from all three patients.

Copper Determinations.—Because the etiology of the illness was unknown at the time of hospitalization, multiple blood samples were drawn from each patient and stored. When it became possible to obtain measurements of copper, samples obtained at the following times were still available for analysis: patient 1, day 2 only; patient 2, before and after dialysis (day 0), day 2 after the inciting dialysis (immediately before hemodialysis, exchange transfusion, and peritoneal dialysis), day 2 (from blood withdrawn during exchange transfusion), and day 4; and patient 3, before and after dialysis (day 0), and days 1, 2, and 3. Copper concentration in whole blood cells, red blood cells (RBC) or serum obtained at these times, as well as in a dialysate sample (patient 3), was measured by the method of Gubler et al (normal < 140μg/100 ml).[10]

Effect of Initial Water pH on Final pH of Dialysate.—Tap water was adjusted with 0.1N or 1.0N hydrochloric acid to pHs of 1, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0, measured by a pH meter (model 26, Radiometer Copenhagen). An aliquot of water of each of the various pHs was mixed in a ratio of 34:1 (as for regular hemodialysis) with dialysate concentrate, and the pH of the mixture was determined.

Test for Myoglobin.—Serum from a patient with hemolysis, serum from patient 1, serum to which hemolysed blood had been added, and serum to which purified myoglobin had been added were all subjected to paper electrophoresis.[11] The strips were tested to identify heme-containing proteins (benzidine reaction).

Patient Summaries

PATIENT 1.—A 53-year-old Negro man complained of headache and diarrhea during his 219th hemodialysis (day 0). A predialysis hematocrit reading was 22%, and after the transfusion of 1 unit of packed RBC the postdialysis hematocrit reading was 26%. After dialysis the patient felt well enough to return to his home, 55 miles away. There, the headache abated but diarrhea continued for 24 hours. Thirty-six hours (day 2) after his dialysis he became stuporous and was returned to the VA hospital for admission. He was comatose, and responded only to painful stimuli and abdominal palpation. No history could be obtained concerning the presence of abdominal pain, myalgia, or testicular symptoms. The pulse rate was 100 beats per minute; respiratory rate, 12/min; and blood pressure, 120/70 mm Hg. There were no focal neurological signs. The hematocrit reading, 22% on admission, decreased to 17% over the next eight hours. The leukocyte count was 67,000/cu mm with 94% granulocytes and three nucleated erythrocytes per 100 leukocytes. The reticulocyte count was 3.4%; other laboratory values were serum bilirubin, 6.3 mg/100 ml; serum amylase, 600 Somogyi units (SU)/100 ml; serum glutamic oxaloacetic transaminase (SGOT), > 250 Karmen units (KU)/100 ml; lactic dehydrogenase (LDH), >350 Wacker units (WU)/100 ml; creatinine phosphokinase (CPK), 177 international units (IU)/100 ml (normal values: amylase, < 150 SU; SGOT, < 45 KU; LDH, < 120 WU; and CPK, < 20 IU); and a Coombs' test was negative. The serum was reddish brown and reacted positively with benzidine. Electrophoresis of the serum showed free hemoglobin and myoglobin.

Six hours after admission (day 2) the patient's blood pressure fell to 90/60 mm Hg and arterial pH was 7.3. Treatment was begun with pressor agents, hypertonic sodium bicarbonate, oxygen, exchange transfusion (9 units), hemodialysis, and concurrent peritoneal dialysis. The exchange transfusion was accomplished by removing blood during hemodialysis from the arterial line and administering blood bank blood into the venous line. During exchange transfusion the hematocrit reading rose to 30%. With these measures blood pressure and pH were restored to normal, but the patient remained comatose. An electroencephalogram was nonspecifically abnormal; cerebrospinal fluid was normal. On the third hospital day (day 5) a muscle biopsy was performed. Examination of the specimen by light microscopy showed normal muscle.

The patient remained in a coma, but his clinical condition was otherwise stable from the second hospital day (day 4) to the fourth hospital day (day 6). At this time the leukocyte count was 11,000/cu mm (90% granulocytes); other laboratory values were amylase, 127 SU/100 ml; bilirubin, 3.3 mg/100 ml; SGOT, 53 KU/100 ml; LDH, 560 WU/100 ml; and CPK, 300 IU/100 ml. On the fourth hospital day (day 6) he became hypotensive, and cardiac arrhythmia developed. He did not respond to therapy and died the next day, seven days after the onset of his illness during hemodialysis. Necropsy disclosed acute necrotizing hemorrhagic pancreatitis and acute and chronic orchitis.

PATIENT 2.—A 52-year-old white man manifested nausea and vomiting during his 35th hemodialysis (day 0). Immediately after dialysis the blood pressure was 120/60 mm Hg with a postural fall to 90/50 mm Hg. Diffuse abdominal pain and generalized abdominal tenderness were present, but bowel sounds were normal. The testes were small and firm. The hematocrit reading was 21%. Over the 12 hours abdominal pain persisted and the patient passed 15 to 20 loose, watery, nonbloody stools. He complained bitterly of severe myalgia which had developed during this same period. At this time (day 1) the hematocrit reading was 15%. All serum samples were noted to be red. The leukocyte count was 60,100/cu mm with 92% granulocytes. There were 5 nucleated erythrocytes per 100 leukocytes. The level of SGOT was 187 KU/100 ml. Arterial pH was 7.2; level of serum amylase, 600 SU/100 ml. Over the next eight hours (day 1) his body temperature rose to 105 F (40.6 C). The SGOT value rose to 567 KU/100 ml on day 2. Patient 2 was treated with sodium bicarbonate and pressor agents but became progressively hypotensive and died 38 hours (day 2) after the end of dialysis. At necropsy acute necrotizing hemorrhagic pancreatitis, and acute and chronic orchitis were present. The histology of the pancreas was the same as that reported for patient 1. The testes were atrophic, and

Table 1.—Results of Copper Determinations on Whole Blood, Erythrocytes, Serum, and Dialysate of Three Hemodialysis Patients

Patient	Days After Dialysis	Type of Sample	Copper Concentration (μg/100 ml)
1	0*	Predialysis serum	202
	0	Postdialysis serum	2,655
	2	Erythrocytes †	4,956
	2	Serum †	326
	2	Unit from exchange transfusion‡	841
	4	Serum	224
	4	Erythrocytes	182
2	2	Whole blood	647
3	0	Predialysis serum	66
	0	Postdialysis serum	1,312
	1	Serum	148
	2	Whole blood	325
	3	Serum	105
Dialysis bath of patient 3	0	Dialysate before passage through copper coil	5.6
Normals		Blood or serum	<140

* Day 0 signifies the day of dialysis and onset of illness.
† Cells and serum from one sample.
‡ Hemodialysis and peritoneal dialysis begun at this time. Sample was obtained from arterial dialysis line during exchange transfusion (see patient summaries). Determination was performed on whole blood.

chronic inflammatory cells were predominant over acute inflammatory cells.

PATIENT 3.—A 35-year-old white man developed nausea, malaise, diarrhea, and postural hypotension with syncope during his 38th hemodialysis. At that time (day 0) hematocrit readings before and after dialysis were 20% and 18%, respectively. The next morning (day 1) the blood pressure was 180/100 mm Hg; pulse rate, 120 beats per minute; respirations, 40/min. He was quite pale and markedly tachypneic, but alert and oriented. He complained of generalized, severe myalgia. Right upper quadrant abdominal pain and tenderness were present. There were no testicular signs or symptoms. The leukocyte count was 27,000/cu mm with 92% granulocytes. The hematocrit reading was 17% and the serum noticeably red. Sixteen hours later (day 2) the hematocrit reading had fallen to 8%. Serum electrophoresis revealed free hemoglobin. The serum amylase level was 200 SU/100 ml and rose over the next two days to a peak of 2,250 SU/100 ml. Serum bilirubin level was 1.65 mg/100 ml; SGOT, 200 KU/100 ml; and LDH, 680 WU/100 ml. Severe metabolic acidosis was present (day 1); arterial pH, 7.18; bicarbonate, 7.6 mm/liter; and arterial carbon dioxide pressure (P_{CO_2}), 19 mm Hg. He was treated with hypertonic sodium bicarbonate, peritoneal dialysis, and blood transfusions. He improved gradually and was discharged 12 days after the onset of his illness.

Patient 3 died nine months later following unsuccessful cadaveric renal transplantation. The pancreas and testes were normal at postmortem examination.

Results and Comment

In addition to the determinations of copper concentration in serum and blood of the cases reported here, a number of other studies were performed. The osmolality and the concentration of sodium, potassium, and chloride in dialysate samples, both at the time of the acute illness and from prior dialyses, were all within the expected range. Serum, dialysate, and returns from peritoneal dialysis were all negative for methanol and formaldehyde. No pathogens were isolated from cultures of blood, urine, sputum, dialysate, and cerebral spinal fluid.

Results of copper analyses of serum, blood, and dialysate are shown in Table 1. Although only one sample from patient 2 was assayed for copper, the blood copper concentration two days after the inciting dialysis was almost five times normal. The results of copper determinations before and after dialysis in patient 1 demonstrate a mildly elevated serum copper level before dialysis with an increment in serum copper of over 2,400μg/100 ml after dialysis. Results of additional measurements of copper in whole blood and serum at two-day intervals after the day of the inciting dialysis confirmed the persistence of significant hypercupremia at least until the time of hemodialysis and exchange transfusion on day 2. Values reported on day 4 are slightly elevated. Similarly, the results in patient 3 document an increase in serum copper concentration of 1,240 μg/100 ml following hemodialysis and a persistence of hypercupremia for at least 48 hours. Prior to contact with the copper heating coil, the concentration of the water used for dialysis in this patient was 5.6μg/100 ml. Samples of dialysate after exposure to copper were not available for copper analysis.

From the data in Table 1 it can be seen that significant hypercupremia after dialysis was present in all three patients for at least 48 hours after the inciting hemodialysis. The peak values for each patient ranged from 4.7 to 35 times the upper limit of normal.

Copper ion, in concentrations equal to or less than those found in our patients, has been shown to alter significantly the metabolism of RBC in vitro. These changes include accelerated loss of reduced glutathione, accumulation of hydrogen peroxide, increased tendency to form Heinz bodies, increased autohemolysis, and inhibition of glutathione reductase and glucose-6-phosphate dehydrogenase. Copper and other heavy metals may also directly injure the RBC membrane and result in enhanced agglutination.[7,8] The relative importance of these various effects in producing hemolysis in vivo is not known, but the direct association between high blood levels of copper and hemolysis appears to be unequivocal.[1,8]

Of the patients reported by Ivan-

ovich et al,[7] one had only a slightly elevated serum copper level but severe hemolysis, while another with a serum copper level almost three times normal had no hemolysis. They did report a fatal case of copper toxic reaction in which the "copper level" was 4,840μg/100 ml, but it is not clear whether this was measured in serum, whole blood, or erythrocytes, and no clinical details were given. The inability to establish a direct relationship between the level of serum copper and the presence of hemolysis may be explained by the observations of Chuttani et al[1] who have stressed the importance of relating the clinical findings to the whole blood or RBC copper concentration rather than to the serum copper concentration. The copper value of 4,956μg/100 ml on erythrocytes of patient 1 at 5 PM on day 2 (48 hours after hemodialysis and the onset of symptoms) is contrasted with the value of 326μg/100 ml in the serum. The importance of measuring copper concentrations in the whole blood cells or erythrocytes in suspected instances of copper intoxication should be emphasized.

The ability of the RBC to concentrate copper has been documented previously in experimental situations and in the unique clinical setting of Wilson's disease. In dogs given copper sulfate, as much as 89% of the total copper in the blood may be bound by erythrocytes.[19] In Wilson's disease, with a deficit of the major copper-carrying protein in the plasma and a high tissue level of copper, it has been shown that mobilization of tissue copper leads to hemolysis.[14,20] In such instances RBC copper concentration was again higher than that in the serum.

Dialysis-induced copper intoxication occurs in the setting of a high copper concentration in the dialysis fluid. One mechanism of copper entry into the dialysate is the oxidation of metallic copper to cupric ion. This could take place if copper parts on the dialysis machine are in contact with a sufficient concentration of free hydrogen ion in the presence of oxygen. Heat would facilitate this reaction. The general reaction can be written as

$$Cu + 2H^{+} + \tfrac{1}{2} O_2 \rightarrow Cu^{++} + H_2O.$$[21]

Tap water or water from exhausted ion exchange columns are two possible sources of acidic (low pH) water. Matter et al have demonstrated an exponential rise in dialysate copper concentration as the pH of water exposed to the copper and used for dialysis falls from 6.5 to 2.5.[7] Ivanovich et al[7] and Matter et al[7] measured copper concentrations of 844μg/100 ml and 3,100μg/100 ml, respectively, in dialysate prepared from copper-free water of pH 2.9 and 2.85. Once the copper is released into the dialysate it is bound rapidly by the artificial membrane and then released into the blood.[22]

An alternate mechanism to explain a copper-rich dialysate is the contamination of tap water directly from copper pipes. However, the low concentration of copper in the tap water used to prepare the dialysate for patient 3 (Table 1) does not support tap water as a copper source. In addition, the tap water copper concentrations have been low in the other reports of hemodialysis-acquired copper intoxication.[7,8]

In the dialysis system employed for our patients, it was the dialysate rather than water which came into contact with the copper heating coil. Matter and co-workers have demonstrated previously the ability of tap water with a pH of less than 6.5 to leach copper from dialysis fittings. Since the dialysate used is buffered with acetate, it was important to determine what effect variations in initial water pH would have on the final pH of the dialysate (Table 2). It should be noted that the buffering capacity of sodium acetate in the dialysate is not sufficient to maintain the pH of the dialysate above 6.5 if added to water with initial pH below 4. Interestingly enough, it is at pH 6.5 and below that Matter et al demonstrated significant copper oxidation potential for water. Therefore, the addition of the dialysate concentrate to the water prior to its passage through the heating coil did not prevent the fall in pH with its attendant oxidation of copper if the initial water pH was less than 4 (and, therefore, dialysate pH less than 6.5). A fall in the pH of water issuing from exhausted ion exchange columns or spontaneous variations in the pH of tap water could create the proper setting for oxidation of copper in the dialysis equipment. The buffering ability of the acetate in the bath does not appear adequate to stabilize pH and protect against the production of a copper rich dialysate.

Table 2.—Effect of Initial Water pH on Final pH of Dialysate

pH to Which Tap Water Adjusted	Final Dialysate pH*
6.0	6.5
5.0	6.5
4.0	6.45
3.0	5.8
2.0	4.6
1.5	1.85
1.0	1.0

* 34 parts water + 1 part chemical concentrate.

The following is a summary of the clinical and laboratory features of our patients.

Clinical findings
- Gastrointestinal symptoms including diarrhea and abdominal pain
- Myalgia
- Pancreatitis (confirmed at autopsy)

Laboratory features
- Marked leukocytosis (range, 26,000-67,000 WBC/cu mm)
- Elevated serum amylase (range, 600-2,250 SU; normal, < 150 units)
- Acidosis, metabolic (pH range, 7.1-7.3)
- Hemolysis
- Myoglobinemia and elevated CPK (1 patient)

We can find no report of pancreatitis or myoglobinemia occurring as the result of acute copper intoxication in man. The second patient of

Matter et al did have abdominal pain and intestinal ileus, but the serum amylase activity was normal.[9] Pancreatitis was not present at postmortem examination (BJ Matter, personal communication). Pancreatitis may result from the intravenous rather than the more common oral route of administration of the toxin. Although increased serum amylase activity may be observed in uremic patients, the pancreatitis was documented grossly and microscopically at autopsy in two of the cases reported here. In the two years that have elapsed since these patients became ill, we have obtained serum for amylase determinations on any dialysis patients with abdominal pain or an unexplained acute illness. There has been no other evidence of pancreatitis or increased amylase in 14 such instances.

Although a muscle biopsy specimen was normal, we are inclined to attribute significance to the myoglobinemia in the light of severe myalgia reported by two patients and the markedly elevated level of CPK in the patient with myoglobinemia. The serum and erythrocyte copper concentrations in the patient with myoglobinemia were so high that it is not unreasonable to postulate the inhibition of many enzyme systems. This, in turn, could lead to muscle cell breakdown and release of myoglobin.

To our knowledge, orchitis is not a recorded feature of copper intoxication. The presence, in two patients, of chronic as well as acute orchitis raises the possibility that the orchitis may have been part of a recurring disease process unrelated to the acute copper intoxication. Possible etiologies for the orchitis would include the uremic state or long-term hemodialysis, or both. The meaning of the orchitis is unclear.

It is no longer permissible to have copper parts in the hemodialysis system and the effluent of the ion exchange column must be used only if its resistance is greater than 1 million ohms. Certain dialysis systems still contain copper or brass parts, the latter being a potential source of copper ion. The effluent of the ion exchange column should be monitored and there should be a mechanism to signal impending column exhaustion. Physicians using dialysis must be aware that the symptom complex of myalgia, abdominal pain, diarrhea, acidosis, pancreatitis, methemoglobin formation, and hemolysis is indicative of acute copper intoxication. The adage "new therapies create new diseases" remains pertinent.

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Direct Determination of Serum Zinc and Copper by Atomic Absorption Spectrophotometry[1]

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A variety of procedures have been developed to measure zinc and copper in biological fluids, including chemical (1–16), fluorometric (17), polarographic (18, 19), neutron activation (20, 21), and flame atomic absorption spectrophotometry (A.A.S.). Published A.A.S. methods for serum and plasma require one or more preparatory steps, including deproteinization (22–25), extraction procedures (26–28), ashing (29), addition of albumin or Dextran to the standard solutions (28, 30), or dilution (utilizing water or dilute hydrochloric acid) (31–38). Of these methods, the latter are preferred, as they are simple to use and reduce sharply the risks of contamination.

The method presented here involves analysis on undiluted serum. The minimum of sample handling greatly decreases the time for analysis and the chances of contamination. The object of the work reported in this paper was to develop a simple, rapid, and accurate method, using a commercial flame atomic absorption system, for determining zinc and copper in serum and to establish pediatric normals, which may be used in investigations of metabolic disorders in children.

METHODS

Collection and Sample Handling

Blood was collected with disposable needles and polypropylene syringes. The blood was transferred to test tubes cleaned as described below and allowed to clot. The tubes were then centrifuged at 2500 rpm for 10 minutes. The serum was transferred by Pasteur disposable pipettes to screw-capped vials and frozen until analysis. Preliminary investigations

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showed no significant changes in the concentration of zinc or copper in samples frozen, thawed, and refrozen up to 24 times within a 2-year period. Disposable syringes and needles were tested and found to be free of extractable zinc and copper.

Preparation of Reagents

Commercially prepared stock solutions of zinc[1] and copper[2] of 1000 μg/ml were stored at room temperature in polyethylene bottles. Working standard solutions were prepared from these stock solutions in synthetic serum of the following composition: 8.768 gm NaCl; 0.373 gm KCl; 100 ml of 1000 μg/ml calcium stock solution;[4] and 20 ml of 1000 μg/ml magnesium stock solution,[5] made up to one liter with demineralized water. The working standards were stored at 5° in polyethylene containers, with no significant change in concentration observed due to storage.

All glassware used was washed with detergent (Haemo-sol[6]) in tap water, rinsed 10 times with tap water, three times with distilled water, and oven-dried at 200°. It was then immersed for 24 hours in a solution of one part redistilled nitric acid[7] and one part demineralized water. It was rinsed four times with demineralized water and then placed upside-down in a specially prepared glassware basket to protect it from air contamination. The basket was lined with absorbent paper with the polyethylene lining towards the inside of the basket. The glassware was covered with polyethylene paper until used.

Instrumentation and Techniques

Analyses were performed with a Bausch and Lomb[8] AC 2-20 atomic absorption spectrophotometer fitted with a premix, laminar flow burner system, 10-cm slot air/acetylene burner, and a manually variable aspirator.

For zinc analyses, acetylene pressure was 6 psig and the flow rate was 2.5 scfh; air pressure was 20 psig with a flow rate of 19 scfh. The burner height was 0.5 cm. The 213.9-mμ zinc line was used and the wavelength was peaked daily by rotating the wavelength selector until a maximum signal was obtained. Slit width was 0.5 mμ; the zinc hollow cathode

[1] Cat. #36305, Instrumentation Laboratory Inc., Lexington, MA.
[2] Cat. #36295, Instrumentation Laboratory Inc., Lexington, MA.
[4] Cat. #36205, Instrumentation Laboratory Inc., Lexington, MA.
[5] Cat. #36125, Instrumentation Laboratory Inc., Lexington, MA.
[6] Scientific Products, Evanston, IL.
[7] G. Frederick Smith Chemical Company, Columbus, OH.
[8] Bausch and Lomb, Rochester, NY.

lamp[9] was run at 12 mA. A sensitivity of 4 μg/100 ml/1% absorption was obtained.

For copper analyses, the acetylene and air pressures, as well as the flow rates, were the same as for zinc; the burner height was 0.65 cm. The 324.8-mμ copper line was used, with daily peaking. The slit width was 0.5 mμ; the copper hollow cathode lamp[10] was run at 15 mA. A sensitivity of 7 μg/100 ml/1% absorption was obtained.

The desired aspiration rate was obtained by rotation of the cannula screw of the aspirator. Demineralized water was aspirated first to rinse the burner. One milliliter of each standard was aspirated, its aspiration rate measured to the nearest 0.1 second, and its absorbance read to check machine stability and to insure proper operation. Each standard was followed by a rinse of demineralized water. One milliliter of serum was aspirated, its aspiration rate measured and its absorbance read; each sample was also followed by a demineralized water rinse.

For the analysis, the cannula screw on the aspirator was adjusted manually such that the standard solutions in the synthetic serum aspirated at the same aspiration rate as the serum sample. At least three

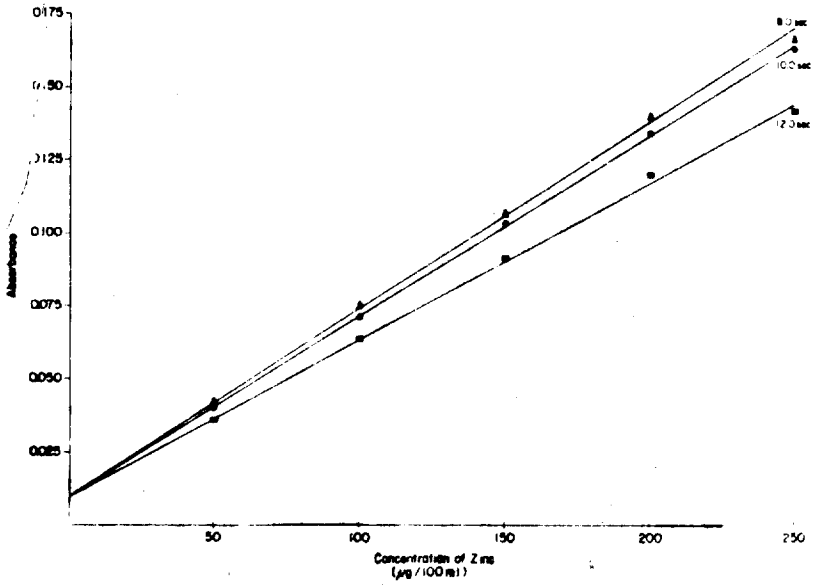

FIG. 1. Absorbance vs concentration in μg/100 ml of zinc at varying aspiration rates based on an average of 10 determinations.

[9] Cat. #62811, Instrumentation Laboratory Inc., Lexington, MA.
[10] 33-38-13; Bausch and Lomb, Rochester, NY.

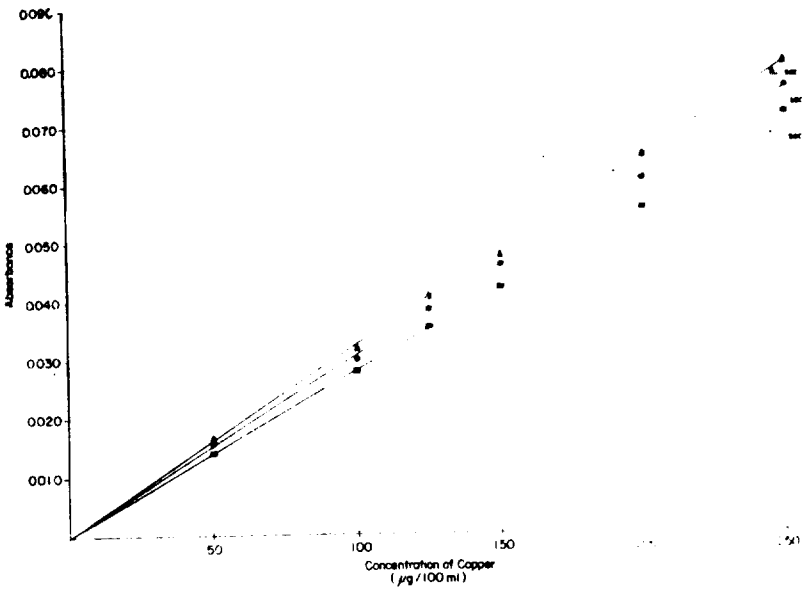

FIG. 2. Absorbance vs concentration in μg/100 ml of copper at varying aspiration rates based on an average of 10 determinations.

standard solutions were run at this aspiration setting to establish a working standard curve. Thus mineral levels in samples aspirating at 12 seconds/1.0 ml were computed from a curve of standard solutions aspirating at 12 seconds/1.0 ml.

Sample sizes less than 1.0 ml can be used, provided that the volume is known and the aspiration rate is accurately measured. An appropriate correction of the aspiration rate makes this smaller sample comparable to a 1.0-ml sample (i.e., a 0.5-ml sample is aspirated in 6 seconds; this rate would be multiplied by two and the result would be obtained from the 12-second curve).

The plots of absorbance vs copper and zinc concentrations in the working range of 50 to 250 μg/100 ml are linear, as shown in Figs. 1 and 2. The concentration of the unknown solutions are computed from each graph at the appropriate aspiration rate.

RESULTS AND DISCUSSION

It is known that aspiration rate differs between serum or plasma samples and water standards (30). In fact, in the method described in this paper, undiluted serum was found to have a much slower aspiration rate than the standards in synthetic serum, due to the differences in

viscosity, which were as great as 0.8 cp at 25° (serum: 1.8 cp; standards: 1.0 cp).[11] In order to avoid analytical error, these varying aspiration rates were taken into account. While this correction could have been made by equalizing viscosity (28, 30), this technique involves a risk of contamination. Use of the method as described in this paper corrects for aspiration rate difference using the graphs shown in Figs. 1 and 2. The statistical significance of the regression lines of these graphs is shown in Table 1 ($N = 10$). The standard errors of estimate indicate only a small amount of overlap in the 0–50 μg/100 ml range for each mineral.

Results obtained by this method for adults are comparable to the adult values reported in the literature, as shown in Table 2, Collected Data on Serum Zinc and Copper Levels in Normal Subjects. Pediatric values obtained by this method fall within the accepted range of pediatric normals.

The self-consistency of this method was examined by the recovery of added zinc and copper to a specimen, and the measurement of the same sample at several dilutions. Recovery studies were performed by adding 50 μl each of zinc and copper standards ranging from 100 μg/100 ml to 4000 μg/100 ml to 1.0 ml of undiluted serum. This resulted in no significant change in aspiration rate. The mean recovery for zinc was 103.4% and for copper was 103.3%. The results are shown in Table 3. Dilutions of 1:3,1:2, and 1:1 at measured aspiration rates on a sample gave zinc and copper assay values 25, 33, and 50% of the value obtained on the undiluted serum.

TABLE 1
STATISTICS FOR MINERAL REGRESSION LINES[a]

Regression line	Slope	Y intercept OD units	SE of estimate (biased)
Zinc:			
8.0 second	0.00057	0.0194	±0.0052
10.0 second	0.00055	0.0147	±0.0014
12.0 second	0.00050	0.0142	±0.0036
Copper:			
8.0 second	0.00033	−0.0005	±0.0005
10.0 second	0.00031	0.0004	±0.0003
12.0 second	0.00029	−0.0011	±0.0014

[a] N = 10 SD range = ±0.0000 to ±0.0017; SD average = ±0.00050.

[b] Wells Brookfield micro cone-plate viscometer, Model LVT, Brookfield Engineering Laboratories Inc., Stoughton, MA.

TABLE 2
COLLECTED DATA ON SERUM ZINC AND COPPER LEVELS IN NORMAL SUBJECTS

Reference	Method	Adult (A) or Pediatric (P) No. of subjects	Mean μg/100 ml	Range SD or SE
Zinc:				
E. M. Butt (3)	Chemical	122 A	119	7.0 SE
		48 A	130	6.0 SE
J. W. Gofman (5)	Chemical	39 A	98	2.0 SE
H. L. Helwig (6)	Chemical	64 A	91	±16.7 SD
F. M. Smit (11)	Chemical	55 A	138.7	±28.73 SD
H. Wolff (16)	Chemical	1550 A	166	±22 SD
M. M. Parker (23)	A.A.S.	23 A	89.9	±10.3 SD
B. Foley (40)	A.A.S.	14 A	115.0	
K. Fuwa (33)	A.A.S.	A	122.7	±3.19 SD
This method	A.A.S.	11 A	110.0	±20.7 SD
		37 P	128.2	±15.6 SD
Copper:				
E. M. Butt (3)	Chemical	122 A	106	2.0 SE
		48 A	110	4.0 SE
J. W. Gofman (5)	Chemical	39 A	129	5.0 SE
A. L. Neilson (10)	Chemical	100(f) A	123	±16.0 SD
		30(m) A	110	±12.0 SD
J. J. Theron (12)	Chemical	18(f) A	93.0	±19.9 SD
		33(m) A	95.0	±21.2 SD
B. L. Vallee (15)	Chemical	—	110	±13 SD
M. M. Parker (23)	A.A.S.	10(f) A	102.6	±16.4 SD
		18(m) A	105.1	±15.9 SD
J. B. Dawson (31)	A.A.S.	24 A	108	70–165 range
This method	A.A.S.	45 P	125.2	±20.7 SD

TABLE 3
RECOVERY OF ADDED ZINC AND COPPER

Zinc in pooled serum μg/100 ml	Zinc added μg/100 ml	% zinc recovered	Copper in pooled serum μg/100 ml	Copper added μg/100 ml	% copper recovered
92.0	2.0	104.3	98.0	2.4	104.6
92.0	14.3	103.5	98.0	7.1	106.5
92.0	33.4	103.1	98.0	11.9	104.6
92.0	47.6	104.3	98.0	23.8	102.6
92.0	91.0	106.0	98.0	47.6	103.0
92.0	143.0	100.0	98.0	143.0	101.2
92.0	190.0	102.8	98.0	190.0	100.3

SUMMARY

The method for the determination of zinc and copper in serum which has been described in this paper is simple and rapid, requiring only undiluted serum. Absorbance is measured on a commercially available atomic absorption spectrophotometer. Mean reproducibility and recovery of this method for serum zinc is 103.4% and for serum copper is 103.3%. The results on normal adult and pediatric subjects are comparable to those reported in the literature.

Preliminary studies show that this method lends itself to analyses of those trace minerals which are in low concentrations in human biological specimens, since no dilution factor is present. The addition of a direct concentration computer readout to the A.A.S. system simplifies determinations in a low concentration range. The most recent development in nonflame atomic absorption spectrophotometry, the carbon rod atomizer (CRA), is a promising technique for minerals in low ranges (39). However, the use of xylene impregnation to prevent sample absorption into the rod is not yet perfected and incomplete recovery remains a problem.

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Intracellular Distribution of Copper in the Liver during Chronic Administration of Copper Sulfate to the Rat

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Intracellular Distribution of Copper in the Liver during Chronic Administration of Copper Sulfate to the Rat. LAL, S., and SOURKES, T. L. (1971). *Toxicol. Appl. Pharmacol.* 18, 562–572. The intracellular distribution of copper was studied in groups of male Sprague-Dawley rats injected ip with 1.25, 2.5, or 3.75 mg Cu/kg daily for periods of 6, 12, or 18 wk. Control animals were given 0.9% NaCl. The initial body weight was approximately 50 g. The partition between the nuclear and mitochondrial fractions was related to the actual hepatic concentration of the metal rather than to the dose and duration of copper loading. At liver concentrations between 30 and 100 μg Cu per gram of fresh tissue, a larger proportion of the metal occurred in the mitochondrial component. At 100 μg/g the distribution was approximately equal in these 2 fractions. With further increase in hepatic concentration, the quantity of copper retained by the mitochondrial fraction tended to plateau. On the other hand, the nuclear portion continued to accumulate the metal without evidence of any decline in storage capacity. Accumulation in the supernatant fraction, in relation to hepatic concentration of the metal, varied in a complex fashion with dose and duration of administration. Very small increments occurred in the microsomal portion compared with the other fractions, and the pattern of uptake was inconstant. The present findings are discussed in terms of Wilson's disease and current ideas of the mode of cytotoxicity of copper. It is suggested that useful information might accrue from attempts to correlate subcellular distribution of the metal with histologic changes. More attention should be paid to the intracellular distribution in all subcellular components rather than focusing on a single organelle.

Several reports have appeared on the intracellular distribution of copper in livers containing elevated levels of the metal: in the newborn (Porter *et al.*, 1961, 1964; Porter, 1964; Gregoriadis and Sourkes, 1967; Evans *et al.*, 1970); in copper-loaded animals (Hermann and Kun, 1961; Fare and Woodhouse, 1963; Verity *et al.*, 1967; Gregoriadis and Sourkes, 1967; Milne and Weswig, 1968; Evans *et al.*, 1970); in Wilson's disease (Porter, 1964); and in various experimentally induced states (Brinkman *et al.*, 1961; Hermann and Kun, 1961; Fare and Woodhouse, 1963; Gregoriadis and Sourkes, 1967). In most cases investigators have assigned to the mitochondrial fraction a preeminent role in retention of excess copper. However, Gregoriadis (1968) noted a changing pattern of uptake of the metal by the nuclear and mitochondrial components, respectively, with progressive increase in hepatic concentrations from 5 to 136 μg/g fresh liver. He

speculated that the mitochondrial fraction may reach a saturation level, and additional metal reaching the liver is diverted to the nuclear fraction.

A review of the literature reveals but a single investigation of the chronically copper-loaded animal with respect to partition of the metal among intracellular organelles (Fare and Woodhouse, 1963). The present experiments were carried out to study the effects of dose and duration of copper treatment on intracellular distribution of copper in the liver of rats. In addition, an evaluation of the relation of hepatic concentration of copper to distribution within the cell was carried out.

METHODS

Male Sprague-Dawley rats weighing initially 50 g were used. The diet consisted of Purina Chow (copper content, 10–12 μg/g) and tap water (<0.2 μg copper per 20 ml). The animals were injected with 1.25, 2.5, or 3.75 mg Cu/kg body weight ip daily in the form of copper sulfate dissolved in 0.9% NaCl. A total volume of 1.0 ml was injected. A control group received an equal volume of saline. The animals were weighed weekly and the quantity of copper injected was adjusted accordingly. Groups of animals were killed by decapitation at 6, 12, or 18 wk. The number of animals in each group is indicated in Table 1.

The procedure for subcellular fractionation and the determination of copper was carried out as described by Gregoriadis and Sourkes (1967), except that the nuclear fraction was washed twice and the washings were combined for further centrifugation. The recovery of copper in all 4 fractions in relation to the total homogenate was 89.1% ± 1.17 (SE).

RESULTS

The growth of animals was affected by the copper treatment. Whereas the dose of 1.25 mg/kg had no significant effect, the higher doses caused a significant depression in body weight in rats treated for 18 wk (Table 1). In the shorter periods the effect on weight was less consistent.

The significant decrease in liver weight at 6 wk in animals treated with 2.5 and 3.75 mg Cu/kg paralleled body weight changes. With the same 2 doses at 18 wk, despite the significantly lower body weights, the liver weights were not significantly different from controls (Table 1).

All animals treated with 3.75 mg/kg for 12 and 18 wk developed ip adhesions and megacolon. The latter condition was thought to result from the adhesions but, while this study was in progress, Wiederanders *et al.* (1968) reported similar findings which they attributed to destruction of the myenteric plexuses.

The control rats had liver concentrations of 3.8–5.7 μg Cu/g fresh weight. The pooled controls revealed the following intracellular partition (mean % of hepatic copper ± SE): nuclei 18.4 ± 0.88, mitochondria 23.1 ± 0.97, microsomes 5.4 ± 0.88, and supernatant 5.3 ± 1.31. The range of copper concentrations in the loaded animals was 31–964 μg/g fresh liver. The intracellular distribution for the different time periods is shown in Table 2. It can be seen that the concentration increases with time and dose but that there is a large individual variation between animals within each subgroup. In rats beyond the period of neonatal copper storage, the supernatant fraction normally contains about

TABLE 1

EFFECT OF COPPER LOADING ON BODY AND LIVER WEIGHT OF RATS

Dose of Cu[a] (mg Cu/kg/day)	Duration of treatment: Outset	6 Wk		12 Wk		18 Wk	
	Body weight[b] (g)	Body weight (g)	Liver weight[b] (g)	Body weight (g)	Liver weight (g)	Body weight (g)	Liver weight (g)
Controls	51 ± 2.7 (15)[c]	263 ± 5.6 (4)	11.4 ± 0.25	346 ± 29.0 (6)	13.2 ± 0.77	463 ± 12.8 (5)	15.1 ± 0.72
1.25	51 ± 3.6 (18)	236 ± 10.1 (5)	10.5 ± 0.66	330 ± 2.9 (7)	12.6 ± 0.55	431 ± 26.2 (6)	14.8 ± 0.82
2.5	46 ± 3.5 (15)	208 ± 17.2[d] (5)	8.8 ± 0.66[d]	332 ± 6.2 (5)	12.2 ± 0.48	353 ± 29.7[e] (5)	12.7 ± 0.89
3.75	57 ± 1.1 (20)	224 ± 6.1[e] (7)	9.2 ± 0.51[d]	292 ± 7.0 (6)	12.3 ± 0.58	367 ± 13.0[f] (7)	14.6 ± 1.00

[a] Rats received daily injections of copper sulfate solutions by the ip route.
[b] Expressed as mean ±SE.
[c] Number of rats.
[d] Significantly less than control values ($P < 0.05$).
[e] Significantly less than control values ($P < 0.01$).
[f] Significantly less than control values ($P < 0.001$).

TABLE 2

EFFECT OF DURATION AND DOSE OF COPPER ADMINISTRATION ON INTRACELLULAR DISTRIBUTION OF HEPATIC COPPER IN RATS

Dose of Cu[a] (mg Cu/kg/day)	Duration of treatment														
	6 Wk					12 Wk					18 Wk				
	Hepatic Cu[b] concentration	N[c]	Mt	Mc	S	Hepatic Cu concentration	N	Mt	Mc	S	Hepatic Cu concentration	N	Mt	Mc	S
Controls	4.2 ± 0.07 (4.1–4.4)	19.1[d] ± 2.49	24.9 ± 1.71	4.9 ± 1.50	51.5 ± 1.85	4.0 ± 0.07 (3.8–4.2)	18.7 ± 1.21	24.6 ± 1.71	3.9 ± 0.92	52.8 ± 2.74	4.7 ± 0.29 (4.2–5.7)	17.4 ± 1.35	20.3 ± 0.84	7.5 ± 1.90	54.8 ± 1.82
1.25	42.4 ± 3.31 (35–54)	22.4 ± 2.17	59.7 ± 1.66	5.4 ± 1.97	12.5 ± 2.37	134 ± 31.2 (31–276)	33.7 ± 2.79	44.9 ± 3.86	2.9 ± 0.26	18.5 ± 2.39	212 ± 15.9 (140–254)	42.3 ± 4.20	35.1 ± 2.36	3.9 ± 0.50	18.7 ± 1.98
2.5	242 ± 30.7 (162–316)	43.6 ± 3.66	38.1 ± 1.87	2.8 ± 0.54	15.5 ± 2.38	287 ± 64.1 (166–534)	47.5 ± 1.79	36.1 ± 2.92	2.5 ± 0.44	13.9 ± 3.16	394 ± 72.0 (131–559)	61.2 ± 3.55	22.6 ± 2.50	3.4 ± 0.48	12.8 ± 1.91
3.75	278 ± 55.9 (41–485)	49.1 ± 1.70	33.8 ± 1.49	2.3 ± 0.19	14.7 ± 1.98	535 ± 84.5 (164–692)	61.7 ± 2.38	23.4 ± 1.59	2.8 ± 0.37	12.1 ± 2.40	721 ± 68.3 (486–964)	70.0 ± 2.99	17.3 ± 1.45	2.4 ± 0.37	10.3 ± 2.38

[a] Fifty-three male Sprague-Dawley rats (see Table 1) were injected daily with solutions of $CuSO_4$ by the ip route. Fifteen animals were given 0.9% NaCl only.
[b] Expressed as mean ±SE in μg Cu per gram fresh weight of liver. Range in parentheses.
[c] N = nuclear, Mt = mitochondrial, S = supernatant, Mc = microsomal fraction.
[d] Percentage of hepatic concentration in different fractions expressed as mean ±SE.

half the hepatic copper, but this proportion decreases sharply with each of the copper dosage-schedules used. Mitochondrial copper, about 20–25% of the total in the controls, increases initially in percentage with the lowest dose of copper sulfate; the percentage then declines with increasing dose and duration of treatment. In fact, the decline from the maxima attained with 1.25 mg Cu/kg is more intense the greater the accumulation of copper in the liver (Table 2). In contrast to this, the nuclear fraction acquires the progressively higher proportion of the hepatic copper with increasing dose and duration of treatment.

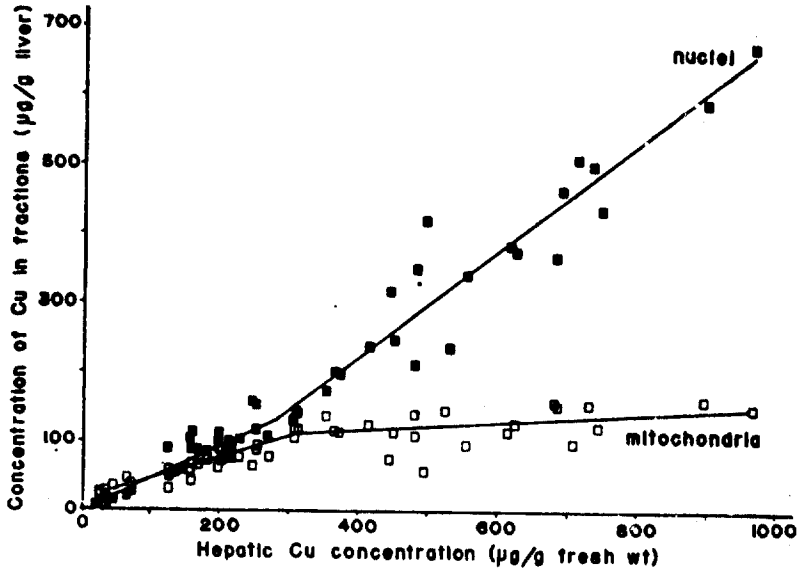

FIG. 1. The partition of Cu between nuclear and mitochondrial fractions in relation to hepatic Cu concentration. Rats were injected daily ip with Cu in doses of 1.25, 2.5, or 3.75 mg/kg body weight and sacrificed in groups at 6, 12, or 18 wk. The data are plotted as concentration of Cu in nuclear (—■—) and mitochondrial (—□—) fractions against total hepatic Cu concentration irrespective of dose or duration of Cu loading. Each pair of symbols represents 1 animal. The total number of animals depicted is 53. Regression lines were drawn (least squares method) for values between 30 and 350 and 300 and 1000 µg/g liver.

Because of the striking variation between rats in the dosage groups, the data were reassessed on the basis of hepatic concentration achieved without respect to specific treatment. The results are shown as plots of the copper concentration in nuclei, mitochondria (Fig. 1), and supernatant fractions (Fig. 2) as a function of total liver concentration. In Fig. 1 it is evident that the distribution of copper to the 2 organelles is directly and simply related to the hepatic concentration. For hepatic concentrations between 30 and 100 µg Cu/g liver a somewhat higher mean proportion of the metal is localized in mitochondrial than in the nuclear fraction. At approximately 100 µg Cu/g liver the partition between these 2 components is equal, but beyond this level the accumulation in the 2 fractions diverges. The mitochondrial portion approaches a limiting value near 175 µg/g liver, as seen in the scatter of points at high concentrations (Fig. 1). On the other hand, deposition in the nuclear fraction increases progressively without sign of plateau, at least up to a concentration of about 1000 µg/g liver.

In contrast to copper deposition in the nuclear and mitochondrial fractions the process in the supernatant portion of the cell is affected by the dose of copper sulfate administered and, at the highest dosage, by time. In Fig. 2 it can be seen that the curves for copper concentration in the supernatant fraction in relation to total hepatic copper concentration shift to the right as the dosage increases. This is true not only for the 2 lower doses (Fig. 2A), where the pattern of uptake is independent of duration of treatment, but also for the highest dose (Fig. 2B), where the accumulation of the metal is clearly a function of duration of copper administration.

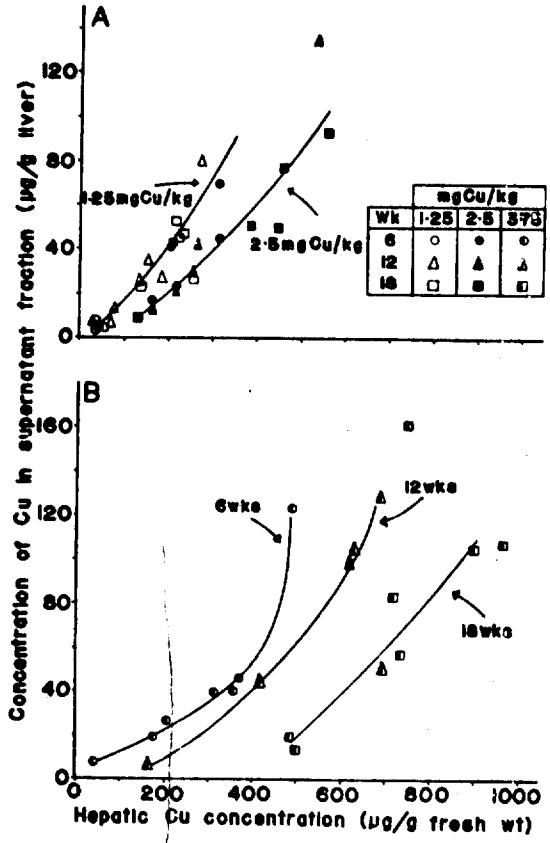

FIG. 2. The effect of dose and time on concentration of Cu of the supernatant fraction in relation to total hepatic Cu. Animals were injected with 1.25, 2.5 (A) or 3.75 mg Cu/kg body weight (B) ip daily and killed in groups at 6, 12, or 18 wk. The concentration of Cu in the supernatant fraction is plotted against total hepatic copper concentration. The symbols used are explained in the inset of panel A. Each symbol represents 1 animal.

This means that for identical hepatic concentrations of copper the absolute quantity of the metal in the supernatant is higher the lower the dose (Fig. 2). There is an additional factor with the highest dose, 3.75 mg/kg (Fig. 2B); in this case the copper content of the supernatant at a given total hepatic copper concentration tends to be lower the longer the treatment has been going on. There appears to be a limiting level of accumulation of copper in this fraction, lying between 135 and 160 μg Cu/g liver.

The storage of copper in the microsomal fraction is very small compared with the other fractions; in the heavily loaded liver it is reduced to 2–3%, compared to 5.4% in the saline-treated controls. No clear pattern of accumulation emerges apart from a general tendency to increase in quantity with the progressive elevation of hepatic copper concentration (Table 2).

DISCUSSION

The present findings bear out Gregoriadis's speculation (Gregoriadis, 1968) in regard to an apparent saturability of the mitochondrial fraction with copper. In the heavily loaded animal the nuclear component plays a predominant role in copper storage. The present values are in close agreement with the data of Gregoriadis and Sourkes (1967) over the limited range in which they worked. The slight differences between the nuclear and mitochondrial control values can be attributed to the fact that in the present study the nuclear fraction was washed twice. Evans *et al.* (1970) found that the copper concentration increased linearly in all fractions with increasing total hepatic concentration for values up to 80 μg/g fresh tissue. The predominant intracellular accumulation was in the mitochondrial fraction. In the present study the predominant storage was also in the mitochondrial fraction for hepatic concentrations of 30–100 μg/g. Both Fare and Woodhouse (1963) and Verity *et al.* (1967) observed a striking percentage increase of copper in the mitochondrial fraction at the expense of the other fractions, for hepatic concentrations of 800 and 125 μg Cu/g, respectively. The former authors obtained a diametrically opposite partition between the nuclear and mitochondrial components compared with the present study. Moreover, their control rats had a much larger percentage of copper in the nuclear and mitochondrial particles and a correspondingly reduced supernatant concentration than that found here. As they used female rats, there may be a sex difference that would account for at least some of the discrepancies. Milne and Weswig (1968) studied relatively small increments in hepatic copper in the brown rat, and found a preferential uptake in the microsomes; the latter contained a much higher copper content than reported in other strains of rat (Thiers and Vallee, 1957; Hermann and Kun, 1961; Brinkman *et al.*, 1961; Edwards *et al.*, 1961; Gregoriadis and Sourkes, 1967; Gregoriadis, 1968; Evans *et al.*, 1970). A recalculation of the data of Hermann and Kun shows that they obtained a predominant increase of copper in the nuclear fraction even with an hepatic concentration of only 80 μg Cu/g in the Long-Evans strain. Their control values in 2 strains, for both sexes, showed a high mitochondrial copper level. This may be related to the unusually elevated copper concentrations in their Long-Evans rats and, possibly, contamination in their Sprague-Dawley strain, as evidenced by a recovery of 126%.

Much evidence based primarily on histological methods has accumulated for deposition of copper in the lysosomes in the copper-loaded animal (McNary, 1963; Barka *et al.*, 1964; Wessel, 1965; Goldfischer, 1967; Lindquist, 1967; Scheuer *et al.*, 1967), in Wilson's disease (Schaffner *et al.*, 1962; Sternlieb and Scheinberg, 1963; Goldfischer, 1963, 1965; Goldfischer and Moskal, 1966; Goldfischer and Sternlieb, 1968) and in the newborn (Goldfischer and Sternlieb, 1968). Verity *et al.* (1967) were able to demonstrate directly the association of copper with these organelles by partial separation of the lysosomes from the mitochondrial fraction using differential centrifugation. In the present

study, as in previous reports on subcellular distribution (Porter *et al.*, 1961, 1964; Hermann and Kun, 1961; Fare and Woodhouse, 1963; Porter, 1964; Gregoriadis and Sourkes, 1967; Gregoriadis, 1968; Milne and Weswig, 1968; Evans *et al.*, 1970), the lysosomes were not isolated from the crude mitochondrial component. Hence, saturation of the mitochondrial fraction described in this study may involve saturation of the lysosomal bodies.

Though the nuclear fraction appears to take up very large quantities of copper, stainable deposits in the nucleus are little mentioned in the literature (cf., Uzman, 1956; Vogel, 1959). In fact, Howell (1959) stated that copper granules are never seen in the nucleus. It is possible that copper is actually deposited there but in a form unreactive with the histochemical agents used. The limitation of staining methods and even the failure to demonstrate copper histochemically in livers with distinctly elevated concentrations of the metal by chemical analysis is well documented (Levi *et al.*, 1967; Goldfischer and Sternlieb, 1968; Lindquist, 1969).

Though the major portion of injected copper is in a non-dialyzable form in all fractions (Hermann and Kun, 1961), little information is available about the manner of its binding. A copper protein, hepatomitochondrocuprein, is said to account for the deposition of much of the elevated copper in the newborn (Porter *et al.*, 1962, 1964; Porter, 1966), and may be induced in Wilson's disease (Porter, 1964). Whether such an induction accounts for the deposition of the metal in the loaded rat is unknown. An intramitochondrial polypeptide with high chelating properties for copper similar to the one isolated from rat spleen (Vogel and Kemper, 1966) is also a possible sequestering agent for copper in this fraction.

In regard to the nuclear fraction, Wacker and Vallee (1959) have shown that copper is associated with nucleic acids whose phosphate groups are probable sites of interaction with metals. However, the extent to which the phenomenal increase in nuclear copper is bound to DNA is unknown. There is also little information about the nature of copper binding in the supernatant fraction. Morell *et al.* (1961) have isolated a cuproprotein containing most of the copper in this fraction. This protein contained a large number of —SH groups which were considered probable binding sites. Current work (Bloomer and Sourkes, unpublished data) indicates that much of the copper in the supernatant layer of the liver homogenate following loading is bound to a single protein. The present report indicates that there is a limited capacity of the supernatant to retain copper. The effects of dose and time noted suggest that some adaptive process is taking place to eliminate the metal from this fraction when excessive amounts of the element enter the hepatocyte. An alternative hypothesis is that large amounts of copper depress hepatic synthesis of soluble copper-binding proteins.

The present findings may be relevant to the intracellular distribution of copper in Wilson's disease. Scheinberg and Sternlieb (1959) found copper concentrations in patients with this disorder equivalent to 16–737 μg/g fresh tissue. In a later study of asymptomatic cases (Sternlieb and Scheinberg, 1968) they found values of 38–457 μg/g fresh liver, with a mean value of 246 $\pm$ 15 (SE). The partition among organelles as recorded by Porter (1964) must therefore be reconsidered in the context of changing distribution of the metal with hepatic concentration, in addition to other variables such as progress of the cirrhotic state of the liver. Protection of the cell from copper has been attributed to binding of the excess metal to a mitochondrial protein (Porter, 1964) or a

polypeptide (Vogel and Kemper, 1966), or to storage in the lysosomes (Goldfischer, 1965). On the other hand, the mechanism of copper cytotoxicity has been attributed to the release of copper from the lysosomes (Goldfischer, 1965) or to the release of hydrolytic enzymes secondary to peroxidation of lysosomal membranes (Lindquist, 1967, 1968). The present findings indicate that the crude mitochondrial fraction, which includes lysosomes, has a large but not unlimited capacity to store copper. If these organelles are the chief structures protecting the cell by deactivating the metal, one would expect cellular damage to appear prominently as they become saturated with copper and as the metal is consequently deposited in large quantities in other parts of the hepatocyte. The high concentration of copper in the nuclear component does not necessarily indicate a locus of cytotoxicity, but it is nevertheless interesting to note that one of the earliest histologic changes in Wilson's disease, occurring before cirrhotic changes are evident, is in the nuclei (Anderson and Popper, 1960).

Some light may be shed on the problem of copper cytotoxicity by studies correlating histological changes with the subcellular distribution of the metal. Clearly, any theory of cytotoxicity must take into account the distribution of the metal in all cellular components rather than focusing on a single organelle, and due consideration should be paid to the parameters influencing this distribution.

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Note added in proof: The authors have now seen the paper of Roussanov, E., and Balevska, P., (*Dokl. Bulg. Akad. Nauk* **20**: 241–243, 1967), which also suggests changes in distribution of copper with respect to the mitochondrial and nuclear fractions as a function of hepatic concentration of copper.

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Deposition of Copper in Rat Tissues—The Effect of Dose and Duration of Administration of Copper Sulfate

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Deposition of Copper in Rat Tissues—the Effect of Dose and Duration of Administration of Copper Sulfate. LAL, S. and SOURKES, T. L. (1971). *Toxicol. Appl. Pharmacol.* **20**, 269–283. Two hundred and twenty-five rats were injected ip with copper sulfate in doses of 0.625, 1.25, 2.5, and 3.75 mg Cu/kg body weight or with saline daily. The animals were sacrificed at 24 hr, 1, 2, 6, 12, or 18 wk, and copper was measured in 13 tissues. With the highest dose, all tissues accumulated the metal. The data suggest that increase in hepatic copper occurs without saturation of the excretory capacity of the rat.

The concentration of the metal in the liver at 12 and 18 wk increases linearly with dose; minimum doses of 0.5 and 0.3 mg Cu/kg, respectively, are needed to elevate hepatic levels. The percentage of injected metal retained in the liver or kidney varies with the dose and duration of loading. Deposition of copper in extrahepatic tissues is unrelated to the storage capacity of the liver.

Normal bone loses copper with age. This decline is less marked in the loaded animal and can be reversed with a high dose (3.75 mg/kg), or with prolonged loading at a lower dose (2.5 mg/kg). Data suggest that increases in bone copper may stimulate a mechanism involved in elimination of the metal from this tissue. Progressive increases of copper occur in spleen and lung with both time and dose. With the highest dose the increase is more marked and suggests an overwhelming of factors regulating tissue levels of the metal.

Brain and testis showed an early increase, but deposition in those organs is limited, and the values tend to plateau. Skeletal and cardiac muscle as well as skin are relatively resistant to copper accumulation. The increase in hair is less consistent.

The findings are discussed in relation to Wilson's disease.

Chronic copper poisoning in farm animals (Todd, 1962, 1969) and in Wilson disease (Walshe, 1966) is well known. In both conditions the liver is the major organ of copper deposition, although other tissues also accumulate the metal. The mechanism and time factors involved in accumulation of copper in Wilson's disease are unknown (Walshe, 1967), but Osborn and Walshe (1967), Osborn *et al.* (1969) and Sternlieb and Scheinberg (1968) have suggested that extrahepatic deposition of the metal occurs consequent upon saturation of hepatic binding sites for the element. Innumerable copper-loading experiments have been conducted in the sheep (Eden, 1940; Edgar, 1942; Marston and

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Lee, 1948; Dick, 1954; Sutter *et al.*, 1958; Todd *et al.*, 1962; Barden and Robertson, 1962; Hemingway and MacPherson, 1967; McCosker, 1968; Doherty *et al.*, 1969), pig (Lucas and Calder, 1957; Allcroft *et al.*, 1961; Allen and Harding, 1962; Suttle and Mills, 1966; Gipp *et al.*, 1967; Beames, 1969), rat (Flinn and Inouye, 1929; Lindow *et al.*, 1929; Herkel, 1930; Cunningham, 1931; Boyden *et al.*, 1938; Gubler *et al.*, 1954; Fare and Woodhouse, 1963; Barka *et al.*, 1964; Gregoriadis, 1966; Williams, 1966, 1967; Owen and Hazelrig, 1968; Wiederanders *et al.*, 1968; Milne and Weswig, 1968; Russanov and Balevska, 1968; Beames, 1969), rabbit (Eden, 1940; Herkel, 1930), calf (Shand and Lewis, 1957), turkey (Wiederanders, 1968), chicken (Goldberg *et al.*, 1956) and goldfish (Vogel, 1959). However, investigators have paid little systematic attention to the effects of such basic parameters as dose and duration of administration on the pattern of deposition of copper in tissues other than liver and blood. In order to understand more fully factors which influence this distribution, experiments were designed to study the effect of these 2 variables on the concentration of the metal in rat tissues.

METHODS

Male Sprague-Dawley rats with an initial weight of 50 g were used. The animals were injected ip with 0.625, 1.25, 2.5, or 3.75 mg Cu/kg body weight daily as copper sulfate dissolved in 0.9% NaCl. The injection volume was 1.0 ml. Control rats received an equal volume of NaCl. The rats were weighed weekly, and the quantity of copper administered was adjusted accordingly. The diet consisted of Purina Chow and tap water (copper content, less than 0.2 μg/20 ml). According to the statement of the manufacturer (Ralston Purina Co., St. Louis, Missouri), the chow contains the following ingredients in the indicated concentrations (ppm): Cu, 15.1; Fe, 197; Zn, 30.3; Mn, 54.4; Co, 0.37.

The animals were sacrificed by decapitation at 24 hr, 1, 2, 6, 12, or 18 wk. The copper concentration was estimated in 13 tissues. In a few cases serum copper levels were also measured. The method of copper determination was that used by Gregoriadis and Sourkes (1967).

Because of possible regional variation of copper deposition in the liver (Howell, 1959), this organ was cut into small pieces, and aliquots were taken for each sample. In most cases, approximately 0.5–1.0 g of liver was analyzed. In some livers the subcellular distribution was also studied (Lal and Sourkes, 1971); in such cases part of the homogenate was used to estimate copper. One gram of skin, 0.5 g hair, and 2 g leg muscle were sampled. The whole tibia or femur served as the source of bone; in small animals both tibias and femurs were combined for each measurement. Only the muscular portion of the diaphragm was used. With other tissues the whole organ was taken for analysis, but in young rats heart, spleen, tongue, testis, and diaphragm were pooled from 2–3 animals, and in the case of kidney both organs were utilized. Hair and skin were thoroughly washed with acetone and glass-distilled water. Two to three milliliters of serum was taken for copper estimation. Tissues not examined immediately were frozen and stored for later analysis.

Variation between duplicate liver values was less than 5%. Hepatic copper was also measured in 14 rats dying prematurely but free of any chronic infective condition; the values were within the expected range. In all, 225 rats were used (Table 1).

RESULTS

Effect of Cu on Growth and Mortality

Only the high doses, 3.75 and 2.5 mg/kg, had a consistent depressant effect on growth (Table 1). This was evident at 6 and 16 wk, respectively. All rats treated beyond 10 wk with the highest dose had adhesions and megacolon. The findings of megacolon were considered related to the ip adhesions, but while this study was in progress Wiederanders *et al.* (1968) reported similar findings which they were able to attribute to destruction of the myenteric plexuses. Mortality at dose levels of 0.625, 1.25, and 2.5 mg/kg was 16.1, 7.5, and 19%, respectively. Though the mortality rate was higher with the 3.75 mg/kg treated animals (35%) compared to the controls (20%), this was not statistically significant (Chi square = 3.68).

Effect of Cu on Liver Weight

Within 24 hr the liver weight was significantly increased with all copper doses except the lowest (Table 1). Thereafter, except for the 6 wk groups the liver weights did not differ significantly from controls even where body weight was significantly lower.

The effect of age on normal tissue copper levels has been discussed in another report (Lal and Sourkes, 1970) and will be alluded to only briefly.

Accumulation of Cu in Tissues

Liver. Control rats injected with saline had about 4 μg Cu/g fresh liver except in the initial group where the concentration was higher (Table 1): this reflects neonatal storage of the metal (Cunningham, 1931; Evans and Wiederanders, 1967). Administration of copper caused an increase in concentration of the metal with all doses even after a single injection (Table 1). With the lowest dose, however, the mean increase (37%) was not statistically significant; after 1–6 wk the concentration increased 2.1–3.2 times control values. A more dramatic increase (14.2 times) was evident at 18 wk. Increments were much more striking with the higher doses (Table 1). With the 1.25 mg/kg dose there was an initial increase followed by a plateau between weeks 1 and 6. Thereafter the increase was linear. With the 2 highest doses the concentration increased linearly for the first 6 wk, and the values were similar for the 2 doses. Beyond this point the patterns diverged. With the 2.5 mg/kg dose, the rate of accumulation declined; the metal, however, continued to increase in a linear fashion. With the 3.75 mg/kg dose, the rate of deposition did not taper off until after 12 wk of loading. It is interesting to note that after 12 wk all doses cause accumulation of hepatic copper at the same rate.

After 12 and 18 wk the copper concentration in the liver is related linearly to the dose of the metal administered. On extrapolation of these data it can be shown that threshold values for hepatic accumulation of 0.5 and 0.3 mg Cu/kg are needed for the 12- and 18-wk period, respectively. For shorter periods of loading, the relationship of concentration to dose is more variable.

After a single injection of copper, a high percentage of the metal is retained in the liver (Table 1). During the period of 6–18 wk, the proportion retained with the lower doses is virtually constant: 2–3% at the dose level of 0.625 mg/kg and 6–8% with the 1.25 mg/kg dose. At the 2 highest dose levels the percentage accumulated continues to decrease with time. The lowest dose at every time interval studied produces a lower

TABLE 1

EFFECT OF DAILY IP CU LOADING ON BODY WEIGHT, LIVER WEIGHT, AND HEPATIC CU[a]

Duration (wk)	Dose Cu (mg/kg)	N	Total Cu injected (mg)	Body wt. (g)	Liver wt. (g)	Hepatic Cu (μg/g wet wt)	Hepatic Cu[b] retention (%)
1/7	Saline	5	—	48 ± 1	2.15 ± 0.13	6.76 ± 0.87	—
	0.625	5	0.0325	48 ± 1	2.60 ± 0.16	9.34 ± 1.69[c]	22.2 ± 9.6
	1.25	5	0.0625	49 ± 1	2.73 ± 0.07[d]	15.80 ± 2.02	38.5 ± 8.4
	2.5	5	0.125	48 ± 2	2.56 ± 0.06[c]	30.0 ± 4.7	46.7 ± 8.7
	3.75	6	0.190	49 ± 1	2.70 ± 0.13[c]	32.8 ± 6.2	35.1 ± 7.3
1	Saline	5	—	81 ± 3	3.64 ± 0.14	4.18 ± 0.17	—
	0.625	5	0.26	75 ± 2	3.38 ± 0.10	8.78 ± 1.14	6.0 ± 1.6
	1.25	5	0.53	72 ± 4	3.46 ± 0.18	34 ± 4.0	19.9 ± 3.0
	2.5	5	1.05	72 ± 3	3.31 ± 0.14	69 ± 7.2	20.1 ± 3.4
	3.75	4	1.58	72 ± 3	4.06 ± 0.14	69 ± 12.9	16.6 ± 2.9
2	Saline	8	—	101 ± 2	4.29 ± 0.26	4.30 ± 0.16	—
	0.625	4	0.6 ± 0.03	113 ± 7	5.35 ± 0.56	10.55 ± 1.69	5.3 ± 1.5
	1.25	7	1.1 ± 0.05	104 ± 6	4.63 ± 0.40	33 ± 8.5	11.0 ± 2.2
	2.5	5	2.3 ± 0.06	102 ± 4	4.99 ± 0.33	101 ± 16.6	20.8 ± 3.4
	3.75	5	3.5 ± 0.10	95 ± 5	4.90 ± 0.28	117 ± 13.3	16.1 ± 2.3

6	Saline	8	—	275 ± 9	11.93 ± 0.51	4.24 ± 0.06	—
	0.625	3	3.6 ± 0.1	238 ± 8	9.45 ± 0.45[f]	13.57 ± 2.51	2.5 ± 0.6
	1.25	6	6.1 ± 0.2	230 ± 10[g]	9.95 ± 0.78[f]	46 ± 4.8	6.7 ± 0.5
	2.5	5	13.3 ± 0.5	208 ± 17[g]	8.76 ± 0.65[g]	242 ± 30.7	15.8 ± 2.6
	3.75	8	20.4 ± 0.2	224 ± 5[g]	7.24 ± 0.44[g]	266 ± 49.7	11.6 ± 2.3
12	Saline	8	—	357 ± 23	13.65 ± 0.68	4.01 ± 0.07	—
	0.625	5	10.8 ± 1.0	350 ± 24	12.38 ± 1.04	22 ± 6.5	2.0 ± 0.8
	1.25	7	21.3 ± 0.7	332 ± 3	12.58 ± 0.55	134 ± 31.2	7.5 ± 1.7
	2.5	7	43.8 ± 1.2	340 ± 9	13.00 ± 0.63	327 ± 53.5	9.9 ± 2.0
	3.75	8	59.6 ± 1.6	297 ± 6[f]	12.56 ± 0.59	593 ± 73.4	12.2 ± 1.6
18	Saline	7	—	443 ± 16	14.50 ± 0.63	4.34 ± 0.29	—
	0.625	4	20.8 ± 0.3	394 ± 14	12.01 ± 1.10	62 ± 16.6	3.2 ± 0.8
	1.25	7	42.5 ± 2.6	434 ± 18	14.47 ± 0.78	215 ± 13.9	7.2 ± 0.6
	2.5	7	76.6 ± 4.9	375 ± 26[f]	13.52 ± 0.97	426 ± 56.7	7.7 ± 1.3
	3.75	8	118.5 ± 2.4	367 ± 13[f]	14.11 ± 0.98	710 ± 60.1	8.4 ± 0.8

[a] Results expressed as mean ± SE.
[b] Refers to accumulated hepatic Cu, calculated as a percentage of total copper injected.
[c] Not significant. Other values for Cu concentration significantly greater than controls.
[d] Significantly greater than controls ($P < 0.01$).
[e] Significantly greater than controls ($P < 0.05$).
[f] Significantly less than controls ($P < 0.05$).
[g] Significantly less than controls ($P < 0.01$).

retention than for the other doses. After 18 wk the percentage retained in the liver for the 3 highest doses is almost identical (7.2–8.4%) despite the vast difference in amount of copper injected (Table 1).

Kidney. In general, kidney wt in loaded animals did not differ significantly from control values (Table 2) even where body wt was significantly lower. In control animals copper increases with age (Table 2). After a single injection of copper sulfate, the renal copper concentration increased significantly only with the 2 highest doses. The rate of

TABLE 2

EFFECT OF DAILY IP COPPER LOADING ON RENAL COPPER[a]

Duration (wk)	Dose Cu (mg/kg)	*n*	Renal wt. (g)	Cu concentration (μg/g wet weight)	Renal Cu[b] retention (%)
1/7	Saline	4	0.27 ± 0.01	3.05 ± 0.38	—
	0.625	5	0.32 ± 0.01[c]	3.10 ± 0.33[f]	0
	1.25	5	0.31 ± 0.01[d]	3.40 ± 0.16[f]	0.22 ± 0.08
	2.5	5	0.27 ± 0.00	7.54 ± 0.79	0.99 ± 0.16
	3.75	5	0.30 ± 0.02	10.62 ± 1.73	1.16 ± 0.20
1	Saline	5	0.42 ± 0.02	3.42 ± 0.06	—
	0.625	5	0.39 ± 0.01	4.02 ± 0.09	0.10 ± 0.01
	1.25	5	0.37 ± 0.02	4.92 ± 0.25	0.11 ± 0.01
	2.5	5	0.36 ± 0.00	6.76 ± 0.75	0.08 ± 0.02
	3.75	4	0.43 ± 0.01	16.23 ± 2.64	0.35 ± 0.08
2	Saline	8	0.43 ± 0.01	4.30 ± 0.23	—
	0.625	4	0.52 ± 0.03[c]	7.38 ± 0.84	0.25 ± 0.07
	1.25	6	0.49 ± 0.04	5.43 ± 0.56[f]	0.09 ± 0.04
	2.5	5	0.44 ± 0.02	18.43 ± 2.90	0.27 ± 0.05
	3.75	4	0.45 ± 0.02	25.14 ± 4.49	0.28 ± 0.06
6	Saline	8	0.98 ± 0.06	7.11 ± 0.45	—
	0.625	3	0.87 ± 0.06	13.70 ± 1.43	0.16 ± 0.03
	1.25	6	0.88 ± 0.05	16.80 ± 1.95	0.15 ± 0.03
	2.5	5	0.76 ± 0.06[e]	59 ± 15.0	0.29 ± 0.08
	3.75	8	0.80 ± 0.04[e]	73 ± 20.6	0.26 ± 0.09
12	Saline	8	1.06 ± 0.09	9.65 ± 0.91	—
	0.625	5	1.04 ± 0.07	14.38 ± 2.39[f]	0.05 ± 0.02
	1.25	7	1.12 ± 0.04	36 ± 5.5	0.14 ± 0.03
	2.5	7	1.18 ± 0.07	110 ± 21.9	0.28 ± 0.06
	3.75	8	0.99 ± 0.05	230 ± 59.3	0.37 ± 0.10
18	Saline	7	1.24 ± 0.05	10.07 ± 0.80	—
	0.625	3	1.08 ± 0.09	21.8 ± 3.94	0.06 ± 0.02
	1.25	7	1.31 ± 0.07	69 ± 13.0	0.19 ± 0.04
	2.5	7	1.30 ± 0.09	177 ± 32.0	0.29 ± 0.06
	3.75	8	1.17 ± 0.06	212 ± 75.3	0.28 ± 0.07

[a] Results expressed as mean ± SE.
[b] Refers to accumulated Cu per kidney as a percentage of total Cu injected.
[c] Significantly greater than controls ($P < 0.01$).
[d] Significantly greater than controls ($P < 0.05$).
[e] Significantly less than controls ($P < 0.05$).
[f] Not significant. Other values for Cu concentration significantly elevated.

accumulation was found to increase with dose. With a dose of 3.75 mg/kg the rate of copper deposition increased progressively until 12 wk, at which time the mean concentration leveled off. With the 2.5 mg/kg dose the concentration after the first week increased linearly. A consistent elevation with the 1.25 mg/kg dose was evident only beyond 2 wk of loading. With the lowest dose, increments were small and reached twice the control levels only after 18 wk of treatment.

The percentage of injected copper retained by the kidney is small (Table 2). With the 2 highest doses the maximum is only about 1 % and rapidly declines to a stable value of approximately 0.3 % between the weeks 2 and 18. With the 2 lowest doses the percentage retained is less.

Bone. The copper content of normal bone declines with age (Table 3). The decline is less in the loaded animals and is actually reversed with the 3.75 mg/kg dose or with prolonged loading with the 2.5 mg/kg dose. With the highest dose there is an initial peak, then a lowering in concentration which in turn is followed by a progressive increase. It is interesting that more copper accumulates in 1 wk on the 3.75 mg/kg dose (total 1.6 mg Cu injected) than after 18 wk with the 2.5 mg/kg dose (total 76.6 mg Cu injected). With the lowest dose the injection of 21 mg Cu over 18 wk failed to cause a significant increase.

If at the 6-, 12-, and 18-wk periods the copper concentration is plotted against dose, the values are found to increase exponentially.

Spleen. Elevation of splenic copper occurs with all doses used and increases progressively with duration of loading (Table 3). With the 3.75 mg/kg dose this elevation is evident at 1 wk (total of 1.6 mg Cu injected); with the lowest dose a significant augmentation is apparent only after 18 wk (total of 21 mg Cu injected). At the 6-, 12-, and 18-wk period the copper concentration increases steadily with dose. The increases are most striking with the highest dose.

Lung. Elevation of lung copper only occurs with the 2 highest doses and is first evident at 6 wk of treatment (Table 3). Beyond this time the rate of accumulation is much greater with the higher dose. The relationship of concentration of copper to dose after 6-, 12-, and 18-wk loading is similar to that found in spleen.

Brain. Significant, though quantitatively small, increments of copper occur in brain with all doses except the lowest (Table 3). Despite the early deposition of metal after 2 wk of loading with the 3.75 mg/kg dose little further elevation occurs over the subsequent 16 wk. Treatment with the 2.5 mg/kg dose results in comparable brain copper concentrations at 12 and 18 wk.

Testis. Significant, though quantitatively small, increases of copper occur in the testis with all doses used (Table 3). As in the case of brain, following initial increases in concentration the values level off. With the highest dose accumulation of the metal was evident at 6 wk; lower doses took longer to effect an increase.

Skin and hair. Copper increased in skin and hair only with the 2 highest doses (Table 4). In the case of hair the elevation was less consistent. With the highest dose copper concentration in hair leveled off but increased progressively in the skin.

Muscle. All 3 skeletal muscle tissues showed an increase in copper levels with prolonged loading. In the case of leg muscle this was evident only with the highest dose after 18-wk treatment compared with 12 wk for tongue and diaphragm. Significant elevation in cardiac muscle was present at 12 wk with the 2 highest doses. The maximum

TABLE 3

EFFECT OF DAILY IP COPPER LOADING ON TISSUE COPPER CONCENTRATIONS[a]

Duration (wk)	Dose Cu (mg/kg)	n	Bone	n	Spleen	n	Lung	n	Brain	n	Testis
1/7	Saline	5	1.74 ± 0.27	2[b]	1.45	2[b]	1.39	2	1.85	2[b]	1.65
	1.25	5	1.76 ± 0.19	2[b]	1.55	—	—	—	—	—	—
	2.5	5	2.06 ± 0.13	2[b]	1.75	—	—	—	—	—	—
	3.75	5	1.70 ± 0.28	2[b]	1.85	—	—	—	—	—	—
1	Saline	5	1.10 ± 0.16	4	1.40 ± 0.21	—	—	4	1.93 ± 0.05	—	—
	1.25	5	1.16 ± 0.10	4	1.78 ± 0.13	—	—	—	—	—	—
	2.5	4	1.68 ± 0.34	4	2.13 ± 0.08[c]	—	—	—	—	—	—
	3.75	4	3.23 ± 0.13[d]	4	2.53 ± 0.27[c]	—	—	4	1.95 ± 0.03	—	—
2	Saline	7	1.14 ± 0.14	6	1.48 ± 0.13	6	1.48 ± 0.13	6	1.88 ± 0.04	4	1.50 ± 0.14
	1.25	5	1.10 ± 0.10	5	1.40 ± 0.15	5	1.50 ± 0.08	5	1.88 ± 0.09	—	—
	2.5	5	1.80 ± 0.16[c]	5	2.40 ± 0.22[d]	5	1.72 ± 0.22	5	1.86 ± 0.10	—	—
	3.75	5	3.82 ± 0.30[d]	5	2.72 ± 0.15[d]	5	1.76 ± 0.12	5	2.12 ± 0.09[c]	4	1.50 ± 0.16
6	Saline	6	0.80 ± 0.11	6	1.43 ± 0.12	5	1.42 ± 0.09	5	1.74 ± 0.07	5	1.38 ± 0.10
	0.625	3	0.80 ± 0.12	3	1.53 ± 0.24	3	1.37 ± 0.12	3	1.87 ± 0.06	3	1.70 ± 0.10
	1.25	5	1.12 ± 0.19	5	1.74 ± 0.07	5	1.52 ± 0.09	5	1.88 ± 0.06	5	1.46 ± 0.07
	2.5	5	1.48 ± 0.08[d]	5	1.94 ± 0.11[c]	5	1.92 ± 0.06[d]	5	1.86 ± 0.10	5	1.58 ± 0.09
	3.75	5	2.30 ± 0.23[d]	5	4.92 ± 1.21[c]	5	2.02 ± 0.13[d]	5	2.20 ± 0.10[d]	5	1.96 ± 0.15[c]
12	Saline	5	0.46 ± 0.02	5	1.46 ± 0.10	5	1.28 ± 0.06	7	1.87 ± 0.05	5	1.46 ± 0.09
	0.625	5	0.64 ± 0.09	5	1.46 ± 0.14	5	1.14 ± 0.08	5	1.88 ± 0.06	5	1.70 ± 0.08
	1.25	5	0.80 ± 0.07[d]	5	2.34 ± 0.36[c]	5	1.36 ± 0.10	5	1.96 ± 0.08	5	2.06 ± 0.13[d]
	2.5	5	1.30 ± 0.11[d]	5	4.48 ± 0.70[d]	5	1.74 ± 0.15[c]	5	2.28 ± 0.08[d]	5	2.04 ± 0.12[d]
	3.75	5	3.52 ± 0.31[d]	5	9.72 ± 1.99[d]	5	3.08 ± 0.35[d]	5	2.28 ± 0.11[d]	5	2.22 ± 0.13[d]
18	Saline	6	0.52 ± 0.06	5	1.70 ± 0.06	5	1.38 ± 0.10	5	1.82 ± 0.06	5	1.60 ± 0.04
	0.625	4	0.63 ± 0.07	4	1.95 ± 0.09[c]	4	1.55 ± 0.14	4	1.83 ± 0.06	4	1.95 ± 0.10[c]
	1.25	5	0.94 ± 0.05[d]	5	3.04 ± 0.32[d]	5	1.78 ± 0.25	5	2.20 ± 0.04[d]	5	1.94 ± 0.12[c]
	2.5	5	1.96 ± 0.22[d]	5	5.64 ± 0.98[d]	5	2.22 ± 0.27[c]	5	2.58 ± 0.07[d]	5	2.02 ± 0.12[d]
	3.75	5	5.50 ± 1.05[d]	5	26.54 ± 13.66[c]	5	6.98 ± 2.78[c]	7	2.50 ± 0.07[d]	5	2.22 ± 0.21[c]

[a] Values expressed as μg Cu/g fresh tissue, mean ± SE.
[b] Two to three organs pooled for each determination.
[c] Significantly greater than controls ($P < 0.05$).
[d] Significantly greater than controls ($P < 0.01$).
[e] Significantly greater than controls following logarithmic transformation ($P < 0.05$).

TABLE 4

EFFECT OF DAILY IP COPPER LOADING ON TISSUE COPPER CONCENTRATIONS[a]

Duration (wk)	Dose Cu (mg/kg)	*n*	Skin	*n*	Hair	*n*	Heart	*n*	Diaphragm	*n*	Tongue	*n*	Leg muscle
6	Saline	5	1.04 ± 0.11	5	7.70 ± 0.64	5	5.00 ± 0.25	5	1.92 ± 0.22	6	2.10 ± 0.19		—
	0.625	3	0.93 ± 0.12	3	7.67 ± 2.29	3	4.53 ± 0.09		—	3	1.87 ± 0.23		—
	1.25	5	0.80 ± 0.05	4	8.03 ± 1.02	5	4.66 ± 0.09		—	5	2.04 ± 0.12		—
	2.5	4	0.98 ± 0.06	5	8.88 ± 0.45	5	4.28 ± 0.27		—	5	2.36 ± 0.16		—
	3.75	5	1.00 ± 0.09	5	11.54 ± 0.58[b,d]	5	4.98 ± 0.40	5	1.62 ± 0.17	5	2.60 ± 0.15		—
12	Saline	5	0.90 ± 0.15	5	7.12 ± 1.01	5	4.44 ± 0.32	5	1.76 ± 0.18	5	1.98 ± 0.19	5	0.46 ± 0.10
	0.625	5	0.90 ± 0.11	5	7.02 ± 1.06	5	4.92 ± 0.07	5	1.74 ± 0.21	3	1.80 ± 0.15	5	0.50 ± 0.04
	1.25	5	0.70 ± 0.03	4	7.75 ± 1.25	5	5.18 ± 0.10	5	1.76 ± 0.20	5	2.24 ± 0.11	5	0.52 ± 0.10
	2.5	5	1.12 ± 0.07	5	11.92 ± 0.46[d]	5	5.34 ± 0.09[c]	5	1.90 ± 0.20	5	2.58 ± 0.18	5	0.66 ± 0.14
	3.75	5	2.30 ± 0.49[c]	5	11.22 ± 1.20[c]	5	5.26 ± 0.12[c]	5	4.08 ± 0.90[c]	5	2.92 ± 0.21[c]	5	0.68 ± 0.10
18	Saline	5	0.66 ± 0.15	5	9.00 ± 0.57	6	4.23 ± 0.36	5	1.68 ± 0.25	5	1.92 ± 0.14	5	0.52 ± 0.10
	0.625	4	0.75 ± 0.06	4	7.10 ± 1.25	3	4.53 ± 0.18	4	2.13 ± 0.64	4	2.00 ± 0.18	4	0.48 ± 0.06
	1.25	5	0.82 ± 0.05	5	8.22 ± 1.36	5	5.24 ± 0.28	5	2.66 ± 0.42	5	2.20 ± 0.10	5	0.64 ± 0.09
	2.5	5	1.46 ± 0.24[c]	5	8.66 ± 0.92	5	5.24 ± 0.65	5	2.92 ± 0.84	5	3.24 ± 0.58[c]	5	0.54 ± 0.05
	3.75	5	3.76 ± 0.88[d]	5	11.26 ± 1.32	5	6.34 ± 0.61[c]	5	6.52 ± 1.54[c]	5	3.70 ± 0.36[d]	5	0.88 ± 0.07[c]

[a] Values expressed as μg Cu/g fresh tissue, mean ± SE.
[b] Values at 2 wk, 8.00 ± 0.88.
[c] Significantly greater than controls ($P < 0.05$).
[d] Significantly greater than controls ($P < 0.01$).
[e] Significantly greater than controls following logarithmic transformation ($P < 0.05$).

increase was 1.5 times the control values compared with 4 and 2 times control values in the case of diaphragm and tongue, respectively.

Serum. Only a few values were determined. The mean of 6 controls was 88.3 ± 13 (mean ± SE) μg Cu/100 ml serum. At a dose of 3.75 mg/kg, the means of 3 values were 148.3 and 131.6 μg Cu/100 ml at 12 and 18 wk, respectively.

DISCUSSION

It is evident that all tissues examined are capable of accumulating copper although the influence of the two factors, dosage of metal and duration of loading, on the degree of deposition varies with the individual tissue.

Evidence for a threshold dose of hepatic copper accumulation has been provided in sheep (Dick, 1954), in the brown and cotton rat (Milne and Weswig, 1968), and in the pig (Allcroft *et al.*, 1961; Lucas and Calder, 1957). In the present study a linear relationship between hepatic concentration and dose following 12 and 18 wk loading was noted. On extrapolation of these data it is apparent that doses of 0.5 and 0.3 mg Cu kg are needed to elevate hepatic copper levels over periods of 12 and 18 wk, respectively. This suggests that the dose required to ensure net deposition varies with the duration of loading. It is obviously difficult to extrapolate these data to the human organism, where the parameters affecting accumulation of copper are unknown. It is nevertheless interesting to note that if a similar order of threshold operated in man a 70-kg man would need to absorb 21–35 mg of the metal daily to increase liver copper. In Wilson's disease, Tu *et al.* (1965) have estimated that a dietary intake of 0.6 mg Cu is sufficient to result in a positive copper balance.

Milne and Weswig (1968) attributed the increase in liver copper, once a threshold dosage was exceeded, to overloading of the excretory capacity. Scheuer and Barka (1964), however, provided evidence that retention of the metal may be due not to saturation or impairment of the normal excretory mechanism, but to diversion of the metal to copper-rich granules from which it would only be slowly eliminated. The present results also show that accumulation of hepatic copper occurs independently of saturation of the normal excretory mechanisms. Thus, it can be calculated (from tissue copper retained) that a mean of 108 mg of copper was excreted by rats treated with 3.75 mg/kg over 18 wk. This represents 5.2, 2.5, and 1.8 times the total quantity of the metal injected into animals treated with 0.625, 1.25, and 2.5 mg/kg over the same period. In the copper-loaded rat, quantitatively very little is excreted in the urine (Owen and Hazelrig, 1968). This, together with the present experiments, suggests then that the biliary route of excretion has a vast capacity to adjust elimination of the metal. In Wilson's disease it is controversial whether biliary excretion of copper is normal (Cartwright *et al.*, 1954; Denny-Brown and Porter, 1951) or impaired (Gitlin *et al.*, 1960).

In sheep, hepatic copper concentration increases linearly with dose. After 3–6 mo loading 4.4–5% of the ingested element was retained in the liver (Dick, 1954; Hemingway and MacPherson, 1967). In the pig, a value of 2.02% was noted (Suttle and Mills, 1966). In the present study a linear relation between concentration and dose was evident only at 12 and 18 wk. The percentage of hepatic copper retained changes with duration of loading; in addition, a smaller proportion is retained with the lowest dose. The dose-time patterns of accumulation suggest that the liver can achieve several equilibrium

states in regulation of hepatic copper concentration, and it is not possible to adduce from the data a saturating concentration of the metal. It is evident, however, that extrahepatic deposition of the metal occurs even before the liver becomes heavily loaded. This conclusion is contrary to what has been postulated to occur in Wilson's disease (Osborn and Walshe, 1967; Osborn *et al.*, 1969; Tauxe *et al.*, 1966; Sternlieb and Scheinberg, 1968).

In Wilson's disease many data have been gathered on increased copper levels in the liver (Scheinberg and Sternlieb, 1959) and in the brain (Cumings, 1948, 1959). From the scant data available for other tissues, it would appear that copper may increase in all tissues except possibly hair and nails (Cartwright *et al.*, 1954; P'ei-En, 1957; Bearn and McKusick, 1958; Butt *et al.*, 1958; Sass-Kortsak *et al.*, 1959; Bickel *et al.*, 1957; Bottiger and Möllerberg, 1959; Rice and Goldstein, 1961; Martin, 1964; Spillane *et al.*, 1952; Fell *et al.*, 1968; Gibbs and Walshe, 1965; Plooij *et al.*, 1960; Tu *et al.*, 1963; Leu *et al.*, 1970). In terms of the diversity of tissues accumulating the metal the copper-loaded rat resembles the patient with Wilson's disease. In the present study the hepatic copper concentrations cover the range found in Wilson's disease (Scheinberg and Sternlieb, 1959). However, in contrast to that disorder, only small increases of the metal occur in the brain. Similar limited increments were also found by Gregoriadis (1966) and Russanov and Balevska (1968). On the other hand, Williams (1966, 1967) was able to obtain levels of 39 μg Cu/g, which were 3.5 times his control values. In other species an increase in brain copper has been produced in goldfish (Vogel, 1959) but not in the turkey (Wiederanders, 1968). In sheep with chronic copper toxicosis, tremors and spasticity are occasionally observed (Kowalczyk *et al.*, 1964) as well as pathological changes in the brain (Doherty *et al.*, 1969). Studies on brain copper levels, however, are surprisingly few. The limited reports on this topic (Eden, 1940; Barden and Robertson, 1962; Todd, 1969) indicate that excess copper is not deposited in this organ.

Apart from the liver, the kidney was the only organ to accumulate sizable quantities of the metal. In Wilson's disease, the kidney also appears to be a major site for concentrating the metal (Aspin and Sass-Kortsak, 1966; Wolff, 1964; Reynolds *et al.*, 1966; Sass-Kortsak *et al.*, 1959). A similar affinity has been noted by other authors in the copper-loaded rat (Gubler *et al.*, 1954; Lindow *et al.*, 1929; Wiederanders *et al.*, 1968; Williams, 1966; Herkel, 1930; Gregoriadis, 1966; Russanov and Balevska, 1968), pig (Allen and Harding, 1962; Allcroft *et al.*, 1961), calf (Shand and Lewis, 1957), and goldfish (Vogel, 1959). A less marked affinity was found in the turkey (Wiederanders, 1968). A raised kidney level has been noted in sheep (Eden, 1940; Barden and Robertson, 1962) but Todd (1969) considers that extrahepatic deposition of copper in this species occurs only after the animal has undergone a hemolytic crisis, and then only in kidney and spleen.

Raised levels of spleen copper have been obtained in the loaded rat (Lindow *et al.*, 1929; Boyden *et al.*, 1938; Gregoriadis, 1966), sheep (Barden and Robertson, 1962), and pig (Allen and Harding, 1962). Such an increase has also been noted in the lung of the rat (Gregoriadis, 1966) and pig (Allen and Harding, 1962). In the present study both these tissues show a similar pattern of deposition. The sharp increase in concentration with the 3.75 mg/kg dose for the 6-, 12-, and 18-wk periods suggests an overwhelming of the cellular mechanisms regulating copper levels in these tissues.

Little information is available on copper accumulation in bone. Some increase was

noted in the rat (Lindow *et al.*, 1929), but no change in the pig (Allen and Harding, 1962) or rabbit (Herkel, 1930). It is evident from Table 3 that there is a tendency for normal bone to lose copper with age. As bone and marrow were not separated it is not clear from which element the decrement occurred. In the copper-loaded animal levels of bone copper also decline with age of the animal, though the decrease is less than in control animals, and further, the decline can be reversed with high doses of the metal (3.75 mg/kg) or prolonged loading with a lower dose (2.5 mg/kg). With the dose of 3.75 mg/kg there is an initial rise in bone copper which is followed by a temporary decline. This suggests that increase in bone levels of copper may stimulate the process of elimination of the metal from this tissue. As in the case of spleen and lung, prolonged loading with the 3.75 mg/kg dose appears to overwhelm factors regulating tissue copper levels.

Reports on testicular copper are few. In the copper-loaded rat, Lindow *et al.* (1929) found an increase in the mean concentration. Gregoriadis (1966) found an increase in some animals but not in others. In the present study all 4 doses increased the concentration. Dose had some effect on onset of loading, though the final concentrations reached were similar for all doses.

Skeletal muscle has in general been found resistant to copper loading in the rat (Gregoriadis, 1966; Wiederanders *et al.*, 1968; Lindow *et al.*, 1929), turkey (Wiederanders, 1968), and sheep (Eden, 1940; Barden and Robertson, 1962). An increase has been noted in the pig (Allen and Harding, 1962) and substantial increases in goldfish (Vogel, 1959). Data for tongue and diaphragm are lacking. In the present study all 3 skeletal muscle tissues accumulated the metal though only after prolonged loading with high doses.

Elevated copper levels may be found in cardiac muscle of the rat (Gregoriadis, 1966; Wiederanders *et al.*, 1968), pig (Allen and Harding, 1962), and sheep (Barden and Robertson, 1962). In the present study, the relative resistance to loading resembled that of skeletal muscle.

Skin and hair have been little studied under conditions of loading. The data of Lindow *et al.* (1929) show a mean increase in concentration in these tissues in the rat, but Cunningham (1931) could not confirm this. Gregoriadis (1966) found an inconstant increase in hair. In the present study both tissues took up excess copper, but in the case of hair this was quite variable. The loss of copper in hair must be considered to be a minor form of excretion of the metal.

Although elevated levels of serum copper were found in loaded animals, it is unlikely with the degree of increase noted that residual blood in the tissues investigated influenced the copper determinations significantly. The method of sacrifice also makes this unlikely. The close duplicate values in the case of the liver, indicate the reliability of the methods employed. The large individual variation between animals is a regular finding in the literature. Hepatic levels determined in animals dying prematurely indicate that selection of animals storing low amounts of copper was not an important factor.

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"If you follow the diet carefully, your baby should do fine. Getting adjusted to the diet does take time. And it requires careful attention in the supermarkets."

Treating Infant Celiac Disease

By Jean Mayer

Q. My 10-month-old baby has just been diagnosed as having celiac disease. The doctor assures me he will be fine, but I'm quite concerned because he is small and rather thin. Can you tell me whether his growth will be permanently affected by being somewhat malnourished so early in life?

A. Be reassured that your doctor is giving you sound information. Once celiac disease is diagnosed and children are put on gluten-free diets—that is a diet which contains no wheat, rye, oats or barley—them seem to make up for lost time quite quickly.

Recently, a group of investigators studied the growth of infants with celiac disease. The children were between the ages of nine and 15 months of age when the characteristic diarrhea and other symptoms first appeared. And it took anywhere from two to eight months to make the diagnosis.

At the time of the diagnosis, all of the children were considerably below normal in terms of weight, height and bone development. Once on the diet, however, they began catching up quite quickly. The most rapid increase was in weight. Within six months, they had caught up to the average weight of other children. It did take somewhat longer for height and bone development to reach normal levels.

If you follow the diet carefully, your baby should do fine. Getting adjusted to the diet does take time. And it requires careful attention in the supermarkets. Since many processed foods contain small amounts of wheat flour, it's important to read the ingredients listed on food labels very carefully.

You many want to contact the nutrition section of your State Health Department for materials to help you plan and prepare your youngster's food.

Q. After reading in your column that a reduced salt intake appears to prevent hypertension, our family has really cut way down on salt. The salt we do use is iodized, but I'm concerned that we now may not be getting enough iodine. Is it possible that we may need an iodine supplement?

Some individuals on extremely low salt diets may need an additional source of iodine. But this is something for the doctor to decide. Self-dosing with iodine compounds or dried seaweed preparations rich in iodine over a long period of time can be dangerous.

Q. Some time ago I read an article that said copper kitchen utensils should not be used because they were hazardous. Since I regularly use a tea kettle with a copper bottom, I'm particularly concerned to know whether this is true.

A. During the 18th con-

See NUTRITION, K18, Col. 1

THE WASHINGTON POST
K 18 Sunday, June 2, 1974

Treating Celiac Disease

NUTRITION, From K17

tury, copper cooking utensils were widely used in some countries and copper poisoning was common. In fact, because of the high incidence of copper poisoning, the Swedish Senate, in 1753, prohibited their fleets and armies to cook with utensils made from anything but iron.

Today, however, few copper pots are used, and most of the ones that are used are lined with tin. So the food never comes in contact with copper anyway.

While we recognize that very excessive intakes of copper over long periods of time can be dangerous, little is known about whatever minimum levels of dietary copper can induce copper toxicity in normal indivduals. Nor do we understand the influence of other dietary factors on the development of copper poisoning.

Copper toxicity is quite rare. It is only seen among people exposed to extreme industrial contamination, or among individuals with a rare inherited disorder of copper metabolism called Wilson's disease.

There have been, however, extensive studies of copper toxicity in animals. Since it is believed that man handles copper in much the same way as other one-stomach species, it is estimated that man can tolerate 20 times his normal intake over a prolonged period before toxicity develops. In other words, there is a wide margin of safety between your normal intake from food, water, and your tea kettle and any amounts likely to produce toxic symptoms.

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A STUDY OF ERYTHROCYTIC GLUTATHIONE IN ACUTE COPPER SULPHATE POISONING

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The use of copper sulphate as a suicidal agent is surprisingly common in the lower strata of people at Agra. The severity of symptoms varies depending on the amount of copper sulphate ingested and the time interval between the ingestion of the poison and admission to the hospital (Wahal et al, 1963). The presence of spontaneous haemolysis of red cells immediately after collection of blood or on storage has been frequently observed by previous workers but the mechanism responsible for haemolysis remains obscure. (Chowdhury et al, 1961; Gupta et al, 1962; Wahal et al, 1963). The occurrence of haemolysis in some cases, while not in others, cannot be explained at present. The present study was initiated to find out whether some metabolic pecularity of erythrocytes was responsible for the haemolytic phenomena frequently observed. Erythrocytic glutathione in reduced form has been shown to play an important role in the preservation of red cell integrity. A decrease in the glutathione content of red cells predisposes them to haemolysis. (Fegler 1952, Sheets et al, 1956). The probable relationship of erythrocytic glutathione to intravascular haemolysis following ingestion of copper sulphate was investigated.

MATERIALS AND METHODS

Fifty cases of acute copper sulphate poisoning admitted to the wards of S. N. Hospital, Agra, from September 1963 to October 1964 were investigated in detail. A record of important presenting symptoms, the amount of copper sulphate ingested and the time interval between the ingestion of the poison and admission to the hospital was made. A thorough clinical examination was performed and the course of the disease was followed at least for a week. A battery of laboratory investigations were done on the day of admission. Haematological tests included complete blood picture with haematocrit, reticulocyte count, osmotic fragility and autohaemolysis tests. Liver function tests,

blood urea and complete examination of urine were also performed in every case. In all the cases of acute copper sulphate poisoning, red cell glutathione was estimated according to the method of Grunert and Phillips (1951) as modified by Beutler et al (1955). In the later part of the study, erythrocytic glutathione stability test according to Beutler's technique was performed (Beutler et al, 1957). Erythrocytic glutathione and glutathione stability tests were also performed in 50 normal individuals who acted as controls. The stability test was considered to be positive when there was a reduction of the glutathione content of red cells to 30 mg per cent or less in the post-incubation samples containing acetyphenylhydrazine and glucose.

Further samples of blood and urine were analysed on alternate days for a minimum period of a week to find out any evidence of haemolytic phenomenon and to follow the course of the disease.

OBSERVATIONS

The present series of 50 cases of acute copper sulphate poisoning consisted of 34 males and 16 females. Their ages varied from 13 years to 60 years with a mean of 23.5 years. 92% of the cases belonged to second or third decades of life.

TABLE I

Age and sex distribution in acute copper sulphate poisoning

Age group (Years)	Sex		Total
	Male	Female	
0—10	—	—	—
11—20	16	8	24
21—31	15	7	22
31—40	2	—	2
41—50	1	—	1
51—60	—	1	1
	34	16	50

Gastrointestinal symptoms were the commonest manifestations. Metallic taste in the mouth, burning sensation in the epigastrium with pain in abdomen and nausea and vomiting were the usual presenting symptoms. Renal and neurological symptoms were also encountered and a few cases had symptoms of peripheral shock.

Only an approximate idea about the amount of copper sulphate ingested could be made from the data obtained from the patients or their relatives. In a good number of cases for obvious reasons no information regarding the amount of copper sulphate ingested could be procured (Table II).

TABLE II

Showing amount of copper sulphate ingested

Quantity of copper sulphate ingested	Number of Cases
Upto ½ oz.	16
½ oz to 1 oz.	18
More than 1 oz.	5
Quantity not known	11

Table III shows the important complications encountered in cases of copper sulphate poisoning. Haemolytic episodes were the commonest complications being present in 19 cases. Laboratory investigations in these cases revealed an increase in reticulocyte counts following ingestion of the poison. The haematocrit values fell, sometimes as much as 10% within a week, there was increased excretion of urinary urobilinogen and haemoglobinuria was frequent. van den Bergh's reaction was indirect positive with an increase in the icteric index. Saline osmotic fragility was not significantly altered. In all the cases with evidence of haemolysis, except 4 cases, liver function tests were found to be within the normal range.

TABLE III

Complications encountered in acute copper sulphate poisoning

Complication	Number of cases
Haemolytic episodes	19
Clinical juandice	9
Neurological symptoms	7
Renal complications	6
Cardiovascular symptoms	5
Deaths	3

Levels of erythrocytic glutathione in 50 normal individuals ranged from 41.0 to 127.2 mg per 100 ml. of erythrocytes with a mean of

69.1 mg% (±16.60). In cases of acute copper sulphate poisoning the values ranged from 32.2-83.6 with a mean of 57.6 mg% (±10.74). The erythrocytic glutathione content was found to be significantly low in patients who had evidence of haemolysis as compared to the normals, the value being 21.03 at 1% level (P>0.1).

TABLE IV

Erythrocytic glutathione levels in normal and acute copper sulphate poisoning.

Group	Number of cases	Range	Mean	Standard deviations
Normal	50	41.0 — 127.2	69.1	±16.60
Copper sulphate poisoning cases	50	32.2 — 83.6	57.6	±10.74
a) without haemolysis	31	33.3 — 83.6	59.6	±10.29
b) with haemolysis	19	32.2 — 70.0	46.8	±11.20

Instability of erythrocytic glutathione was demonstrated in 5 of the 50 normal persons in whom glutathione stability test was performed. The test was performed only in 24 cases of acute copper sulphate poisoning and was found to be positive in 13 cases. A breakup of these cases showed that out of 12 cases showing haemolysis 9 had instable glutathione in red cells.

TABLE V

Glutathione stability test in normal and acute copper sulphate poisoning

Group	Number of cases	Preincubation Value		Postincubation Value		No. of cases with glutathione instability
		Mean	Range	Mean	Range	
Normal	50	69.1	41.0—127.2	47.0	16.3—72.7	4
Copper sulphate Poisoning	24	51.6	32.2— 77.3	31.0	14.1—48.4	13
a) without haemolysis	12	54.2	35.5— 77.3	37.7	25.0—48.4	4
b) with haemolysis	12	49.1	32.2— 64.7	25.4	14.1—43.2	9

DISCUSSION

Although a large number of chemical agents have been recognised to induce haemolytic anaemia in human beings, copper sulphate has not been included in the group so far (Wintrobe, 1960). Administration of copper salts in experimental animals for prolonged periods leads to a haemolytic state (Albiston et al, 1940; Gubler et al, 1953 and Goldberg et al, 1956) but the experimental production of a state in animals resembling copper sulphate poisoning in man has not been possible as yet.

Recent reports of acute copper sulphate poisoning from various centres in India have emphasized the occurrence of haemolytic episodes in a large number of cases. The frequency of haemolytic reactions in these reports has varied from 13 per cent to 50 per cent. (Table VI).

TABLE VI

Incidence of haemolytic reactions in acute copper sulphate poisoning

Author	Year	Total No. of cases	Number of cases with haemolysis	Percent
Chowdhury et al	1961	20	10	50
Gupta et al	1962	150	38	13
Wahal et al	1963	50	10	20
Present series	1964	50	19	38

The mechanism responsible for haemolysis in cases of copper sulphate poisoning remains obscure. The in vitro studies of Goldberg and colleagues (1956) have shown that copper in low concentrations causes agglutination and/or haemolysis when incubated with chicken, rabbit or human erythrocytes suspended in saline. But no such effects are observed even up to a concentration of 5000 microgram when the red cells are suspended in serum. On the basis of their experimental study in chickens Goldberg et al (loc cit) have suggested that the erythrocytes are destroyed in liver rather than due to an increase in the plasma copper levels.

Gubler and associates (1953) suggested that the concentration of copper in the red cells is increased. This results in increased susceptibility of the cells to destruction. An increase in the saline osmotic fragility of red cells from individuals having haemolytic phenomena is

to be expected but significant alteration in osmotic fragility was not observed by us in all the cases who had haemolytic episodes. Similar have been the observations of previous workers.

Chowdhury and associates (1961) have pointed out that the haemolytic reactions may occur in acute copper sulphate poisoning when the plasma copper level is much elevated in the presence of deranged liver functions. The study of liver function tests in patients with haemolytic reactions in the present series does not confirm this concept. Gupta et al (1962) also did not observe any derangement of liver functions in 8 cases of haemolytic jaundice.

Erythrocytic glutathione in reduced form has been shown to play an important role in the preservation of red cell integrity particularly under stressful situations of exposure to relevant agents. The preincubation values of erythrocytic reduced glutathione in normal cases in the present series ranged from 41.0 to 127.2 mg per 100 ml. of erythrocytes with a mean of 69.1 mg. percent. These are in close agreement with those of previous workers who found an average of 68.9 mg.% in males and 72.9 mg.% in females (Swarup et al, 1962). The preincubation values of erythrocytic glutathione in acute copper sulphate poisoning were significantly diminished in the present series with a mean of 57.6 mg. percent. A decrease in the glutathione content of red cell predisposes them to haemolysis. Fegler (1952) produced haemolysis of red blood cells in vitro by oxidation of glutathione with iodine. The haemolysis could be inhibited by the addition of reduced glutathione.

In the present series 8% of apparently normal cases had instability of erythrocytic reduced glutathione. Chaterjea (1964) on analysis of 110 subjects found unstable glutathione pattern in 10.9% cases. A number of structurally unrelated drugs have been reported to induce intravascular haemolysis in persons having instability of erythrocytic glutathione (Tarlov et al, 1962). Our studies indicate that acute copper sulphate poisoning is associated with instability of erythrocytic glutathione. Out of 24 cases of acute copper sulphate poisoning in which glutathione stability test was performed in 13 cases the test was positive and 9 of these had intravascular haemolysis.

Three patients of copper sulphate poisoning who had normal glutathione stability of red cells developed haemolytic anaemia. De Leeuw et al (1961) have also reported a number of patients who developed Heinz bodies in the presence of adequate and stable reduced glutathione. Desforges (1962) has suggested that in the normal individuals critical

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levels of oxidant drugs may also exist above which the biochemical trauma may be sufficient to produce haemolysis. Jandl (1963) has suggested that factors other than erythrocytic glutathione should be considered in the mechanism of drug-induced haemolysis. These include simultaneous administration of other drugs, the effect of blood transfusion, intercurrent bacterial and viral infections, immune factors or serum abnormalities and abnormalities of glucose metabolism.

Erythrocytic glutathione instability may be associated with a deficiency of glucose-6-phosphate dehydrogenase (G-6-PD), glutathione reductase (G-R) or glutathione peroxidase. In some cases the glutathione instability has been demonstrated in a variety of haematological disorders and in normal persons even in the absence of deficiency of these enzymes (Chatterjea, 1964). In a preliminary study, normal G-6-PD activity has been observed in three cases of acute copper sulphate poisoning who had instability of erythrocytic reduced glutathione (Mital, 1965). Moreover the high incidence of glutathione instability in acute copper sulphate poisoning cannot be explained on the basis of natural distribution of the defect in general population.

Desforges (1962) has shown that even glutathione stable red cells may be rendered unstable because of various factors including degree of oxygenation and changes in glucose metabolism. Zinkham et al (1959) have shown that when the glucose concentration is extremely low, normal erythrocytes behave similarly to primaquine sensitive cells in that the glutathione is unstable when incubated aerobically in the presence of acetyl phenylhydrazine or other haemolytic drugs. It is possible that copper sulphate makes the erythrocytic glutathione unstable by interfering with its glucose metabolism. Whether or not activity of enzyme glutathione reductase is inhibited by the chemical has to be worked out.

SUMMARY

The present studies reveal that 38% cases of acute copper sulphate poisoning had haemolytic episodes. In the majority of these cases significantly low erythrocytic glutathione levels were present. Erythrocytic reduced glutathione instability was very frequently observed. The possible relationship between erythrocytic glutathione and glutathione stability to acute copper sulphate poisoning has been discussed.

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Neutron Activation Analysis of Copper by Substoichiometric Extraction with Neocuproine

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COPPER IS ONE of the seven essential trace elements necessary for normal animal and plant metabolism. Copper deficiency in humans causes Wilson's disease. Copper acts as an activator of lipid enzyme systems, particularly those concerned with phospholipid synthesis. At the same time copper is an important metal in various industries and technologies, and mineral prospecting for copper is important geochemically. Nondestructive activation analysis of copper is not feasible since ^{64}Cu being a positron emitter, any other activity in the irradiated material which decays by positron emission will interfere seriously in this determination. Hence, radiochemical separation of copper is necessary for its determination. By incorporating the substoichiometric principle in the radiochemical separation scheme, several purification steps can be eliminated, and the time of separation shortened, provided a suitable reagent is available. Dithizone (*1*) and diethyldithiocarbamate (*2*) are two reagents which have been used for substoichiometric activation analysis of copper. As is well known, the former is a very unstable and sometimes unpredictable reagent, while in the case of the second reagent, there is interference from several elements which can be avoided only by employing a multichannel analyzer. Neocuproine has been known for long time as an almost specific spectrophotometric reagent for copper (*3*, *4*). We have developed a method for the determination of trace amounts of copper based on neutron activation and substoichiometric extraction of copper with neocuproine in chloroform.

EXPERIMENTAL

Copper standards were prepared by dissolving 99.999% pure copper metal in HNO_3 and diluting by weight to the desired concentration. Aliquots of this solution were taken in quartz vials and dried. The samples were dried (except for blood serum which was freeze-dried) at 80 °C for several hours. About 50-200 mg of the sample and about 10 µg

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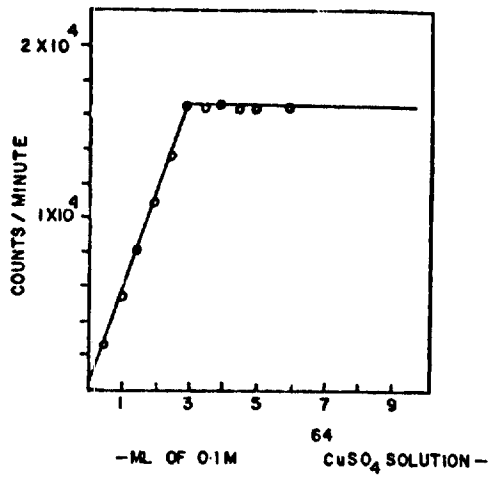

Figure 1. Substoichiometric extraction of Cu(II) with neocuproine in chloroform

of the copper standard were sealed in quartz vials, and units of 2 samples and 1 standard were irradiated in the CIRUS reactor of Bhabha Atomic Research Centre, Trombay, for 24 hours in a thermal neutron flux of 1.5×10^{12} n · cm^{-2} sec.$^{-1}$ After radiochemical separation the ^{64}Cu activity was measured either on a single channel analyzer connected to a well-type 1.5-in. × 1.5-in. NaI(Tl) detector or on an end-window type GM counter.

Other reagents used were 0.1*M* $CuSO_4$ carrier solution, carrier free ^{64}Cu isotopic tracer solution, 30% sodium citrate, 10% hydroxylamine hydrochloride, and 0.05*M* neocuproine solution in ethanol.

Substoichiometric Extraction of Cu(I) with Neocuproine. Effect of Variables. Extraction of copper with neocuproine takes place from pH 1 to 10.5; however, quantitative extraction occurs only at pH 1.5 to 4. Hydroxylamine hydrochloride was used as the reducing agent for converting Cu(II) to Cu(I). Sodium citrate was used as a buffer as well as masking agent for any possible interference from other metals.

Reproducibility of the Method. To a series of solutions containing increasing amounts of labeled 0.1*M* $CuSO_4$ (^{64}Cu) were added 5 ml of hydroxylamine hydrochloride and 10 ml of citrate buffer. The pH of the solutions was adjusted to 2.5–3, and 6 ml of 0.05*M* neocuproine were added to each of them. The solutions were shaken for a minute with 10 ml of chloroform, and a 7-ml aliquot of the organic phase was taken for counting. The plot of amount of labeled ^{64}Cu taken *vs.* the activity in organic phase (Figure 1) shows linear increase till all of stoichiometric copper is extracted, after which the amount of copper extracted remains constant. The complex of Cu:neocuproine is seen to be 1:1.

Recommended Procedure. The irradiated and weighed samples are dissolved after addition of 1 ml of 0.1*M* $CuSO_4$ solution and about 5 mg each of other carriers. The geological samples are dissolved in HNO_3 + H_2SO_4 + HF mixture, while biological samples are dissolved in HNO_3 + $HClO_4$ mixture. To this solution is added 10 ml of 10% hydroxylamine hydrochloride, and 10 ml of 30% sodium citrate. The pH of the solution is adjusted to 2.5–3, and 1 ml of 0.05*M* neocuproine solution is added to it. The red color is extracted with 10 ml of chloroform. The organic phase is washed with 20 ml of water and a 7-ml aliquot of the organic phase is taken in a glass planchet, dried under a heat lamp, and the activity counted either on a gamma-ray spectrometer at the channel corresponding to 0.51 MeV peak of ^{64}Cu, or on an end window type GM counter. The irradiated copper standard is processed exactly the same way. The amount of copper in the sample is calculated in the usual fashion.

Table I. Decontamination Studies in the Substoichiometric Extraction of Copper with Neocuproine in Chloroform

Element	Decontamination factor[a]	Element	Decontamination factor[a]
^{24}Na(I)	1.58×10^6	^{32}P(V)	7.22×10^4
^{42}K(I)	1.09×10^6	^{59}Fe(III)	6.87×10^4
^{56}Mn(II)	6.61×10^5	^{99}Mo(VI)	4.72×10^4
^{186}Re(VI)	5.16×10^5	^{192}Ir(III)	3.10×10^4
^{82}Br(I)	4.51×10^5	^{124}Sb(III)	2.74×10^4
^{95}Zr(V)	2.83×10^5	^{185}W(VI)	2.48×10^{4c}
^{46}Sc(III)	2.44×10^5	^{35}S(VI)	2.04×10^4
^{137}Cs(I)	1.80×10^5	^{203}Hg(II)	1.11×10^{4d}
^{113}Sn(II)	1.45×10^{5b}	^{75}Se(IV)	6.07×10^3
^{110m}Ag(I)	1.36×10^5	^{65}Zn(II)	4.51×10^3
^{144}Ce(III)	9.81×10^3	^{60}Co(II)	2.36×10^3
^{103}Ru(III)	8.72×10^4	^{51}Cr(III)	2.22×10^3

[a] Decontamination Factor = Activity taken/Activity in organic phase.
[b] Oxalic acid used as the masking agent.
[c] Phosphoric acid used as the masking agent.
[d] Potassium iodide used as the masking agent.

RESULTS AND DISCUSSION

On thermal neutron irradiation ^{63}Cu (69.2% abundance) by (n, γ) reaction produces ^{64}Cu. This reaction has a cross section of 4.7 barns, and ^{64}Cu has a half-life of 12.8 hours and emits gamma rays of 0.51 (by positron emission) and 1.35 MeV, and betas of 0.65 MeV energy. The same isotope can also be produced by the reaction ^{64}Zn(n,p)^{64}Cu which has a cross section of 390 millibarns and ^{64}Zn has 48.9% natural abundance. Since most geological or biological materials contain amounts of zinc comparable with those of copper, this reaction can pose some interference in the determination of copper. From calculations of amounts of copper activity that will be produced at a given flux by both (n,γ) and (n,p) reactions, it is seen that 1 mg of zinc corresponds to 5.36×10^{-3} mg of copper in terms of the activity produced (5). This correction is not appreciable unless the ratio of the amount of zinc to that of copper is very unfavorable in the material to be analyzed; however, since zinc content of the materials analyzed was known to a fair degree of certainty, this correction was applied to our copper results.

Table I gives the decontamination factors obtained by using various isotopes in the substoichiometric extraction of copper with neocuproine in chloroform. The values obtained are satisfactory for most of the isotopes studied. When Hg(II), W(VI), and Sn(II) are present, KI, H_3PO_4, and oxalic acid have to be used as masking agents to suppress their interference.

Table II gives the results of determination of copper in several geological and biological materials. The standard deviations given are from the replicate determinations calculated at 95% confidence limits. For the samples for which the data are available in literature, the agreement between our results and the reported or recommended values in literature is, for most part, excellent. Allende is a C_3 carbonaceous chondrite and our value of 78.3 ppm is comparable with ~100 ppm as the average value for copper in meteorites

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Table II. Determination of Copper by Substoichiometric Neutron Activation Analysis

Material	Cu found,[a] ppm	Cu reported,[b] ppm
T-1	65.7 ± 2.4 (3)	62 (*6*)
W-1	104 ± 3 (2)	110 (*7*); 114 ± 4 (*8*) 116 ± 10 (*9*)
Sye-1	29.3 ± 2.9 (2)	23 (*10*); 25 (*11*)
PCC-1	9.23 ± 0.09 (2)	10.4 (*12*), 8 ± 2 (*9*)
G-1	12.8 ± 1.2 (2)	13 (*7*)
GR	358 ± 19 (2)	345 (*13*)
Allende meteorite	78.3 ± 2.9 (3)	...
Bowen's kale	5.20 ± 0.06 (2)	4.81 ± 0.74 (*14*)
1R1 tobacco	70.1 ± 1.6 (2)	...
Human blood serum	1.65 ± 0.05 (2)	...
Artificial mixture	0.0083 μg	0.0077 μg
Artificial mixture	0.0427 μg	0.0446 μg

[a] Figures in parentheses indicate the number of replicate determinations carried out.

[b] Figures in parentheses indicate the reference numbers.

matter (*15*). 1R1 tobacco is an international cigarette tobacco standard manufactured and distributed by the University of Kentucky, Lexington, Ky. Our value of 70.1 ppm may be compared with the values 76.7 (*16*), 32.4 (*17*), 17–32 (*18*), and 15–21 (*19*) ppm in various commercial tobaccos reported in the literature. The wide variation in the values is a result of the tobacco crops grown on different soils. In freeze-dried human blood serum, we found 1.65 ppm of copper. Earlier values for copper in human blood serum were 1.54 (*20*) and 2 (*21*) ppm. To find out the experimental limits of copper determination by this method, two artificial mixtures containing 0.0077 μg and 0.045 μg of copper were analyzed and the values found were 0.0083 μg and 0.043 μg, respectively.

Five samples containing the same amounts of copper were extracted by the procedure given, and the replicate results were found to agree within ±1.84 relative per cent at 95% confidence limits. Two samples and a standard can be analyzed by this method in 45 minutes after receiving the irradiated material in our laboratory. The radiochemical purity of the isolated ^{64}Cu activity was checked by measuring its gamma-ray spectra, beta maximum energy, and half-life, and in all cases the samples were found to be highly pure. Thus, the proposed method can be used for the determination of trace amounts of copper in geological or biological materials with good degree of precision and in a very short time.

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5

Thrombocytopenic Purpura Following Copper Sulphate Therapy

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During recent years a large number of chemical agents have been reported to be capable of producing thrombocytopenia and the haemorrhagic manifestations which accompany it (Wintrobe, 1961). While some of these chemical agents, viz., nitrogen mustards can regularly produce thrombocytopenia provided a large dose is given, there are many others which produce this defect only occasionally depending upon the sensitivity of the host (Schen and Robinowitch, 1958). The mechanism of thrombocytopenia developing as a result of sensitivity reaction depends mainly on the destruction of the platelets without producing any deleterious effect on the megakaryocytes. Thrombocytopenic purpura has been reported to occur following the use of gold salts (Thomson *et al.*, 1954), mercurials (Butt and Simonsen, 1950), iodides (Davis and Saunders, 1964, cited by Wintrobe, 1961), bismuth and arsenicals (Falconer and Epstein, 1940), but so far there has been no reported instance of its occurrence after the use of copper salts. The purpose of this communication is to report a case of thrombocytopenic purpura following the use of copper sulphate in the treatment of vitiligo.

Case Report

L. A., a seventeen-year old Moslem male, developed depigmented patches on the ankles which were gradually progressive. He attended the dermatology outpatient of S. S. Hospital, Varanasi, where he was diagnosed to have leucoderma and was prescribed 1 per cent solution of copper sulphate in the dose of 1 drop twice a day (2 mg./day). Continuing this treatment for nearly 2 months he noticed some red spots over the shins on both sides. A few days later he developed bleeding from the gums and epistaxis. He was shown to E. N. T. specialist who put adrenaline packs in the nose and over the gums, but the bleeding did not stop. He was referred to the medical outpatient and was admitted to the medical ward. During this period the patient had not taken any other medication.

Physical examination revealed a young boy of average build, markedly anaemic but without any jaundice or cyanosis. There was no lymphadenopathy, ankle oedema or clubbing of nails. There were generalised purpuric spots all over the body. Gums were spongy and bled

on pressure. No bleeding points were observed in the nasal mucosa. Pulse was 22/minute, temperature 98.8°F and B. P. 115/80 mm. of Hg. Liver and spleen were not palpable. Examination of the cardiovascular, respiratory and central nervous systems was negative.

Investigations — Total leucocyte count 16000/c.mm. with polymorphs 77 per cent, lymphocytes 18 per cent and monocytes 5 per cent. Haemoglobin 7 g. per cent. Erythrocyte sedimentation rate (Wintrobe) 66 mm. Bleeding time above 10 minutes and coagulation time 3 minutes (capillary tube method). Tourniquet test positive, poor clot retraction, prothrombin time 22 seconds (normal control 20 seconds) and platelet count 2400/c.mm. Peripheral blood smear showed erythrocytes of normal shape and size, apparently normal leucocytes and absence of platelets. Bone marrow smear showed mild degree of erythroid hyperplasia with normal myeloid-erythroid ratio, megakaryocytes normal in shape and absence of any abnormal cells. Slide test for lysis of platelets on addition of 1 per cent solution of copper sulphate was positive. Urine analysis, stool examination and fluoroscopy of the chest revealed no abnormality.

The chief abnormality in this patient with bleeding diathesis was low platelet count. His prothrombin time was not significantly higher than that of the control and could not account for the bleeding. A diagnosis of secondary thrombocytopenic purpura was made and copper sulphate medication was stopped. He was initially given 325 ml. of fresh blood transfusion and then prednisolone 30 mg. daily was started. As the patient was anaemic, oral medication with ferrous sulphate 200 mg. thrice daily was also given. Bleeding from the gums stopped after 5 days and gradually the purpuric spots also disappeared. Platelet count increased to 1,20,000/c.mm. and bleeding time returned to normal after 15 days' time. The dose of prednisolone was gradually tapered off. At this stage readministration of copper sulphate was contemplated but the patient refused to co-operate. Follow-up of the patient for nearly six months has not shown any recurrence of the bleeding diathesis or reduction in platelet count. The haemoglobin also returned to normal. The presence of anaemia (Hb. 7 g. per cent) in this patient needs some clarification. It could not have been caused by the bleeding which was small in amount and of short duration. In all probability, there was co-existing iron deficiency which was corrected after oral iron medication. Ankylostomiasis which is another common cause of iron deficiency anaemia was not present in this case.

Discussion

Haematological complications after the use of copper salts are extremely rare. Intravascular haemolysis with haemoglobinuria has been reported after acute copper sulphate poisoning (Chowdhury *et al.*, 1961; Gupta *et al.*, 1962). Although the exact mechanism of haemolysis in these cases is not fully understood, it is thought to be due to the direct action of copper on red blood cells. However, no haematological complication following the use of small amounts of copper salts has been reported in the literature. The paucity of reports on the subject may be due to the fact that copper salts are seldom used now in the therapy of diseases. During recent years, small doses of cupric chloride for prolonged period have been used in the treatment of leucoderma (Behl, 1961). There were no toxic reactions in well over 1000 cases of vitiligo treated by the above regimen. Similarly, no adverse effects were encountered when small doses of copper sulphate were employed in the treatment of vitiligo (Singh, 1966).

There is strong evidence to suggest that the occurrence of sudden thrombocytopenia in the case reported here was due to copper sulphate. The patient was taking small doses of copper sulphate and has not been exposed to any other chemical or physical agent which is known to produce this disorder. The clinical course and haematological investigations were consistent with the picture of drug induced thrombocytopenic purpura. As only a small quantity of the drug was ingested, thrombocytopenia must have developed as a sensitivity reaction. In the absence of any quantitative or morphological change in the megakaryocytes the thrombocytopenia probably occurred due to the destruction of the platelets in the peripheral blood. Once the patient had recovered from this illness attempts were made to test the effects of readministration of the drug but the patient and his relatives refused to co-operate. Thus we have been deprived of another valuable evidence to substantiate our claim. However, we feel reasonably certain that copper sulphate like several other chemical agents (Ball, 1960, Falconer and Epstein, *loc cit.*) can occasionally produce thrombocytopenia in sensitive individuals.

Summary

A case of thrombocytopenic purpura following copper sulphate therapy of vitiligo is reported. The probable mechanism of thrombocytopenia in the case is discussed.

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Acute Copper Sulfate Poisoning with Jaundice

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ACUTE copper sulfate poisoning is common in India (Chowdhury *et al.*, 1961; Chuttani *et al.*, 1965; Wahl *et al.*, 1963) where it is frequently employed as a suicidal poison by poor people because it is cheap.

Jaundice has been reported in copper sulfate poisoning, due either to liver damage or to haemolysis, attributed by Fairbanks (1967) to glucose-6 phosphate dehydrogenase (G-6-P-D) deficiency. He suggested that persons with erythrocyte G-6-P-D deficiency are more susceptible to the haemolytic effect of copper sulfate while Chuttani *et al.* (1965) have suggested that an increased concentration of copper in the erythrocytes may make them vulnerable to haemolysis.

We present a case of fatal suicidal copper sulfate poisoning which was associated with liver damage and haemolytic anemia in a patient with a gastrectomy.

Case Report

A 36 year-old man was admitted on 22 November 1966 in a coma. The relatives stated he had swallowed 250 g of copper sulfate in the form of powder with water 3 hours prior to admission in a suicide attempt. He had had a gastrectomy for a duodenal ulcer. There was no history of anemia or liver disease.

Tendon and cutaneous reflexes were absent and the patient looked severely dehydrated. The pulse was not palpable and the blood pressure could not be recorded. He was slightly cyanosed and the respiration rate was 30/min. There was oozing from his mouth and the digital rectal examination revealed fresh blood. The tongue was covered with a bluish fur.

Laboratory examinations carried out on admission were as follows: Haematocrit 52 per cent, Haemoglobin 15.2g per cent, White Cell Count 20,400/cmm. Differential count: Polymorphs, 94 per cent, Lymphocytes, 5 per cent and Monocytes, 1 per cent. The E.S.R. 8 mm (1st hour). The urine had a portwine colour. Specific gravity 1055. There was no proteinuria, but it contained 5 g per cent of sugar. Urine was negative for bile for two days but positive for haemoglobin. Examination of the sediment was negative for pus cells and erythrocytes. The electrocardiogram was within normal limits. Serum potassium was 7.7 mEq/L and sodium 126 mEq/L. Traces of copper were found in the vomit (method after Stewart and Stolman, 1961).

An intravenous drip was set up, initially he was given 5 per cent dextrose with noradrenaline (solution 0.2 per cent) and metaraminol followed by blood transfusion. Hydrocortisone was administered in a dose of 50 mg daily and the blood pressure rose to 130 mm Hg. Then dimercaptopropanol was administered i.m. 150 mg×6 daily and the patient regained consciousness.

On the day following admission there was a tendency for the blood pressure to fall, he had 8 bloody watery movements and developed pyrexia. The urine volume was reasonable (1150 ml per day) but towards evening jaundice was noticed. A fall of the haematocrit was detected (36 per cent), the reticulocytes were 2.5 per cent and his blood sugar 99 mg per cent. The intravenous administration of fluids was continued and the electrolyle imbalance corrected.

Serum bilirubin was found to be 4 mg per cent, blood fibrinogen 95 mg per cent, and the G-6-P-D test and the Coomb's test were normal.

During the following days the patient's condition was much the same. The gastrointestinal bleeding continued, and jaundice deepened and the anemia became worse gradually. On the 5th day of his admission the haemoglobin fell to 6.6 g per cent, despite repeated transfusions. He received a total of 5 L. of whole blood and the haemoglobin rose to 10 g per cent. Haemolysis decreased but the bilirubin rose to 6.5 mg per cent on the 9th day. On the 7th day the G-6-P-D test was normal and the liver tests as follows: Zinc-sulfate 5.7 units, thymol turbidity 10.5 units, cephalincholesterol (++++). S.G.O.T. 190 units. Total protein 5.2 g per cent, albumen 3.7 g per cent, globulin 1.5 g per cent.

On the 8th day the patient complained of abdominal discomfort with a greenish vomit. There was no bowel movement. The bowel sounds were not heard and the abdomen was tender. Peritonitis was suspected due to possible intestine ulceration and perforation. He was treated with antibiotics and his pyrexia decreased but the leucocytosis initially found was about the same. Despite the treatment the patient deteriorated and died on the 2 December 1966, 11 days after admission.

Autopsy findings: Ulcers varying in size (figure 1) were

Figure 1. Small intestine. Small ulcerous lesions with infiltration of the wall.

Figure 2. Liver showing cholestasis in centrilobular areas and portal areas infiltrated with mononuclear cells.

found throughout the length of the small intestine. One had perforated into the peritoneum and was covered with numerous pseudomembranes. Microscopic examination showed necrosis of the mucosa of the intestine and accumulation of inflamed connective tissue.

The liver (figure 2) revealed infiltration with mononuclear cells microscopically, particularly in the portal areas. Necrosis was not seen but considerable cholestasis could be detected in centrilobular areas. Bile thrombi lay in moderately dilated canaliculi. Occasionally bile was seen in portal zones.

The kidney contained microscopic areas of interstitial infiltration with lymphocytes in cortical and medullary areas. Some tubules contained casts of bile.

The remainder of the organs were essentially normal in both gross and microscopic appearance.

Discussion

Copper sulfate stimulates the vomiting reflex (Soine and Wilson, 1967), incomplete in our patient due to the previous gastrectomy. The absence of the stomach resulted to a rapid passage of the poison into the intestine. This might explain the severe ulceration in the gut, similar ulcers have not been found in other cases. Chuttani *et al.* (1965) reported microscopical mucosal ulcers but not large ulcers with perforation as found in this patient.

The jaundice which developed was no doubt initially caused by the intense haemolysis, as it was detected by a variety of tests, but later should be attributed to the hepatocellular damage, as revealed by the liver function tests and confirmed by the histological examination of the liver. Haemolysis, as has been suggested by Chuttani *et al.* (1965), is caused by copper itself although the absence of G-6-P-D may contribute (Fairbanks 1967). The estimation of G-6-P-D in our case could not be evaluated because of the large transfusions. The hypothesis expressed by Fairbanks (1967) that haemolysis is dependent from the amount of copper ingested could not be substantiated, since there was no quantitative estimation of the whole blood copper which Chuttani *et al.* (1965) recommend. However the mechanism of this haemolytic process is not well understood (1966).

It is not clear if the low plasma fibrinogen found was caused by the toxic effect of copper sulfate or resulted from extensive haemorrhage.

Death was most likely caused by the perforation of the gut and the accompanied peritonitis.

In the cases presented by Chuttani *et al.* (1965) there was a distinct difference between cases presenting jaundice. In our case there was a combination of the cause of the jaundice. Jaundice of mixed origin has been reported by Wahl *et al.* (1963).

The glycosuria found on admission might be due to liver damage or to a lower threshold due to renal tubular changes as suggested by Chowdhury *et al.* (1961). It is interesting that more severe alterations of kidney structure were not found, despite the fact that our patient remained in shock for several hours. It is worth noting that the cardiovascular collapse which is a frequent cause of death in these patients was successfully treated.

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Chronic Copper Poisoning Presenting as Pink Disease

Copper toxicity is extremely rare, and Sternlieb and Scheinberg (1964) suggest that this is due to excretion or incomplete absorption of the metal rather than to an inherent lack of toxicity in copper. It is only in Kinnier-Wilson's disease (hepato-lenticular degeneration) that progressive copper toxicity is likely to occur due to defective copper balance from deficiency of copper-binding caeruloplasmin. Acute copper poisoning (usually copper sulphate in weed-killers) is not infrequently encountered but, as far as we are aware, no case of poisoning due to chronic ingestion of copper has so far been reported. With increased use of copper for hot-water pipes, further examples of copper poisoning must be anticipated.

Case Report

A 15-month-old infant was admitted to the Children's Unit after a 5-week history of behaviour change, diarrhoea, and progressive marasmus. Before admission developmental progress had been entirely satisfactory and there was nothing of significance in the birth or family history. When first seen the clinical picture was that of pink disease with prostration, misery, red extremities, hypotonia, photophobia, and peripheral oedema (Fig. 1). The body weight was only 7·48 kg (weight at 1 year 10·09 kg). The liver was palpable 2 cm below the costal margin.

Investigations. The outstanding finding was a raised serum copper level of 286 µg/100 ml (normal range taken as 164±70 µg/100 ml (Zak, 1958)), with a urinary copper level of zero (normal range 48±16·3 µg/100 ml (Eden and Green, 1940)). The serum and urinary copper levels were measured in a laboratory where these estimations are routinely performed, and where a high level of accuracy can be expected. All tests for mercury were negative. Other investigations which gave abnormal values were: serum aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) both 180 Karmen units/100 ml. Alkaline phosphatase 33 KA units. Lactic dehydrogenase, γ-glutamyl transpeptidase, and pseudocholinesterase activity were not increased. Total plasma protein was 4·0 g/100 ml with [illegible] level of 2·2 g/100 ml.

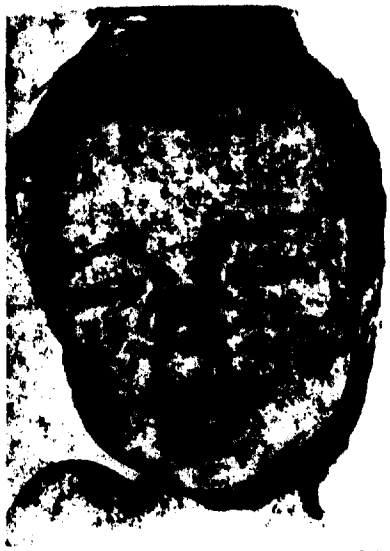


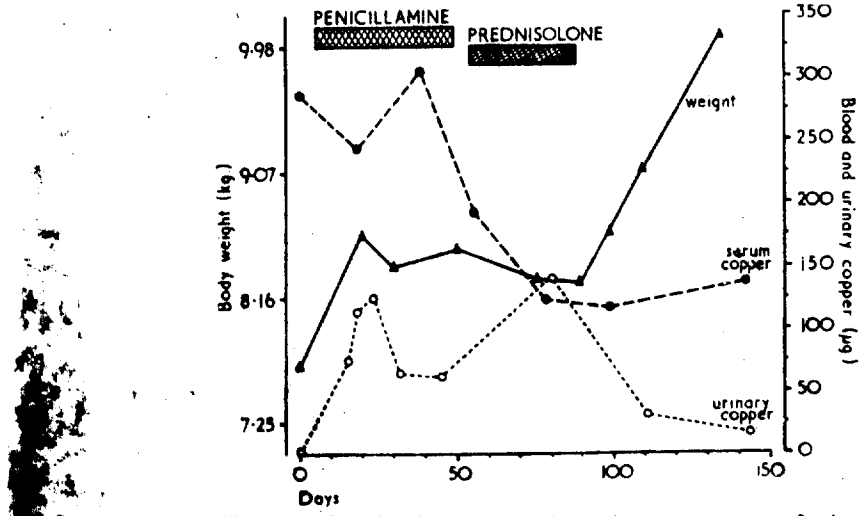
FIG. 1.—*Photophobia, misery, and flushed facies.*

The peripheral blood picture, bleeding and clotting times, urea, cholesterol, total serum lipids, uric acid, faecal porphyrins, urinary coproporphyrins, urine chromatography for aminoaciduria and skeletal survey were all within accepted normal limits. Copper oxidase activity was 0·10 units (normal range 0·14–0·57 units (Ravin, 1961)).

Treatment. From the data available it was thought that this infant showed signs of copper poisoning simulating classical pink disease. Walshe (1956) first showed the cupruretic effect of D-penicillamine which renders protein-bound copper filtrable by the renal glomeruli, and this was prescribed in a daily schedule of 150 mg t.d.s. Almost immediately there was a deterioration in his clinical status which became critical. Oedema became massive and there was apathy and early signs of bronchopneumonia. The dosage of D-penicillamine was reduced to 75 mg t.d.s. for 5 weeks, and he improved. Serial estimations of serum and urinary copper are shown in Fig. 2; on clearing the excess copper his bodyweight immediately increased and recovery began. Generalized purpura in the presence of a normal platelet count and coagulogram occurred during the phase of recovery. It was thought to be a side effect of D-penicillamine therapy (Corcos *et al.*, 1964; Conway and Walker, 1962; Cramér and Selander, 1965; Sternlieb and Scheinberg, 1964), and this, together with probable liver involvement in the primary illness, prompted the use of prednisolone in small doses over a 3-week period.

Discussion

Three months before this patient's admission, the family moved to a new house in which the hot-water system was entirely composed of copper. The hot water was stored in a 40-gallon 'Primatic' tank made of copper and at night the temperature in the tank reached about 93 °C. An idiosyncrasy of this family was that all water for cooking or beverages invariably came from the hot-water tap, and after only two months the electric kettle was coated inside with a thick green film of a copper complex. Estimations of the copper content of the tap water, both hot and cold, were made on samples



Effect of penicillamine and prednisolone on patient's weight, serum copper, and urinary copper concentrations (µg/100 ml).

from the patient's home and that of a neighbour whose hot water system also used copper piping. The results were compared with samples from the hospital (mixed copper and other piping) and North London (no copper piping) (Table).

TABLE

Source of Water	Cold Water Tap (Cu μg 100 ml)	Hot Water Tap (Cu μg/100 ml)
Patient's home	35	79
Neighbour's home	30	68
Hospital	4	30
North London	8	16

From these results it is seen that the copper content of 'hot' water is significantly higher than water from the cold tap. The presence of copper piping apparently leads to a significant increase in the copper content of the water.

As was mentioned earlier the copper oxidase activity was only 0·10 units, and because of this the caeruloplasmin (α-copper binding protein) level was estimated at 22·5 mg/100 ml (Dr. J. Walshe). The determination was based on the enzyme assay on whole plasma and compared with results obtained from similar assays using purified caeruloplasmin. This result effectively eliminates hepato-lenticular degeneration from the differential diagnosis. Low levels of caeruloplasmin have also been reported in portal cirrhosis (Gubler *et al.*, 1957) and in nephrosis (Cartwright, Gubler, and Wintrobe, 1954), but in this case these conditions can be ignored. During the convalescent phase liver biopsy was performed and histology revealed a normal architecture. Copper assay on biopsy material gave levels of 4·7 and 5·1 μg g wet weight, which are at the lower end of the normal range. It thus appears that he had cleared his stored copper very well. It is disappointing not to have the results of liver biopsy before treatment was started as this would have made the diagnosis of copper poisoning wholly tenable. The infant's condition on first admission was such that biopsy was never considered. The critical change in the clinical status after starting D-penicillamine was thought to be due to mobilization and excretion of stored copper. In fact it constituted acute copper toxicity superimposed on a more chronic picture.

In conclusion it is suggested that this child was subjected to a high copper intake over a period of three months and showed abnormal sensitivity to the metal. The clinical picture was that of pink disease. From the progress following chelation with D-penicillamine and the fact that mercury was not found, we put forward the suggestion that in some children excess copper can cause a clinical picture that cannot be distinguished from pink disease. The long-term prognosis for this child is probably good.

Summary

A case of chronic copper poisoning in a male infant of 15 months is reported. It is believed to be the first case report. The infant presented with a picture indistinguishable from that of classical pink disease (acrodynia). The copper had been ingested over a period of 3 months from contaminated water. Treatment consisted of D-penicillamine and prednisolone. Recovery was slow and at one time the patient's condition became critical.

We thank our Ward Sister, Miss B. M. Barch[illegible].

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Review: Relationships of copper and molybdenum to iron metabolism

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This paper surveys the literature on the influence of copper and molybdenum on the transport and utilization of iron, and considers how their interaction with one another can affect iron utilization. Evidence as to the role of copper in the transport of iron, and of molybdenum in its absorption and release is considered, as are data on interrelationships of copper and molybdenum, when one is present in excess and the other is marginal or deficient in supply. It is possible that a relative excess of one of these elements may induce a conditioned deficit of the other, with resultant interference with normal iron metabolism.

Copper in iron metabolism

Copper deficiency-induced abnormalities in iron kinetics and enzymes have been studied in rats and swine by the investigators of the Cartwright and Wintrobe team (1–4). Reviews by Adelstein and Vallee (5), Cartwright and Wintrobe (6, 7), Marston (8), and Scheinberg and Sternlieb (9, 10) provide summations of the experimental (animal) evidence that induced copper deficiency causes hypochromic microcytic anemia that is morphologically indistinguishable from that caused by iron deficiency. Conversely, chronic ingestion of excess copper has been shown to cause microcytic anemia, hemolytic anemia, hemoglobinuria, and liver damage in rats and swine (9, 11–13). Exposure to excess copper has caused similar abnormalities in man (14–19). The participation of copper directly in heme synthesis and erythropoiesis will not be considered in this report as this area has been reviewed by others (1, 6, 12, 20).

Copper, ceruloplasmin, and iron transport

Ceruloplasmin, the copper-carrying plasma protein, has recently been shown to be a link between copper and iron metabolism (20–24). Frieden et al. (20–22) demonstrated that the oxidation of ferrous iron is greatly accelerated (10- to 100-fold) by ceruloplasmin, which they suggested might better be termed "ferroxidase." It seems to be rate-determining, in the formation of Fe^{3+} transferrin, in the absence of which the rate of iron oxidation in the plasma is not adequate to meet the Fe^{3+} transferrin demands of the erythropoietic tissues. Lee et al. (24) found that copper deficiency (in swine) is associated with abnormal iron metabolism. Copper-deficient swine, given iron, developed hypoferremia even though iron stores were normal or elevated. On administration of copper to such animals, there was a prompt increase in plasma iron. This was interpreted as indicative that plasma iron levels reflect a balance between the availability of iron from the reticuloendothelial (RE) cells and the rate at which iron is removed. In copper deficiency, the release of iron from RE cells and hepatic cells is impaired. Injection of pig ceruloplasmin, but not copper sulfate, into copper-deficient pigs with adequate iron stores, caused an immediate elevation in plasma iron (23). Ferrokinetic studies indicate that the increase in serum iron results from an increase in its

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rate of flow into plasma. They suggest that ceruloplasmin mediates RE iron release. These findings were not duplicated in normal rats that were neither copper nor ceruloplasmin depleted (25). Serum has been shown to contain two ferroxidase factors, ceruloplasmin and citrate (26), only the former of which is copper protein. In the absence of ceruloplasmin, ferrous iron may not be as rapidly oxidized to ferric iron, in which form it is transported by transferrin.

Copper in cytochrome system

Cytochrome *c* is an intracellular iron enzyme (that participates in the electron transport chain) that has been shown to be markedly decreased in iron deficiency (27). Beutler (27) has attributed some of the symptoms of iron-deficiency anemia to the malfunctioning of such intracellular enzymes. Like iron, copper is also a component of some of the enzymes that mediate oxidation–reduction in the mitochondrial electron transfer chain; it has been shown, for example, to be a component of cytochrome oxidase (28). As early as 1934, it was known that tissue levels of several components of the cytochrome system are markedly diminished in copper-deficient rats (29–31).

Molybdenum in iron metabolism

The biologic importance of molybdenum has been recognized since it was found to be a component of the intracellular iron-containing enzymes: xanthine oxidase and aldehyde oxidase (32–60). Study of these enzymes suggests that molybdenum may play an important role in iron utilization, as well as in cellular metabolism (47–49, 51, 58). The two molybdoflavin enzymes are involved in the electron transport chain (45–55). The evidence that xanthine oxidase may be involved in the uptake and release of iron from ferritin in the intestinal mucosa and in the mediation of the release of iron from ferritin in the liver, in erythropoietic tissue, and in the placenta is reviewed in the discussion of xanthine oxidase and iron-ferritin.

Molybdenum: component of flavoprotein enzymes

Xanthine oxidase. DeRenzo et al. (32) first identified molybdenum as the "liver residue factor" that Westerfeld and Richert (33, 34) had shown to increase the level of xanthine oxidase in liver and intestinal mucosa of weanling rats. Addition of as little as 1 mg of sodium molybdate, or the injection of 10 μg subcutaneously, gave values of xanthine oxidase equal to that obtained when 10% liver residue was fed to weanling rats (32). When such rats were made molybdenum deficient (35), their intestinal xanthine oxidase increased rapidly following subcutaneous injection of 40 μg molybdenum (36, 37) (Table 1). The rapid response was interpreted as indicating direct participation of molybdenum in the formation of the enzyme (36).

Verification that molybdenum is the dietary factor required for the deposition and maintenance of normal levels of intestinal xanthine oxidase in rats was soon provided in other studies (38–42) that showed molybdenum was necessary for the activity of xanthine oxidase of the intestinal mucosa and of the enzyme contained in milk and in other foods.

It has been postulated that the function of molybdenum may be to promote the formation of, or to stabilize, flavin-free radicals in the enzyme-catalyzed reactions (43). Each molecule of xanthine oxidase has been shown to contain two molecules of flavin adenine nucleotide (FAD) and one atom of molybdenum (44). Furthermore, molybdenum participates in the reaction of xanthine oxidase with cytochrome *c* and may function as an obligatory electron transport bridge to 1 electron acceptors such as cytochrome *c* (45). The molybdenum of xanthine oxidase is associated with iron at the active site (46), the iron having a great affinity for certain valence states of the molybdenum. In the internal

TABLE 1

Intestinal xanthine oxidase after molybdenum injection[a]

After 40 μg Mo injection, hr	Xanthine oxidase, rat intestine (μl O_2 uptake/20 min/250 mg intestine
0	8.3
1	14.3
4	17.7
8	20.8

[a] Adapted from (36).

electron transport sequence of the enzyme, iron is the carrier closest to the site of oxygen reduction, whereas molybdenum is the most remote (47). It has also been suggested (48) that flavin, molybdenum, and iron participate in oxidation–reduction, with transport of electrons in the order: substrate → Mo → FAD → Fe. Handler et al. (49) have observed that the fact that, kinetically, the Mo atoms of xanthine oxidase (and of aldehyde oxidase) are the first components of these enzymes to be reduced must relate to the manner of oxidizing attack on their substrates. Both enzymes function as "hydroxylases" rather than as "dehydrogenases." The oxidation of purines to uric acid by xanthine oxidase seems to be accomplished by successive withdrawal of a hydride ion and replacement by a hydroxyl ion (see review by Spence (50) for an analysis of the biochemical reactions of molybdenum in association with xanthine oxidase and aldehyde oxidase, in the transfer of electrons).

Aldehyde oxidase. Molybdenum is also a component of and is required for the activity of aldehyde oxidase (51–55). It participates in the catalytic action of liver aldehyde oxidase (51–53). The mode of binding the molybdenum and its function in the reduction of 1 electron acceptors (cytochrome, ferricyanide, nitrate) and the requirement for inorganic phosphate are properties common both to xanthine oxidase and aldehyde oxidase. In addition to FAD and molybdenum, aldehyde oxidase has a hematin component associated with it. This enzyme, like xanthine oxidase when complexed with molybdenum, possesses remarkable ability in facilitating the interaction with cytochrome *c* (54). Both in aldehyde oxidase and xanthine oxidase, the molybdenum component is present at the substrate-binding site (49, 55).

Effect of molybdenum deficit on tissue flavoprotein enzymes

Xanthine oxidase in molybdenum deficit. Before molybdenum was known as the "liver residue factor" (32–34) and after it was proved to be a component of xanthine oxidase (32–38, 41, 42), it was shown that neonatal rats lack this enzyme, and that weanling rats, which were fed molybdenum-free but otherwise presumably adequate purified diets, failed to develop normal levels of this enzyme in the mucosa and in the liver. The same increase in intestinal xanthine oxidase can be achieved in rats by administering molybdenum, as by feeding liver or liver extracts.

Although xanthine oxidase levels were low in rats and chicks on Mo-deficient diets, they did not manifest any overt abnormalities (56). Three successive generations of Mo-deficient rats, when evaluated according to growth rate, reproduction, purine oxidation, and tissue levels of xanthine oxidase and molybdenum, showed only abnormally low intestinal levels of the enzyme. They apparently were able to obtain sufficient molybdenum, even from their purified diets, to prevent overt pathology. As tungsten-feeding had been shown to inhibit the response of intestinal xanthine oxidase to molybdenum, Higgins et al. (56) intensified the effects of dietary molybdenum deficiency with tungsten in both rats and chicks. All such animals had depressed growth rates, mortality rates of 25% in 5 weeks, and marked depletion of tissue molybdenum and xanthine oxidase. The amount of uric acid excreted by the chicks was reduced by one-half, and was replaced by xanthine and hypoxanthine. Significantly higher liver and intestinal xanthine oxidase was also demonstrable in turkey poults on a purified diet when it was supplemented with 0.0254 ppm molybdenum than when it was not (57). A diet adequate in all respects, including supplementation with iron (100 ppm) and copper (10 ppm), but lacking in molybdenum influenced both hemoglobin formation and xanthine oxidase in chicks (58). When 5 ppm of molybdenum was added to the diet, there was a threefold increase in liver xanthine oxidase; 1.2 ppm molybdenum produced a substantial increase in intestinal xanthine oxidase. The usual decrease in hemoglobin (to one-third the level seen at hatching) during the first week of life, was prevented by adding 5 ppm molybdenum to the diet.

Molybdenum depletion has also been shown to influence uric acid formation in the rat (59). In the absence of xanthine oxidase in rat liver, such as was seen in molybdenum-deficient rats, the uric acid and allantoin

normally excreted by rats was not formed. On the other hand, rats on a molybdenum-deficient diet had higher serum uric acid levels than did those supplemented with 1 ppm Mo (60).

Livers from molybdenum-deficient rats oxidize acetaldehyde at a slower than normal rate, possibly due to the molybdenum dependence of aldehyde oxidase (52).

Xanthine oxidase and iron-ferritin

Ferritin in iron absorption and storage. Because the xanthine oxidase step that oxidizes xanthine or hypoxanthine to uric acid converts Fe^{3+}-ferritin at the same time to Fe^{2+}-ferritin and as this enzyme is a molybdoflavoprotein, the role of ferritin in iron metabolism must be considered here. The structure and synthesis of ferritin and its precursor, apoferritin, and their participation in the regulation of absorption of iron and in its storage, have been reviewed recently by Crichton (61). Ferritin is the primary iron storage protein of the body, and is in dynamic equilibrium with plasma iron (61). Hahn et al. (62) first suggested that the rate of absorption of iron is probably governed by a process in the gastrointestinal mucosa and that when the "mucosal acceptor" (possibly apoferritin) is fully saturated with iron, its further uptake might be blocked (63). Support for this concept was provided by Granick (64), whose work indicated that the uptake of ferrous iron by the mucosal cells stimulates these cells to produce apoferritin, and that a part of the absorbed iron becomes attached to apoferritin to form ferritin (65), which is found not only in the intestinal mucosa but also in liver, bone marrow, spleen, and other tissues.

There is controversy as to whether ferritin functions primarily to block or to mediate iron absorption. After refuting the mucosal block theory on the basis of their iron-loading human studies (66), Moore and Dubach (67) evaluated the evidence up to 1962 and concluded that there is a "mucosal block," but that it is transient. They pointed out that, although increased iron stores (in mucosa or elsewhere) tend to retard absorption, in some conditions iron continues to be absorbed despite adequate stores.

More recent studies indicate that the regulation of iron absorption still remains an incompletely resolved question. Crosby and his co-workers (68–73) have presented evidence that the formation of ferritin within the villous epithelial cells (of man (68) as well as of experimental animals (71, 72)) provides the most important mechanism for control of iron balance. In rats, iron absorption shows two phases: *1*) an initial period of rapid absorption lasting up to 2 hr, during which 60 to 80% of absorption takes place, and *2*) a delayed phase, taking 12 to 20 hr (71). The amount of iron moved into the carcass during the phase of rapid absorption depends upon the amount of iron stored, with a ratio of 1:2:14 for retention of iron in iron-loaded, normal, and iron-depleted rats. Wheby and Crosby (71) concluded that the second delayed phase of absorption may be mediated by a mechanism for putting iron into a more stable, slowly released form within the intestinal mucosal cells. However, they suggest that the iron incorporated into epithelial ferritin cannot be released and therefore is lost when the cell is lost at the end of the cell's life cycle in about 3 days (70). Recent studies with rats given specific anti-ferritin serum support the concept that ferritin functions chiefly as a barrier to excess iron absorption and as an important mechanism for its excretion (73).

Beutler et al. (74) question whether it is the formation of ferritin that is most important in the control of iron absorption. They consider it likely that as iron can be absorbed across the intestinal mucosa, even when the ferritin is fully saturated, it is probably absorbed as iron, bound or chelated to protein (possibly apoferritin), or to some other substance. They suggest that at least three types of stimuli may exert a significant effect on iron absorption: *1*) the level of the iron stores, *2*) hypoxia, and *3*) erythropoietic activity. It remains to be elucidated how the message that the body stores are deficient or replete in iron is conveyed to the absorptive site in the intestinal mucosa. Höglund (75) could find no significant correlation (in young women and in blood donors with low stores of iron) between iron absorption and the hemoglobin, packed cell values, serum iron,

transfer iron, or plasma iron clearance. Only in young, healthy men were the transferrin and iron stores significantly correlated to iron absorption. Their studies confirm the relation of iron absorption to the amount of storage iron in the body, but the way in which the mucosal cells are informed about iron needs remains unclear.

Ferritin and release of iron: mediation by xanthine oxidase. The analysis by Bothwell and Finch (76) suggests that the formation of apoferritin and the release of iron from ferritin may play an important mediating role through which the need for iron is conveyed to the mucosal cell. They have evaluated the evidence as to the role of ferritin as an iron-releasing, as well as an iron-binding compound, not only in the intestinal mucosa but also in erythropoietic and iron storage tissues. They call attention to the evidence that *1*) iron stimulates the formation of apoferritin within liver and bone marrow cells of the erythropoietic tissues, and *2*) this process is not limited to cells of the intestinal mucosa. Cellular levels of apoferritin decrease when iron is mobilized from the tissues. Only ferrous iron crosses the cell membrane and is taken up by apoferritin and incorporated into ferritin, although the iron is transported to the tissues (by transferrin) as the ferric ion and is stored as tissue iron (complexed as ferritin) as the ferric ion. They point out that iron has been demonstrated to be released from ferritin in the presence of ascorbic acid, glutathione, cysteine, or anaerobic liver slices, and that xanthine oxidase may participate in the release mechanism. The substrates for the action of xanthine oxidase on ferritin are the purines, i.e., inosine, xanthine, or hypoxanthine (Fig. 1).

The release of iron from ferritin of spleen, liver, bone marrow, and placenta has been demonstrated by Mazur et al. (77–79). Most

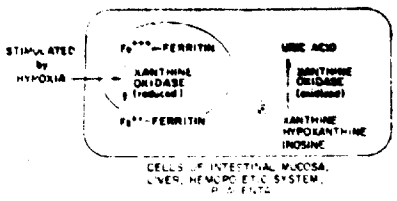

FIG. 1. Mobilization of iron from ferritin. Based on (77–79).

of the iron in ferritin is in the ferric state and not available for reaction with iron-binding agents (77); only that small proportion of ferritin iron that is present in the ferrous state (at the surface of the protein) is readily available for reaction with the plasma iron-binding protein, transferrin. Anaerobic liver slices and bone marrow release more ferrous iron than do aerobic liver slices, a finding that suggests that local conditions of hypoxia favor the conversion of ferric to ferrous iron in liver and in bone marrow ferritin, with the release of ferrous iron, which can then be utilized in heme synthesis. Local intermittent hypoxia in human placenta in which ferritin has been demonstrated, results in the reduction to the ferrous form, which can then be transferred across the placental barrier to the fetal blood stream (77). Green and Mazur (78, 80) then demonstrated that the reduction of ferritin iron takes place anaerobically in the presence of xanthine oxidase. Within the liver cells, the reduction of ferritin iron takes place in the presence of hypoxanthine or xanthine, which xanthine oxidase converts to uric acid. Thus, lowered oxygen tension is a stimulus for this reaction, in which ferritin acts as an electron acceptor and its iron is reduced to the ferrous state. The mechanism by which xanthine oxidase affects the release of iron from ferritin is indicated by the elevation in plasma uric acid and iron in dogs subjected to hemorrhage (77), and in dogs, rabbits, and guinea pigs given substrates of the xanthine oxidase–ferritin reaction (xanthine, hypoxanthine, and inosine) (79). Administration of uric acid caused a decrease in plasma iron. Hepatic ferritin is the major source of iron liberated to the plasma, following purine administration, or after hemorrhage. These results show that xanthine oxidase oxidizes substrates in the absence of oxygen, provided ferritin iron is present, and suggest that the site of ferritin iron reduction is associated with the nonheme iron of the enzyme and takes place by direct electron transfer. Because the physiologic stimulus to acceleration of release of iron from ferritin stores to the plasma is tissue hypoxia, and as lowered oxygen tension also stimulates erythropoiesis and therefore the incorporation of iron into hemoglobin, Mazur et al. (79) proposed that the

ferritin–xanthine oxidase system is part of the homeostatic mechanism for the regulation of iron in the circulation.

Subsequent work by this group has demonstrated that ascorbic acid and adenosine triphosphate (ATP) are required for conversion of ferric iron from plasma transferrin to the ferrous form that is taken up by apoferritin for storage as hepatic ferritin (81–83). It is of interest that the step carrying iron from plasma protein-bound iron to protein-bound storage iron is an oxygen-dependent process. Thus, storing iron takes place with normal tissue oxygenation, such as is seen with normal hemoglobin. Anemia, with resultant tissue hypoxia, allows the anaerobic process, xanthine oxidase-mediated iron release from ferritin, to function. Bothwell et al. (84, 85) showed that after hemorrhage the reticuloendothelial cell, the gastrointestinal mucosal cell, and the placental cell all show an increased release of iron. Cheney and Finch (86) verified the influence of administered xanthine oxidase substrate on iron levels by showing that inosine increased iron transport across the intestinal mucosa of rats by 50 to 100%.

It cannot be assumed from the foregoing that the interrelationships of xanthine oxidase and iron are fully resolved. Iron-deficiency anemia itself has been shown to influence xanthine oxidase activity. Srivastava and Tewari (87) showed that rats made anemic by milk diet had lowered levels of two iron enzymes including (hepatic) xanthine oxidase. Awai and Brown (88) demonstrated that severe iron deficiency in rats is associated with a diminution in the xanthine oxidase activity of the duodenal mucosa, but with an increase in its activity in the liver. Hypoxia has been shown by Strohmeyer et al. (89) to cause a decrease in xanthine oxidase activity of the bowel of iron-deficient rats, but an increase in the intestinal enzyme activity in iron-loaded rats. No change was seen, however, in hepatic xanthine oxidase, in response to hypoxia, either in iron-loaded or iron-depleted rats (89), an observation seemingly at variance with the in vitro findings of increased xanthine oxidase-mediated reduction of ferritin iron under anaerobic conditions (78, 80). Depending upon the technique used, lowering xanthine oxidase activity has been shown to increase the level of iron in the liver (of ethionine-treated rats) (90), or to have no effect on hepatic iron (of rats given sodium tungstate to decrease xanthine oxidase activity) (91).

Allopurinol, a specific inhibitor of xanthine oxidase, has been used to determine more directly the effect of xanthine oxidase on iron movement. Following Ayvazian's (92) association of the excessive storage of iron of hemochromatosis with a genetic xanthine oxidase deficiency, the possibility that pharmacologic inhibition of this enzyme might cause aberrations in iron metabolism was explored in man and animals, with equivocal results. Significantly increased hepatic storage iron was observed in rats consuming iron plus allopurinol, as compared with iron-supplemented rats not given the enzyme inhibitor (93). These results were not confirmed by others (94–96) who studied the effect of allopurinol in rats on different intakes of iron. Both intestinal absorption and storage of iron were diminished in anemic rats given allopurinol, but this was not seen in comparably treated normal rats (88). In evaluating their findings, in light of the conflicting reports of others as to the role of xanthine oxidase in iron mobilization and absorption, Awai and Brown (88) suggested that only the later phase of iron absorption (that influenced by the effect of xanthine oxidase on ferritin) is affected by the enzyme-inhibitor. Their 11-day studies showed that, with the exception of an increase in serum iron levels in normal rats given allopurinol, and less tissue uptake of radioiron in the iron-deficient rats given allopurinol, the enzyme inhibitor caused little change in the distribution of iron. Thus, it is not surprising that clinical studies (97, 98) of the effect of allopurinol on radioiron absorption in normal subjects were not illustrative of a significant effect on blood.

Animal studies demonstrate that xanthine oxidase functions within the mucosal cell, within the spleen and other erythropoietic tissues, and in the liver, at each site of the $Fe^{3+} \rightarrow Fe^{2+}$-ferritin interaction. It also functions in the placenta, which probably participates in the transfer of maternal iron

to placental ferritin, to the fetal circulation. Pollycove (99) considers it more plausible to regard mucosal ferritin formation as a process that facilitates the absorption of iron, rather than as a process that blocks its absorption. He observes that the experimental evidence indicates that mucosal formation of ferritin increases the rate of removal of intracellular ferrous ions by binding them as ferric ferritin, thereby increasing the rate of transfer of ferrous ions from the lumen into the cell. He refers also to the very active aerobic metabolism of the intestinal mucosa, which would make it susceptible to tissue hypoxia in the event of low hemoglobin content in the blood. Pollycove (99) cites the evidence by Mazur et al. as being indicative of the role of the xanthine oxidase–ferritin reaction in the release of ferrous iron from stores of iron in the tissues to the blood, under conditions of tissue hypoxia.

Molybdenum/copper interrelationships in iron utilization

In considering the effect of copper and molybdenum on iron utilization, it is necessary to take into account the influence of disproportionate intakes or disproportionate levels of each metal in plasma and tissue. Each mineral seems to be necessary for the body's handling of iron: molybdenum to mediate the release of iron from intestinal mucosal and storage ferritin (predominantly in the liver), and copper to mediate the uptake of iron by transferrin in the plasma. Figure 2 depicts the absorption, transport, storage, and release of iron, and indicates the sites at which molybdenum and copper act. Although each is necessary for steps in the metabolism of iron, an excess of either copper or molybdenum causes anemia by interfering with the absorption and utilization of the other (8, 11, 100–103). Thus, the characteristics of molybdenosis, a disease reported among grazing animals on forage rich in molybdenum and poor in copper, is associated with signs of copper deficiency and responds to treatment with copper (8, 104–110). Conversely, ruminants on forage rich in copper and poor in molybdenum develop chronic copper toxicity, and show a therapeutic response to increased intakes of molybdenum (8, 11, 106, 111, 112).

Such extremes of nutritional imbalance are probably not a clinical problem, except in farm animals (sheep, cattle). However, there are physiologic and pathologic situations in which a relative nutritional imbalance between molybdenum and copper may contribute to abnormalities in iron metabolism, and possibly in erythrocyte survival and in uric acid production. There is evidence that in

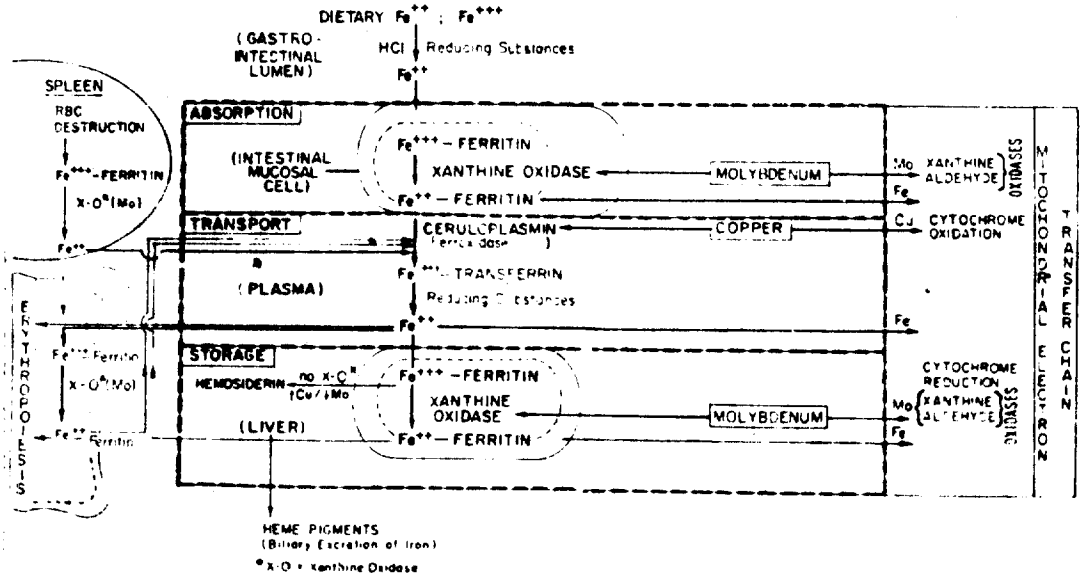

FIG. 2. Iron absorption, transport, storage, and utilization as effected by molybdenum and copper.

such situations, there may be some risk in ingesting even moderate excesses of copper. For example, customary intakes of copper are known to be considerably in excess of requirements, and high copper levels have been reported in a number of iron-deficiency anemias, and in some iron-storage diseases (113). It is not possible to make definitive statements about the status of molybdenum in these conditions, as levels of molybdenum have been determined only rarely in diseases associated with abnormalities in iron metabolism. However, in the one paper (113) found that reported molybdenum blood levels in three types of iron-deficiency anemia, the plasma and erythrocyte levels of molybdenum were about one-half normal levels.

As indicated in the foregoing section, the evidence of molybdenum's role in iron metabolism is derived from work showing that it is needed for xanthine oxidase activity (33–50, 56–60) and from laboratory and clinical work suggesting that xanthine oxidase plays a role in the release of iron from ferritin (77–80, 86, 88, 90, 92, 99). These findings, it must be noted, have not been demonstrable in all test systems (89–91, 94–98).

Copper/molybdenum complexes

Considerable evidence has been presented that the mutual interference of copper and molybdenum is caused by the formation of a Cu–Mo–S complex that is relatively undissociable and from which neither copper nor molybdenum is readily mobilized (53, 103, 104, 111, 115–123). Excess molybdenum interferes with removal of copper from blood to liver in sheep and pigs (117, 118). In sheep, molybdenum interferes with copper uptake by the liver, and with the incorporation of copper in ceruloplasmin, and it also increases the urinary excretion of copper (121, 123). An important concomitant finding is that the toxic effect (of molybdenum in rats) is more difficult to produce unless there is a dietary deficiency of copper (122, 124). That when such a complex is formed it can also interfere with the availability of molybdenum within the tissues is suggested by demonstration that the ratio of copper to molybdenum in the complex found in liver is 4:3 (117). As the range of copper found in liver tissue from apparently healthy American adults has been shown to be 5 to 20 times higher than that of molybdenum (Table 2) (125); should such a complex be formed in human disease, a functional deficit of molybdenum is far more likely than is a functional deficit of copper.

Copper is plentifully supplied in the American diet to the extent that copper excess is considered a significant risk (10). In contrast, the possibility of a dietary copper deficit is considered exceedingly unlikely (6, 7, 126). Far less is known of the adequacy of molybdenum intakes. Preliminary studies, however, suggest that molybdenum intake may not be optimal in many areas of the United States, and that molybdenum blood levels tend to be low (60, 127, 128). Thus, the possibility exists that there may be a conditioned molybdenum deficiency in certain clinical conditions, particularly in those associated with elevated copper levels, which may affect iron metabolism adversely.

Copper intakes and levels in man

Despite the fact that copper has been shown to be necessary for the absorption and utilization of iron (in experimental copper deficiency in rats and swine (1–4, 23, 24)), there is no evidence that copper deficiency contributes to human iron-deficiency anemia. It is generally agreed that the average dietary intake of copper in the United States considerably exceeds the requirement. Cartwright and Wintrobe (6, 7) have shown that adult subjects are in copper balance, that on the 2 to 5 mg copper ingested daily, 0.6 to 1.6 mg is absorbed. They have commented that copper is widely distributed in foods and that a diet of even mediocre quality contains 2 mg. In-

TABLE 2
Tissue levels of molybdenum, copper, iron, zinc, and lead in organs of American adults[a]

	Liver[b]	Kidney[b]
Molybdenum	0.33–1.5	0.21–0.50
Copper	5.0–21	3.0–5.4
Iron	95–430	72–200
Zinc	34–92	49–120
Lead	0.29–1.2	0.21–1.1

[a] Adapted from (125). [b] μM g tissue ash.

fant requirements are difficult to estimate because of the large stores of copper present in the newborn (6, 7). During fetal life, the concentration of hepatic copper reaches a value, at term, which is 5 to 10 times that of adult liver. Values comparable to those in adults are not reached until after the fifth year. Cartwright and Wintrobe (6, 7) have estimated that the dietary requirements of copper for infants and children is probably 0.05 to 0.10 mg/kg per day, a requirement that is met by most diets, quite apart from the body stores of copper available for utilization should the intake fall below this amount. Gubler (126), in his report for the Council on Foods and Nutrition, concurred that it is extremely unlikely that copper deficiency could occur in normal humans even on suboptimal diets.

Scheinberg (10) has observed that, in view of the excess over that required of dietary copper, there is considerably more risk of copper toxicity than of copper deficit in man. He has pointed out that if we make the reasonable assumption that the body content of each metal is an index of the respective requirement for copper, or for iron, the body has approximately 40 times as much iron as copper. An average diet, however, delivers only four times as much iron as copper. Therefore, relative to need, there is about ten times as much copper as iron in our food. This disproportion is intensified with hemorrhage, as much more iron than copper is lost with blood. He concluded that this explains why copper deficiency does not occur in human beings. In contrast, the large dietary intake of copper necessitates excretion of practically all we ingest, predominantly through the feces. In 1 year the diet supplies about 10 times as much copper as the total amount in the body, in addition to which we ingest copper from water delivered in copper plumbing and in alcohol from copper stills.

Molybdenum intakes and levels in man

There has been little research on the supply of molybdenum, its requirements, or its metabolism in man. Only two studies of molybdenum balance have been found, that by Engel et al. (127) in preadolescent girls, and by Tipton et al. (60, 128). There was storage of molybdenum when the intake approximated 100 μg/day (127). This sort of retention of an element suggests either repair of a prior deficit or the meeting of growth needs. (In contrast, their metabolic balance findings with copper suggested that the intake considerably exceeded the requirements, which confirms the earlier findings demonstrating clinical copper excess.) One of three subjects on diets delivering approximately 100 μg/day of molybdenum was in negative Mo-balance (60, 128). The subject receiving 210 μg/day and two of those on the 100-μg daily intake were in equilibrium. The subject given a diet containing 460 μg/day was in positive balance.

Analyses of the molybdenum content of many common foods have revealed that purine-containing foods (including liver, kidney, spleen, scallops, dark green vegetables, legumes, and cereals), which are rich in xanthine oxidase, are particularly rich in molybdenum (40, 60, 110). However, four purine-free foods that contain considerable molybdenum are fruits, eggs, butter, and milk. Schroeder et al. (60) have commented that, although the probable daily intake of molybdenum on standard diets might average about 350 μg, marginal or deficient intakes of molybdenum are a distinct possibility, particularly among those ingesting a high percentage of refined foods.

Studies of the metabolic fate and blood and tissue levels of molybdenum in man are also rare, but data are beginning to be accumulated. Molybdenum levels in human organs and the urinary excretion of the element in normal subjects were reported first by Schroeder et al. (60, 129), who also later performed a more extensive analysis. They cite the participation of molybdenum in the xanthine and aldehyde oxidases, and have considered the variation in tissues by age and by geographic areas. Perry and Perry (130) studied the urinary excretion of molybdenum in different population groups and found that the molybdenum in 24-hr urine specimens ranged from 12 to 150 μg/liter, with a mean of 40.8 ± 4.32. There were slight differences between the means of the 14 white and the 10 American black subjects (29.0:44.7). The mean molybdenum level of

single urine specimens from 12 hospitalized African patients was 77.5 ± 24.6, suggesting that the difference seen in the American subjects may have been real, despite the small number studied. This finding is of interest in view of the high copper levels seen in patients with sickle cell anemia (131, 132).

Blood levels of molybdenum are beginning to be reported in the literature now that procedures for its measurement have been developed: colorimetry (133, 134), photoelectric spectrometry (130, 135), emission direct reading spectrometry (136–138), and neutron activation analysis (139, 140). The fragmentary data depicted in Table 3 indicate the paucity of knowledge of molybdenum in whole blood and serum. From preliminary clinical data (114, 136) it appears that patients with chronic disease, or with anemias, have lower serum levels of molybdenum than do healthy subjects. In 181 of 229 samples analyzed by Allaway et al. (134), most of the blood donors tested (134, 136) had low molybdenum levels. In 8 of the 122 samples analyzed by Butt et al. (136) the molybdenum levels were so low as to be undetectable. Allaway et al. (134) stated that molybdenum at concentrations of more than 0.5 μg/100 ml was found in less than one-fifth of the blood samples; only six samples contained more than 10 μg/100 ml, and five of these were collected in one western town. That there was a local factor in the area that necessarily caused an increase in molybdenum retention is negated by the fact that 15 of the 26 samples collected had serum levels so low as to be undetectable.

There is still much to be done to elucidate the biologic fate of administered molybdenum. It has been shown that ^{99}Mo is cleared rapidly from the blood in man (141, 142), and that the main pathway of molybdenum excretion in man is via the kidney (142). The cumulative urinary ^{99}Mo excretion in five days was 16.6 to 27.2% in four patients with neoplasms who had been given a single intravenous dose; it was 24 to 29% in the two patients followed for 10 days. The cumulative fecal excretion of ^{99}Mo for the two patients followed for 10 days was 6.8 and 1%. Although this study was designed merely to show the pathways of excretion of molybdenum, the fact that only approximately one-third of the administered dose was excreted by 10 days indicates body retention, which is suggestive of prior insufficiency.

TABLE 3
Normal blood levels of molybdenum, copper, and iron, reported from the literature

Investigator	Technique	Healthy				Blood donors			
		No. of subjects	Mo	Cu	Fe	No. of subjects	Mo	Cu	Fe
Brune et al. (140)	Activation analysis								
	μg g whole blood	8	0.0033	0.70	430				
Butt et al. (136)	Emission spectrometry								
	mg 100 ml whole blood	47	0.0072	0.0711	38.8				
	μg 100 ml serum	48	3.4	110	171	122	1.8 (8)[a]	106	159
Allaway et al. (134)	Colorimetry								
	μg 100 ml serum					229	0.5–15.7 (181)[a]	ND[b]	ND
Iwata and Ifshits (114)	Fractional spectrography								
	Whole blood								
	μg 100 ml	ND	1.47	ND	ND				
	Erythrocytes								
	μg whole blood		0.75						
	Plasma		0.72						

[a] Figures in parentheses = number of subjects with undetectable Mo levels. [b] ND = no data.

Possible consequences of molybdenum deficiency

Is there malfunction of the electron transport chain? The effects of presumed molybdenum deficiency are still speculative, as there are so few data on intake, levels, and requirements. From the standpoint of molybdenum being a component of two of the iron-containing enzymes in the electron transport system (45–55), a deficit in molybdenum may well find expression in the malfunction of these intracellular enzymes. Many of the symptoms of iron-deficiency anemia, such as fatigue, headache, dyspnea, and paresthesia may be caused by malfunctioning intracellular enzymes, such as cytochrome *c* among other iron enzymes (27, 70, 145). Gillman and Hathorn (144, 145) have further suggested that hemochromatosis and siderosis may also be a consequence of a disruption of iron-containing enzymes. Copper is necessary for cytochrome oxidase activity (29–31); molybdenum is necessary for cytochrome reduction by purine oxidases (45, 54). Because molybdenum has been shown to be necessary for the interaction of xanthine oxidase and aldehyde oxidase with cytochrome *c* in the electron transport chain of reactions (45, 46, 51, 54), a relative molybdenum deficit might contribute equally to the generalized symptoms associated with iron-deficiency anemias.

Is mobilization of iron decreased? That molybdenum plays a role in the utilization of iron is suggested by the fact that it is a component of xanthine oxidase (32–38, 41, 42), which may be involved in the mobilization of iron from ferritin stores (61, 76–80, 86). The studies of molybdenum deficiency in animals have concentrated on the levels of the xanthine and aldehyde oxidase and on the decrease in oxidation of the purines (xanthine and hypoxanthine) to uric acid as a determinant of the deficit (32–42, 52, 56, 60). Except for one report (58) of the prevention by molybdenum feeding of a decline in hemoglobin in newly hatched chicks, study of the influence of molybdenum deficit on iron utilization has been neglected.

Other abnormalities possibly related to deficit in molybdo-metalloenzymes. Schroeder et al. (60) have considered other possible consequences of deficits in the metalloenzymes that are dependent on molybdenum. They call attention to the nephrotoxicity of hypoxanthine, and suggest that xanthine oxidase, which is necessary for its oxidation to uric acid, may thereby function to protect renal integrity. The possibility that xanthine renal calculi may also be a consequence of molybdenum deficit was considered. They have also suggested that aldehyde oxidase may function in circulatory homeostasis, as most aldehydes are vasodilators.

Discussion

Copper and molybdenum, as components of enzymes that mediate the oxidation and reduction of iron, are intimately involved in the utilization of iron. The copper protein, ceruloplasmin, is invoived in the oxidation of iron to the ferric form, for transport in the plasma by transferrin (20–24). Ceruloplasmin has recently been shown to function as a ferroxidase, converting the Fe^{2+}, as it is released from ferritin, to Fe^{3+} (20–22). Although there is evidence of an alternative ferroxidase system in the blood (26), ceruloplasmin has been shown to increase greatly the rate at which transferrin can remove iron from the cells (20–22).

There is evidence that molybdenum, as a component of xanthine oxidase, may be necessary for the conversion of ferric-ferritin to ferrous-ferritin, the step immediately preceding the release of iron from ferritin in intestinal mucosal cells or from liver or hemopoietic storage sites (76–80, 88, 90, 92, 93). Normal xanthine oxidase activity in the intestinal cells may participate in the absorption of iron (delayed phase) by increasing the rate of iron release from the intestinal cellular ferritin (77, 86, 88, 99). It may also participate in the mobilization of iron from the liver stores of ferritin (77–79). As the Fe^{3+}-ferritin $\xrightarrow{Mo\text{-}x\text{-}ase}$ Fe^{2+}-ferritin step is an anaerobic one (76–80), this may be one means of mediation of the release of iron under conditions of relative tissue hypoxia. Because such a condition prevails in anemia, one might expect the normal response to be increased xanthine oxidase activity, with consequent increased release of iron to the blood.

Xanthine oxidase, as well as aldehyde oxidase, also participates in the oxidation-reduc-

tion reactions of the intracellular electron transfer chain (43–55). The molybdenum atoms of these intracellular iron flavoprotein enzymes are the first components of these enzymes to be reduced (49). It is possible that some of the systemic characteristics of iron-deficiency anemias that may respond promptly to iron therapy may be caused by malfunctioning of intracellular iron enzymes of the electron transfer chain. That the relative amounts of copper and molybdenum, present intracellularly, are also important in the functioning of the mitochondrial oxidation–reduction system is indicated by the need for copper for cytochrome oxidase activity (28–31) and the need for molybdenum in cytochrome reduction (45–50).

When there is a relative excess of either copper or molybdenum, there is interference with the activity of the other (8, 11, 100–103). Apparently, a Cu–Mo–S complex is formed when an excess of either copper or molybdenum is present (in the gut, or in the tissues), from which neither metal can be readily mobilized (53, 103, 104, 111, 115–123). In that event, a conditioned deficiency of the metal that is in relatively limited supply is established. An excess of molybdenum over copper, producing signs of copper deficiency, has been reported to occur naturally only in grazing animals on copper-poor, molybdenum-rich forage (8, 104–110). Iron-deficiency anemia is a characteristic finding. In other geographic areas, where the forage is rich in copper and poor in molybdenum, the resultant condition is hypercuprosis (8, 11, 106, 111, 112). In this disease, not only is there hypochromic anemia, but the blood picture is complicated by hemolytic crises, with resultant hemoglobinuria, as well as by xanthinuria and xanthine calculi.

In man, there is considerable evidence that copper excess can present clinical problems (10, 14–19), whereas there is little evidence of clinically verifiable copper deficiency (6, 7, 126). The molybdenum story is not as well developed. There are a few findings that suggest that clinical molybdenum deficit is far more likely than is an excess of this metal (60, 114, 127, 128, 134, 136, 142). In view of the paucity of clinical data on the role of molybdenum in human physiology, it has been necessary to correlate the laboratory findings on the participation of molybdenum in the iron flavoprotein enzymes, particularly xanthine oxidase, with the laboratory and clinical knowledge of the role of this enzyme in the iron–ferritin system. When to this basic knowledge is added the data on the interference by high copper levels with the availability of molybdenum, new insight may be gained into anemias that do not respond optimally to iron, and to iron-deficiency anemias that persist despite high stores of tissue iron. The influence of imbalance in Cu/Mo on abnormalities of purine metabolism requires further elucidation.

Summary

Both copper and molybdenum influence the internal transport and release of iron. There is evidence that copper, as a component of ceruloplasmin, increases the rate of oxidation of ferrous to ferric iron for transport by plasma transferrin; and that molybdenum, as a component of xanthine oxidase, participates in the reduction of cellular ferric to ferrous ferritin. An excess of either copper or molybdenum, by giving rise to a relatively undissociable Cu–Mo–S complex, may produce a deficit of the metal in marginal supply. As dietary excess of copper is known to be common in the United States, and preliminary data suggest that molybdenum intake may not be optimal, it is possible that a conditioned deficit of molybdenum may contribute to abnormalities of iron metabolism and utilization.

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ACUTE COPPER POISONING

An Outbreak Traced to Contaminated Water from a Corroded Geyser

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DURING the past eighteen years, the number of cases of food poisoning from all causes has risen from 119 in 1941 to 7300 in 1958. Although the majority of these were attributed to salmonellæ, many (21,501) causes remained unidentified, and only 27 during that period were attributed to chemicals.

We have recently investigated an outbreak of 18 cases of acute gastroenteritis traced to copper contamination of water from a canteen geyser. The mode of this contamination leads us to believe that this form of poisoning may be more common than is supposed.

THE OUTBREAK

On June 10, 18 of 150 workers in a Liverpool factory collapsed with moderate to severe gastroenteritis after drinking tea in the factory canteen. Their symptoms, consisting of severe abdominal pain with vomiting and diarrhœa, headache, and dizziness, which occurred either immediately or within five to ten minutes of drinking tea, suggested an acute chemical poisoning.

2 of the victims were so collapsed that they were admitted to hospital for twenty-four to thirty-six hours. Chemical analysis of their fæces showed the presence of copper in concentrations of 80 and 50 parts per million (p.p.m.). A diagnosis of copper poisoning was made. Over the next four weeks, regular chemical analysis of their fæces showed decreasing quantities of copper, confirming the diagnosis of acute rather than chronic copper poisoning.

SOURCE OF COPPER POISONING

The tea was prepared in large aluminium teapots, each holding sufficient tea for 18 cups; the water was boiled in a gas-heated geyser. All 18 victims had drunk tea from the same teapot; this being the last pot of tea made that morning. Unfortunately, the residue of the teapot had been discarded, but the geyser which supplied the water was tested. 3 pints of water were drawn off and macroscopically this sample showed a brownish tinge due to suspended particles of matter with a similarly-coloured sediment. Chemical analysis showed that this was due to suspended particles of copper sulphate. The water was very slightly acid (pH 6·7) and the total copper in suspension was 44 p.p.m.

The geyser was now dismantled and examined. It consisted of a gas-heated all-purpose water boiler holding 10 gallons. Externally it was chromium-plated, but the inside part of the boiler and lid was made of copper. Examination of the internal parts showed a blue-grey deposit of copper sulphate encrusted over the top of the boiler lid and around the opening for the float control arm. The boiler was cleaned and re-assembled. The result of chemical analysis of water from the geyser after cleaning was negative for copper.

DISCUSSION

We can trace only three recent references to illness attributable to copper contamination of water from geysers:

Griffin [1] reported an outbreak in which drinking water had become contaminated from a corroded geyser. Copper in drinking water was responsible for an earlier outbreak where the water had been boiled in a large untinned and unlined copper.[2] More recently, in 1956, 5 cases of acute copper poisoning occurred in a factory canteen and this outbreak was apparently due to copper salts from a gas geyser, which had contaminated water stored in it overnight and used to make tea the following day. In this outbreak it was established that copper salts from the flue could have fallen into the water. The water used for the tea was not available for examination, but a further sample yielded 10 p.p.m. of copper.[3]

In this Liverpool outbreak, drinking water was again contaminated by copper salts from a corroded geyser, and it is interesting to examine the reason why only 18 persons were affected out of the 150 who were at risk.

For the purpose of tea making, there were six 4½ pint aluminium teapots in use and tea was prepared by putting a 1½ oz. packet of tea in each teapot and adding boiling water from the geyser as required. When the geyser was dismantled for examination, a heavy deposit of copper sulphate was found on the upper and inner cover of the copper boiler. To one side of this cover there was a circular hole (2 in. in diameter) to give access to the arm of the float. Close to this hole there was a bare patch from which it was concluded that a portion of the copper sulphate had recently broken off. This chemical deposit may have been dislodged from the lid by some sudden disturbance such as the jarring of the teapot against the boiler outlet-tap, so causing the copper sulphate to fall through the hole into the boiling water, and, the tap being

1. Griffin, A. J. B. *J. R. sanit. Inst.* 1951, 71, 1.
2. Hebblethwaite, A. S. *Publ. Hlth*, 1928, 41, 276.
3. *Mon. Bull. Minist. Hlth Lab. Serv.* 1957, 16, 241.

open, the current of flow may have enabled the chemical to pass from the boiler through the tap into the teapot. If this is the correct explanation of the presence of copper in the teapot, it would account for there being only 18 victims out of 150 who were served with canteen tea on that particular morning.

The geyser in this canteen was of a type that is in common use. The makers state in their maintenance manual that in hard-water districts the interior surface of the boiler would require cleaning every three to four months to remove the scale deposited. In soft-water districts, they state, there is rarely any necessity for internal cleaning; servicing, when required, is reduced to the cleaning of burner and flue-ways, renewal of all valve washers, regreasing of gas cocks, and to adjusting the automatic gas-valve. No mention is made specifically of the formation of copper-sulphate deposits. Inquiry showed that this particular geyser had never been dismantled for cleaning or scaling, and it had been in constant use since 1949.

SUMMARY AND CONCLUSIONS

An outbreak of 18 cases of gastroenteritis occurred in a Liverpool factory canteen owing to the contamination of tea by copper from a corroded geyser.

We recommend that this type of geyser should carry a revised maintenance instruction booklet, indicating the possibility of the formation of copper-sulphate deposits and the methods by which these deposits can safely be removed. This form of maintenance should be carried out every three to six months.

We wish to thank Mr. J. F. Clark, Analyst for the City of Liverpool, for the chemical analysis of the tea water and the fæcal specimens.

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ORIGINAL ARTICLES

Biochemical Changes in Blood in Cases of Acute Copper Sulphate Poisoning

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The frequent occurrence of acute copper poisoning, following the ingestion of copper sulphate, either accidentally or intentionally, in India, is now a well-known fact. The clinical manifestations were lucidly described by Modi (1952), Roy Chowdhury *et al.* (1961) and Gupta *et al.* (1952). Roy Chowdhury *et al.* (*loc. cit.*) also described some of the biochemical changes in these cases, and attempted to establish a correlation between these and the haemolysis and jaundice often present in them.

This paper deals only with the biochemical changes in blood occurring in acute copper sulphate poisoning, with special emphasis on the study of copper in serum and whole blood and the serum transminases.

METHODS AND MATERIALS

In all, 40 patients were studied, 27 males and 13 females, of ages between 13 and 85 years. In the first 16 patients was estimated only whole blood copper, in the next 4 cases only serum copper—total as well as direct-reacting. In the subsequent 20 cases both blood copper as well as serum copper estimations were carried out. Serum glutamic oxalacetic transaminase (SGOT) level was determined in all the cases, and serum glutamic pyruvic transaminase (SGPT) only in the last six cases.

Serial studies of copper contents as well as transaminase level were carried out in acute cases only. Besides these, serum bilirubin, plasma protein—total as well as differential, zinc sulphate turbidity, serum alkaline phosphatase, prothrombin time and blood urea estimations were also carried out.

In the samples obtained for copper and enzyme studies, a note was made of any haemolysis and/or icteric tinge, occasionally using the spectroscope for the confirmation of haemolysis. Further, the haemolysis observed in the serum samples was real and not due to faulty collection of blood. During precipitation of proteins, in the course of serum copper estimations, a definite yellow to green colour was given by the icteric sera as opposed to the normal ones. The methods used and normal values were as follows:

(*i*) *Copper estimations*—For total and direct reacting serum copper and whole blood copper, the techniques employed were, in principle, the same as proposed by Gubler *et al.* (1952, 1953). 8 ml. of blood was collected in a plain tube, dried after washing with all-glass redistilled water, for serum copper estimations and 2 ml. of blood in oxalated bottles for whole blood copper estimation. Attempts were made to collect the first

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samples as soon as possible after admission, and to ascertain the time that elapsed after the ingestion of the poison. In a few cases the estimation of direct-reacting copper was made impossible by the severe jaundice. Normal values (Singh and Singh, 1962) are given in Table 1.

TABLE 1—SHOWING NORMAL VALUES OF COPPER

Copper	No. of individuals	Age range (years)	Range of values (μg. %)	Mean values with S.D.
Total serum copper ...	35	10-60	56-400	151·6 ± 75·1
Direct reacting serum copper ...	24	11-53	0-64	21·8 ± 20·0
Whole blood copper ...	22	10-60	144-288	217 ± 58·2

(ii) *SGOT and SGPT*—The levels of these enzymes were estimated in the serum by a colorimetric technique standardised in our own laboratory (Singh and Singh, *loc. cit.*). The normal range for SGOT, as determined from 61 individuals, was 10-100 units/ml. and for SGPT, from 20 individuals, was 10-90 units/ml.

(iii) *Liver function tests and blood urea*—Serum proteins were estimated by Biuret method, serum bilirubin by diazotisation method, $ZnSO_4$ turbidity by the usual technique, serum alkaline phosphatase by the method of King and Armstrong, and blood urea by urease-nesslerisation method, as described by Harrison (1957) and the normal values were also taken from the same source. Prothrombin time was done by using Quick's one-stage technique, a control being put up simultaneously every day.

RESULTS

The results of copper studies and serum transaminase levels along with the serum bilirubin values and notes on haemolysis and icterus are given in Table 2.

(A) *Copper studies and observations on haemolysis and icterus*—The earliest time when we could collect a blood sample was 3 hours after ingestion of copper sulphate (Case 33) and that was the case which, incidentally, showed the highest value for direct reacting copper, the same constituting the major fraction of total serum copper. Two more cases (Nos. 31 and 32) in which the blood had been collected 3½ hours and 5 hours respectively after ingestion of $CuSO_4$ also showed high levels of direct-reacting copper, again constituting a major fraction of the total serum copper. The latest time when we noted a high level of direct-reacting serum copper was 24 hours after ingestion, in Case 38. In Case 20, high level of direct-reacting copper was seen even on the 6th day, but this was probably due to copper contamination. In all, only 6 of the 24 cases, in whom serum copper estimations were done, showed high serum copper levels mainly due to the raised direct-reacting fraction in their first samples collected between 3 and 24 hours after ingestion of $CuSO_4$. No case showed any increase in indirect-reacting fraction of serum copper except when haemolysis was there in which case copper-proteins (e.g. erythrocuprein) released from erythrocytes were obviously the responsible factors. In Case 20, the indirect reacting fraction was found to be higher on the 13th day as compared to the preceding values, but in another case (No. 23) no such secondary rise was seen even on the 24th day.

Whole blood copper levels were found to be increased in 28 out of the 36 patients in whom this estimation had been carried out. The earliest time when the increase was noted appeared to be 3 hours after ingestion of the poison and the latest was noted on the 10th day. In all those patients which showed normal blood copper values throughout, blood had been collected very late after ingestion. The highest values were found in those cases in whom the blood had been drawn within 48 hours. In Case 35, there was a secondary rise in blood copper, the value on 10th day being higher than the value on 2nd day. This patient expired on the 12th day. None of the other patients showed any secondary rise in whole blood copper. The normal blood copper levels were reached, within 17 hours to 7 days in the majority of the patients. The highest value for blood copper was noted in Case 34 who expired due to severe intravascular haemolysis.

The appearance and extent of haemolysis bore a positive correlation with blood copper levels except at the start of illness. The fall in copper levels was attended with disappearance of haemolysis. Icterus, however, usually appeared later than haemolysis and often outlasted it, although in some patients icterus persisted just for a few hours and haemolysis did not appear at all or *vice versa*. Haemolytic sera were obtained in 19 patients and icteric sera in 14 cases, about half the number showing both.

(B) *Serum transaminases*—Out of 40 patients only 7 showed normal SGOT levels, and they exhibited neither haemolysis nor icterus and were very mild cases. In the rest of the patients the SGOT levels varied from 106 to 429 units per ml.,

TABLE 2—SHOWING BLOOD BIOCHEMICAL CHANGES

Case No. and name	Duration after ingestion of copper sulphate in days (approx.)	Serum copper (μg. per cent)		Whole blood copper (μg. per cent)	SGOT level (units/ml.)	SGPT level (units/ml.)	Haemolysis in serum	Icterus in serum	Serum bilirubin (mg. per cent)	Blood urea (mg. per cent)	Remarks (clinical toxicity)
		Total	Direct								
1	2	3 A	3 B	4	5	6	7	8	9	10	11
1 P	3	—	—	—	—	—	—	—	0·6	38	Moderate
	4	—	—	480	84·6	—	No	Yes	—	—	
2 SU	2	—	—	—	—	—	—	—	0·4	38	Moderate
	10	—	—	265	45	—	No	No	—	—	
	14	—	—	—	—	—	—	—	0·4	—	
	16	—	—	180	—	—	No	No	—	—	
3 S	2	—	—	—	250	—	Yes	Yes	1·4	40	Severe
	4	—	—	—	180	—	Yes	Yes	—	—	
	7	—	—	303	200	—	Yes	No	—	—	
	8	—	—	—	—	—	No	No	0·2	—	
	10	—	—	180	100	—	No	No	—	—	
4 SK	2	—	—	—	95	—	—	—	—	—	Mild
	5	—	—	340	—	—	—	—	—	—	
5 JK	27/24	—	—	380	220	—	Yes	No	—	37	Moderate
	5	—	—	280	143	—	No	No	—	—	
6 MC	1	—	—	320	144	—	No	No	0·2	—	Mild
7 BP	4½	—	—	292	96	—	No	No	0·2	—	Mild
8 KL	2	—	—	210	33	—	No	No	—	30	Mild
9 BS	15/24	—	—	—	—	—	—	—	1·5	—	Severe
	39/24	—	—	436	212	—	Yes	Yes	—	—	
	5½	—	—	—	—	—	—	—	1·2	—	
10 DC	20/24	—	—	174	106	—	No	No	0·4	—	Mild
11 ML	1½	—	—	220	112	—	No	No	0·3	—	Mild
12 AK	1	—	—	—	—	—	No	No	0·6	—	Mild
	2	—	—	300	112	—	No	No	0·6	38	
13 D	17/24	—	—	274	275	—	Yes	No	—	—	Moderate
	4	—	—	—	148	—	No	No	0·4	—	
14 CL	26/24	—	—	360	120	—	No	No	0·2	—	Mild
15 DR	2	—	—	527	240	—	Yes	No	—	32	Moderate
	6	—	—	350	130	—	No	No	—	—	
16 RK	4	—	—	380	85	—	No	No	2·0	—	Mild
17 BB	11/24	289	30·4	—	146	—	No	No	0·2	33	Mild
18 SD	1	—	—	—	264	—	Yes	Yes	0·8	41	Severe
	2	266·6	—	—	288	—	Yes	Yes	—	—	
	5	277·5	—	—	180	—	No	Yes	1·2	—	
19 BL	2	105	30·9	—	220	—	No	No	—	—	Mild
20 MR	1	—	—	—	378	—	Yes	Yes	—	70	Severe
	2	—	—	—	—	—	No	Yes	2·0	—	
	5	—	—	—	143	—	No	Yes	—	—	
	6	190	95	—	220	—	No	No	0·2	—	
	8	220	0	—	110	—	No	No	—	—	
	10	173	6·6	—	44	—	No	No	—	—	
	13	248	9·5	—	36	—	No	No	—	—	
21 JK	1	99	4·5	550·2	120	—	Yes	No	0·2	—	Mild
22 NM	8/24	142	10·0	307·0	150	—	No	No	—	—	Mild
23 Rag	3	114	41·2	—	154	—	No	Yes	—	47	Moderate
	6	116	0	282	80	—	No	No	—	—	
	24	116	13·0	133	165·6	—	No	No	0·4	—	

TABLE 2—(*Contd.*)

Case No. and name	Duration after ingestion of copper sulphate in days (approx.)	Serum copper (μg. per cent)		Whole blood copper (μg. per cent)	SGOT level (units/ml.)	SGPT level (units/ml.)	Haemolysis in serum	Icterus in serum	Serum bilirubin (mg. per cent)	Blood urea (mg. per cent)	Remarks (clinical toxicity)
		Total	Direct								
1	2	3 A	3 B	4	5	6	7	8	9	10	11
24 PB	1	131	33	342	120	—	No	No	0·4	23	Mild
25 SD	21/24	127	27	647	121	—	Yes	Yes	1·75	37	Moderate
	2¼	109·5	20·5	208	132	—	No	Yes	—	—	
	4	132·5	220	—	110	—	No	No	—	—	
26 SD	29/24	113·0	6·5	888	248·4	—	No	Yes	—	—	Moderate
	2¼	210·5	—	—	72·0	—	No	No	—	—	
27 GD	18/24	79	0	200	77	—	No	No	—	33	Mild
28 YR	13½/24	—	—	709	88	—	No	No	—	—	Mild
	2	—	—	371	88	—	No	No	—	—	
	5	95	21	257	80	—	No	No	0·25	37	
29 RK	35/24	211	0	800	165	—	No	No	0·2	—	Mild
30 SD	7/24	242·2	61	284	132	—	Yes	No	—	—	Mild
	4	134·5	8·1	—	121	—	No	No	0·2	—	
31 VD	3½/24	444	150	449	165	—	Yes(±)	Yes(±)	—	—	Moderate
	8	165	35	169	93·15	—	No	No	0·2	—	
32 NK	5/24	413·5	298	483	150	—	No	No	—	—	Severe
	1	—	—	—	—	—	—	—	0·4	40	
	63/24	178	64	166	429	—	No	Yes	—	—	
	10	—	—	—	130	—	No	No	—	37	
33 RS	3/24	848	557	1047	154	—	No	No	—	—	Severe
	25½/24	139·9	0	176	154	—	No	Yes	—	47	
	3½/24	70·0	0	216	146	—	No	No	0·2	—	
34 CD	25/24.	199	15	3240	143	—	No	Yes	—	—	Expired (severe intravascular haemolysis)
	50/24	—	—	—	270	—	Yes	Yes	—	30	
35 JS	1	—	—	—	120	—	Yes	No	—	35	Expired (hepatorenal failure)
	1½	—	—	310	190	—	Yes	Yes	—	73	
	2	—	—	—	250	—	Yes	Yes	—	380	
	4	177	—	—	310	—	Yes	Yes	—	318	
	10	240	—	500	540	—	Yes	Yes	30	262	
	12	129·8	—	—	238	224	Yes	Yes	—	—	
36 GR	11/24	977·6	277	277·3	364	187	Yes	No	—	—	Expired (severe intravascular haemolysis)
37 MR	3	205·2	43	603·7	128	66	Yes	No	—	—	
38 BL	1	410	150	2150	220	429	Yes	Yes	—	—	Severe
	2¼	—	—	309·3	400	440	Yes	Yes	—	—	
	4	131·5	—	280	280	440	No	Yes	—	—	
	6	200	43·1	113·2	308	429	No	Yes	—	—	
	10	172·3	74·2	175·3	145	364	No	Yes	—	—	
	11	57·0	0	167·2	198	428	Yes	No	1·2	—	
39 Zi.	16/24	—	—	—	88	132	No	No	—	—	Mild
	40/24	123	5	100	116	139	Yes	No	—	—	
40 KL	2	302	—	1200	264	228	Yes	No	—	—	Expired (haemolysis)

the rise being found as early as 3 hours after, and as late as the 24th day after ingestion of poison. In Case 23, SGOT levels had come down to normal on the 6th day, when he was discharged, but had risen again on the 24th day when he was readmitted with pain in the abdomen.

Of the six patients in whom SGPT levels were also determined, Case 37 showed normal SGPT level but raised SGOT level (serum haemolysed but not icteric); Case 35 showed on the 12th day, that is, the day of his death, almost equal rise of SGOT and SGPT level (both haemolysis and icterus present). Case 36 showed, 11 hours after ingestion of poison, a much greater rise of SGOT than SGPT (serum very much haemolysed). This patient also showed persistently raised SGOT and SGPT levels, the SGPT level always remaining higher than the SGOT level. Case 39 showed almost normal SGOT in the first 40 hours but definitely raised SGPT level, and Case 40 showed almost equal rise of SGOT and SGPT.

(C) *Liver function tests and blood urea*—A tendency to reversal of albumin/globulin ratio was noted, as reported by Roy Chowdhury *et al.* (*loc. cit.*), but high alkaline phosphatase (18 K.A. units) and $ZnSO_4$ turbidity (10 units) were noticed only in one patient each. Serum bilirubin levels varied between 0·2 mg. per cent and 30 mg. per cent. Serum bilirubin estimations were made difficult by the haemolysis and therefore recourse had to be taken to making a note of icteric tinge in sera apart from doing serum bilirubin estimations, as and when possible.

Prothrombin time was on the high side of normal in most of the cases, and blood urea was found to be raised in four cases, the highest value of 390 mg. per cent being found in Case 35. Poor separation of serum, attributed to poor clot retraction, was observed in some cases.

(D) *Mortality*—Four patients in this series died (Cases 34, 35, 36 and 40).

Discussion

The observation of not only a high value of direct-reacting copper but also a greatly increased whole blood copper within 3 hours of the ingestion of copper sulphate shows: (a) Inorganic copper is absorbed quickly from the intestines. (b) From the serum it moves at a fast rate into red blood corpuscles, where it attains a high concentration.

Gubler *et al.* (1953), on injecting inorganic copper intravenously into mice, observed a rise in the direct-reacting copper of plasma which fell quickly to come down to normal in 4 hours' time. They also observed a rise in erythrocyte copper in 10 minutes' time and its fall to normal level in 24 hours' time.

The subjects in this series had all taken copper sulphate orally, often in solid from and in large amounts. Considering these facts, one would not be surprised to find raised direct-reacting copper in serum as late as 24 hours, even though there is enough evidence to show that ionic copper disappears from serum quite fast. The high levels of whole blood copper attained as well as their perpetuation for many days would also be explicable on the same grounds. The copper moving into the erythrocytes was obviously held quite firmly, because in haemolysed samples it was the 'indirect copper' rather than the direct fraction in serum which was elevated. The secondary rise of serum copper, because of the elevation of indirect-reacting fraction reported in studies with Cu^{64} (Adlstein and Vallee, 1962), was observed only in one case (No. 20) on the 13th day, but in another case (No. 23) there was no such rise even on the 24th day although evidence of copper toxicity was still there.

Possibly secondary rise of serum copper occurs after a long time.

The accumulation of copper in blood cells would appear to be the responsible cause for haemolytic tendency. Goldberg *et al.* (1956) found that copper salts in higher concentrations precipitated haemoglobin out of the solution *in vitro* experiments. This is what might be happening *in vivo* also, because, even though haemoglobin has got much greater avidity for iron, a great excess of copper ions will shift the balance in favour of cuprohaemoglobin due to the law of mass action, and, following the dictates of the same law, precipitation will ensue. This would naturally make the erythrocytes far more amenable to destruction though not necessarily fragile. Gubler *et al.* (1953), Bush *et al.* (1955) and Goldberg *et al.* (*loc. cit.*) have also propounded that increased concentration of copper in erythrocytes makes them more destructible.

Haemolysis was noted only once in an early sample and that too to a slight degree (Case 31) but in most of the cases it appeared 12 to 24 hours after ingestion of the poison, the time that appears necessary for the derogatory changes to reach an optimal pitch. The haemolytic tinge, when present, was noticeable in most cases, immediately after the withdrawal of blood, and might indicate intravascular haemolysis rather than extravascular. Three of the four mortalities in this series were definitely the result of severe intravascular haemolysis (Cases 34, 36 and 40).

The subject of jaundice in copper poisoning is a rather complex one. Haemolysis must be a

causal factor, but is surely not the whole cause. Persistently raised SGOT and SGPT, especially the latter, along with jaundice even when haemolysis was absent, would show that jaundice is, at least partly, hepatic in origin. Wolff (1960), Saltman *et al.* (1959) and Bal and Haldar (1961) showed accumulation of copper in the rat liver cells, accompanied by cell necrosis. This can be applicable to human beings too. The available data on serum transaminase levels would tend to show that the nature of hepatic lesion is both cellular as well as obstructive. In Case 38, SGPT level remained persistently higher than SGOT, a fact characteristically encountered in obstructive jaundice (Wroblewski, 1958). This may mean that the obstructive factor is predominant in copper toxicity as opposed to other types of toxic hepatitis especially carbon tetrachloride poisoning. The intrahepatic obstruction could be the result of haemolysis.

In interpreting the serum transaminase levels, we have ignored any possible contribution from heart cell necrosis because there is no evidene, at least clinically, of any direct effects of copper toxicity on heart. The main brunt of copper toxicity is borne by erythrocytes, the liver and kidneys, in descending sequence. Kidney cell necrosis must be contributing a little to the rise of SGOT levels, because transaminase content of kidney tissue is less than that of the heart, liver and skeletal muscle only, but the major contribution must come from the liver and from erythrocytes, if the haemolysis is gross. SGPT rise is, however, a better index of hepatic insult than the SGOT rise.

A fall in blood copper level is presumably attended with rise in copper content of tissues, specially the liver and kidneys, which would explain the high serum transaminase levels long after the blood copper levels have come down to normal. In Case 23, a raised SGOT level even on the 24th day, with normal blood copper level, would mean continuing tissue damage by deposited copper.

The conventional liver function tests are, in our opinion, of very little importance, either in diagnosis or prognosis of copper sulphate poisoning. For diagnostic purposes, total and direct-reacting serum copper along with whole blood copper or erythrocyte copper estimations on a sample collected as early as possible in illness can be of help. Only a very rough estimate of the dose of copper sulphate can be made from blood copper studies, keeping in view the fate of copper ions entering the serum. A diagnosis of copper poisoning after 10th day would be possible only by the demonstration of raised copper content of the liver or kidney, which has obvious difficulties, dealing as we are with human beings. For prognostic purposes, serial studies of SGOT and SGPT levels along with blood urea estimations appeared to be the best.

Summary

An analysis of biochemical changes in blood in 40 patients with acute copper sulphate poisoning in which the fate of ingested copper as copper sulphate is discussed.

The probable factors responsible for the haemolysis and jaundice, encountered in this condition, are analysed.

The usefulness and limitations of serum and whole blood copper estimations in the diagnosis of copper poisoning are discussed.

The importance of serial serum transaminase determination in the prognosis of the disease is emphasised.

The possible nature of hepatic lesion is surmised on the basis of serum transaminase figures.

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Chronic Hepatitis As a First Manifestation of Wilson's Disease

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In four males and three females, from 12 to 28 years of age, chronic hepatitis was diagnosed because of manifestations indistinguishable from those generally associated with this disease. From 3 months to 4½ years after the onset of hepatic illness, persistence of the hepatic disorder for 4 years in one boy, a diagnosis of Wilson's disease in one girl's sibling, the development of neurologic symptoms in four of these patients, and the accidental discovery of Kayser-Fleischer rings in a man led to the suspicion of Wilson's disease in each of the seven patients. Although copper toxicity is etiologic in probably no more than 5% of patients with chronic hepatitis, it is susceptible to specific and effective pharmacologic therapy, unlike most other forms of hepatitis. Consequently, the disease should be borne in mind and the concentration of ceruloplasmin in serum measured in every child or young adult under 30 years of age with idiopathic chronic hepatitis.

WILSON ORIGINALLY described progressive lenticular degeneration as the "constant. . .association of a. . . profound degree of cirrhosis of the liver" with neurologic disorders (1). The liver disorder has ever since been generally characterized as cirrhosis, in part, no doubt, because of Wilson's description. In the six decades since the publication of Wilson's monograph it gradually has become apparent that copper toxicity is the cause of the disorder, that clinical manifestations of copper-induced damage to the liver not infrequently antedate any neurologic symptom or sign (2-6), and that several of the clinical and pathological forms of hepatic disorder reported in patients with indubitable Wilson's disease are distinguishable from cirrhosis (7-14). One of these is the entity termed "chronic active hepatitis." In this paper we consider the clinical significance of the case histories of seven patients who sought medical care for what was initially diagnosed as chronic hepatitis but later proved to be Wilson's disease.

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Patients and Methods

The diagnosis of Wilson's disease was confirmed in six of our patients by the demonstration of Kayser-Fleischer rings (15) and in the seventh patient (Patient 3) by a serum ceruloplasmin concentration of 1 mg/100 ml (normal, 20 to 40 mg/100 ml) and a hepatic copper concentration of 1,531 μg/g dry liver (normal, less than 50 μg/g dry liver). Every patient, except Patient 6, had deficiency of ceruloplasmin (16, 17); every one excreted more than 100 μg of copper per day in the urine (11, 18) (normal, less than 50 μg/24 hr); and the hepatic concentration of copper exceeded 250 μg/g dry liver in each of the three patients in whom it was measured (10, 18, 19) (Table 1).

CASE REPORTS

Patient 2: This Irish girl had a normal childhood and development until 1966, when, at age 11, abdominal distention, jaundice, and anemia appeared rather acutely. She was found to have ascites, splenomegaly, hypochromic anemia, and laboratory evidence of liver dysfunction (Table 1). Because an intra-abdominal tumor was suspected, exploratory laparotomy was carried out. The liver was seen to be grossly and histologically abnormal, with acute and chronic hepatitis (Figure 1). With a low-salt diet and 15 mg of prednisone daily, her ascites diminished, and her ability to tolerate activity increased.

In January 1967 she was reevaluated after a younger brother (who had had "hepatitis" 2 years earlier), who had started to drool, tremble, and manifest progressive incoordination and inattention in school, was shown to have Wilson's disease. Ultimately, four of the six children of this family, including Patient 2 (Table 1), proved to have this illness.

Patient 6: "Infectious mononucleosis," with apparent hepatic involvement, was diagnosed in an 18-year-old Puerto Rican college student in 1966. She recovered. In 1968 jaundice appeared, lasted several weeks, and was attributed to "infectious hepatitis." Eight months later fatigue, weakness, ascites, and splenomegaly were noted, and she was found to be anemic and leukopenic and exhibited laboratory evidence of liver dysfunction (Table

1). Lower esophageal varices were demonstrated. Liver biopsy was not considered advisable because of a prolonged prothrombin time, but a diagnosis of chronic hepatitis was made on clinical grounds.

With nonspecific therapy she recovered sufficiently to be married in March 1969, only to have ascites and edema recur shortly. In October 1969, 2 months after peritoneoscopy had shown ascites and a shrunken liver, a temper tantrum and the appearance of tremors led to the suspicion of Wilson's disease, which was confirmed when bilateral Kayser-Fleischer rings were seen on slit-lamp examination. There was a normal serum ceruloplasmin concentration of 25 mg/100 ml but an abnormally high 24-hour urinary copper excretion of 295 μg. Serum gamma globulin was abnormally elevated to 40.9%.

Specific treatment with D-penicillamine, in doses as high as 3 g daily, together with appropriate attempts at diuresis and correction of electrolyte imbalance, was followed by transient improvement in the patient's hepatic and neurologic disorders and by a fall in serum ceruloplasmin to 6.8 mg/100 ml (Figure 2). Within 2 months, however, she rapidly worsened, to die of pneumonia and renal insufficiency in September 1970.

Patient 7: In 1962, his medical history having previously been uneventful, lymphadenopathy, epigastric pain, fever, hepatomegaly, and splenomegaly developed in this Jewish medical student at age 20. An initial diagnosis of infectious mononucleosis was made, despite a heterophil agglutination positive only in a titer of 1:14. A serum glutamic-pyruvic transaminase (SGPT) level of 219 Karmen units/ml was also reported. He recovered completely, although splenomegaly persisted.

In November 1968 renal colic was followed by passage of a calcium oxalate stone, at which time the transaminase values were normal. One month later malaise and a sore right upper quadrant were accompanied by slight jaundice and transaminase elevations (800 to 1,000 Karmen units/ml). Hospitalized briefly, he was found to be mildly febrile and obtunded, with diarrhea and urinary frequency but no nausea, vomiting, or bleeding. He convalesced slowly, with minimally abnormal tests of liver function, such as SGPT and serum glutamic-oxalacetic transaminase (SGOT) values of 60 Karmen units/ml.

Persistence of mild malaise, slight elevation of alkaline phosphatase, and Australia antibody in his serum justified liver biopsy in the fall of 1969. The histologic appearance of the sample was interpreted as "resolving chronic active hepatitis" (Figure 3). No treatment was prescribed, and symptoms and laboratory findings changed little in the ensuing year.

In November 1970, because of mild conjunctivitis, he consulted a fellow resident—an ophthalmologist—who discovered bilateral Kayser-Fleischer rings. Deficiency of serum ceruloplasmin concentration (15.1 mg/100 ml) and a 24-hour urinary copper excretion of 390 μg confirmed the diagnosis of Wilson's disease.

Discussion

"Chronic hepatitis" is an ill-defined syndrome, with insidious onset and a prolonged course of chronic parenchymal disease and inflammation (19-22). Necrosis of hepatocytes, erosion of the limiting plates, and mononuclear cell infiltrations, generally most intense at the peripheries of lobules, are frequent histologic findings. Deficient synthesis of several plasma proteins, including coagulation components, and hypergammaglobulinemia, transaminase elevations, and hyperbilirubinemia reflect parenchymal damage. The precise pathogenic significance of the presence of Australia antigen; of heterophilic antibodies or of antibodies to Australia antigen, nuclear material, mitochondria, or smooth muscle; of a positive latex fixation test; or of lupus erythematosus cells is obscure, although any of these may be demonstrable in a patient with chronic hepatitis (22).

Our seven patients had enough of these features

Figure 1. Surgical biopsy specimen of liver obtained from Patient 2. There is marked distortion of the lobular architecture and evidence of parenchymal collapse. The remaining hepatocytes are variable in size, with some showing various degrees of cytoplasmic degeneration. There are areas of focal necrosis and scattered eosinophilic cells resembling Councilman bodies (*arrow*). Inflammation, with round cells and histiocytes, is prominent. There is some fibrosis and marked proliferation of Kupffer cells. (Hematoxylin and eosin; magnification, × 100.)

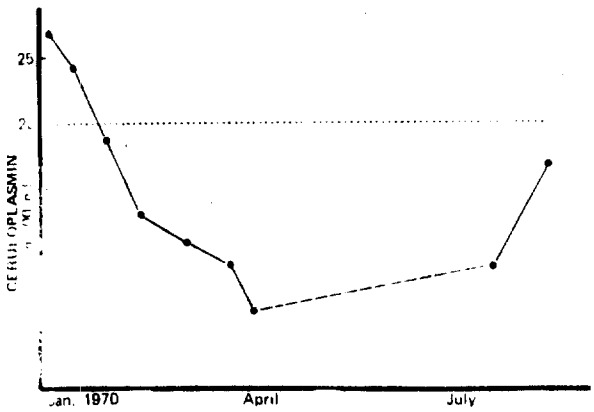

Figure 2. Variations in serum ceruloplasmin concentrations in Patient 6 over an 8-month period, during which improvement in her liver disease was accompanied by a fall in serum ceruloplasmin to levels characteristic of patients with Wilson's disease. The lower limit of the normal range of serum ceruloplasmin concentration (20 mg/100 ml) is indicated.

to justify the diagnosis of chronic hepatitis. The four liver biopsy samples available were generally compatible with this diagnosis (Table 1, Figures 1 and 3), and in only one of them might a pathologist have been expected to attribute the peculiar fatty infiltration adjacent to an area of collapse to Wilson's disease (4, 8, 13). At first, infectious mononucleosis or viral hepatitis was considered as the probable cause of the chronic disorder in all seven patients, because no distinctive clinical features were noted before tests specific for Wilson's disease were performed.

Once Wilson's disease was suspected—because of a 4½ year course of "chronic hepatitis" (Patient 1); when a sibling was found to have Wilson's disease (Patient 2); when the development of neurologic signs (Patients 3 to 6) was noted (1, 2, 11); or because of the accidental discovery of Kayser-Fleischer rings (Patient 7)—it was easily confirmed by finding one or more of the following five tests or abnormalities of copper metabolism to be diagnostically positive.

First, the unequivocal demonstration of Kayser-Fleischer rings confirmed the diagnosis of Wilson's disease (15) in Patients 1, 2, and 4 to 6, and, of course, in Patient 7.

Second, the presence of less than 20 mg of ceruloplasmin per 100 ml of serum in a patient with chronic hepatitis made Wilson's disease highly probable (Patient 1-5, 7). But deficiency of ceruloplasmin, although found in more than 95% of all patients with Wilson's disease (11, 23), may temporarily give way to a normal concentration of the protein, as in Patient 6 (Figure 2), because of hepatic inflammation or necrosis or an elevated plasma level of estrogen, each of which is frequently associated with hepatitis of any cause and each of which can increase the synthesis of ceruloplasmin, even in patients with Wilson's disease (24, 25). Consequently, a normal or even an elevated serum ceruloplasmin level, which would virtually rule out the diagnosis of Wilson's disease in a patient with predominantly central nervous system symptoms, cannot be so conclusive when hepatitis is the presenting disorder. Conversely, severe hepatitis and liver failure not caused by Wilson's disease may rarely be associated with low concentrations of ceruloplasmin (26) as well as of many other plasma proteins whose synthesis may be impaired nonspecifically. Thus, deficiency of ceruloplasmin is neither a necessary nor a sufficient condition for the diagnosis of Wilson's disease in a patient with chronic hepatitis, although it almost always is a necessary one in a patient in whom this diagnosis is suspected because of the family history or of neurologic or psychiatric disease.

Third, if biopsy is not interdicted by clotting abnormalities and quantitative analysis of the sample shows its copper content to exceed 250 μg/g dry liver, the patient with chronic hepatitis can be assumed to have Wilson's disease (Patients 2 to 4) (23, 27). The

Figure 3. Fragment of needle biopsy specimen of liver from Patient 7. Hepatocytes are of variable size and density. There is some mononuclear cell infiltration and some fibrosis "consistent with chronic hepatitis." (Hematoxylin and eosin; magnification, × 180.)

Table 1. Clinical and Laboratory Data in Seven Patients with Chronic Hepatitis

Patient	Sex	Age at Onset of "Chronic Hepatitis"	Sequential Clinical Manifestations	Initial Laboratory Results*
		yr		
1	M	8	Fever, splenomegaly, malaise; icterus, edema, gynecomastia	Hb, 12.4; bilirubin, 2; SGPT, 58; cephalin flocculation, 3+; globulins, 3
2	F	11	Fatigue, icterus, ascites, splenomegaly	Hb, 9.6; bilirubin, 3.9; SGOT, 119; SGPT, 75; albumin, 2.1; globulins, 2.9
3	M	12½	Abdominal pains, nausea, vomiting, epigastric discomfort and tenderness, hepatosplenomegaly	Hct, 41; bilirubin, 0.3; SGOT, 91; SGPT, 180; alkaline phosphatase, 32.5 K.A.U.; IgG, 2,500; heterophil positive
4	M	12	Hepatosplenomegaly, found when brother died of "hepatitis"	Hb, 12.2; bilirubin, 1.8; SGOT, 50; SGPT, 52; cephalin flocculation, 4+; albumin, 3.7; globulins, 2.2; alkaline phosphatase, 19.5 B.U.; prothrombin time, 22/15 sec
5	F	14	Fatigue, edema, ascites, hepatosplenomegaly	Hb, 10; bilirubin, 1.5; SGOT, 56; albumin, 2.5; gamma globulin, 2.6 (41%); prothrombin time 22/13 sec; thymol turbidity, 9.7; BSP, 43% retention; latex fixation, positive
6	F	20	Amenorrhea; "infectious mononucleosis"; jaundice; ascites, splenomegaly, esophageal varices; anasarca	Hb, 10.9; bilirubin, 3.4; SGOT, 82; SGPT, 100; albumin, 2.7; globulins, 4; prothrombin time, 21/13 sec; cephalin flocculation, 4+; latex fixation, positive
7	M	26	"Infectious mononucleosis" at age 20; splenomegaly; malaise, right upper quadrant soreness, jaundice, fever	Hb, 13.8; bilirubin, 4.5; SGOT, 800; SGPT, 875; alkaline phosphatase, 21 K.A.U.; albumin, 3.1; gamma globulins, 1.8 (28%)

* Hb = hemoglobin, SGPT = serum glutamic-pyruvic transaminase; SGOT = serum glutamic-oxalacetic transaminase; Hct = hematocrit; K.A.U. = King Armstrong units/100 ml; B.U. = Bodansky units/100 ml; BSP = sulfobromophthalein. Units of measure are, for albumin, g/100 ml; for bilirubin, mg/100 ml; globulins, g/100 ml; hemoglobin, g/100 ml; IgG, mg/100 ml; SGOT and SGPT, Karmen units/ml; and thymol turbidity, Maclagan units.

only other disorder of which we are aware that can be accompanied by a hepatic copper concentration of this magnitude is biliary cirrhosis (28, 29), and patients with this form of liver disease have normal concentrations of serum ceruloplasmin and characteristic hepatic histopathology (30). There is the possibility that electron-microscopic examination of the liver may prove in the future to be diagnostically specific for Wilson's disease (13, 31), although this does not appear to be true of light microscopy.

Fourth and fifth, are evidence of a marked diminution of the incorporation of radioactive copper into ceruloplasmin (32-34) and of the urinary excretion of more than 100 μg of copper in 24 hours (Patients 1 to 7) (11, 27, 34). The former test is particularly helpful in patients with normal serum ceruloplasmin concentrations in whom liver biopsy cannot be performed.

We have seen about 200 patients with Wilson's disease, of whom about a dozen presented with chronic hepatitis. These figures probably lead to a falsely low estimate of the incidence of chronic hepatitis in Wilson's disease, since there have been patients who have died of progressive chronic hepatitis and in whom a postmortem diagnosis of Wilson's disease is made, occasionally long after a living sibling has been found to have Wilson's disease (4, 35).

There are only scant data about the converse situation. Thus, in one group of 54 (36) and in another of 23 patients (37) with chronic active hepatitis, a patient with Wilson's disease was found. We believe it is unlikely that more than 5% of such patients, even if properly studied, will be found to have Wilson's disease. But since they are virtually the only patients with "chronic hepatitis" for whom specific and effective therapy is available, it is mandatory for the physician to be certain that, in *his* patient with chronic hepatitis, Wilson's disease is not the underlying cause. This means that one or more of the five diagnostic tests listed above should be performed in every patient under 30 years of age with "chronic hepatitis."

Although most patients with Wilson's disease first seek medical care because of neurologic or psychiatric symptoms, almost all of them also have liver disease

Table 1. (Continued)

Histopathologic Findings	Events Leading to Diagnosis of Wilson's Disease	Age at Diagnosis of Wilson's Disease	Serum Ceruloplasmin	Hepatic Copper
		yr	*mg/100 ml*	*μg/g dry wt*
—	Persistence of hepatic abnormalities	12	15	
See Figure 1	Diagnosis of Wilson's disease in a sibling	12	3	892
	Dysphagia	13	1	1,531
—	Dysarthria, clumsiness, and deteriorating school work	16	5.9	687
Stromal collapse; irregular regeneration of hepatocytes; round-cell infiltration; proliferated bile ductules.	Dysarthria	16	5	
Distorted lobules; fibrosis; moderate round cell infiltration; marked bile ductular proliferation.	Temper tantrums, tremors	22	25.2 (*See* Figure 2	
See Figure 3	Accidental finding of Kayser-Fleischer rings	28	15.1	

(4-9, 27, 34). Furthermore, there is no reason to doubt that the liver lesion in every patient with Wilson's disease is caused by copper. Thus, a positive heterophil test, unaccompanied by any other characteristic features of infectious mononucleosis (Patients 3, 7), is more likely a reflection of this test's nonspecificity than it is an indication of coexistent mononucleosis. Similarly, there is no reason to indict hepatitis virus as a necessary adjuvant to copper in causing the chronic hepatitis seen in these patients.

Treatment with D-penicillamine (38) and moderate restriction of dietary copper has led to dramatic, marked, and sustained improvement in six of our patients, followed from 1 to 4 years. The seventh patient (patient 6) was diagnosed only after severe and irreversible damage to the liver had already occurred.

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Determination of Nanogram Amounts of Copper by Activation of Insolubilized Apo-polyphenol Oxidase

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Summary Chemically-insolubilized apo-polyphenol oxidase is shown to be a very selective, sensitive and stable means of collecting and determining ng amounts of copper(II).

It has recently been demonstrated that zinc can be reproducibly re-incorporated into apo-alkaline phosphatase, thus providing a means of determining ng amounts of zinc.[1] This enzyme system however, has many disadvantages when applied to trace metal analysis, for example susceptibility to inhibiting and activating metal ions, and the instability and time-consuming preparation of the apo-enzyme.

Insolubilization of a metallo-enzyme is found to eliminate the last two disadvantages, and judicious choice of enzyme can minimize inhibition and activation effects. Polyphenol oxidase (from mushrooms), a copper-containing enzyme, has been covalently linked to the polyacrylamide derivative Enzacryl AA.[2] The copper is readily removed by washing the insolubilized enzyme with a 0·1M cyanide solution, and the insoluble apo-enzyme collected by centrifugation, after washing. This apo-enzyme is reactivated by copper(I) or copper(II); maximal activity is reached after 2 h incubation at 25 °C in pH 7·0 tris maleate buffer solution. After such incubation, the activity of the reactivated enzyme, as measured by its catalysis of the oxidation of catechol, is reproducible and is linearly dependent upon copper(II) concentration over the range 10^{-7} -2×10^{-6}M. This indicates a measurable response to as little as 10 ng of copper(II).

No other metal ions at the 2×10^{-6}M level cause any activation of the insoluble apo-enzyme; activation by a mM concentration of Ni^{2+}, Fe^{2+}, Sr^{2+}, and Mg^{2+} of magnitudes similar to that given by 5×10^{-7}M copper(II), are the only effects observed at this higher level. However, a number of metal ions inhibit the uptake of copper by the apo-enzyme. Large concentrations (mM) of Zn^{2+}, Cd^{2+}, Hg^{II}, Hg^{I}, and Ag^{+} completely prevent incorporation of copper from a 5×10^{-7}M solution; but only Ag^{+} at the

2×10^{-6}M level inhibits copper uptake (by 50%). Polyphenol oxidase was chosen for this study because of its reported freedom from inhibition and activation by metal ions,[3] which is confirmed in the present study. Only vanadium(V) was found to inhibit the insolubilized enzyme (I_{50} *ca.* 3×10^{-3}M).

The apo-enzyme is readily prepared and isolated, and may be stored as a suspension in the pH 7·0 buffer solution for three months at 6 °C without noticeable deterioration. It provides a very sensitive, highly selective means of collecting *and* monitoring traces of copper in aqueous solutions; and it is hoped that it will be applicable to the determination of copper at levels as low as 10^{-12} g ml^{-1} in large volumes of sample.

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Nature of copper compounds in liver supernate and bile of rats: studies with ^{67}Cu

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TERAO, TOSHIO, AND CHARLES A. OWEN, JR. *Nature of copper compounds in liver supernate and bile of rats: studies with ^{67}Cu.* Am. J. Physiol. 224(3): 682-686. 1973.—Copper compounds in liver supernatant fraction and in bile, obtained from normal male rats after ^{67}Cu injection, were separated by Sephadex column chromatography. In the liver supernate, three different radiocopper compounds with molecular weights of about 150,000, 31,000, and 11,000 were found. Shortly after injection of ^{67}Cu, approximately 40% of the radioactivity in liver supernate was found in the copper compound with the smallest molecular weight. The radioactivity of this fraction decreased rapidly and that of the molecular weight 31,000 compound increased steadily. After 24 hr the second peak (mol wt 31,000) accounted for 90% of the radioactivity in liver supernate. Most of the stable copper in the liver supernate coincided with this large radioactive peak, but absorbance at 280 nm was small in this peak and negligible in the 11,000 peak. Oxidase activity toward *p*-phenylenediamine paralleled the protein but not the copper content of the fractions. Biliary radioactivity consisted of a large molecular substance (over 800,000) and of a smaller complex with a molecular weight less than 5,000. The ratio of the ^{67}Cu in these two fractions changed with time.

ceruloplasmin; cuproproteins; biliary proteins; biliary copper; liver hepatic copper

SINCE THE FIRST cuproprotein of the liver was discovered in 1938 (15), several cuproproteins have been identified in the liver. These include hepatocuprein (20, 32), mitochondrocuprein (30, 31), a colorless copper protein (21, 34), and metallothionein (4). It is also known that the liver synthesizes ceruloplasmin (13, 18, 25).

By means of radioactive copper, it has been shown that bile is the major route for copper excretion (2, 14, 22). However, little is known regarding the handling of copper in the liver, the mechanism of its excretion into bile, and the nature of biliary copper (3, 23, 25, 27).

MATERIALS AND METHODS

Adult male Sprague-Dawley rats kept on a normal diet (Purina Laboratory chow ad libitum) were used; most of them weighed 250-300 g.

Carrier-free ^{67}Cu as $CuCl_2$ was obtained from the Atomic Energy Commission, Oak Ridge, Tenn. From 30 to 100 μc were injected into the rats intravenously. The rats were killed by exsanguination via cardiac puncture at 30 min or 1, 6, 24, and 48 hr after injection. The liver was perfused in situ with 25 ml of cold 0.25 M sucrose and then removed, minced, and homogenized for 60 sec in a VirTis 45 homogenizer with 0.25 M sucrose (pH 7.5), 9 ml/g of liver. The homogenate was centrifuged at 4 C at 105,000 × g for 1 hr in a Beckman Instruments model L ultracentrifuge. Thus, 1 ml of supernate represented 0.1 g of liver.

Sometimes a Teflon homogenizer or a Polytron (Brinkmann Instruments) homogenizer was used for comparison. Although less ^{67}Cu was recovered in such supernates, the distribution of ^{67}Cu in fractions at 15 min, 30 min, 24 hr, and 48 hr was indistinguishable from the distribution of fractions from supernate obtained by more rigorous homogenization.

To obtain bile, a rat's bile duct was cannulated with a polyethylene no. 50 catheter on the afternoon before the injection of ^{67}Cu. After the injection, bile was collected at various intervals up to 1 week. During the collection of bile, copper-free saline was continuously infused into a tail vein. The bile was centrifuged at 700 × *g* for 15 min in a refrigerated centrifuge (International Equipment Co.).

The liver supernatant fraction was subjected to column chromatography on Sephadex G-200 (mol wt fraction range, 4,000-150,000) or Sephadex G-100 (mol wt fraction range, 5,000-80,000). For the bile samples, Sephadex G-50 and G-25 also were used.

The chromatographic column size was 1.9 x 55 cm. Gel filtration was carried out at 22 C with a flow rate of 0.[illegible] ml/min, with 0.05 M Tris buffer, pH 7.5, containing 0.[illegible] NaCl used for elution. Fractions of 4 ml were collected. The void volume was defined by the elution of Blue Dextran. To determine the elution volume of the reference proteins and Blue Dextran, 1- to 2-ml fractions were collected.

Radioactivity of each fraction was determined in an automatic γ-counter, and the protein was measured by absorbance at 280 nm in a Beckman Instruments spectrophotometer.

Stable copper was measured by atomic absorption spectrometry (Perkin-Elmer type 303). Oxidase activity of the samples was measured as previously described (26).

Acrylamide gel (containing sodium dodecylsulfate) electrophoresis was carried out by the method of Weber and Osborn (35).

All chemicals and reference proteins were obtained commercially from the following sources: Sephadex G-200, G-100, G-75 (40-120), G-50 (particle size 50-150, medium) and Blue Dextran, Pharmacia Fine Chemicals Inc., U.S.A.

sala, Sweden; bovine albumin, myoglobin (sperm whale), chymotrypsin, and chymotrypsinogen, Mann Research Laboratories, New York, N.Y.; ovalbumin, Pentex, Kankakee, Ill., or Mann Research Laboratories; cytochrome *c* (equine heart), Calbiochem, Los Angeles, Calif.; alcohol dehydrogenase, Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Liver supernatant fraction. When the hepatic supernates obtained from livers removed between 15 min and 48 hr after administration of ^{67}Cu were filtered through Sephadex G-100, the ^{67}Cu was always present in three peaks (Fig. 1). At 15 and 30 min, *peak 3* (effluent volume, 140–144 ml) usually predominated. *Peak 2* (effluent volume, 108 ml) and *peak 1* (near the void volume) were present but were smaller than *peak 3*. In some cases, *peak 2* was slightly larger than *peak 3* at 30 min. At 1 hr after injection, *peak 2* had become the major one, followed by *peak 3* and *peak 1* in decreasing order. By the 6th hr and thereafter, *peaks 1* and *3* steadily diminished and *peak 2* accounted for most of the radioactivity in the supernate.

The distribution of the radioactivity among the peaks is summarized in Table 1. Approximately 50% of the radioactivity was in the second peak at 1 hr after ^{67}Cu administration. However, at 6 hr 85% and thereafter more than 90% of radioactivity in liver supernate was found in the second peak. The percentage of the dose in the total supernate obtained from 1 g of liver increased slightly from 30 min to 24 hr and then decreased.

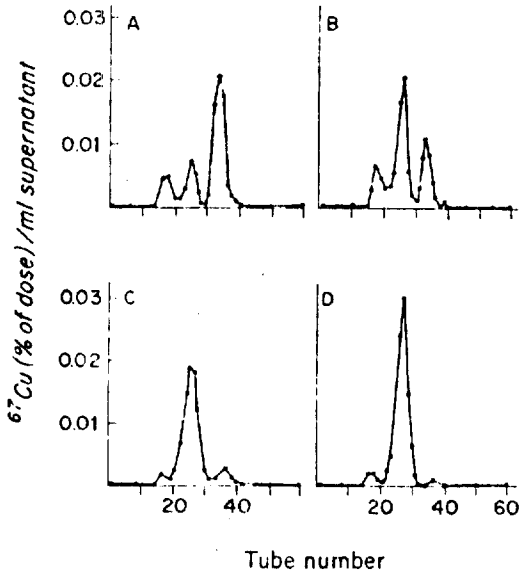

FIG. 1. Radioactivity in fractions (4 ml/tube) from Sephadex G-100 chromatography of liver supernatant fraction from normal rat at various times after intravenous injection of carrier-free ^{67}Cu. *A*: 30 min. *B*: 1 hr. *C*: 6 hr. *D*: 24 hr.

TABLE 1. *Distribution of ^{67}Cu in rat liver supernate at various time intervals after ^{67}Cu administration*

Hours After ^{67}Cu	No. of Animals	% of Dose in Supernate (From 1 g Liver)	% of Total Radioactivity		
			Peak 1	*Peak 2*	*Peak 3*
.5	5	1.26 ± 0.11	21.0 ± 8.2	37.6 ± 11.8	41.5 ± 15.4
1	5	1.38 ± 0.14	19.8 ± 5.8	49.6 ± 3.7	30.6 ± 7.3
6	5	1.36 ± 0.04	4.7 ± 0.9	85.2 ± 4.6	10.1 ± 4.9
24	5	1.36 ± 0.16	5.1 ± 0.9	92.8 ± 1.2	2.2 ± 1.0
48	6	0.91 ± 0.25	4.7 ± 2.1	93.4 ± 3.9	1.9 ± 1.9

Values are means ± SD.

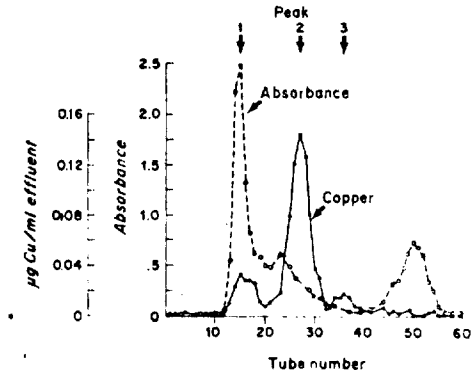

FIG. 2. Distribution of stable copper and of absorbance at 280 mμ in Sephadex G-100 fractions (4 ml/tube) from normal rat liver supernatant fraction. Arrows indicate locations of ^{67}Cu peaks.

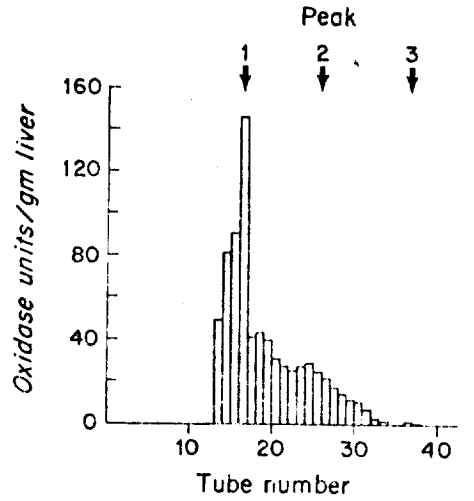

FIG. 3. Distribution of *p*-phenylenediamine oxidase activity in Sephadex G-100 fractions (4 ml/tube) from normal rat liver supernatant fraction. Arrows indicate locations of ^{67}Cu peaks.

To compare the three radiocopper peaks with the distribution of stable copper in the liver supernate, the supernatant fraction from a normal rat liver, without ^{67}Cu injection, was chromatographed on a Sephadex G-100 column, and the copper concentration in each fraction was measured (Fig. 2). At the same position as the radioactive *peak 2*, a high copper peak was also seen. However, at the positions of the first and third peaks of radioactivity, only minor stable copper peaks were found. Absorbance at 280 mμ was low at *peaks 2* and *3* but high at *peak 1*.

The results of experiments to measure the oxidase activity of the three peaks (ceruloplasmin has been characterized by its oxidase activity (8, 9, 28)) are shown in Fig. 3. *Peak 1* had the most oxidase activity, *peak 2* had less activity, and *peak 3* had virtually no activity. Ceruloplasmin is eluted in the same position as *peak 1*.

An attempt was made to estimate the molecular weights

of the three copper-containing peaks from the liver supernate by means of gel filtration and acrylamide gel electrophoresis. Alcohol dehydrogenase, bovine albumin, ovalbumin, myoglobin, and cytochrome *c* were used as reference proteins for Sephadex chromatography. As shown in Fig. 4, *peak 1* had a molecular weight of approximately 150,000, similar to the value reported for ceruloplasmin (7, 11). *Peaks 2* and *3* had molecular weights of about 31,000 and 11,000, respectively. Comparable values for the molecular weights of *peaks 2* and *3* were obtained from acrylamide gel electrophoresis (Fig. 5).

Bile. Sephadex of various fraction ranges was used for the chromatography of the bile. Fig. 6 shows the chromatograms of rat bile collected between 6 and 12 hr after ^{67}Cu injection on different types of Sephadex.

Each chromatogram on the various types of Sephadex showed at least two major radioactive peaks. The first was eluted in the void volume and the second was eluted between 140 and 200 ml of elution volume on Sephadex G-200, G-100, and G-75. In the Sephadex G-200 chromatogram, no marked radioactive peak was noticed at the point of elution of ceruloplasmin (*tubes 25* and *26*).

Occasionally, a small radioactive peak was observed between the two major peaks on Sephadex G-100 chromatography. The elution point of this small peak was exactly the same as that of *peak 2* of liver supernate in the same system.

The best separation of the smaller biliary copper components was obtained by Sephadex G-50 chromatography. The broad second peak, which was eluted between 104 and 250 ml, was partially resolved into two peaks (one with an elution volume of 160 ml and the other, 200 ml). In this system, cytochrome *c* (mol wt 13,700) was eluted at 108 ml, indicating that biliary *peak 2* has a molecular weight considerably less than 13,700. On the Sephadex G-50 column, free copper was eluted after 240 ml. In this chromatographic system, *peak 3* of liver supernate was eluted at 128 ml. Hence, there is the possibility that the early part of the second peak in bile might contain a component comparable to *peak 3* of liver supernate. The small biliary peak, which was eluted later at a volume of 200 ml, could be a small molecular weight complex, like amino acid chelates, since free tyrosine is eluted in this range.

To study the nature of the broad second biliary peak (on G-50), the eluant of *peak 2* from Sephadex column was collected, pooled, lyophilized, and examined further on Sephadex G-25 (Fig. 7). Three radioactive peaks were seen. The first peak, eluted between 84 and 172 ml, was the largest; the remaining two peaks had elution volumes of 184 and 212 ml, respectively. When rechromatographed on Sephadex G-10, the first peak from the Sephadex G-25 accounted for the major fraction and the peak was eluted at void volume. Hence, the copper compound in the peak could have a molecular weight of less than 5,000, was also suggested by Evans and Cornatzer (3).

The distribution of the radioactivities in *peaks 1* and 2 (the latter including the two slightly resolved peaks) from Sephadex G-50 columns was examined at various times after injection of ^{67}Cu (Table 2). It was found that up to 30–45 hr, the later the collection of bile after injection of ^{67}Cu, the greater the decrease in the proportion of *peak 2*

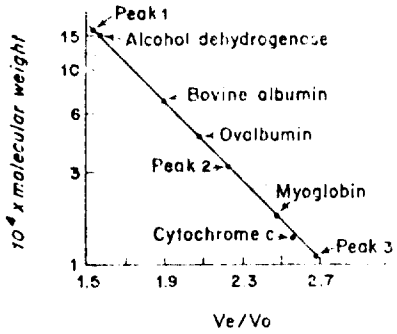

FIG. 4. Molecular weight estimation of hepatic ^{67}Cu *peaks 1*, *2*, and *3* by Sephadex G-200 gel filtration. Elution volume (Ve) is expressed in relation to void volume (Vo).

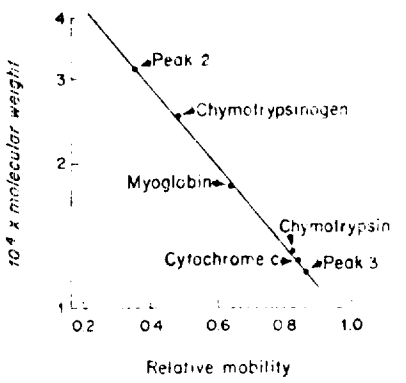

FIG. 5. Molecular weight estimation of hepatic ^{67}Cu *peaks 2* and *3* by migration distances in acrylamide gel containing sodium dodecyl sulfate. Mobilities are in relation to bromphenol blue as unity.

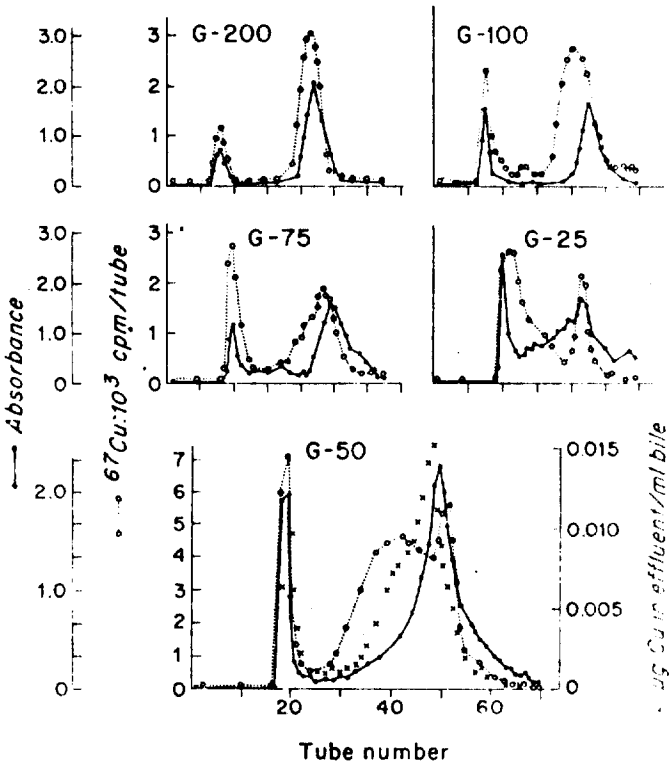

FIG. 6. Radioactive fractions (4 ml/tube) from gel filtration through Sephadex columns of different fraction ranges of normal rat bile collected between 6 and 12 hr after intravenous injection of carrier-free ^{67}Cu.

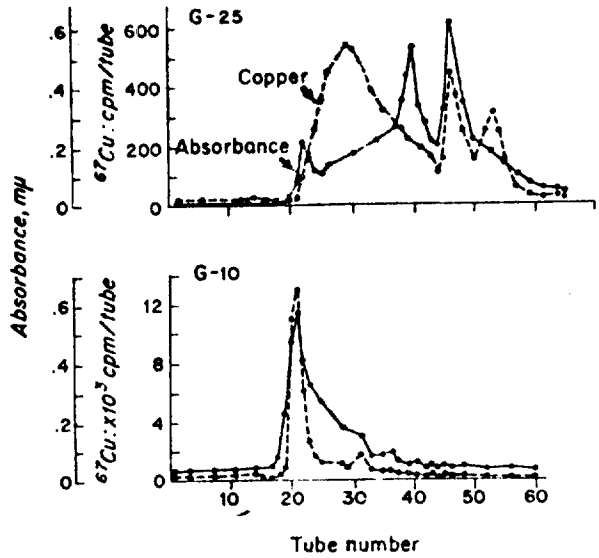

FIG. 7. *Peak 2* of ^{67}Cu from Sephadex G-50 filtration of normal rat bile (see Fig. 6) rechromatographed on Sephadex G-25 (*upper*). First radioactive peak from Sephadex G-25 (shaded area) was rechromatographed on Sephadex G-10 (*lower*). Absorbance was measured at 280 nm.

TABLE 2. *Distribution of radioactivity in bile between peak 1 and peak 2 (Sephadex G-50) at various times after intravenous administration of* ^{67}*Cu*

Hours	% of Radioactivity	
	Peak 1	*Peak 2*
0–1	7.6	92.4
0–1	6.3	93.7
0–1	9.0	91.0
0–1	8.8	91.2
0–1.5	8.9	91.1
0–1.5	14.7	85.3
6–12	11.7	88.3
6–12	16.7	83.3
6–18	15.3	84.7
6–18	25.7	74.3
11–19	23.8	76.2
24–28	22.7	77.3
30–45	20.6	79.4
30–45	21.3	78.7
30–45	26.9	73.1
30–45	33.5	66.5
30–45	35.3	64.7
6–7 days	16.1	83.9

DISCUSSION

Since the liver actively removes copper from the blood, in turn excreting some into the bile (6, 22), secreting some as ceruloplasmin (24) and storing some (25, 30, 31), one might expect to find radioactive copper in these various compartments in homogenized liver and in bile after administration of ^{67}Cu to an animal.

In the supernatant fraction of the liver, which contains more than half the hepatic copper in man (29, 33) as well as in rat (12), a number of small copper-containing proteins have been recognized (4, 21, 30–32, 34). These range

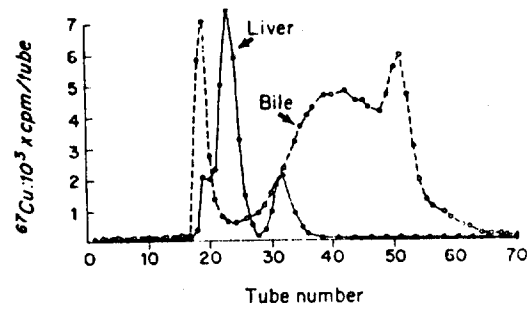

FIG. 8. Superimposition of hepatic ^{67}Cu (solid line) and biliary ^{67}Cu (broken line) elution patterns from Sephadex G-50, 6 hr after intravenous injection of carrier-free ^{67}Cu in normal rats.

from 10,000 (4, 5, 34) to about 40,000 (20, 32) in molecular weight, all far below the 150,000–160,000 of ceruloplasmin. The smallest component (*peak 3*) resembles a sulfhydryl-bound copper compound (4, 21, 34), and the 31,000 component (*peak 2*) is about the same size as erythrocuprein (16), cytocuprein (1), and superoxide dismutase (17), which are apparently all one and the same and present in all tissues.

The first ^{67}Cu found in supernates of rat liver was in the 11,000 mol wt zone. After 30–60 min, this hepatic peak diminished and the 31,000 mol wt component predominated. One might assume a progressive synthesis of the larger fraction from the smaller one or that the latter was being disposed of more rapidly, perhaps into the bile. However, the smaller biliary ^{67}Cu component was significantly smaller than *peak 3* in the liver so that, if this were the pathway, *peak 3* would have to be degraded before or during the transit.

Since little ^{67}Cu occurred in hepatic *peak 1*, of 150,000 mol wt, in which peak ceruloplasmin would be expected to occur, one can only assume that ceruloplasmin was secreted into the blood as rapidly as it was synthesized. One might even suspect that what ^{67}Cu appeared in *peak 1* might represent contamination from radioceruloplasmin in the blood. This seems unlikely, since *peak 1* was at its largest in the liver at .5 and 1 hr. Negligible radioceruloplasmin is demonstrable in the plasma as early as 1 hr after the injection of ^{67}Cu, let alone at .5 hr. Second, the liver was perfused to clear it of blood before homogenization.

The large molecular ^{67}Cu component in the bile (>800,000 daltons) could not be matched with any hepatic component (Fig. 8). Despite the observation by Jeunet *et al.* (10) that there is ceruloplasminic activity in bile, very little biliary radioactivity appeared in the zone where hepatic *peak 1* occurred.

The observed difference in absorbability of copper from "early" and "late" bile (19), representing the time of collection after intravenous administration of radiocopper, seems to parallel the decrease in the second biliary peak (mol wt 5,000) and the increase in the first peak (mol wt >800,000). This suggests that the poor resorbability of biliary copper (22) is related to the *peak 1* complex.

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TABLE 1. DIET COMPOSITION

Item	Percent
Ground yellow corn	80.95
Meat and bone meal	6.0
Soybean meal	12.0
Iodized salt	0.5
Vitamin premix[a]	0.5
Trace mineral premix[b]	0.05

[a] Provided the following per kilogram diet: Vitamin A, 2,200 IU; Vitamin D, 220 IU; Riboflavin, 2.2 mg; d-Calcium pantothenate, 6.6 mg; Niacin, 11 mg; B12 11 μg.
[b] Provided the following per kilogram diet: Zn, 100 mg; Fe 50 mg; Mn, 27.5 mg; Cu, 5.5 mg; Co, 0.5 mg; I, 0.75 mg.

ml of the ice cold sucrose solution. Following homogenization, the samples were filtered through eight layers of cheesecloth and centrifuged at 13,000 x *g* at 0 C for 15 minutes. The supernatant was used to study the *in vitro* desaturation of stearic acid.

The incubation medium which was used in these studies was a modification of those used by Johnson *et al.* (1967) and Paulsrud *et al.* (1970). The incubation medium consisted of 0.40 ml supernatant, 2.5 mM $MgCl_2$, 5.0 mM adenosine triphosphate (ATP) 0.25 mM coenzyme A (CoA), 1.0 mM reduced nicotinamide adenine dinucleotide ($NADH_2$), 100 mM K_2HPO_4, 50 mM lactic acid, 1.0 mM glutathione, 0.15% bovine serum albumin (BSA), and 0.4 mM K-stearate-1-^{14}C (1 μC) in a final volume of 1.0 ml at pH 7.4. The amount of BSA used was less than that used by Johnson *et al.* (1967) because it was found that the lower level was adequate as a carrier of K-stearate. The K-stearate concentration which was used was greater than that used by Johnson *et al.* (1967) and Paulsrud *et al.* (1970) in order to insure first order kinetics throughout the incubation time period.

Incubations were initiated with addition of the supernatant, after which the flasks were flushed with oxygen, stoppered tightly and placed in a 37 C water bath and agitated at 80 cycles/min. for 60 minutes. The reaction was terminated with the addition of 3.75 ml chloroform-methanol (1:2) containing 0.25% HCl. The lipid was extracted in the flask by the method of Bligh and Dyer (1966). The lipid extract was dried over sodium sulfate and methyl esters were prepared according to the method of Glass and Christopherson (1969).

Methyl stearate-1-^{14}C was separated from methyl oleate-1-^{14}C by chromatographing on 1.5 cm x 11.5 cm glass fiber sheets impregnated with silicic acid and previously dipped in a 1% solution of $AgNO_3$ in methanol (Graff, Marcel and Holman, 1969). Chromatograms were developed in a 6 cm x 13 cm glass jar containing 3 ml of hexane-diethyl ether-acetic acid (95:5:0.5). The bands of the saturated and monounsaturated methyl esters were made visible by spraying with 2,7-dichlorofluorescein and examining under ultraviolet light. The bands were marked, cut from the strips, and placed in scintillation vials containing 5 ml of scintillation solution. The radioactivity of each fraction was determined with a Packard liquid scintillation spectrometer. Protein contents of the supernatants were determined by the biuret procedure. The values for K-stearate desaturation were calculated as percentages and then converted to picomoles of oleate/min./mg of soluble protein.

A sample of outer subcutaneous fat was taken dorsal to the last rib and a sample of perirenal fat was taken from each carcass for fatty acid analysis. The lipid was extracted by homogenizing in chloroform and methyl esters were prepared as previously described. Fatty acid composition was determined by means of gas-liquid chromatography as outlined by Hood and Allen (1971). Means for fatty acid ratios and enzyme specific activities from the two groups of pigs were compared with an F-test according to Steel and Torrie (1960).

Experiment 2. A liver from pigs receiving no supplemental copper was prepared as described in experiment 1. Supernatant (500 μl) was incubated in a final volume of 1.0 ml of medium containing copper sulfate at six different concentrations (0.0155, 0.155, 0.332, 0.664, 0.996 and 1.660 mM). The incubation medium with no added copper sulfate served as the control. The incubation medium used in this experiment was changed somewhat from that used in experiment 1. The concentrations of ATP, CoA and $NADH_2$ were increased to 10 mM, 0.5 mM and 2 mM, respectively, and dithiothreitol was substituted for glutathione because it is generally accepted as being more effective in the protection of sulfhydryl groups. The concentration of K-stearate-1-^{14}C was decreased to 0.2 mM to produce a higher percentage of oleate-1-^{14}C during the incubation period. This enabled a more accurate quantitation of the product formed. It was shown by Paulsrud *et al.* (1970) that this level of substrate produced pseudo first order kinetics in the stearic acid desaturase system.

Incubations were carried out in 10 ml

Erlenmeyer flasks which were flushed with oxygen, stoppered with serum caps, and placed in a shaking water bath at 37 C. Single incubations for time periods of 10, 20 and 30 min. were carried out for each concentration of copper sulfate and the control. Extraction, esterification and analysis of the lipid were performed as described in experiment 1. Duplicate chromatograms were used for determining the desaturation of stearate-1-^{14}C to oleate-1-^{14}C. Initial velocities were calculated by regression analysis for each concentration of copper sulfate and the control.

Due to a nonlinear relationship in desaturase specific activity (picomoles oleate/min./mg protein) between the incubation time periods of the *in vitro* copper sulfate study, this study was repeated using a different liver preparation and with triplicate incubations of each sample. The triplicates were averaged for determination of initial velocities. In addition, the control and one level of copper sulfate (0.664 mM) were incubated for 60 min. to determine if the reaction rate was linear for that period of time.

Results and Discussion

Experiment 1. Supplementary copper in the diet produced an increased palmitoleic acid content and a decreased stearic acid content in outer subcutaneous fat (table 2). The other fatty acids were not affected. In perirenal fat, the copper-supplementation increased oleic and decreased stearic acid levels, while the other fatty acids were unchanged. The net effect in outer subcutaneous fat was that the ratios of palmitoleic/palmitic and of oleic/stearic acids were significantly ($P<.01$) greater for the copper-supplemented pigs. Only the oleic/stearic acid ratio was significantly ($P<.01$) greater in perirenal fat.

No significant differences were found in mean linoleic acid contents between the copper-supplemented pigs and the control pigs in either outer subcutaneous or perirenal fat. This result indicates that supplementary dietary copper did not change the absorption, transport or metabolism of linoleic acid. There were also no significant differences between the copper-supplemented pigs and the control pigs for the total quantities of either 16 or 18 carbon fatty acids in either outer subcutaneous or perirenal fat. This result indicates that copper did not produce an increase in elongation of fatty acids.

Data from this study indicate that supplementary copper in the diet influenced the ratios of the mono-unsaturated fatty acids to their saturated fatty acid precursors. Even though there were differences between the copper-supplemented pigs and the control pigs for mean values for particular fatty acids, the ratio of mono-unsaturated fatty acid to its saturated precursor is of particular interest because this ratio can be directly affected by the fatty acyl desaturase system. Because of the relatively high proportion of stearic and oleic acids in pork fat, the overall degree of unsaturation is affected most by changes in these fatty acids between the two groups of pigs. The oleic/stearic acid ratio is affected directly by the desaturation of stearic to oleic acid and indirectly by the desaturation of palmitic to palmitoleic acid if palmitoleic acid is subsequently elongated to oleic acid.

Stearic acid desaturase activity was significantly ($P<.01$) greater in livers of pigs re-

TABLE 2. INFLUENCE OF SUPPLEMENTARY DIETARY COPPER SULFATE ON THE FATTY ACID COMPOSITION OF LIPID IN PORCINE ADIPOSE TISSUE

	Major fatty acids[a]					Fatty acid ratios	
Location and diet	16:0	16:1	18:0	18:1	18:2	16:1/16:0	18:1/18:0
Outer subcutaneous							
Diet A[b]	22.65	3.80	11.62	45.68	11.19	0.17	3.95
	ns	**	**	ns	ns	**	**
Diet B[c]	22.46	4.48	10.46	45.79	11.78	0.20	4.40
Perirenal							
Diet A	27.86	3.32	17.90	38.29	8.63	0.12	2.15
	ns	ns	*	*	ns	ns	ns
Diet B	26.63	3.67	16.17	40.58	9.00	0.14	2.54

* $P<.05$.
** $P<.01$.
ns = nonsignificant ($P>.05$).
[a] Means of 12 observations expressed as weight percent.
[b] Diet A = control diet.
[c] Diet B = control diet plus 250 ppm copper from copper sulfate.

ceiving supplementary copper than in livers from the control pigs (table 3). Specific activity of the desaturase system was not changed in the subcutaneous adipose tissue. This might be expected since it has been shown that the absorbed dietary copper is concentrated in the liver and that very little of the copper accumulates in the fatty tissues (Barber *et al.*, 1957; Bowland *et al.*, 1961). Liver copper levels were not determined in this study. The higher specific activity for stearic acid desaturation in the liver could account for the observed increase in oleic/stearic acid ratios in subcutaneous and perirenal fat of the pigs fed the copper-supplemented diet. Other studies (Reiser and Raju, 1964; Allen *et al.*, 1967) have related the decrease in oleic/stearic acid ratios of tissue lipids to inhibition of stearic acid desaturation by cyclopropene fatty acids.

Experiment 2. Stearic acid desaturase specific activities (picomoles oleate/mg protein/min.) as determined in the first *in vitro* study of copper sulfate were not linear over the three incubation time periods studied. Therefore, this experiment was repeated with triplicate incubations for each incubation time period and each copper sulfate concentration. The triplicates were averaged and in this case there was a linear relationship of desaturase specific activity between all incubation time periods for each level of copper sulfate studied. Initial velocities were calculated for both preparations, but the greatest emphasis was placed on the triplicate determinations since experimental error was reduced by replication.

The supernatant prepared from the first liver contained 18.0 mg protein/500 μl, whereas, that prepared from the second liver contained 12.0 mg protein/500 μl. Therefore, the concentration of copper sulfate relative to the concentration of soluble protein in the incubation medium was greater for the second liver. For this reason, initial velocities (figure 1) were plotted against the ratio of copper sulfate/soluble protein to put the two determinations on an equal basis. The preparation from the second liver had a lower specific activity than that from the first liver, but the initial velocity patterns were basically the same. With both liver preparations, a sharp increase in initial velocity was observed at very low copper sulfate/soluble protein ratios (0.5×10^{-5} mM/mg), but this increased activity was not maintained from $1\text{–}2 \times 10^{-5}$ mM $CuSO_4$/mg protein. This result may suggest two different binding sites on the desaturase enzyme with different affinities for Cu^{++} which could alter the structure and thus affect the activity of the enzyme. At copper sulfate levels greater than 2×10^{-5} mM $CuSO_4$/mg protein, there was a gradual increase in initial velocity to a maximum at about 3×10^{-5} mM $CuSO_4$/mg protein (figure 1). The maximum initial velocity was about 23% greater than that of the controls with both liver preparations. This peak in activity was followed by a gradual decrease until complete enzyme inactivation occurred as a result of high copper sulfate concentration.

The data from the second liver experiment, expressed in terms of the total quantity of oleate produced/mg of soluble protein during the total incubation period of 30 min. are plotted in figure 2. Data in figure 2 support those in table 3, where specific activity was based on the quantity of oleate formed during a 60-min. incubation. These data indicate that $CuSO_4$ has an activation effect on the desaturase system. However, the curve in figure 2 is somewhat different from that of initial velocities (figure 1). The peak in initial velocities at very low levels of copper sulfate (0.0166 mM) is not apparent after a 30-min.

TABLE 3. STEARIC ACID DESATURASE SPECIFIC ACTIVITIES[a]

Treatment	Outer subcutaneous adipose tissue	Liver
Diet A[b]	162.7	16.8
Diet B[c]	169.8	24.7
Statistical significance	ns	**

[a] picomoles oleate/min/mg protein.
[b] Diet A - control diet.
[c] Diet B - control diet plus 250 ppm copper from copper sulfate.
** $P<.01$.
ns - nonsignificant ($P>.05$).

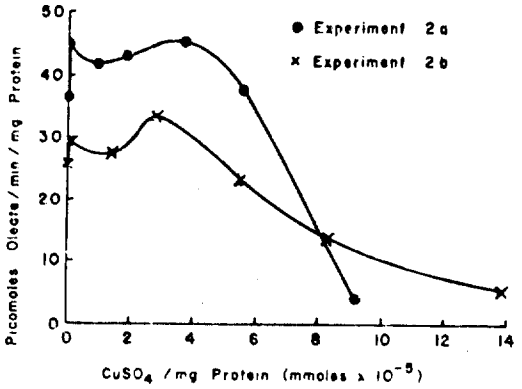

Figure 1. Influence of copper sulfate on initial velocity of the stearate desaturase system.

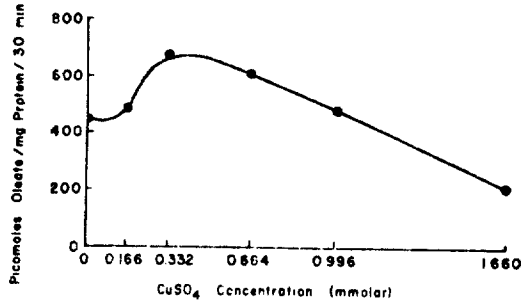

Figure 2. Influence of copper sulfate on the conversion of stearate to oleate during a 30 min. incubation.

incubation. The reason for this may be that even though the initial velocity was relatively high, the rate of decrease in activity with time was also high at 0.0166 mM copper sulfate, thus resulting in less total desaturation over the 30-min. incubation period. This result may suggest that the enzyme had a less stable configuration at this level of copper sulfate. The opposite effect occurred at intermediate concentrations (0.664 mM) of copper sulfate where the initial velocity declined rather rapidly for copper sulfate concentrations greater than that which produced maximum activity, but the total desaturation at 30-min. decreased gradually. This occurred because the rate of decrease in activity with time was less at intermediate levels of copper sulfate than with no copper sulfate (figures 2 and 3). This result may suggest that the enzyme had a more stable configuration at the intermediate levels of copper sulfate.

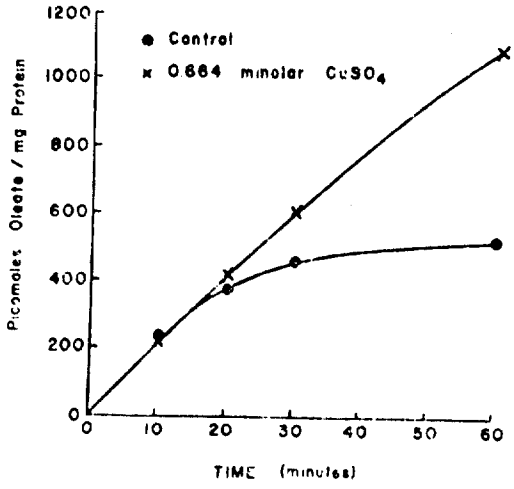

Figure 3. Influence of time and copper sulfate on the stearate desaturase system.

With 60 min. incubations of the samples containing no added copper sulfate and 0.664 mM copper sulfate (figure 3), desaturation proceeded at almost a constant rate for 60 min. in the sample containing added copper. However, with no added copper, desaturation declined very rapidly after 30 minutes. This resulted in about twice as much total desaturation in the copper sulfate sample as in the control sample over the 60-min. incubation period. This also indicates that in addition to the effect on initial velocity, copper may contribute to the stability of the enzyme.

The results of experiment 1 (table 3) may be due to either activation of the desaturase system as shown in figure 1, or to stabilization of the system as shown in figures 2 and 3, or to a combination of these factors. If desaturase enzyme is synthesized at a constant rate in a tissue and copper activates and stabilizes the enzyme, the effective concentration of enzyme in the tissue could be increased, and thus a greater amount of desaturation could occur. This would result in an increased ratio of mono-unsaturated fatty acids to their saturated fatty acid precursors.

The increase in desaturase activity based on the determinations from the second liver (Exp. 2) ranges from about 0.166 mM copper sulfate to about 0.996 mM (figure 2). Based on the amount of copper added within this range and the amount of fresh liver tissue represented in the incubation medium, this corresponds approximately to a range of 100–600 ppm of copper in the liver. This is the general range of copper concentrations that have been reported in livers of pigs receiving 250 ppm of dietary copper sulfate (Barber *et al.*, 1957; Hawbaker *et al.*, 1961; Taylor and Thomke, 1964; DeGoey *et al.*, 1971). Therefore, the *in vitro* increase in velocity of stearic acid desaturation due to copper corresponds with the increase in desaturase specific activity of livers from pigs receiving supplementary copper sulfate at 250 ppm. It seems reasonable to suggest that the increased desaturation of stearic to oleic acid in the liver can be related to the increase in the oleic : stearic acid ratio present in the fatty tissues of the pigs fed the high copper diet.

Since it has been shown in these studies that copper sulfate activates the desaturation of stearic acid at concentrations normally found in the livers of pigs receiving 250 ppm of supplementary copper from copper sulfate, one would expect that the desaturation of palmitic

acid might also be activated. This would be in agreement with the findings of experiment 1 where the palmitoleic, palmitic acid ratio of the fat was greater in pigs receiving supplementary copper than in the controls.

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Determination of Manganese, Copper and Zinc in Serum and Packed Blood Cells by Neutron Activation Analysis

Studies on the metabolism of trace elements

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A method for simultaneous determination of manganese, copper and zinc in serum and packed blood cells by neutron activation analysis is described. After irradiation the samples are wet-ashed. Copper is extracted at pH 2, manganese and zinc at pH 8 with 0.05 mol/l oxine in chloroform. Radioactive measurements are carried out with a 3" × 3" NaI (Tl) well type detector coupled to a 400 channel analyser. Results are reported from 20 individuals.

Es wird eine Methode zur simultanen Bestimmung von Mangan, Kupfer und Zink in Serum und gepackten Blutzellen (Blutkuchen) mit der Neutronen-Aktivierungs-Analyse beschrieben. Nach Bestrahlung werden die Proben feucht verascht. Kupfer wird bei pH 2, Mangan und Zink werden bei pH 8 mit 0,05 mol/l Oxin in Chloroform extrahiert. Radioaktivitäts-Messungen werden mit einem 3" × 3" NaJ (Tl)-Detektor, verbunden mit einem 400-Kanal-Analyzer, durchgeführt. Die Ergebnisse von Bestimmungen bei 20 Probanden werden mitgeteilt.

Manganese, copper and zinc are essential trace elements in man (1—4). Several aspects of their metabolic functions (5—10) and their importance in pathology (11—14) have already been established.

Most published values for serum copper and zinc concentration show good agreement but those for manganese vary over a wide range (15—21).

We developed a sampling and analytical technique which reduces contamination to a minimum.

As it seemed desirable to obtain data concerning the intracellular level, determinations were also made on packed blood cells (blood clot).

Materials and Methods

Collection of samples

Quartz tubes ($\varnothing$ = 16.6 mm, h = 12 cm) for the collection of blood are cleaned with distilled water from a quartz apparatus and then boiled in a mixture of equal volumes of suprapur nitric and sulfuric acid for two periods of 2 h. They are rinsed again with triple distilled water, steamed for 3 h, dried and covered with thoroughly cleaned teflon stoppers until used. All operations are carried out in a "dust-free" room.

Blood samples for trace element analysis are collected after the disposable steel needle has been flushed by approximately 60 ml of blood. It has been demonstrated that even under these conditions the normal serum value for manganese can be increased by up to 10% by contamination, whereas the contaminations of copper and zinc are negligible (22).

The samples are placed in a water bath at 37°C for 3 h. Spontaneous clotting is allowed. Serum and blood clot are separated by centrifugation at 2500 rpm for 1 h and lyophilized.

Obviously, under these conditions, the separation of cellular elements and serum is incomplete. As was shown with albumin —^{131}I as tracer, $\leq 6\%$ of the total extracellular fluid of whole blood is retained in the clot. This results in a negative error of about 7% for the measured intracellular manganese and zinc level and an approximate 5% positive error for the intracellular copper level. The reported values are not corrected for this systematic error.

After lyophilisation about 100 mg of dried serum or about 50 mg of dried packed cells are collected in thoroughly cleaned polyethylene capsules.

Irradiation and nuclear data

The serum capsules are irradiated for 4 h and the packed cells capsules for 6 h in the Thetis reactor of Ghent University. The flux conditions are summarized in table 1 (23).

The nuclear reactions of practical importance are listed in table 2. The only nuclear interference is due to the fast neutron treshold reaction $^{56}Fe(n,p)^{56}Mn$[1]). As the iron content in serum is approximately 1.20 µg/ml, the interference is low at the flux conditions of site 1 ($\leq 3\%$). Because of the high iron content of packed cells (approximately 1 mg/g wet weight) this interference is obvious under these irradition conditions. Therefore the samples were irradiated at a better thermalised reactor position, site 16, where the maximum interference is approximately 7%. A correction is applied based on the separately determined iron content of serum and packed cells.

The flux and spectrum of the reactor being stable, a copper wire co-irradiated with the samples was used as a neutron flux monitor.

Chemical separations

Carrier solution (10 µg Cu, 5 µg Mn and 5 µg Zn) is added after irradiation. The organic material is mineralised by wet-ashing with a 1:1 mixture of 700 g/kg perchloric acid and 14 mol/l nitric acid by heating on a hot plate. The residue is transferred with 10 ml of 1 mol/l perchloric acid into a separatory funnel. 10 ml of a saturated hydrazine sulfate solution are added in order to obtain a quantitative reduction of manganese to the bivalent state. After 2 min, 10 ml of 0.05 mol/l potassium biphthalate buffer are added. Then the solution is adjusted to pH 2 with 14 mol/l ammonia and Cu is extracted with 2 successive 10 ml portions 0.05 mol/l oxine in chloroform (25). Thereafter the pH is adjusted to 8.0 with 14 mol/l ammonia. A temporary flocculation, which appears at

[1]) n = neutron, p = proton.

Tab. 1

Flux in the irradiation positions for serum and packed cells (Thetis reactor, University of Ghent). Fluxes are given as $n \cdot cm^{-2} \cdot s^{-1}$

Material Site irradiation	Thermal flux	Epithermal flux	Fast flux	Thermal/fast flux
Serum 1	$1.10 \cdot 10^{12}$	$0.0435 \cdot 10^{12}$	$0.178 \cdot 10^{12}$	6.18
Packed cells 16	$1.69 \cdot 10^{12}$	$0.0113 \cdot 10^{12}$	$0.0187 \cdot 10^{12}$	90.4

Tab. 2

Nuclear reactions of practical importance in this study. σ_{th} = thermal activation cross section. I_o = resonance integral. $\bar{\sigma}_f$ = fast neutron activation cross section

Nuclear reaction	% isotopic abundance stable form	σth (barn)	I_o (barn) (24)	$\bar{\sigma}f$ (barn)	Half-life (h)
$^{55}Mn(n, \gamma)^{56}Mn$	100	13.3	13.8		2.582
$^{63}Cu(n, \gamma)^{64}Cu$	69.17	4.7	6.1		12.75
$^{68}Zn(n, \gamma)^{69m}Zn$	18.57	0.075	0.23		13.7
$^{23}Na(n, \gamma)^{24}Na$	100	0.53	0.35		15
$^{37}Cl(n, \gamma)^{38}Cl$	24.47	0.435	0.213		0.622
$^{56}Fe(n \cdot p)^{56}Mn$	0.916			$0.97 \cdot 10^{-3}$	2.582

this stage, disappears during the subsequent extraction. Mn and Zn are extracted with two 10 ml portions of 0.05 mol l oxine in chloroform. Since the activity of ^{24}Na is overwhelming in comparison with the ^{56}Mn, ^{64}Cu and ^{69m}Zn activities, traces of the aqueous phase due to incomplete separation cause a serious interference. ^{24}Na however is sufficiently eliminated by washing the organic phases with aqueous solutions of pH 2 and pH 8 respectively.

Radioactive measurements

The measurements are carried out with the following equipment:

1. NaI (Tl)detector, 3″ × 3″, 36.7 ml well type, resolution 10% for the ^{137}Cs line.
2. Intertechnique SA 40 B multichannel analyser, RG 23 magnetic tape unit and HC 20 chronometer.
3. Baird Atomic Sample Changer connected to the SA 40 B.

The sample changer is first loaded with the vials containing ^{56}Mn as this is the shortest lived species. Counting is started 3 h after the end of the irradiation.

For counting ^{56}Mn, ^{69m}Zn and ^{64}Cu respective photopeaks at 846.9 keV, 438.7 keV and 511 keV are chosen. As already mentionned ^{24}Na activity cannot be neglected. Mixed gamma-ray spectrometry was applied choosing respectively 2 (pH 2 phase) and 4 (pH 8 phase) energy regions and solving the equations for respectively 1 and 2 unknowns, i. e. ^{64}Cu with ^{24}Na interference and ^{56}Mn and ^{69m}Zn with ^{64}Cu and ^{24}Na interferences. The net activities are normalised against the copper monitor wire.

Typical spectra with the energy regions chosen are shown in Figures 1a and 1b.

Results

The chemical procedure was tested with tracer amounts of ^{56}Mn, ^{64}Cu, ^{69m}Zn and ^{24}Na. The recoveries are recorded in table 3.

The reproducibility of the method was tested in 4 sera (table 4).

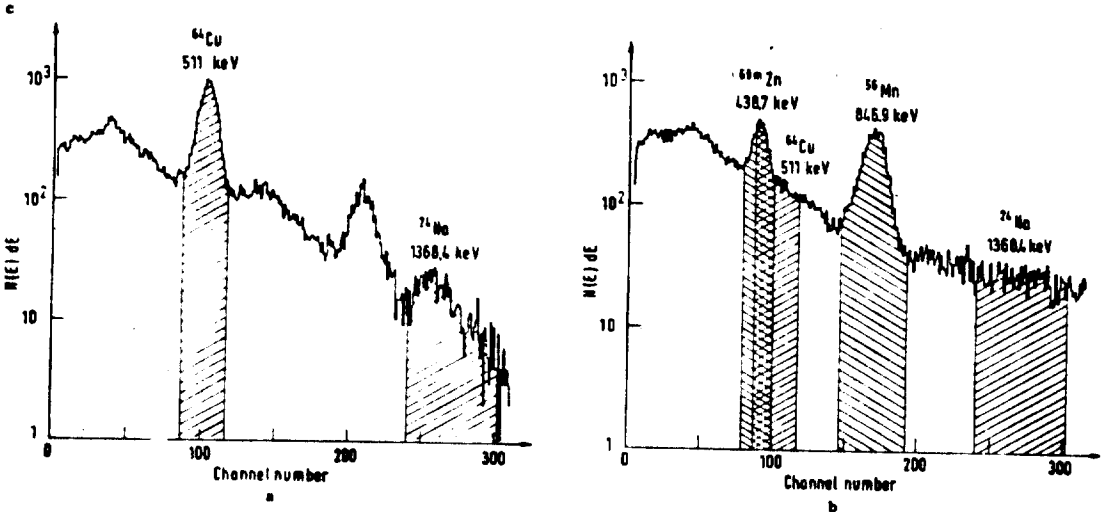

Fig. 1

Gamma-ray spectra recorded with a well-type sodium iodide (thallium activated) detector coupled to a 400 channel analyser
a) Irradiated packed cells. Wet-ashed. Extraction with oxine in chloroform. Phase at pH 2
b) Irradiated packed cells. Wet-ashed. Extraction with oxine in chloroform. Phase at pH 8

Tab. 3

Recovery of added Cu, Mn and Zn

Element	Recovery [%] pH = 2.0 mean	pH = 2.0 range	pH = 8.0 mean	pH = 8.0 range
Cu	99.8	98.7—100.8		
Mn	0.03	0.0—0.05	99.5	98.2—101.0
Zn	0.50	0.0—0.70	98.4	96.2—100.9
Na		~0.02		~0.06

The results obtained for serum and packed blood cells of 20 hospitalized patients are listed in table 5. Two samples of blood were collected from patients 1—19 and handled separately. Manganese, copper and zinc were determined in each sample. The higher manganese

Tab. 4

Reproducibility of the analytical procedure. Determinations in serum of four individuals. Manganese is given as microgram/l serum, copper and zinc as milligram/l serum

Subject	Mn (µg/l)	Cu (mg/l)	Zn (mg/l)
I			
Mean (n = 6)	0.734	1.03	1.01
S. D.	0.051	0.047	0.080
II			
Mean (n = 6)	0.640	1.36	1.00
S. D.	0.060	0.020	0.069
III			
Mean (n = 6)	0.458	1.26	0.928
S. D.	0.032	0.060	0.069
IV			
Mean (n = 6)	0.524	1.14	0.772
S. D.	0.051	0.018	0.032

Tab. 5

Values of manganese, copper and zinc in human serum and packed cells. Of each subject two samples (a and b) or two samples at an interval of two weeks (a, b and c, d) are analysed
Manganese is given as nanogram/ml serum or as nanogram/g packed cells, wet weight
Copper and zinc as microgram/ml serum or microgram/g packed cells wet weight

No.	Subject Age	Sex	Diagnosis	Sample	Mn Serum ng/ml	Mn Packed cells ng/g	Cu Serum µg/ml	Cu Packed cells µg/g	Zn Serum µg/ml	Zn Packed cells µg/g
1	51	♀	Detachment of the retina	a	0.56	13.6	0.93	0.56	0.72	9.9
				b	0.58	14.1	0.93	0.56	0.65	9.8
2	23	♀	Keratitis	a	0.64	15.3	1.54	0.69	0.79	9.6
				b	0.44	15.5	1.63	0.66	0.86	9.4
3	20	♂	Ocular injury	a	0.64	13.8	0.84	0.65	1.14	9.8
				b	0.57	13.7	0.89	0.68	1.10	9.9
4	51	♂	Detachment of the retina	a	0.58	13.1	1.01	0.70	0.90	11.4
				b	0.51	12.7	1.06	0.72	1.05	11.5
5	54	♀	Detachment of the retina	a	0.60	13.6	0.70	0.61	0.78	11.4
				b	0.60	13.8	0.85	0.62	0.74	11.8
6	22	♂	Ocular injury	a	0.72	16.5	1.12	0.70	0.67	11.1
				b	0.60	16.8	1.35	0.68	0.77	11.2
7	45	♂	Detachment of the retina	a	0.44	10.1	1.01	0.61	0.97	10.9
				b	0.49	9.0	1.02	0.64	0.87	10.8
8	32	♂	Detachment of the retina	a	0.57	13.4	0.73	0.73	1.04	11.8
				b	0.70	13.3	0.89	0.69	1.23	11.2
9	54	♂	Ocular injury	a	0.65	12.6	1.00	0.65	1.01	11.4
				b	0.81	13.4	1.18	0.68	1.09	11.2
10	19	♀	Detachment of the retina	a	0.54	17.0	1.01	0.66	0.88	9.7
				b	0.55	17.8	1.03	0.65	0.73	9.4
11	52	♂	Detachment of the retina	a	0.64	13.3	1.01	0.64	1.05	10.1
				b	0.78	13.7	0.99	0.65	0.98	10.2
12	17	♀	Keratoconus	a	1.02	15.7	0.83	0.60	0.90	11.7
				b	1.06	15.2	1.00	0.60	1.07	11.0
13	22	♀	Neurosis	a	0.57	14.7	1.41	0.70	0.97	9.8
				b	0.62	13.7	1.39	0.69	0.90	10.0
14	42	♂	Ocular injury	a	0.61	12.6	1.35	0.62	1.11	10.6
				b	0.55	12.8	1.36	0.66	0.98	10.4
15	16	♀	Ocular injury	a	0.45	15.7	1.26	0.69	0.88	10.8
				b	0.46	16.6	1.29	0.71	0.93	11.2
16	27	♀	Detachment of the retina	a	0.50	13.3	1.13	0.72	0.78	9.5
				b	0.50	13.3	1.15	0.76	0.74	9.5
17	66	♀	Keratitis	a	0.46	17.6	0.95	0.77	0.84	10.2
				b	0.56	19.0	0.96	0.73	0.89	10.4
18	62	♂	Cataract	a	0.76	20.2	1.16	0.82	0.74	11.6
				b	0.63	20.8	1.15	0.77	0.72	11.5
19	29	♂	Ocular injury	a	0.45	16.2	1.12	0.80	0.83	11.1
				b	0.61	15.1	1.09	0.66	0.78	10.0
20	27	♂	Keratoconus	a	1.02	15.2	1.30	0.71	0.96	10.2
				b	0.99	14.7	1.26	0.70	0.93	10.7
				c	1.01	14.6	1.04	0.69	1.03	10.3
				d	1.02	15.8	1.05	0.68	1.02	10.2
			Range from		0.44	9.0	0.70	0.56	0.65	9.4
			to		1.06	20.8	1.63	0.82	1.23	11.8
			Mean		0.64	14.7	1.09	0.68	0.91	10.6
			Standard deviation		0.18	2.3	0.21	0.06	0.14	0.8

concentrations of case 20 (a and b) was checked in samples taken two weeks later (c and d).

Discussion

Reliable results for copper and zinc in serum have been available for many years. Their relatively high concentration is easily determined by many analytical techniques. Adaptions have also been described for other organic material (26). Radioactivation analysis of manganese has been reported by many authors (15, 17, 18, 20, 21, 27, 28, 29), but most probably the only reliable concentration for serum manganese is that reported by Cotzias (16) in 1966. The very low concentration represents a major technical problem: extreme precautions have to be taken to avoid contamination (16, 21, 22).

The reported copper and zinc concentrations in serum are in good agreement with the results obtained by other investigators (3, 5, 11, 12, 13, 30—37). The serum manganese concentrations are comparable to the values obtained by Cotzias (16). In our series two relatively high values were found. In both cases, 12 and 20, the values were obtained in duplicate; in case 20 there was close agreement between samples taken at an interval of two weeks. The concentrations obtained show a relatively small range (table 5). This also corresponds to the findings of Cotzias (16) but is in striking contrast with the data of Mertz et al. (19). In this latter case, sample contamination could be the explanation.

The values of manganese, copper and zinc in packed blood cells also vary within narrow limits. In the literature data are rather scarce (5, 20, 35).

For manganese the ratio packed cells/serum is 24, for zinc 12, whereas the serum concentration of copper is greater than the intracellular (0.64).

As many speculations concerning the biological functions of manganese are based on concentrations that are obviously too high, it may be possible that much earlier work needs revision or confirmation. The available data concerning copper and zinc are not so open to doubt, as earlier determinations seem to have given reliable results.

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