AN ABSTRACT OF THE THESIS OF

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in Foods and Nutrition presented on August 14, 1981

Title: INFLUENCE OF ASCORBIC ACID SUPPLEMENTATION ON COPPER STATUS IN YOUNG ADULT MEN

Abstract approved: Florian Cerklewski

Thirteen healthy adult males, ages 20-40, consuming self selected diets, were given instructions to take one 500 mg tablet of ascorbic acid three times a day with their meals for a period of ten weeks. The effect of this daily supplementation on copper status was investigated. An estimation made from a three day diet record kept by each subject indicated their dietary copper intake to be 1.92 mg per day. Determination of serum ceruloplasmin and serum copper done on the first day of the ascorbic acid supplementation period showed that the subjects fell within accepted ranges of normal. All further determinations of these parameters during the experimental period were compared to initial values so that each subject served as his own control.

At week seven the high ascorbic acid intake significantly decreased ceruloplasmin by 26 percent. At the end of the ten week ascorbic acid supplementation period, serum ceruloplasmin activity was significantly lowered by 20 percent. The slight increase over week seven was attributed to a drop in compliance to taking the ascorbic acid tablets. Serum copper levels were not significantly affected during the 10 week experimental period although a consistent decrease was observed. Two

weeks after acorbic acid was terminated serum ceruloplasmin activity increased but was not significantly different from week ten values. However, when compared to week seven values, a significant increase of 14 percent was observed. Serum copper levels measured two weeks after ascorbic acid supplementation was terminated significantly increased 14 percent over week ten values.

The results of this human volunteer study indicate that taking a megadose of ascorbic acid for ten weeks will significantly decrease serum ceruloplasmin activity much like that observed in laboratory animal studies. Based on this finding, one may question the safety of prolonged self-dosage of high amounts of ascorbic acid by adults as encouraged by the popular press.

Influence of Ascorbic Acid Supplementation on Copper Status in Young Adult Men

by

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a thesis

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed August 1981

Commencement June 1982

APPROVED:

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in charge of major

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Date thesis is presented August 14, 1981

Typed by Opal Grossnicklaus for Elizabeth Bidwell Finley

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to all the people who helped me complete my thesis. I would like to thank my major professor Dr. Florian Cerklewski for his guidance and many hours spent in consultation. I appreciate all the encouragement from my friends. And finally I want to thank my husband for his love and support I received while working on this project.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	3
Absorption	3
Amount of Copper Absorbed	4
Homeostasis	4
Normal Serum Levels of Copper and Ceruloplasmin	5
Tissue Copper	6
Coppper Excretion	6
Copper Containing Proteins	7
Copper Deficiency	10
Copper Toxicity	10
Dietary Copper	11
Copper Antagonists	13
Ascorbic Acid and Copper	14
MATERIALS AND METHODS	18
Subjects	18
Experimental Design	18
Ascorbic Acid Tablets	20
Compliance Determination	20
Dietary Copper Estimation	21
Methods	21
Statistical Analysis	22
RESULTS	23
Subject Compliance	23
Estimated Dietary Copper Intake	23
Effect of Ascorbic Acid Supplementation	23
Termination Period	25
DISCUSSION	32
SUMMARY	36
BIBLIOGRAPHY	39
APPENDIX	45

LIST OF FIGURES

Figure		Page
1	Influence of Ascorbic Acid Supplementation on Serum Copper and Ceruloplasmin	27
2	Effect of Termination of Ascorbic Acid Supplementation on Serum Copper and Ceruloplasmin	31

LIST OF TABLES

Table		Page
1	Serum Ceruloplasmin during Ascorbic Acid Administration	24
2	Serum Copper during Ascorbic Acid Administration	26
3	Serum Ceruloplasmin Following Termination of Ascorbic Acid Supplementation	28
4	Serum Copper Following Termination of Ascorbic Acid Supplementation	30

THE INFLUENCE OF ASCORBIC ACID SUPPLEMENTATION ON COPPER STATUS IN YOUNG ADULT MEN

INTRODUCTION

The first conclusive proof that copper is an essential metal for humans appeared in the literature in 1930 when Mills (1) found that copper was needed for curing hypochromic anemia. Earlier studies demonstrated that rats fed exclusively on a milk diet developed an anemia which was responsive to iron only after the addition of copper (2). Since that time copper has been found to be associated with maintaining iron in the ferric state through ceruloplasmin activity (3). Ceruloplasmin oxidizes the ferrous iron released from liver storage to ferric iron so that it can be incorporated into transferrin for transport. A wide variety of biologic processes have been shown to depend on copper and several investigations have been done to elucidate the metabolism of copper (4).

Intestinal absorption of copper has been found to be decreased in the presence of zinc, molybdenum, cadmium and potassium sulfide (5-9). Findings from several animal studies indicate that ascorbic acid will also decrease intestinal copper absorption (10-14). Investigators in two recent studies using guinea pigs reported that megadoses of ascorbic acid decreased ceruloplasmin and serum copper by 50 percent ($p<0.001$) and liver copper and blood copper by 39 ($p<0.01$) and 52 ($p<0.025$) percent, respectively (15, 16).

The purpose of this study was to investigate the effect of ascorbic acid supplementation on copper status. Healthy adult males

consuming self-selected diets took one 500 mg tablet of ascorbic acid three times per day with their meals for ten weeks.

REVIEW OF LITERATURE

It is the purpose of this review to briefly discuss copper metabolism in man and then concentrate on the interactions of dietary copper with other dietary factors. Copper has been the subject of several review articles (4, 17).

Absorption

The copper available for absorption is that which is released from ingested foodstuffs either as divalent copper or a copper amino acid complex (4). Based on in vivo and in vitro animal studies, the mechanism of copper absorption is thought to involve two processes. One appears to be an energy dependent process involving the absorption of complexes of copper and L-amino acids (18-20). Evidence shows that L-amino acids facilitate the intestinal transport of copper across mucosa membranes and that absorption progressively decreases with increasing molecular size of the copper-amino acid complexes (19). The other mechanism is an enzymatic one involving the binding of copper to and successive release from a macromolecular protein in the mucosal membrane (20).

Within the absorptive cells metallothionein binds copper through the formation of mercaptide bonds and it releases copper to the serosal side of the intestinal cells. Metallothionein may play a passive role in enzymatic copper absorption by providing binding sites within the intestinal mucosa membrane and acting as storage sites for later absorption. Metallothionein may also function as a mucosal block to

protect against absorption of toxic levels of copper due to a three to five day turnover of mucosal cells and subsequent excretion of any unabsorbed copper (19).

Amount of Copper Absorbed

Knowledge of the amount of copper actually absorbed in normal healthy adults is inconclusive and many values have been reported. Measuring fecal copper would provide an unreliable estimate of intestinal absorption as it represents copper from saliva and sloughed-off gastric and intestinal mucosae and biliary copper as well as unabsorbed dietary copper (4). Values for the amount of dietary copper absorbed vary from 32 to 70 percent (21-23). These differences may be explained by the variety of methods used.

Homeostasis

Once copper is absorbed from the intestine it is combined with albumin and amino acids and is transported to the liver through the portal bloodstream (24). The albumin and amino acid bound coppers are referred to as direct reacting copper because they will combine with diethyldithiocarbamate (4). As the key organ in copper homeostasis, the liver allows a portion of these copper complexes to circulate in the systemic circulation where they are maintained at approximately seven percent of total plasma copper (24). The remaining copper is released from albumin and amino acids and taken up by the hepatocyte membrane receptors and transported to the cytosol. In maintaining

copper homeostasis, the liver synthesizes the copper containing protein ceruloplasmin which represents 93 percent of total serum copper (25). In addition, the liver stores copper and excretes copper through the bile. Hazelrig and co-workers (26) concluded from giving rats various levels of ^{64}Cu and measuring it at hour intervals in bile and ceruloplasmin that copper excretion and ceruloplasm synthesis must originate from two different compartments in the liver. The copper in the bile peaked in three hours whereas ceruloplasmin peaked in five hours. They also concluded the liver must store copper as it kept providing copper for ceruloplasmin synthesis past the peak of bile excretion.

Normal Serum Levels of Copper and Ceruloplasmin

A variety of normal values are reported for serum copper and ceruloplasmin. Values for serum copper range from 100 μg/100 ml to 113 μg/100 ml for men and 108 μg/100 ml to 116 μg/100 ml for women (27-29). Ceruloplasmin levels are found to range from 18-45 mg/100 ml (30). It is estimated in man that ceruloplasmin represents 93 percent of serum copper (18). Due to estrogenic effects, women tend to have higher values than men (4). A small but significant increase ($p<0.025$) in serum copper was observed in men but not in women with aging (29). No explanation was offered for this observation.

A small circadian variation in serum copper and ceruloplasmin show a slight rise above the mean values to exist between 10:00 AM and 2:00 PM (31).

Tissue Copper

Autopsies of six men who were healthy prior to accidental deaths revealed the liver and brain to have the highest concentrations of copper over other organs with 8 mg in each (22). Values reported for total body copper range from 80 mg (22) to 150 mg (32).

Copper Excretion

The major pathway for excretion of absorbed copper is through the biliary tract (33). The nature of biliary copper has been investigated by several workers and their findings differ. Gollan examined the binding of copper by human alimentary secretions and observed that bile contains low molecular weight copper binding proteins as well as macromolecular binding species (34). McCullars and co-workers (35) found bile copper to be conjugated to bilirubins. Lewis (36) proposes that the mechanism of copper excretion may involve its complexing with taurochenodeoxycholate in the liver, thereby preventing the copper complex from reabsorption in the upper intestine. Subsequent splitting of the complex in the lower intestine allows reabsorption of the bile acid but not of the copper.

Values for the amount of copper excreted in the bile vary greatly. Copper found in the bile of six patients post mortem ranged from 24-538 mg/100 ml with an average of 329 mg/100 ml (22). Bile copper excretion from a patient through a biliary fistula post obstruction averaged 0.46 mg/100 ml bile per day (22).

The copper excreted in the feces includes copper excreted through

the biliary tract, unabsorbed dietary copper plus salivary copper and copper in the sloughed of cells of intestinal mucosae (34).

Very little copper is excreted in the urine of a normal healthy person. The copper that is found in the urine is that which is dissociated from the copper albumin and copper amino acid fractions (19). Since the copper of ceruloplasmin is tightly bound, it would not be excreted unless the person had significant proteinuria from glomerulonephritis or significant intravascular hemolysis to free red cell copper for urinary excretion.

Profuse sweating could serve as a pathway for copper excretion. Hohnadel and co-workers (37) collected, using an arm bag, sweat from 33 men and 15 women after sauna bathing 15 minutes at 93^{o}C and found that men excreted an average of 55 μg copper in 23 ml sweat and the women excreted an average of 1480 μg copper in seven ml sweat. The sweat from the women was more concentrated in copper.

Copper Containing Proteins

Many copper-protein compounds have been isolated from tissues and have roles identified for them. Several of these copper-protein compounds are enzymes which have oxidative functions. Cytochrome oxidase, lysyl oxidase, superoxide dismutase and ceruloplasmin-ferroxidase have been identified as mammalian copper enzymes (19).

Ceruloplasmin

Ceruloplasmin is a copper containing protein synthesized only in the liver (4). Measuring ceruloplasmin is the best way according

to Danks (38) to determine copper status as a deficiency of copper decreases the production of ceruloplasmin while moderate excesses of copper does not increase the production of ceruloplasmin. The copper of ceruloplasmin is not exchanged with other copper complexes in the serum (39). Hsieh and Frieden conclude that ceruloplasmin must function as a copper transport protein based on their studies in which copper deficient rats had low cytochrome c levels that increased more quickly when given purified rat ceruloplasmin than when given copper amino acid complexes (40). Ceruloplasmin exhibits mild oxidase activity toward a variety of substrates including biogenic amines, amino phenols and paraphenylenediamine (38).

A major role of ceruloplasmin is in mobilization of tissue iron. Ceruloplasmin also is involved in intestinal iron absorption. For both processes ceruloplasmin oxidizes ferrous iron to ferric iron and incorporates it into transferrin (4). It is hypothesized that iron is presented to the cell surface in the form of ferrous iron and must be oxidized to ferric form by ceruloplasmin in order to be bound by transferrin (3). Another suggested mechanism of ceruloplasmin is that it interacts with specific sites occupied by ferrous iron on the reticuloendothelial cell surface and thus removes iron and transfers it to transferrin (3). One other possibility is that ceruloplasmin may be responsible for regeneration of cytochrome oxidase which may be required for intracellular reduction of iron (41). This reaction enhances the formation of a membrane iron pool available to transferrin (41).

Another curious feature of ceruloplasmin and copper is that estrogen elevates the blood levels of both copper and ceruloplasmin often by two to three times above normal (42, 43) without increasing absorption or decreasing excretion. There is no proposed theory for this phenomena; perhaps it is a mechanism for allowing an adequate copper supply for the developing fetus (44).

Cytochrome Oxidase

Cytochrome oxidase is the terminal oxidase of the electron-transport chain in the mitochondria. It permits the reduction of molecular oxygen to water, and energy is preserved in the process through the synthesis of ATP (45). Good and Dallman (46) have concluded that a copper protein, quite possibly cytochrome oxidase, is required for normal mitochondrial iron uptake. This is a step that precedes heme biosynthesis. Cytochrome oxidase by donating electrons to ferrous iron is believed to provide a steady supply of ferric iron to serve as a substrate for heme synthesis (3).

Superoxide Dismutase

Superoxide dismutase catalyzes the transformation of two superoxide anions in the presence of two hydrogen ions to molecular oxygen and peroxide (47). It is capable of protecting cytochrome c from reduction and prevents substances from oxidation by molecular oxygen (47). Superoxide dismutase contains two atoms of both copper and zinc (3).

Lysyl Oxidase

The formation of the cross linking compounds of collagen and elastin is dependent upon the oxidative deamination of the epsilon carbon of specific lysyl residues to form an aldehyde (4).

Copper Deficiency

Copper deficiency has been reported in malnourished infants maintained on milk diets (48), in hospital patients maintained on long term hyperalimentation (49), and in Menkes syndrome (50). Menkes syndrome is an X-linked recessive inherited disease in which blood levels of copper are lower than normal. It is seen only in males and abnormalities are observed usually by one year of age (50).

The symptoms of copper deficiency include anemia, marked neutropenia, scurvy-like bone changes and hypocupremia (49).

In Menkes syndrome intestinal absorption of copper is greatly decreased leaving the patient with progressive cerebral degeneration, pilli torti and scurvy-like bone changes (50).

Copper Toxicity

Copper toxicity is seen in ingestion of more than 15 mg. of elemental copper and in Wilson's disease. The ingestion of more than 15 mg. of elemental copper usually produces nausea, vomiting, diarrhea and intestinal cramps (50). In more severe cases, intravascular hemolysis is seen.

Wilson's disease is an autosomal recessive inherited disease in

which the body retains excessive amounts of copper. It is seen more commonly in males than females (50). The onset of symptoms usually starts in adolescence or early adulthood but have been seen in childhood and as late as the fifth decade (50). Signs and symptoms of Wilson's disease include lenticular degeneration, incoordination, ataxia, progressive mental deterioration, cirrhosis of the liver and Kayser-Fleischer rings of the corneae (51).

Dietary Copper

Copper is found in small amounts in nearly all plant and animal tissue. The copper content in foods varies greatly depending upon the age, genetics and environmental conditions affecting the plant and animals and the methods of handling, processing and cooking the food. The foods in man's diet which contain the highest amount of copper are shellfish and organ meats, such as liver. Other good sources of copper in decreasing order are nuts and seeds, whole grains, legumes, dried fruits and mushrooms. One of the lowest copper containing foods is cow's milk (52).

Older analysis of human diets from which a daily intake of 2 to 5 mg copper was reported are being reexamined. Recent surveys of human dietaries indicate daily copper intakes of below two mg and some below one mg (53, 54). Holden and co-workers (53) analyzed the self-selected diets of 11 men and 11 women and found that diets containing adequate amounts of energy provided adequate amounts of copper and suboptimal amounts of calories contributed to low copper intakes. The average

copper content of Type A school lunch from 300 schools around the USA was found to be 0.34 mg (55). Since this represents one-third of the daily intake, the average copper intake would amount to about 1 mg/day. Hospital diets analyzed for copper provided 0.7 mg/day (56) and 1.05 mg/day (57). In evaluating these data, differences in sample preparation, food preparation and methods of analysis must be considered. These new reports may demonstrate a decrease in copper intake or inaccurate past analysis.

Despite the known essentiality of copper, it has been difficult to determine a recommendation for man because it is unclear how much dietary copper is absorbed and how much absorbed copper is excreted. A provisional NRC-RDA for copper has been set at the range of two to three mg per day (58). Klevay and co-workers (59) believe the provisional recommendation to be too high to be met by average American diets. They estimated a daily requirement of 1.3 mg to replace fecal and urinary losses. On consideration of a calculated surface loss (hair, sweat and skin) of 0.25 mg/day, the requirement to replace losses would be 1.55 mg/day. This is in close agreement with the 1.65 mg/day determined by Hartley and co-workers (60). But these two groups appear to support the provisional recommendation since the requirement is a part of the recommendation. A daily recommendation of 2 mg per day seems to be necessary to replace losses, provide a margin of safety and allow a little for individual variation.

Copper Antagonists

Copper absorption may be decreased in the presence of dietary factors such as excesses of zinc, molybdenum, and cadmium which can compete for binding sites (5-7). Large amounts of potassium sulfide may form unabsorbable copper complexes (8).

In chicks cadmium may replace copper at intestinal absorptive sites and active metabolic sites (5). The effect of high dietary levels of both cadmium and zinc given to rats on a diet marginally adequate in copper was a 40 percent decrease in plasma copper and a 47 percent decrease in liver copper (6). Zinc toxicity in rats interferes with copper metabolism by competing for intestinal absorption sites and increasing the biliary excretion of copper (7). After oral administration of 135 mg/day of zinc for 12 weeks to healthy adult humans, plasma copper levels decreased from 16.5 μmols/liter to 11.2 μmols/liter. This effect was seen after six weeks of excessive zinc intake (61).

In sheep high molybdenum intakes lead to low copper absorption and may induce copper deficient anemia but this effect can be overcome with high intakes of copper. The effect of molybdenum may be due to its nonspecifically binding to sulfur compounds that are specific for copper (62). In the rat molybdenum was found to raise blood levels of molybdenum and copper when copper intake was adequate (9).

Divalent nickel, zinc and cobalt have been found to inhibit the oxidase activity of ceruloplasmin by competing with copper for metal binding sites on the native protein (8).

Potassium sulfide may decrease copper absorption. In one study during administration to humans, it formed copper sulfide complexes and thus increased the excretion of copper in the stools (8).

Ascorbic Acid and Copper

Another dietary component found to affect copper absorption in experimental animals is ascorbic acid. Animal studies have shown that intakes of megadoses of ascorbic acid decrease the absorption of copper.

Carlton and Henderson (10) found that feeding chicks a diet containing 8 ppm copper and supplemented with .5 gm ascorbic acid per kg feed decreased weight, hemoglobin and hematocrit 42, 27 and 20 percent, respectively. Both their unsupplemented copper deficient group and supplemented copper-deficient group died within six weeks of arterial rupture. Two other experimental groups of chicks were given ascorbic acid 5 gm/kg feed with one group receiving 24 ppm copper in the diet and another group 40 ppm copper in the diet. Increased copper protected against hemoglobin and hematocrit depression. Ascorbic acid may have caused a 'conditioned' deficiency as the extra dietary copper seemed to aid in overcoming the ascorbic acid effect. Hill and Starcher (11) observed that the presence of 0.1 percent ascorbic acid decreased growth by 40 percent and reduced aortic elastin by 60 percent in copper deficient chicks. The test chicks were administered radioactive ^{65}Cu either orally or intraperitoneally and given 0.1 percent ascorbic acid. In the test chicks, 25 percent less liver radioactivity was found than in the controls. Since chicks reach

adulthood in a matter of weeks, these investigators were able to see the consequences of copper deficiency within a few weeks.

In studies utilizing rabbits, Hunt, Carlton and Newberne (12) observed weight gains that were one-half normal and severe anemia in a group fed a diet deficient in copper and supplemented with one percent ascorbic acid. The rabbits' hemoglobin decreased 35 percent and hematocrit decreased 37 percent in the first eight weeks by which time they presented the first outward signs of copper deficiency when compared to rabbits with uncomplicated copper deficiency. Activity of cytochrome oxidase was lowered 68 percent in homogenates of heart from the copper deficient ascorbic acid supplemented group. Heart lesions were also seen in these rabbits and are thought to be associated with anemia and reduced cytochrome oxidase. The copper deficient rabbits given ascorbic acid developed bone lesions, and gross evidence of skeletal changes became apparent after five weeks. Supplementation of a diet containing 3 ppm copper with one percent ascorbic acid by Hunt and Carlton (13) to rabbits induced a copper deficiency producing the same myocardial lesions observed in rabbits made deficient by feeding a diet containing 2 ppm copper. In their investigations, Hunt and Carlton found little evidence of a deficiency in rabbits receiving a diet containing 3 ppm copper.

Van Campen and Gross (14) found that ascorbic acid significantly depressed the absorption of ^{64}Cu in rats when the acid was put into a ligated intestinal segment along with the radioactive copper. Rats fed a diet containing one percent ascorbic acid retained less of an

orally administered dose of ^{64}Cu than did the controls. One percent ascorbic acid with a copper deficient diet did not affect liver copper and weight gains.

None of these studies may relate to humans as all of these experimental animals can synthesize vitamin C and these animals were placed on copper deficient diets. But these investigations demonstrate that possibly megadoses of ascorbic acid depress copper absorption.

Recently Milne and Omaye (15) gave male guinea pigs a basal diet adequate in copper and supplemented with an aqueous solution of ascorbic acid 25 mg/100 gm body weight. After 28 days the ascorbic acid supplemental group exhibited a 50 percent ($p<0.001$) reduction in both serum copper and ceruloplasmin.

Smith and Bidlack (16) fed female guinea pigs a diet containing a normal amount of copper (0.5 mg/day) and pharmacologic doses of ascorbic acid (225 mg/day, ten times normal) for 21 days. Copper levels in the liver and the blood were decreased 39 percent ($p<0.01$) and 52 percent ($0<0.025$), respectively, suggesting that ascorbic acid decreased copper absorption in these investigations. These studies relate more to humans not only in that guinea pigs require ascorbic acid but also because they were fed diets containing normal levels of copper.

An explanation for the effect of ascorbic acid is offered by Osterberg who states that at high pHs reduced ascorbate will reduce divalent copper to monovalent copper (62). Since copper appears to

be absorbed in the divalent state a reduction of copper to the monovalent state by ascorbic acid would decrease the efficiency of upper intestinal copper absorption.

Since animal studies have demonstrated that megadoses of ascorbic acid decrease copper absorption this raises the question of: would there be a similar effect in man? Taking megadoses is currently very popular. This popularity may be spurred by Linus Pauling who, in his book Vitamin C and the Common Cold (63), advises intake of ascorbic acid ranging from 250 mg to 10 gm per day. His views have encouraged many to believe that gram quantities of ascorbic acid daily are not only safe but advantageous in promoting good health. But is there a nutrient interaction in humans between megadoses of ascorbic acid and dietary copper as has been found in animal experiments? This question has been raised in recent review articles (4, 64).

MATERIALS AND METHODS

Subjects

The subjects consisted of 13 men ages 20 to 40 who freely answered advertisements placed in the school newspaper and requests made in an introductory nutrition class. Men were chosen as subjects to avoid the influence of estrogen on serum copper and ceruloplasmin. All were in good health as determined by outward appearance. None of the subjects were on any medication prior to or during the study. Also none were taking any nutritional supplements prior to the study. As required by the committee for the Protection of Human Subjects at Oregon State University (which approved this study) the purpose, procedure and risks of the study were explained to all of the subjects before signing a consent form. All of the subjects were told that they could leave the study at any time.

Experimental Design

Each subject was given verbal and written instructions to take one 500 mg ascorbic acid tablet (Cevalin, Eli Lilly) three times per day with their self-selected meals in the morning, noon and evening. It was thought that taking a megadose of ascorbic acid with meals would decrease dietary copper absorption as was demonstrated in experimental animals (14). The subjects took 1500 mg ascorbic acid per day for ten weeks. This was followed by a two week termination period.

One three day diet record was kept by all subjects of their self-selected diets to provide an estimate of their dietary copper intake

as a group. Each subject was instructed to record all food and drink consumed in a 72 hour period. The diet forms also contained instructions for recording all foods eaten. A three day diet record has been found to be a reliable index for intake of nutrients when it is used to assess a group (65, 66).

Over a three month period five 10 ml anticubital blood samples were drawn. Each blood sample was drawn in the morning after an overnight fast. The blood samples were drawn by Linda Barstow, a registered medical technologist and research assistant in the department of foods and nutrition. Becton-Dickinson (Rutherford, New Jersey) serum Vacutainer syringes which are indicated to be metal free were used to draw the blood samples (catalogue number 6526). Analysis of the first blood sample, drawn on day one, showed that the subjects fell within accepted ranges of normal for serum ceruloplasmin and serum copper. All further determinations of these parameters during the experimental period were compared to initial values so that each subject served as his own control. The next four blood samples were taken at 28 days, 52 days, 64 days and 84 days. After day 64 ascorbic acid supplementation was terminated. Analysis from day 84 was used to assess the effect of termination on serum copper and serum ceruloplasmin.

All samples were refrigerated and allowed to coagulate for a period of at least one-half hour. To separate the serum all samples were centrifuged at 2500 rpm for 35 minutes at $4^{o}C$. The serum was removed with Pasteur pipettes and stored refrigerated at $4^{o}C$.

Precautions taken to minimize trace mineral contamination included the use of Pyrex glassware soaked overnight in ten percent reagent grade nitric acid and rinsed with redistilled water.

Ascorbic Acid Tablets

The amount of ascorbic acid given, 1500 mg, was chosen because it was still within a range considered safe (64) and higher than an individual would normally consume from a regular diet.

The ascorbic acid tablets were obtained with the aid of Charles Summy, head pharmacist of the Student Health Center at Oregon State University.

Compliance Determination

The subjects were given a bottle containing a known amount of tablets once a week. Generally each bottle contained 25 tablets and 21 were required to be taken. Extra tablets were given in case a few tablets were lost, dropped or destroyed. The number of tablets left in the bottle was counted and the difference was assumed to be the number of tablets taken for that week. The number of tablets originally in each bottle, the number remaining at the end of the week and the difference were recorded every week. To assess the compliance of the group the number of tablets assumed taken each week by the subjects was totaled and a weekly mean was calculated.

Dietary Copper Estimation

The copper content of the three day diet record was estimated, by hand calculations, using the table of copper content of foods by Pennington (52). The total copper intake was averaged to give a mean copper intake per day for the group. The subjects were asked to include lists of ingredients or recipes for food combinations (all subjects complied). A few assumptions were made for foods that were not fully named such as milk or bread. These foods were assumed to be the most commonly consumed,i.e. "milk" was estimated as whole milk and "bread" as white enriched.

Methods

The blood samples were analyzed for serum copper and ceruloplasmin on the same day they were drawn. Serum copper was analyzed according to the method described by Parker et al. (67). The serum was thoroughly mixed and 1 ml placed in a small test tube to which an equal volume of eight percent trichloroacetic acid was added. The solution was mixed and allowed to stand five minutes before centrifuging for 15 minutes at 2500 rpm at 28^{o}C. The atomic absorption spectrophotometer (Perkin-Elmer, model 403, Norwalk, Conn) was calibrated using known standards of copper. Copper was measured according to Perkin-Elmer's standard methods of analysis (68). To correct for volume change caused by trichloroacetic acid precipitation of serum protein in analysis of serum copper the calculated µg percent value was divided by 0.95 (69).

Ceruloplasmin was assayed as para-phenylenediamine oxidase as described by Houchin (70). Working in duplicate a 0.1 percent para-phenylenediamine solution was equilibrated to 37°C for five minutes in a shaking water bath. One tenth ml of serum was added and allowed to incubate for 15 minutes at 37°C. Sodium azide was added to stop the enzymatic reaction and optical density was read against a reagent blank on a Bausch and Lomb, Spectronic 88 spectrophotometer at 540 nanometers wavelength within one-half hour of analysis. The standard curve was determined using Bandrowski's base and read against a reagent blank.

Statistical Analysis

Statistical analysis of the data was made using one way analysis of variance tested by an F statistic. When the variances were different, differences between the baseline data and each observation were tested for significance by least significant difference. Results are expressed as mean values ± SEM. Linear regression was used when a consistent time related change was observed (71).

RESULTS

Subject Compliance

The mean number of tablets taken per person over the ten week period ranged from 17 to 21 tablets. Group compliance to taking the required number of tablets was at least 90 percent for the first nine weeks with the exception of week two at 85 percent compliance. Week 10 compliance fell to 80 percent.

Estimated Dietary Copper Intake

As calculated from a three day diet record the estimated copper intake from the subjects' self-selected diets was 1.92 mg copper per day. This estimated intake is very close to the provisional NRC-RDA of 2-3 mg copper per day (55) but higher than the 0.5-1.05 mg found by other investigators (51, 52).

Effect of Ascorbic Acid Supplementation

Serum Ceruloplasmin Activity

As shown in Table 1 the overall effect of ascorbic acid supplementation for 10 weeks was a 20 percent decrease ($p<0.005$) in serum ceruloplasmin. The levels for all subjects decreased from initial values. The first observation taken at four weeks showed a 14 percent decrease ($p<0.01$) with 11 out of 13 subjects showing a decrease from initial values. The lowest mean ceruloplasmin value, a 26 percent decrease ($p < 0.005$), was seen in the seventh week. The seventh week values

TABLE 1

Serum Ceruloplasmin during Ascorbic Acid Administration

Subject	Serum Ceruloplasmin, IU			
	initial	4 weeks	7 weeks	10 weeks
1	33.9	44.6	32.7	36.6
2	39.2	39.2	36.6	36.6
3	36.6	31.2	31.4	31.4
4	39.2	29.9	23.5	24.9
5	41.7	28.7	20.7	29.3
6	36.7	31.2	28.7	26.2
7	32.7	24.7	24.7	32.7
8	32.7	28.7	23.5	23.7
9	33.9	28.7	28.7	27.4
10	31.2	28.7	22.2	26.2
11	39.2	33.9	28.7	27.4
12	31.2	28.7	23.5	27.4
13	41.7	26.2	23.5	27.4
mean	36.1	31.1	26.8	29.0
SEM	1.0	1.5	1.3	1.1
cumulative change	0	-5	-9.3	-7.1
P		<0.01	<0.005	<0.005

for all subjects further decreased below all previous values.

Serum Copper

The effect of ascorbic acid supplementation on serum copper is shown in Table 2. Serum copper levels decreased 4.9 percent after four weeks with seven out of 13 subjects showing a decrease from initial values. A 6.9 percent total decrease in serum copper values was seen after ten weeks. The tenth week values for seven out of 13 subjects were decreased below baseline values. A consistent decrease in serum copper values was seen in each observation but was not statistically significant. The seventh week serum copper levels were not included because they were found to be contaminated with an undetermined source of copper.

To summarize, the effect of ascorbic acid supplementation on serum ceruloplasmin, and copper is shown graphically in Figure 1.

Termination Period

One subject became ill and dropped from the study before completion of the termination period.

The effect of terminating ascorbic acid supplementation on serum ceruloplasmin is shown in Table 3. Serum ceruloplasmin levels increased seven percent from week ten to week 12. The difference, however, was not statistically significant. Seven out of 12 subjects showed a rise in ceruloplasmin. The others showed no change or a smaller than previous decrease. Ceruloplasmin increased 14 percent

TABLE 2

Serum Copper during Ascorbic Acid Administration

Subject	Serum Copper µg %		
	initial	4 weeks	10 weeks
1	106.8	132.3	111.2
2	121.4	114.3	111.2
3	113.7	90.2	99.6
4	77.9	90.2	82.2
5	113.7	84.2	93.8
6	99.5	102.2	87.9
7	99.5	90.2	99.6
8	92.1	90.2	87.9
9	113.7	96.2	93.8
10	92.1	102.2	93.8
11	92.1	102.2	93.8
12	84.2	84.2	87.9
13	113.7	78.2	87.9
mean	101.6	96.7	94.7
SEM	±3.7	±3.9	±2.5
cumulative change	0	-4.9	-6.9
P		NS	NS

Figure 1

Influence of ascorbic acid supplementation on serum copper and ceruloplasmin

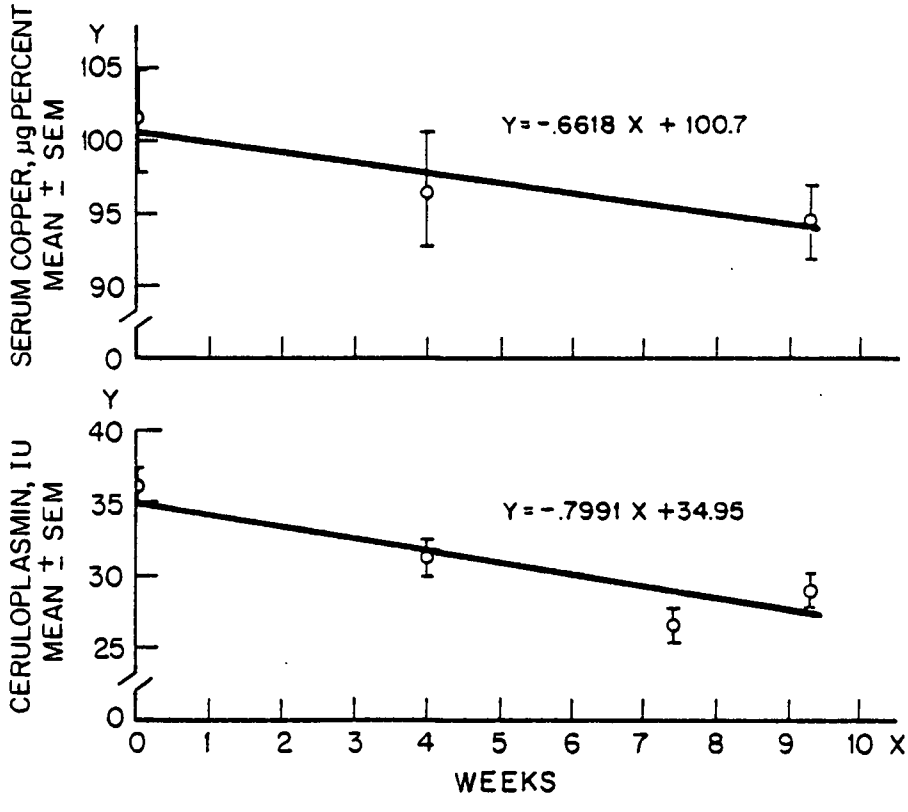


TABLE 3

Serum Ceruloplasmin Following Termination of Ascorbic Acid Supplementation

Subject	Serum Ceruloplasmin, IU		
	10 weeks	12 weeks	Change
1	36.6	36.6	0
2	36.6	39.2	+2.6
3	31.4	31.3	-0.1
4	24.9	18.2	-6.4
5	29.3	26.1	-3.2
6	26.2	31.3	+5.1
7	32.7	31.3	-1.4
8	23.7	23.5	+0.2
10	26.2	37.9	+11.7
11	27.4	36.6	+9.2
12	27.4	30.0	+2.6
13	27.4	39.9	+12.5
mean	29.0	31.3	+2.3
SEM	1.1	0.7	
P		NS	

($p<0.01$) from week seven to week 12.

As seen in Table 4 serum copper increased 14 percent ($p<0.001$) from week 10 to week 12. Serum copper levels for all subjects increased.

Figure 2 summarizes the effect of ascorbic acid termination on serum ceruloplasmin and serum copper.

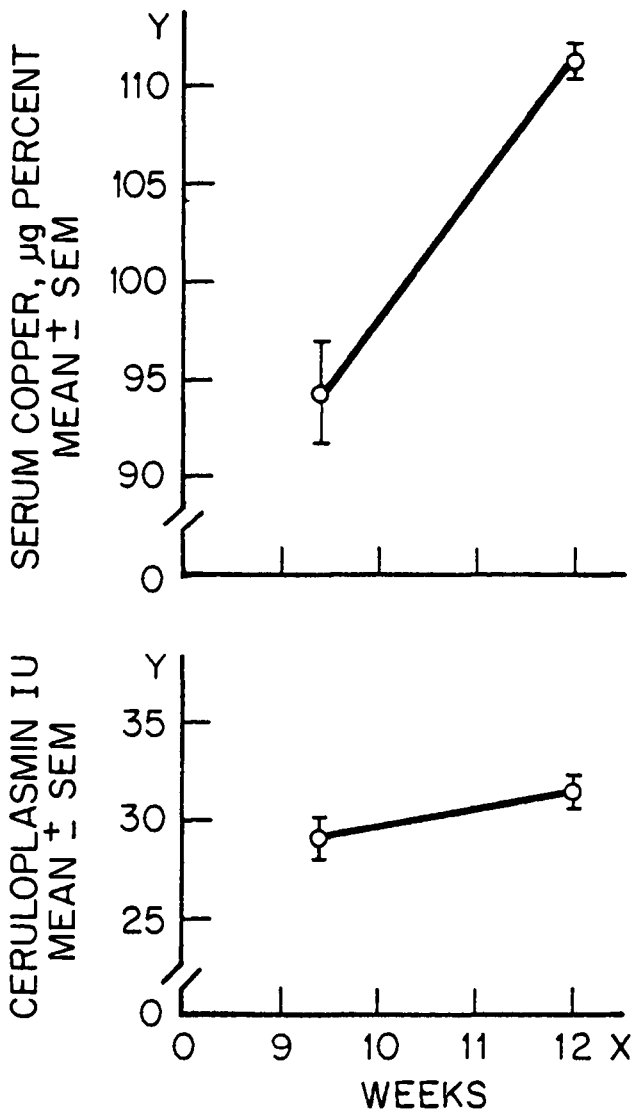
TABLE 4

Serum Copper Following Termination of Ascorbic Acid Supplementation

Subject	Serum Copper, µg %		
	10 weeks	12 weeks	Change
1	111.2	124.5	+13.3
2	111.2	124.5	+13.3
3	99.6	108.7	+9.1
4	82.2	98.2	+16.0
5	93.8	108.7	+14.9
6	87.9	108.7	+20.8
7	99.6	113.9	+14.3
8	87.9	92.9	+5.0
10	93.8	113.9	+20.1
11	93.8	124.5	+30.7
12	87.9	108.7	+21.2
13	87.9	108.7	+21.2
mean	94.6	111.3	+16.7
SEM	±3.7	0.9	
P		<0.01	

Figure 2

Effect of termination of ascorbic acid supplementation on serum copper and ceruloplasmin



DISCUSSION

Copper has been known to be an essential metal for over 50 years. It is involved in a variety of functions including bone formation and heme synthesis. Intestinal absorption of copper is decreased in the presence of zinc, molybdenum, cadmium and potassium sulfide. Results from several animal studies indicate that ascorbic acid will also decrease intestinal copper absorption. This study examined the hypothesis that excess ascorbic acid will decrease the amount of dietary copper absorbed in man which would be reflected in a decrease in copper status. For this reason the subjects of this study were instructed to take the tablets with their meals to maximize a possible decrease in dietary copper absorption by ascorbic acid.

In support of this hypothesis, the subjects' copper status, as indicated by ceruloplasmin, was significantly decreased. The decline in ceruloplasmin values was consistent and progressive over the first seven weeks and at the end of the ten week period was statistically significant. Serum copper values also showed a continual decrease over the ten week period. These results agree with the findings from the guinea pig studies of others (15, 16). The guinea pigs of these workers and the subjects of this study were treated in a similar manner in that they consumed a diet containing a normal amount of copper and were given ascorbic acid in excess of need over a period of weeks. Milne and Omaye (15) report a 50 percent decrease ($p<0.001$) in both ceruloplasmin and serum copper in male guinea pigs after 28 days of

ascorbic acid supplementation. Reversing their procedure and giving an ascorbic acid deficient diet ceruloplasmin and serum copper levels rose to twice the levels found in the controls. Smith and Bidlack (16) found in female guinea pigs after 21 days of ascorbic acid supplementation liver copper decreased 39 percent ($p<0.01$) and blood copper decreased 52 percent ($p<0.025$). Evidence from the guinea pig studies and this present study indicates that over a short period of time excess ascorbic acid will decrease copper status.

The consistent decrease seen in ceruloplasmin and serum copper can be attributed to ascorbic acid supplementation for the following reasons. Physiologic parameters such as estrogen (43) and adolescence (72) influence copper status; therefore, males over 20 years of age were chosen for this study to obtain consistent values. The ceruloplasmin level of an adult has been found to remain constant over time (72) with little diurnal variation (31). All of the subjects but one showed a consistent decrease in ceruloplasmin to week seven. Subject compliance in taking the required number of tablets for the first nine weeks was at least 90 percent with the exception of week two at 85 percent. Week ten ceruloplasmin was slightly higher than week seven but subject compliance dropped slightly. These variations in the last week of supplementation give further evidence to connect the changes in copper status to an influence by ascorbic acid. After ascorbic acid supplementation was terminated, ceruloplasmin and serum copper levels rose. All subjects showed a significant rise in serum copper. Seven out of 12 showed a rise in ceruloplasmin from week ten to week 12.

Ceruloplasmin increased 14 percent ($p<0.01$) from week seven (the lowest point) to week 12.

Since the functions of ceruloplasmin are varied, decreased levels may alter several of the body's metabolic pathways. Ceruloplasmin is the copper transport protein. It transfers copper from the liver to the tissues and specifically to cytochrome oxidase and lysyl oxidase. Copper is released from ceruloplasmin to these tissues through a degradative process. Ceruloplasmin has also been found to oxidize naturally occurring substances such as biogenic amines (38). Copper is associated with maintaining iron in the ferric state through ceruloplasmin activity (3). Ceruloplasmin oxidizes the ferrous iron released from liver storage to ferric iron so that it can be incorporated into transferrin for transport. The results of this study do not define what significant changes would occur when ceruloplasmin levels are decreased. Even though decreased the subjects' serum copper and ceruloplasmin levels remained within the physiologic ranges of normal.

If this subtle reduction in ceruloplasmin continued over time could it decrease the amount of copper delivered to the tissues and eventually alter the activity of other copper containing proteins? If an individual were consuming a marginal level of copper would excess amounts of ascorbic acid significantly depress copper status as was seen in several animal studies? The amount of ascorbic acid given in this study, even though 25 times the RDA, was still within a level considered safe (64). The amount given was not as high as the up to

10 gm amount suggested by Pauling (63). This study indicated that 1500 mg ascorbic acid daily over a relatively short period of time can significantly decrease copper status in healthy adult males. The results of this study call into question safety of prolonged self dosage of ascorbic acid at high levels.

SUMMARY

Intestinal absorption of copper has been found to be decreased in the presence of such factors as zinc, molybdenum, cadmium and potassium sulfide (5-9). Findings from several animal studies indicate that ascorbic acid will also decrease intestinal copper absorption (10-14). Investigators in two recent studies using guinea pigs reported that megadoses of ascorbic acid significantly decreased ceruloplasmin and serum copper by 50 percent and liver copper and blood copper by 39 and 52 percent, respectively (15, 16).

This study examined the hypothesis that excess ascorbic acid will decrease the amount of dietary copper absorbed in man which would be reflected in a decrease in copper status. Thirteen healthy adult males, ages 20-40, consuming self-selected diets took one 500 mg tablet of ascorbic acid three times per day with their meals for ten weeks. An estimation made from a three day diet record kept by each subject indicated their dietary copper intake to be 1.92 mg per day. Determination of serum copper and serum ceruloplasmin done on the first day of the ascorbic acid supplementation period showed that the subjects fell within accepted ranges of normal. All further determinations of these parameters during the experimental period were compared to initial values so that each subject served as his own control. Over a three month period copper status was analyzed five times. Two weeks before the fifth analysis was done ascorbic acid supplementation was terminated. The last analysis was used to assess the effect of terminating ascorbic acid supplementaiton on copper status.

After four weeks of a high ascorbic acid intake ceruloplasmin was significantly decreased 14 percent ($p < 0.01$). The greatest decrease in ceruloplasmin was 26 percent ($p < 0.005$) seen at seven weeks. By the seventh and 10th week observations all subjects showed a decrease from initial values. At the end of the 10 week ascorbic acid supplementation period, serum ceruloplasmin activity was significantly lowered by 20 percent ($p < 0.005$). The slight increase over week seven was attributed to a drop to 80 percent compliance to taking the ascorbic acid tablets. Up to week nine subject compliance to taking the ascorbic acid was at least 90 percent with the exception of week two at 85 percent.

Serum copper levels were not significantly affected although a consistent decrease was observed.

After ascorbic acid supplementation was terminated serum ceruloplasmin activity increased in seven out of 12 subjects but was not significantly different than week ten values. However, when compared to week seven values a 14 percent ($p<0.01$) increase was observed. Serum copper levels rose in all subjects as indicated by a 14 percent ($p < 0.01$) increase over week ten values.

Even though decreased by ascorbic acid supplementation, ceruloplasmin and serum copper levels remained within the physiologic ranges of normal. However, the results of this human volunteer study indicate that taking a megadose of ascorbic acid for a 10 week period will significantly decrease serum ceruloplasmin activity much like that observed in laboratory animal studies. Based on this finding one may

question the safety of prolonged self-dosage of high amounts of ascorbic acid.

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APPENDIX

Thank you for volunteering for this study.

What is asked of you is:

1. Take one vitamin C tablet with your meals--three times per day morning, noon, evening for 3 months.
2. Give two 10 ml blood samples once a month.

   Dates Mon Jan 26

   Mon Feb 23

   Mon Mar 30

   Mon Apr 27

   The blood must be taken before you eat breakfast.
3. Complete one three day diet record to be done at the start of the study and turned in by Friday Jan 30.
4. Come every Friday afternoon, 1:30-4:30 to Mlm 15 and turn in your old bottle of vitamin C tablets and pick up a new bottle.

Consent Form

I have discussed the rationale, procedure, benefits and safety of this investigation with the interviewer. All of my questions have been answered. I understand I am free to withdraw at any time from this investigation. While participating in this study I give my consent to give one three day diet record and two ten ml antecubital venus punctures monthly. I will consume three ascorbic acid tablets daily for a period of three months. I will complete the three day diet record to the best of my ability. I understand my name will remain confidential for the study.

Oregon State University as an agent of the State of Oregon is covered by the state liability fund. If any injury is suffered as a result of the research project, compensation could be available only if it is established that the injury occurred through a fault of the university, its officers or employees.

Subject _____

Witness _____

Date _____

Instructions for recording food

1. Please record each food and beverage you consume on a separate line. Be sure to include all snacks.

2. Record them in reasonably exact amounts: liquids in cups, fluid ounces or milliliters; vegetables and fruits in cups or inches using the ruler on the record sheet; beans, grains and pasta in cups dry or cups cooked; bread in slices, indicate what kind of bread; meats, fish and cheeses in ounces (an average meat portion is 3 oz., a slice of American cheese is about 1oz.) or measure your food with a ruler.

   If it is impractical to measure foods at certain meals, measure a comparable food at least once to establish in your mind the measure of certain quantities. Remember: the more accurate your record the more accurate the analysis will be.

3. Please specify if a food is consumed raw. Also indicate if it was prepared from fresh, canned or frozen products.

4. Indicate how the food was prepared, such as fried, boiled, baked, etc.

5. If the food is a mixture (sandwich, soup, stew) list the major ingredients separately in their proportions or amounts eaten.

6. Use brand names wherever possible, or mention comparable brand name products.

7. Specify if a food is fortified with vitamins and minerals.

8. Provide any other information you feel might be helpful.

9. Indicate if milk is whole, skim, 2% or non-fat dry milk.

10. Be sure to include sauces, gravies, milk in coffe, on cereal, sugar in cereal, etc.
    Everything you eat or drink

    If you have any questions in filling out your record please contact Betsy Finley in Milam Hall room 15 or call 928-4449

DATA RECORD SHEET

CODE NO. _____ DATE CONSUMED: _____

LEAVE A BLANK SPACE BETWEEN EACH MEAL
USE A SEPARATE SHEET FOR EACH DAY

FOOD specify each food or beverage on a separate line	SOURCE canned, dried fresh etc.	BRAND be specific	PREPARATION fried, baked, raw etc.	AMOUNT measure in cups inches etc.	FOR OFFICE USE AMT. code	 WT. code