COPPER AND MANGANESE REQUIREMENTS OF THE BABY PIG

Dissertation for the Degree of Ph. D.

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Anthony Chukwuemeka Okonkwo

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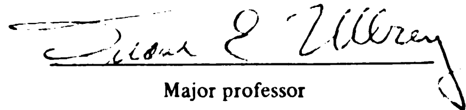

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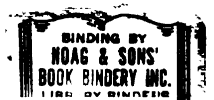

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ABSTRACT

COPPER AND MANGANESE REQUIREMENTS
OF THE BABY PIG

By

Anthony Chukwuemeka Okonkwo

Three experiments involving 52 baby pigs were conducted to determine the minimum copper requirement of baby pigs on semi-purified diets.

In the first experiment 16 week-old baby pigs were fed semi-purified diets supplemented with anhydrous copper sulfate resulting in the following dietary copper levels: diet 1 (basal), 1.3 ppm; diet 2, 3.2 ppm; diet 3, 5.6 ppm; diet 4, 9.3 ppm. Initial weights of pigs were taken and blood parameters including plasma copper were determined at this time and at subsequent 2-week intervals. After 6 weeks, a copper balance trial was conducted. The pigs were killed at the conclusion of the trial and organs and tissues were taken and kept at -20°C until used for analysis.

Ceruloplasmin activity, plasma copper levels, copper balance and hemoglobin were useful indicators of copper status, with all but hemoglobin significantly ($P<0.05$ or $P<0.01$) depressed by the basal diet (1.3 ppm Cu). Hemoglobin, while not significantly influenced by treatments, was consistently depressed by the basal diet, suggesting a sub-clinical anemia as a result of copper deficiency. From this study it appeared that the minimum copper requirement of the baby pig is lower than 5.6 ppm.

In the second experiment, 15 three-day old baby pigs were maintained on three semi-purified diets which analyzed 0.6 ppm, 1.9 ppm and 2.8 ppm copper. The casein used in the basal diet was treated with Na_2EDTA to remove as much copper as possible. The protocol used here was similar to that used in Experiment 1. Hemoglobin was depressed considerably by the basal diet (0.6 ppm Cu) but the effect was not statistically significant. Ceruloplasmin activity and plasma copper showed significant treatment differences ($P<0.05$ or $P<0.01$) particularly between the basal (0.6 ppm) and the other diets (1.9 ppm and 2.8 ppm Cu). Kidney copper concentration and physical measurements of the left femur, with the exception of weight and elasticity showed significant ($P<0.05$ or $P<0.01$) difference due to treatments. Results of the copper balance trial showed that absolute copper retention was significantly ($P<0.05$ or $P<0.01$) influenced by dietary copper levels.

In the third experiment, 21 week-old baby pigs were assigned to four dietary treatments composed of distilled deionized water and semi-purified diets analyzing 0.9 ppm, 2.0 ppm, 4.0 ppm and 4.9 ppm copper. The experimental design was similar to that used in Experiments 1 and 2. Hemoglobin values on weeks 4 and 8 showed significant ($P<0.05$ and $P<0.01$, respectively) differences between diets, with the basal diet inducing a very low hemoglobin value of 7.3 g/100 ml on week 8. The corresponding mean corpuscular hemoglobin concentration (MCHC) was likewise depressed (23.7%). These hemoglobin and MCHC values are indicative of a marginal anemia due to inadequate dietary copper (0.9 ppm). Ceruloplasmin activity was considerably depressed by the basal diet until week 8, after which it rose slightly. There were significant ($P<0.05$ or $P<0.01$) differences in ceruloplasmin activity due to treatments in weeks 4 through 10. After week 2, plasma copper values were consistently and significantly ($P<0.05$

or $P<0.01$) affected by dietary copper levels. Pigs on the basal diet had plasma copper values (5.9 and 5.2 mcg/100 ml) on weeks 8 and 10, respectively, which were much below the critical hemopoietic level of 20 mcg/100 ml.

Data from the above experiments indicate that although anemia and copper deficiency were not grossly evident, the consistent depression of hemoglobin and plasma copper levels by diets low in copper (0.6 ppm, 0.9 ppm and 1.3 ppm) showed that copper deficiency and anemia were biochemically manifested. Such a sub-clinical hypocuprosis was also indicated by the significant depression of ceruloplasmin activity, an observation strongly suggesting that on low dietary copper, ceruloplasmin activity cannot be sustained.

Based on these parameters the minimum copper requirement for the baby pig is very low and is probably between 3.0 and 4.0 ppm on an as-fed basis, or between 3.4 and 4.6 ppm on a dry basis.

As a follow-up to the study previously conducted by Kayongo-Male (1974), a trial was designed to determine the manganese requirement of baby pigs on semi-purified diets. Sixteen 8-day old baby pigs from sows on low-manganese diets (13.9 ppm Mn) were fed diets analyzing 0.9 ppm, 2.2 ppm, 3.8 ppm and 7.4 ppm Mn. Blood samples were taken at the beginning of the trial and subsequently on days 7, 14, 28 and 42 for determination of hemoglobin, hematocrit, alkaline phosphatase activity and serum manganese levels. A manganese balance trial was conducted at the end of the growth trial period. The pigs were killed thereafter and tissues and organs kept at -20°C until used for analysis. Manganese balance, serum manganese concentration and serum alkaline phosphatase activity were indicators of manganese status.

Hemoglobin on day 42 was significantly ($P<0.05$) higher on diet 4 than on diets 1 and 3. Mean corpuscular hemoglobin concentration (MCHC) of pigs on diet 4 was significantly ($P<0.05$) higher than the corresponding values of pigs on diets 1 and 2, while MCHC value for diet 3 was significantly ($P<0.01$) lower than that for diet 4. Initial serum alkaline phosphatase activity was high on all diets, but decreased dramatically throughout the trial; there were, however, no statistically significant treatment differences. Serum manganese concentrations merely fluctuated without showing any statistically significant response to dietary levels of manganese.

Manganese intake, manganese retention (absolute and as percent of intake), and fecal and urinary manganese excretion were significantly ($P<0.01$) influenced by dietary manganese levels. Absolute fecal manganese excretion was significantly ($P<0.05$) different between diets 1 and 2, and between diets 1, 3 and 4 ($P<0.01$). There was a negative absolute manganese retention induced by diets 1 and 2 (-0.05 and -0.004 mg/day, respectively) resulting in negative retention as percent of intake (-16.4 and -0.24%, respectively); thus manganese excretion via urine and feces was greater than manganese absorption on diets 1 and 2.

Heart, thyroid and spleen weights as percent of final body weight were significantly ($P<0.05$ or $P<0.01$) affected by dietary treatments as were liver and kidney manganese concentrations. Manganese intake was significantly ($P<0.01$) correlated with fecal manganese, manganese retention, and negatively but significantly ($P<0.05$ or $P<0.01$) correlated with manganese excretion as percent of intake.

Based on all parameters examined, the dietary manganese requirement for the baby pig is approximately 3.8 ppm (as-fed basis) or 4.3 ppm (dry basis).

COPPER AND MANGANESE REQUIREMENTS OF THE BABY PIG

By

Anthony Chukwuemeka Okonkwo

A DISSERTATION

Submitted to
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Dedicated to

My Beloved Mother

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INTRODUCTION

Although copper was known to occur in the tissues of plants and animals as early as the 1800's its biological importance was not recognized until the reports of McHargue (1925a, 1925b, 1926) and the conclusive evidence furnished by Hart and coworkers (1928) that copper was essential for mammalian hemoglobin biosynthesis. This breakthrough generated much research which resulted in the description of other metabolic functions of copper such as its involvement in cardiovascular structure, connective tissue and bone collagen synthesis, myelin formation, enzyme systems and reproduction. The literature is filled with reports of copper interaction with other minerals such as molybdenum, calcium, iron and zinc, and other dietary factors such as sulfate, proteins and lipids.

Another aspect of research on copper that sparked global interest was the European reports by Barber and coworkers (1955) followed by those of Braude (1965) that a high dietary level of copper up to 250 ppm caused a significant improvement in body weight gain and feed conversion efficiency of swine. Attempts to duplicate these trials resulted in conflicting reports from the United States and Canada. Effects of high level copper feeding ranged from depressed performance, slight improvement in body weight gain accompanied by decreased feed efficiency, to highly significant overall improvement in performance. These contradictory reports notwithstanding, it was clearly established that copper is essential for optimal performance in livestock. However, the minimum

amount of copper required by growing pigs has not been precisely established.

Because of the effects of interaction between copper and other minerals it is difficult to define precisely the minimum copper requirement of baby pigs. It seems therefore that the most practical approach would be to maintain other dietary factors at an optimal level while varying the concentrations of copper in the diets. Semi-purified diets were used in the work reported here, since other dietary factors were easily controlled.

The importance of defining the minimum requirement of copper for swine becomes clear when the side effects of high dietary dosages of copper on the environment are considered. Since copper is not biodegradable and tends to accumulate in the top 8 inches of the soil (Lagerwerff, 1967), it has therefore a very good chance of contaminating water sources and affecting aquatic life. Accumulation of copper in the environment arises from its widespread use as a fungicide in the form of Bordeaux mixture, its high concentration in swine waste from farms using high dietary copper levels, and other agricultural uses such as viniculture, control of potato blight, leaf spot of sugar beets and peach leaf-curl. Although the NCR-42 Committee does not regard "lagoon and lake sterility" as a result of the antibiotic-like effects of copper as a very serious problem, the danger has been recognized. High concentrations of copper in the soil have been reported to interfere with the functions of the soil nitrifying flora (Gilbert, 1952). The use of swine waste containing high levels of copper, if not monitored and modified, produces chronic toxicity in ruminants, particularly sheep.

Although the threat of copper toxicity in humans arising from

ingestion of meat products from animals fed high levels of copper is not very likely, a USDA regulation requires withdrawal of copper from finishing pigs 15 days prior to slaughter. Porcine muscle copper level is low and the effect of dietary copper is negligible; however, the liver concentration of copper is markedly increased by high copper levels in the diet, and the 15-day withdrawal provides sufficient time for depletion of excess copper in this organ.

Based on the studies of Ullrey and associates (1960) the NRC (1973) suggested that 6 ppm copper is the requirement for baby pigs on natural corn-soy diets. Since the mechanism of action of copper at high dietary intakes is not clearly understood but is supposed to resemble the action of antibiotics, it is important that a distinction be made between minimum essential requirement of copper for optimal metabolic functions on the one hand and an antibiotic-like effect on intestinal microflora by high amounts of copper inducing increased performance on the other.

The experiments reported in this dissertation were conducted to determine:

1. The minimum essential requirement of copper for baby pigs, using semi-purified diets and varying the levels of copper.

2. The effects of low levels of copper on body weight gain, feed consumption, feed conversion efficiency, tissue copper levels, some enzyme systems and copper balance in baby pigs.

LITERATURE REVIEW

A. Copper as an Essential Trace Element

In the latter part of the twenties McHargue (1925a, 1925b) provided evidence of the existence of copper in plant and animal tissues indicating that the livers of domestic animals, more than any other organ, contain copper in addition to manganese and zinc. He also showed that at birth bovine liver yields more copper than does the adult liver. At about the same period McHargue (1926) conducted studies using rats and presented data showing the importance of copper as a trace mineral supplement to animal diets. Two years later, Hart and coworkers in Wisconsin (1928) proved conclusively the essentiality of copper and demonstrated its vital role in mammalian hematopoiesis. These researchers induced nutritional anemia in rats by raising them on whole milk diets fortified with iron salts, and then reversed the anemia effectively by administration of iron and copper, but not by iron alone. This copper-iron therapy not only restored hemoglobin to normal levels but also caused increased vigor, increased appetite and smoother hair coat. Although they were not able to define the specific function of copper in hematopoiesis, it was hypothesized that copper, like iron in chlorophyll biosynthesis, might be acting as a catalyst somewhere in the biosynthetic pathway of hemoglobin; since the hemoglobin molecule does not incorporate copper.

Subsequently, the unique position of copper in promoting

hemoglobin synthesis was confirmed by other workers (Elvehjem and Hart, 1929; Waddel *et al.*, 1929; Elvehjem and Sherman, 1932) who showed that iron supplementation by itself failed to restore normal hemoglobin status in anemic rats, thereby establishing the obligatory need for copper. The uniqueness of copper in this role was further lent credence by Waddell *et al.* (1929) who conducted feeding trials using copper and twelve other transition elements. Further studies extended this phenomenon to other species (Elvejhem and Hart, 1932; Schultze *et al.*, 1936a, 1936b; Wilkerson, 1934).

The possibility of other biological roles for copper motivated a great deal of research. Field studies with livestock were being conducted worldwide and simultaneously with laboratory investigations of copper at the cellular level in order to define its mechanism of action. Such laboratory probings uncovered the existence of many copper containing proteins in mammalian tissues and cells. Some of these proteins, such as cerebrocuprein (Porter and Folch, 1957; Porter and Ainsworth, 1959); hemocuprein (Mann and Keilin, 1938); hepatocuprein (Shapiro *et al.*, 1961); and neonatal hepatic mitochondrocuprein (Porter *et al.*, 1962; Porter *et al.*, 1964), were not thought to possess enzymatic activity. They are bluish-green soluble proteins containing two atoms of copper and zinc per molecule (Carrico and Deutsch, 1970). Erythrocuprein, cerebrocuprein and hepatocuprein were later shown by McCord and Fridovich (1969) to exhibit superoxide dismutase activity. Detailed studies of their physical, chemical and immunological properties led Carrico and Deutsch (1969) to report that erythrocuprein, hepatocuprein and cerebrocuprein are identical and should be designated collectively as cytocuprein. Although their specific physiologic functions are unknown they

are thought to represent intermediate compounds in copper metabolism (Li and Vallee, 1973).

Other copper proteins have been shown to exhibit oxidative and a variety of catalytic functions. Such copper-enzymes are monoamine oxidase, ceruloplasmin, tyrosinase, cytochrome oxidase, ascorbic acid oxidase and superoxide dismutase. Studies with copper deficient animals served to reveal the biochemical position of copper in these enzymes.

Meanwhile field studies from different parts of the world implicated copper in some livestock diseases. Since these diseases responded to copper therapy they were regarded to be a consequence of copper deficiency. From Florida, Neal and associates (1931) described the "salt sick" of cattle and sheep, Sjollema (1933) reported the "lecksucht", a disease of sheep and cattle in Holland, "falling disease" of cattle was recorded in south-western Australia (Underwood, 1966), Bennetts and Chapman (1937) labelled a disease of lambs "enzootic neonatal ataxia", some areas of England and Scotland witnessed "swayback in sheep, and there were scattered reports of copper deficient livestock from Scandinavia, tropical Africa, South America, and the Mediterranean regions (Underwood, 1966). Detailed histopathological examinations of these copper deficiency diseases indicated that, in addition to hemoglobin biosynthesis, copper was also involved in cardiovascular function, cerebrospinal myelination, connective tissue and bone formation, growth and reproduction, pigmentation, and adipose tissue metabolism.

In its biological roles, copper was also found to interact with molybdenum and sulfate (Dick and Bull, 1945; Dick, 1952, 1954a, 1954b, 1956), zinc, iron, calcium (Hill and Matrone, 1962; Mills, 1968; Kline *et al.*, 1972) and cadmium (Whanger and Weswig, 1970).

B. Tissue Distribution of Copper

According to the studies of Spray and Widdowson (1951) and Widdowson (1960) the young of most animal species possess a much higher level of copper per unit of body weight than the adults, and this level gradually declines as maturation progresses. Various species of animals have been surveyed and shown (Cunningham, 1931) to contain in their livers a greater fraction of total body copper than in any other organ or tissue at a given point during life. These liver levels can be as high as 79% of the total body supply in ruminants, especially in sheep (Dick, 1954). Tissue analyses by Cunningham (1931) and Smith (1967) of various species established that the liver, brain, kidneys, heart, and hair contain high concentrations of copper, while spleen, pancreas, muscles, skin, and bone are of intermediate copper levels, and prostrate, pituitary, thyroid and thymus glands show low levels. Analysis of samples from liver, spleen, and aorta show a fall in copper content as the adult stage is attained, whereas adult brain concentrates almost twice its fetal copper stores (Schroeder *et al.*, 1966).

Variations in dietary copper concentrations exert a direct effect on the levels of copper in blood, liver, kidney and spleen, but the bone shows a more significant reduction in forms of copper particularly the chicken bone as reported by Rucker *et al.* (1969). Different species show a wide variety of eye copper levels. Examination of fresh water fishes, frogs and mammals (Bowness *et al.*, 1952b; Bowness and Morton, 1952a) indicated that the highest levels of eye copper are found in the pigmented areas like choroid, retina and iris. These levels range from 13.5 ppm (on the dry basis) in sheep choroid to 105 ppm in the iris of

freshwater trout. Hair copper concentrations have been reported to range from a mean value of 7.8 ppm in black hair of dairy cattle (Anke, 1967) to 10 to 47 ppm in black and white hair from ten different species (Goss and Green, 1955). Levels below 8 ppm copper in cattle hair were associated with copper deficiency by Van Koetsveld (1958), but this association did not hold true in studies with copper-deficient cattle (Cunningham and Hogan, 1958) and rats (Dreosti and Quicke, 1966). Depigmentation of hair or achromotrichia is a condition exhibited by a wide range of species including rats, guinea pigs, rabbits, sheep, cattle and goats at dietary intakes low enough to induce copper deficiency; this phenomenon of loss of hair color has not been noticed in pigs (Underwood, 1971). A breakdown in synthesis of melanin from tyrosine, a reaction catalyzed by a copper enzyme, has been suggested as a probable cause of achromotrichia (Roper, 1928). Supplementation of the deficient diet with adequate copper quickly reverses this condition.

In blood, a great proportion of copper (85-90%) is bound with an α-2 globulin as ceruloplasmin (Wintrobe *et al.*, 1953), and about 10 to 15% is associated with red blood cells (Kimmel *et al.*, 1959, Markowitz *et al.*, 1959). About two-thirds of total erythrocyte copper is bound to a protein formerly known as erythrocuprein (superoxide dismutase) in humans (Shields *et al.*, 1961), and hemocuprein (superoxide dismutase) in bovine erythrocytes (Mann and Keilin, 1938). In addition to ceruloplasmin and erythrocuprein, the blood contains copper enzymes such as monoamine oxidase and cytochrome oxidase. The normal blood levels of copper vary from 0.5 to 1.5 mcg/ml in species like rats, dogs, pigs, sheep, cattle and humans (Beck, 1961).

C. Metabolism of Copper

1. Absorption

Various workers have shown that the region(s) of the gastrointestinal tract from which copper is absorbed varies among species of animals. Bowland *et al.* (1961) reported that most of the copper ingested is absorbed from the small intestine and colon in pigs; in the rat, absorption is from the stomach and small intestine (Van Campen and Mitchell, 1965); from the jejunum in dogs (Sacks *et al.*, 1943); and in chicks from the duodenum (Starcher, 1969). In man the net percentage of copper absorbed is slightly over 30% of the amount ingested (Cartwright and Wintrobe, 1964).

Absorption of copper from the gastrointestinal tract has been shown to be affected by several factors. Starcher (1969), using radioactive copper, reported that, in the chick, binding of copper to a duodenal protein constitutes an important phase in copper absorption. As the calcium content of the diet increases, a marked decrease in utilization of dietary copper results (Kirchgessner and Grassman, 1970). This is because high dietary intakes of calcium salts raise the pH of intestinal contents, thereby depressing copper absorption as shown in sheep (Dick, 1954). Ferrous sulfide also depresses copper absorption by inducing formation of insoluble copper sulfide.

Studies on the availability of copper from various copper compounds indicate that for pigs (Bowland *et al.*, 1961) and sheep (Lassiter and Bell, 1960) the water soluble forms of copper, particularly sulfate, nitrate, carbonate and chloride, are more effectively utilized than are copper sulfide, cuprous oxide and cupric oxide.

Copper absorption is also depressed by certain organic substances which form complexes with copper, rendering it unavailable for absorption. Phytate present in soybean proteins was shown by Davis and coworkers (1962) to reduce absorption of copper. In chicks, increased dietary ascorbic acid (Hill and Starcher, 1965) exacerbates copper deficiency by suppressing intestinal absorption of the element. This phenomenon also occurs in rabbits (Hunt and Carlton, 1965) and in rats (Van Campen and Gross, 1968).

Interference with normal absorption of copper from the gut has been reported to involve other minerals such as mercury (Van Campen, 1966), silver (Hill *et al.*, 1964), cadmium (Hill *et al.*, 1963; Van Campen, 1966), zinc (Starcher, 1969), molybdenum (Matrone, 1970) and sulfate sulfur (Dick, 1954). The mode of action of some of these elements has not been elucidated (e.g., mercury and molybdenum), however zinc and cadmium have been reported by Starcher (1969) to depress copper absorption by binding competitively to the duodenal mucosal protein, thereby displacing copper. Binding to mucosal protein is an essential step in copper absorption (Starcher, 1969). Molybdenum probably acts as a result of formation of a copper-molybdenum complex which is absorbed, transported and excreted undissociated (Matrone, 1970); in this bound form, the copper of the copper-molybdenum unit becomes unavailable for utilization by the animal.

Amino acids were shown by Kirchgessner and Grassman (1970) to affect the absorption of copper from rat gastrointestinal tract. Combination of amino acids with copper results in the formation of copper-amino complexes which are then transported uncleaved to the point of absorption. These researchers further indicated that the ease of absorption decreased with increased complexity of the amino acid

polymers, monomeric copper-amino acids being more easily absorbed than dimeric and so on. Configuration of amino acids was also shown to affect copper absorption, the copper-L-amino acid complexes being better absorbed than D-forms.

2. Transport and Tissue Storage

Upon absorption from the gut, copper becomes bound loosely to serum albumin (Bearn and Kunkel, 1954) forming the small direct-reacting fraction of serum copper. This copper-albumin forms a pool which assumes pivotal importance in that it receives copper from many sources; absorbed copper from the intestine enters into it before being distributed to the liver, bone marrow, kidneys, erythrocytes and other tissues (Cartwright and Wintrobe, 1964); the pool also receives copper from these tissues (Bush *et al.*, 1956b). On reaching the liver parenchymal cells, copper is taken up by hepatic mitochondria, microsomes and soluble fractions for storage or for synthesis of erythrocuprein, ceruloplasmin, and other cellular copper-enzymes (Underwood, 1971).

Ceruloplasmin synthesis goes on in the liver (Markowitz *et al.*, 1955; Sternlieb *et al.*, 1962) after which it is released into the blood-stream; while erythrocuprein, according to Bush and coworkers (1956), is probably synthesized in the bone marrow normoblasts. Under normal physiological conditions, ceruloplasmin copper has been shown (Bush *et al.*, 1956b) not to exchange with the copper in the direct-reacting fraction in the blood.

3. Excretion

The biliary system is the major pathway for the excretion of copper in pigs and dogs (Mahoney *et al.*, 1955; Bowland *et al.*, 1961), in mice (Gitlin *et al.*, 1960), in humans (Van Ravestyn, 1944) and in

chickens (Beck, 1961). Liver copper is secreted through bile, back into the intestine, and small quantities of copper are excreted directly into the gastrointestinal tract - from whence they are voided as fecal copper along with a high percentage of unabsorbed dietary copper (Cartwright and Wintrobe, 1964); a smaller amount is excreted through the urinary system.

Biliary-obstruction studies performed on dogs (Mahoney *et al.*, 1955) and man (Bush *et al.*, 1955) show that of the excreted copper about 80% is biliary, 16% is from direct secretion into the gut, and about 4% is urinary. In man, negligible amounts of copper are lost through sweat (Hamilton and Mitchell, 1949).

Estimated copper turnover in man is as follows (Cartwright and Wintrobe, 1964): of the 2.0 to 5.0 mg copper ingested daily, 0.6 to 1.6 mg (32%) is absorbed; 0.5 to 1.2 mg is excreted in the bile, 0.1 to 0.3 mg passes directly into the intestines, and 0.01 to 0.06 mg appears in the urine. The daily turnover of copper through ceruloplasmin is of the order of 0.5 mg (Sternlieb *et al.*, 1961).

D. Metabolic Roles of Copper

1. Hematopoiesis

Following the pioneer studies of Hart (1928) and others in the early thirties, in establishing the role of copper as promoter of hemoglobin synthesis, more detailed research has described other aspects of copper function in blood metabolism. These aspects were studied by means of experimental induction of copper deficiency in various animal species.

The serum copper level below which normal hematopoiesis is arrested has been determined as 0.10 to 0.12 mcg/ml in sheep (Marston *et al.*, 1948) and 0.2 mcg/ml in pigs (Schultze, 1936b; Lahey *et al.*, 1952). The mechanism by which copper deficiency caused subnormal hemoglobin levels eluded early workers, until Chase and coworkers (1952a) provided evidence that, in copper-deficient rats, absorption of iron from the gut is impaired. In a copper-deficient state, depressed hemoglobin levels can result from impairment of iron metabolism, protoporphyrin and heme synthesis or biosynthesis of globin. But since it has been shown that in copper deficiency there is normal protein synthesis and hence normal production of globin (Gallagher *et al.*, 1956; Dreosti and Quicke, 1968), and heme (Lee *et al.*, 1968), the cause of impaired hemoglobin synthesis must be associated with iron metabolism. This observation was already confirmed by Gubler *et al.*, (1952) and Chase *et al.* (1952b) who induced copper deficiency in swine on milk diets and showed impaired iron absorption from the gastrointestinal tract, incomplete mobilization of iron from the tissues and marked inability of the animals to utilize parenterally administered iron for hemoglobin biosynthesis even when the iron was presented to bone marrow in normal quantities. Wintrobe and coworkers (1953) reported a major depletion of total body iron from the hemoglobin compartment in copper deficiency by reason of a depressed movement of iron from tissues to plasma; administration of copper resulted in a sharp rise in plasma iron.

The postulation by some researchers (Gubler *et al.*, 1952; Chase *et al.*, 1952a, 1952b; Wintrobe *et al.*, 1953) that copper deficiency induced hypoferremia arose from impaired mobilization of iron from tissues to plasma was later confirmed by others, and more recently by

Osaki *et al.* (1956) and Ragan *et al.* (1969) who obtained data to show that iron transfer from the tissues to plasma requires the enzymatic conversion of iron from the ferrous to the ferric state by ceruloplasmin. Ability of ceruloplasmin to convert iron into the ferric state is by virtue of its feroxidase activity (Evans and Abraham, 1973; Gray and Daniel, 1973). Oxidation of ferrous to ferric ions occurs before iron is incorporated into the carrier-protein apotransferrin for delivery to the site of hemoglobin synthesis. Failure of this oxidation step because of lack of ceruloplasmin inhibits iron transfer (Wintrobe *et al.*, 1953; Lee *et al.*, 1968), and it accumulates as non-hemoglobin iron in bone marrow, liver and the reticuloendothelial system, thereby becoming unavailable for hemoglobin formation (Cartwright *et al.*, 1956). To increase the circulating and available iron from its storage sites, a release must be effected by copper (Marston and Allen, 1967).

A consequence of this deranged hemoglobin production is anemia. In swine, rabbits and rats, anemia is microcytic and hypochromic (Foster, 1931; Fitz-Hugh *et al.*, 1933; Smith and Ellis, 1944; Smith *et al.*, 1944a, 1944b; Lahey *et al.*, 1952), accompanied by leukopenia, neutropenia and hypocupremia (Wintrobe *et al.*, 1953). Copper deficiency anemia in swine is indistinguishable from anemia of iron deficiency (Lahey *et al.*, 1952). In dogs and chicks the anemia is normocytic and normochromic (Van Wyk *et al.*, 1953; Maas *et al.*, 1944; Matrone, 1960); in ruminants grazing copper-deficient pastures, e.g., cattle and sheep, macrocytic and hypochromic (Cunningham, 1946; Bennetts *et al.*, 1941; Marston *et al.*, 1948); in lambs, microcytic and hypochromic (Beck and Bennetts, 1942). In addition to anemia, the bone marrow undergoes erythroid (normoblastic) hyperplasia with the exception that, in the pig (Baxter and Van Wyk, 1953), there is

depressed reticulocytosis (Lahey *et al.*, 1953).

Unless adequate copper levels are administered, copper deficiency anemia shows no reaction to iron therapy (Cartwright, 1956; Schultze *et al.*, 1936a; Elvehjem, 1932).

2. Cardiovascular Integrity

Severe copper deprivation in cattle was reported by Bennetts and associates (1942a, 1948) to cause myocardial fibrosis which leads to sudden death, known as "falling disease". Believed to be a consequence of cardiac failure precipitated by physical exertion, falling disease occurs almost exclusively in Western Australia. The myocardium becomes infiltrated by collagen, thus causing a loss of its architecture, and adversely affecting its function. Species specificity seems the case with this disease since it is not observed in sheep and horses exposed to the same conditions (Davis, 1950); however, in pigs cardiac hypertrophy has been observed as a result of copper deficiency, but it is not identical to nor a typical characteristic of falling disease (Gubler *et al.*, 1957). Cardiac hypertrophy seems to be due to reduced cytochrome oxidase activity which causes myocardial enlargement and, according to Li and Vallee (1973), is a compensatory mechanism for the reduced respiratory activity.

Studies done on other species have produced cardiovascular malfunction. In chicks raised on low copper diets (0.8 ppm Cu) there was widespread subcutaneous and internal hemorrhage (Carlton and Henderson, 1963; Savage *et al.*, 1966), the aorta showed greatly thickened walls, a small lumen, fragmented elastic laminae and dissecting aneurysms; mortality rate was high (Simpson and Harm, 1964; O'Dell *et al.*, 1961a; Carlton and Henderson, 1963). There were also cases of spontaneous aortic rupture,

loss of elastic fibers, fibrosis of myocardium and vessels, and hemopericardium, due to unsupported vasa vasorum, by diapedesis or rhexis (Carlton and Henderson, 1963). In turkeys, dietary copper levels below 1.0 ppm resulted in frequent aortic rupture and internal hemorrhage from smaller vessels (McSherry *et al.*, 1954). Pigs showed high incidence of aortic rupture (Weissman *et al.*, 1961; Carnes *et al.*, 1961; Weissman *et al.*, 1963), aortic fragility and reduced aortic tensile strength (Coulson and Linker, 1968; Smith *et al.*, 1968). Aneurysms and loss of aortic structure were demonstrated by Everson and coworkers (1967) in guinea pigs, and O'Dell and coworkers (1961b) produced failure of elastic tissue in newborn rats.

Histopathological analyses of affected tissues revealed diminished elastin and increased lysine contents of elastin (Kimball *et al.*, 1962; Starcher *et al.*, 1964). Increased elastin lysine is due to failure of conversion of lysine to desmosine and isodesmosine, the cross linking residues of elastin (O'Dell *et al.*, 1966) and a failure of the incorporation mechanism to attach newly formed proelastin into aortic fibers (Partridge *et al.*, 1964). These abnormalities result in accumulation of lysine, the precursor of proelastin. The lysine to desmosine conversion is catalysed by copper containing amine oxidase whose activity is depressed in aorta, serum and heart of copper-deficient animals (Bird *et al.*, 1966; Blaschko *et al.*, 1965; Mills *et al.*, 1966; Hill *et al.*, 1967). Amine oxidase acts by oxidatively deaminating the epsilon-amino group of lysine residues in elastin (Hill *et al.*, 1968). Other workers (Partridge *et al.*, 1964; Starcher *et al.*, Weissman *et al.*, 1963) reported increased swelling and solubility in hydrolytic agents of aortic and heart valvular elastin from copper-starved animals, and accumulation of non-elastin,

non-collagen protein and soluble collagen. Significantly lower concentrations of desmosine and isodesmosine were found by O'Dell and associates (1966) in copper-deficient animals.

3. Bone and Connective Tissue Metabolism

Copper-deficient animals have been shown to display abnormal bone conditions. Teague and Carpenter (1951) described unusual leg formations, loss of rigidity in leg joints, excessively flexed hocks and crooked forelegs in young pigs as signs that were reversed by copper therapy. Similar bone defects resulting in fractures and animals with squatty and shorter than normal body structures were reported by other workers in swine (Lahey *et al.*, 1951; Follis *et al.*, 1955). In chicks there were unsteady gait, complete paralysis and sharp bending of the proximal end of the metatarsus (O'Dell *et al.*, 1961a; Gallagher, 1957; Rucker *et al.*, 1969a, 1969b). In rabbits, Hunt *et al.* (1966) reported medial curvature of radius and ulna, cortical thinning, increased width, distortion of epiphyseal cartilage and bone destruction; in turkeys, the bones were fragile and deformed (Rucker *et al.*, 1969a). To a lesser extent there were various forms of defects observed in mice (Guggenheim *et al.*, 1964), dogs (Baxter and Van Wyk, 1953) and foals (Bennetts, 1932). In ruminants grazing copper-deficient pastures, Cunningham (1950) and Davis (1950) observed brittle bones that fractured easily even though they appeared normal in form and shaft thickness.

Upon histochemical examination, affected bones showed osteoporosis (Carlton and Henderson, 1964; Rucker *et al.*, 1969a), failure of bone deposition in cartilage matrix, unusually thin cortices, wide epiphyses, deficient trabeculae, and spontaneous fractures due to inadequate stabilization of fibrous proteins (Baxter and Van Wyk, 1953; Carnes,

1971). Osteoporosis was a consequence of deranged osteoblastic activity accompanied by cessation of osteoclastic function (Carlton and Henderson, 1964).

Gross bone lesions have been shown to be due to metabolic defects at the cellular level which are caused by copper deficiency. The cuproenzymes that maintain the structural integrity of bone and connective tissue are known to be markedly reduced; depressed cytochrome and amine oxidase activities have been reported by several workers (Hunt *et al.*, 1966; Rucker *et al.*, 1969a; Waino *et al.*, 1959). Chou and associates (1968) described decreased amine oxidase activity in tendon and cartilage of copper-deficient chicks. Subnormal activities of these enzymes result in a derangement in connective tissue metabolism. Rucker and coworkers (1969b) stated that, in copper-deficient chick bone, amine oxidase which functions in oxidative deamination of lysyl residues to α-amino adipic acid-Δ-semialdehyde residues in collagen synthesis is inhibited, much like in vascular tissues. This inhibition results in a concomitant decrease in intramolecular cross-linking of elastin (O'Dell *et al.*, 1966; Kim and Hill, 1966; Hill *et al.*, 1967). Consequently the copper-deficient bones yielded more collagen which contained more aldehyde and was more soluble than did control bones (Rucker *et al.*, 1969a).

Connective tissue abnormality was initially identified by O'Dell and associates (1961) in young chicks, and in pigs by Shields and coworkers (1962). That deranged biosynthesis of collagen and elastin culminates in connective tissue and bone lesions has been confirmed by several workers using swine (Weissman *et al.*, 1963, 1965; Smith *et al.*, 1968), chicks (O'Dell *et al.*, 1966; Miller *et al.*, 1965; Chou *et al.*, 1968; Rucker *et al.*, 1969a, 1969b, 1969c) and turkeys (Savage *et al.*,

1966).

More recently Carnes (1971) summarized these observations: "...in copper deficiency the connective tissue showed diminished tensile strength of elastin and collagen due to impaired polypeptide chain cross-linkages, ... the proelastin lacked crosslinks and had increased lysine residues, diminished aldehyde precursors of cross links and an inhibition of a late step in biosynthesis of elastin and collagen as already reported by Hill (1969) and Carnes (1968)."

4. Enzyme Activity

a. Ceruloplasmin. This enzyme, also known as polyphenol oxidase, was described by Holmberg and Laurell (1947) as a serum copper-containing alpha-globulin, blue in color, with a molecular weight of 151,000. It contains 8 atoms of copper and 4 units of the hemocuprein of Mann and Keilin (1938). Of the two types of copper present in serum, direct and indirect acting, ceruloplasmin contains indirect reacting copper, which is tightly bound and will not react with chelating agents unless the molecule is destroyed (Milne and Weswig, 1968; Cartwright and Wintrobe, 1964) to liberate the copper from the protein by acid hydrolysis (Wintrobe *et al.*, 1953; Marceau and Aspin, 1972). It was shown by Wintrobe and associates (1953) that, irrespective of copper status in animals, the ratio of direct reacting copper and ceruloplasmin copper is the same.

About 80% of copper in serum is in the form of ceruloplasmin in most animals such as rats, pigs, sheep, dogs and humans (Butler, 1963; Milne and Weswig, 1968; Wintrobe and Cartwright, 1953; Cartwright, 1950). Thus, Todd (1970) concluded, the activity of ceruloplasmin gives a strong indication of serum and whole blood copper concentration. In chicks,

only a small portion of plasma copper is contained in ceruloplasmin (Starcher and Hill, 1965); and turkeys have no measurable ceruloplasmin oxidase activity (Wiederanders, 1968) and in fact possess the lowest reported serum copper levels (Evans and Wiederanders, 1967). Ceruloplasmin functions in:

1. transfering iron from cells to plasma by promoting the rate of iron saturation of transferrin and stimulating utilization of iron (Osaki *et al.*, 1966). This function has been confirmed by Ragan and coworkers (1969) and by Gray and Abraham (1973) who established that ceruloplasmin facilitates iron transfer as a result of its ferroxidase activity.

2. donating copper to extrahepatic tissues (Owen, 1965).

3. maintaining hepatic copper homeostasis as shown in cases of post-adrenalectomy and post-hypophysectomy states; in rats increases due to adrenalectomy were reported in hepatic copper concentration and serum ceruloplasmin (Evans *et al.*, 1970; Gregoriadis and Sourkes, 1970). The hepatic copper level increases were a consequence of decreased biliary copper excretion (Evans *et al.*, 1970).

4. oxidation of various substances such as polyphenols (Holmberg and Laurell, 1948), serotonin and epinephrine (Martin *et al.*, 1964). *In vitro* reactions indicate that the best substrate with which to measure oxidase activity is p-phenylenediamine. In addition to these functions, ceruloplasmin has been shown by Hampton *et al.* (1972) and by Gary and Daniel (1973) to possess histaminase activity.

Evans and Wiederanders (1967) established a correlation between total plasma copper and ceruloplasmin oxidase activity; lower plasma copper concentration was associated with low oxidase activity of

ceruloplasmin. In rats kept on diets unsupplemented with copper, ceruloplasmin activity was significantly depressed when compared to control values (Milne and Weswig, 1968; Starcher and Hill, 1965; Evans and Abraham, 1973). In a study with swine raised on a copper-deficient milk diet, Williams and associates (1975) reported that ceruloplasmin concentration at 8 weeks was 8% of control values, and decreased further to less than 1% of controls at 12 weeks.

b. Superoxide Dismutase. McCord and Fridovich (1969) described an enzyme purified from bovine erythrocytes which catalyzes the disproportionation or dismutation of univalently reduced oxygen or superoxide radicals. Known as superoxide dismutase (SOD), it functions according to the following:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2$$

The enzyme contains two equivalents of copper per mole (McCord and Fridovich, 1969) and an equal molar level of zinc (Carrico and Deutsch, 1970). It has been shown to be identical to the previously isolated copper-containing human erythrocuprein (Markowitz *et al.*, 1959, Kimmel *et al.*, 1959), bovine hemocuprein (Mann and Keilin, 1939), and equine hepatocuprein (Mohamed and Greenberg, 1953).

Superoxide dismutase is widely distributed within mammals and among microbes (McCord *et al.*, 1971; Keele *et al.*, 1970); in fact all cytochrome containing aerobes and some aero-tolerant anaerobes (except *Lactobacillus plantarum*) exhibit superoxide dismutase activity (McCord *et al.*, 1971).

It has been shown by McCord and Fridovich (1969) that in catalyzing the oxidation and reduction of the superoxide radical to H_2O_2 and

O_2, superoxide dismutase enables the organism to survive in the presence of molecular oxygen. This is achieved by prevention of $O_2^{\cdot -}$-induced membrane damage. An organism that lacks superoxide dismutase could survive in a molecular oxygen environment provided the superoxide radical does not accumulate in lethal amounts (McCord *et al.*, 1971).

The dependence of enzymatic activity of superoxide dismutase upon adequate dietary copper was demonstrated by Williams and associates (1975). Using an evaporated milk diet, these researchers induced copper deficiency in swine and reported an 85% drop in hepatic and erythrocyte superoxide dismutase activity in the copper-deficient animals whose plasma copper was 5 to 10% of controls.

c. Cytochrome Oxidase. As the terminal member of the cytochrome system, cytochrome oxidase is capable of reducing oxygen. Basing their conclusion on a strong positive correlation between copper content, heme content and enzyme activity, several researchers (Eichel *et al.*, 1950; Wainio *et al.*, 1959) reported that cytochrome oxidase contains copper as a functional component of its structure, participates in electron transfer in the terminal steps of the respiratory chain, and contains a lipid (Wainio *et al.*, 1959), present as mitochondrial lipid (White *et al.*, 1959). Cytochrome oxidase was described by White and associates (1959) as a polymer of subunits with a molecular weight of 72,000, with each subunit containing one heme and one copper atom, and its activity due to its polymeric form.

Cohn and Elvehjem (1934) reported a marked reduction in cytochrome oxidase in heart and liver of milk-anemic rats, and a restoration of its activity to normal following copper administration. Similar reductions in cytochrome oxidase activity in liver, heart and bone

marrow of copper-deficient rats were shown by Schultze (1939, 1941) and Gallagher *et al.* (1956a, 1956b) who also reported that copper therapy caused restoration of enzymatic function. In copper-deficient swine, an eight-fold reduction in heart cytochrome oxidase and three-fold decrease in liver cytochrome oxidase was observed by Gubler and coworkers (1957). Other investigators reported depressed brain cytochrome oxidase in lambs suffering from copper deficiency-induced swayback (Howell and Davison, 1959; Mills and Williams, 1962); in copper-deficient cattle (Mills *et al.*, 1963; Poole, 1970); and in copper-deficient chickens (Hill and Matrone, 1961).

Loss of cytochrome oxidase function has been attributed by Gallagher and associates (1956a) to failure of synthesis of heme α prosthetic group due to copper deficiency.

d. Other Enzymes. That copper deficiency affects the activity of other copper-containing enzymes has been reported by several workers. Schultze and Kuiken (1941) and Adams (1953) fed rats diets deficient in copper and reported that catalase activity of liver, kidney and blood showed a significant decrease when compared to controls, and supplementation of diets with copper restored activity of catalase to normal. In one set of experiments with swine, liver catalase activity was not affected by subnormal copper levels (Lahey *et al.*, 1952), whereas Gubler and associates (1957) reported decreased catalase activity in copper-deprived swine; however, in both experiments low copper levels did not influence activity of catalase in kidney and erythrocytes.

Monoamine oxidase activity was reported to be undetectable in plasma of copper-starved pigs, and its activity was normalized by dietary copper supplementation (Blaschko *et al.*, 1965). The function of amine

oxidase was described by Partridge and coworkers (1964) as catalysts of oxidation of the epsilon amino group of lysine to aldehyde prior to condensation to desmosine and isodesmosine (the crosslinkage group in elastin synthesis); and it has been established that amine oxidase contains copper (Yamada and Yasunobu, 1962; Hill and Mann, 1962; Buffoni and Blaschko, 1964). Kim and Hill (1966) subsequently confirmed that the role of copper in elastin synthesis is related to its role in amine oxidase activity. They reported that in copper deficiency amine oxidase activity was reduced in the aorta and addition of amine oxidase to a copper-deficient system stimulated synthesis of desmosine from lysine. In ewes and lambs, Mills and associates (1966) observed that low plasma amine oxidase activity was not associated closely with copper deficiency.

Several researchers reported that copper is an essential functional component of the enzyme tyrosinase (Kubowitz, 1937, 1938; Allen and Bodine, 1941; Sreerangacher, 1944) and that the enzyme exhibits oxidase activity (Brown and Ward, 1959). The removal of copper from tyrosinase by cyanide treatment followed by dialysis resulted in a loss of 85% of its activity, and addition of cupric ions restored activity of the enzyme to 90% of original values (Lerner *et al.*, 1950). In copper deficiency in all animal species except swine, failure of pigmentation in hair or wool (achromotrichia) seems to be a consequence of deranged conversion of tyrosine to melanin mediated by copper-containing tyrosinase (Underwood, 1966). In humans, albinism is characterized by a lack of detectable tyrosinase activity (Harris, 1959).

Butyryl coenzyme A dehydrogenase was described by Mahler (1954) as a cuproflavoprotein since it contains riboflavin and copper. However, it was reported to be unaffected by copper starvation (Gubler *et al.*,

1957) since upon dialysis to remove copper the copper-free butyryl co-enzme A dehydrogenase was still actively reduced by butyryl coenzyme A (Mahler, 1954). Subsequently, studies by Scheinberg and Sternlieb (1960) showed that copper was not a functional component of butyryl coenzyme A dehydrogenase, but probably was a contaminant.

Uricase activity was studied in swine liver and was thought to be related to the presence of copper in the enzyme. The enzyme catalyzes conversion of uric acid to allantoin.

5. Nervous System Myelination

A disorder of the nervous system in young and newborn lambs known as enzootic neonatal ataxia was first described in Australia by Bennetts (1932), and was known to be associated with tissue hypocuprosis. This condition can be prevented by administration of copper to the pregnant ewe (Bull *et al.*, 1938). Later a clinically similar disease called "swayback" and exhibiting identical morphological signs as ataxia was reported by Innes (1936). Pathological signs include diffuse symmetrical degeneration of cerebral white matter, cerebral cavitation, secondary motor tract degeneration into the spinal cord, and degenerative changes in the neurons of the brain stem nuclei and spinal cord. There were also chromatolysis, myelin degeneration and cell necrosis (Innes and Shearer, 1940; Barlow *et al.*, 1960a). That swayback and enzootic ataxia are not caused by a failure in the intermediary metabolism of copper or by unavailability of copper in pastures as previously postulated (Eden *et al.*, 1945) was conclusively demonstrated by workers (Bennetts and Beck, 1942b; Barlow *et al.*, 1960b) who reported the diseases in Australian and English pastures with excessively low copper levels.

Earlier views that swayback was essentially a demyelinating

encephalopathy (Innes, 1936; Innes and Shearer, 1940) were challenged by the findings of Behrens and von Schulz (1959) and von Schulz and Behrens (1960). These researchers suggested that swayback was caused by venous stasis, edema, and perivascular cuffing. However, later investigations confirmed the reports of earlier workers and stated that cerebral lesions were not essential features of swayback, but rather scattered lesions of hyaline neuronal necrosis and nerve fiber degeneration in the brain and spinal cord (Spais *et al.*, 1961; Fell *et al.*, 1961; Howell *et al.*, 1964). In the brain, neural lesions were found to persist after birth (Barlow, 1963a); and lesions in the white matter of the spinal cord indicate aplasia of myelin rather than demyelination (Howell *et al.*, 1964).

Mills and Fell (1960) examined lambs from ewes on a high sulfate-molybdenum diet and reported degeneration of cells in motor neurons of the red nucleus and ventral horns of gray matter in the spinal cord; there was demyelination in the cerebral cortex and spinal cord. These researchers state that ataxia was likely to develop in lambs if liver copper levels of ewes fell below 20 mcg/g and especially below 10 mcg/g. Butler and Barlow (1963) were unable to produce swayback in lambs by administering high levels of molybdenum and sulfate to pregnant ewes.

A nervous disease was described by Roberts and associates (1963a, 1963b) in lambs, characterized by nervous lesions, cerebral edema, tissue hypocuprosis, and minor degrees of cavitation in cerebral white matter. Histopathological findings confirmed acute cerebral edema with severe widespread neuronal involvement, perivascular cuffing, cortical necrosis and cerebellar compressive effects attributable to pressure and or anoxia. The hypocuprosis was reversed by copper therapy.

Sheep are not unique in showing nervous disorders due to

hypocuprosis. In guinea pigs fed a copper-deficient diet, Everson and coworkers (1967, 1968) reported ataxia, missing or malformed cerebellar folia, cerebellar agenesis, widespread hypomyelinogenesis as indicated by phospholipid determinations, and soft translucent areas of the cerebral cortex. In rats and chickens, copper deficiency was shown to cause two major metabolic disturbances: loss of cytochrome oxidase activity and suppression of phospholipid synthesis by the liver (Gallagher *et al.*, 1956a; 1956b; Gallagher, 1957). In pigs with ataxia, low liver copper levels were found (Wilkie, 1959) and although no pathological reports were made, there was demyelination of all areas of the spinal cord except dorsal areas. The author reported ataxia in 2- to 3-week-old pigs, incoordinated hind quarters, and liver and blood copper levels of 9.8 and 0.25 ppm, respectively; these conditions were reversed by administration of copper. McGavin and associates (1962) made field observations in Australia and reported that pigs with liver copper levels of 3, 9, 10 and 14 ppm showed marked spinal demyelination which affected the dorsal spinocerebellar tract, dorsal, lateral and ventral funiculi extending to the medulla oblongata, cerebellum and posterior cerebellar peduncle. In goats, signs of swayback have been described by Hedger *et al.* (1964) and by Owen *et al.* (1965). At necropsy, cells and tracts of the central nervous system showed typical swayback lesions, and cytochrome oxidase activity was low, accompanied by low blood and brain copper levels.

Decreased cytochrome oxidase activity in brain and liver as a result of hypocuprosis was reported by Howell and Davison (1959) in swayback lambs, by Schultze (1939, 1941) in rats, and by Gubler *et al.* (1957) in pigs. The relationship between demyelination and cytochrome

oxidase was established by Gallagher (1957): low brain copper leads to decreased cytochrome oxidase activity (since the enzyme contains copper) in motor neurons which in turn leads to demyelination since phospholipid synthesis is depressed in copper deficiency; phospholipids are a component of myelin.

6. <u>Fatty Acid Metabolism in Depot Fat</u>

Following reports by Barber *et al.* (1957) and Bellis (1961) that high dietary levels of copper adversely affected pork carcass grade, Taylor and Thomke (1964) fed 250 ppm copper to bacon pigs to determine the quality and properties of porcine depot fat. They observed a highly significant difference in iodine number of backfat between treated (250 ppm Cu) and control groups. Using vacuum techniques, the researchers showed that dietary copper caused softer backfat (i.e., with a lower melting point), and found this observation compatible with iodine values. The authors therefore speculated that high levels of dietary copper may affect absorption and transport of dietary fat in such a way as to cause changes in composition of depot fat, and that high liver copper levels may interfere with liver functions in fat metabolism. Experiments by Bowland and Castell (1964, 1965) yielded data to indicate that copper supplementation softens porcine backfat. However, their papers made no mention of specific biochemical processes involved, but alluded to a possibility of an interaction between copper and protein source to cause soft fat.

Investigations by Braude (1965) failed to confirm these findings. However Elliot and Bowland (1968) reported that copper supplementation at 280 ppm caused a significant increase in proportion of unsaturated

fatty acids (UFA) in the outer and inner backfat, and perinephric fat at 26, 47 and 70 kg liveweight. This increase was accompanied by a corresponding decrease in proportion of saturated fatty acids (SFA), and there were no significant differences in fatty acid composition of these fats at 90 kg liveweight. The observed increase in proportion of UFA was probably due to increases in the 16:1 and 18:1 fatty acids with decreases in 16:0 and 18:0 acids. Softness of depot fat due to high levels of dietary copper was attributed by some workers (Moore *et al.*, 1969; Christie and Moore, 1969) to changes in structure of triglycerides, and by others (Elliot and Bowland, 1968, 1969, 1970) to increases in major saturated fatty acids. Other investigations showed that in the depot fat of pigs on diets supplemented with 250 ppm copper there was a slightly higher concentration of oleic acid and lower concentration of stearic acid (Moore *et al.*, 1968), a low proportion of stearic acid, more palmitoleic acid and a higher oleic:stearic acid ratio (Castell *et al.*, 1975).

Several researchers (Amer and Elliot, 1973a, 1973b; Myres and Bowland, 1972, 1973, 1975; Ho *et al.*, 1973, 1974, 1975; Castell *et al.*, 1975) published confirmatory reports that porcine depot fat softness induced by diets containing high levels of copper was caused by increased proportions of unsaturated fatty acids and a concomitant decrease in proportions of saturated fatty acids. In addition to increased percentages of UFA in pig backfat resulting from feeding 6 levels of copper (125, 150, 175, 200, 250 ppm), soft fat has been linked to an increase in oxidative susceptibility and decreased keepability of fatty pork (Amer and Elliot, 1973b), a condition which was countered by supplemental vitamin E (Amer and Elliot, 1973a).

These depot fat changes were thought by Myres and Bowland (1973)

to be related to a disturbance in the balance between lipolysis and re-esterification of fatty acids. More specifically, Ho and Elliot (1973) presented data to suggest that increased 18:1 and decreased 18:0 fatty acids in depot fat of copper-supplemented pigs were related to an enhancing effect of copper on specific activities of both hepatic and adipose stearoyl-CoA desaturase systems. Later, Ho and Elliot (1974) implicated copper involvement in the entire fatty acyl desaturase system, and more recently the authors suggested that UFA increases were due to the role of copper in desaturation reactions as a component of some cuproprotein enzyme system or metalloprotein (Ho and Elliot, 1975). It appears from the work done so far that copper is definitely involved in fatty acid metabolism in porcine depot fat, but the exact mechanism is yet to be clearly defined.

7. Reproduction

Although the mechanism by which copper influences fertility and reproduction in animals is not well understood, various workers have shown that subnormal levels of dietary copper adversely affect reproduction. Keil and Nelson (1931) placed rats on milk diets supplemented with iron in form of ferric chloride and observed that reproduction occured only when copper sulfate was added to the diet. Bennetts and associates (1942a, 1948) reported that low copper status of dairy cows resulted in a high incidence of infertility, decreased milk production and retarded growth and development of the young. Dutt and Mills (1960) raised female rats on copper-deficient diets and observed that the rats had normal estrous cycles; out of 18 pregnant rats, only 3 produced litters; necropsy showed unmistakable evidence of fetal resorption, and

uterine nodules representing previous sites of implantation of embryos were found in a majority of the rats. Studies by Hall and Howell (1969) and Howell and Hall (1969) showed that copper-deficient rats were successfully mated after exhibiting normal estrous periods but failed to produce litters. Pregnancy was not inhibited, but normal fetal development ceased on day 13 of pregnancy, fetal tissues disintegrated and the placenta underwent necrosis. These studies show conclusively that copper deficiency does not affect the estrous cycle, mating and conception in the rat, but for maintenance of pregnancy until production of normal litters, there must be adequate intakes of copper, otherwise the fetuses undergo resorption.

In chickens, copper deficiency has been shown to affect reproductive perfomrance. Savage (1968) placed hens on low copper diets for 20 weeks and observed decreased egg production and copper content of eggs, plasma and liver; hatchability was decreased and approached zero at 14 weeks. There was early embryonic mortality following anemia and widespread hemorrhage, due probably to defective red blood cell and connective tissue formation (Simpson *et al.*, 1967) during the early stages of embryonic life.

E. Interaction of Copper with other Minerals

1. Iron

The fact that copper deficiency produces anemia in animals provides evidence for an interaction between copper and iron. Effects exerted upon iron metabolism by copper have been extensively investigated by many researchers (Lahey *et al.*, 1952; Cartwright *et al.*, 1952, 1955, 1956; Gubler *et al.*, 1952; Bush *et al.*, 1955, 1956a; Chase *et al.*, 1952a,

1952b; Jensen *et al.*, 1956).

In rats, copper increased iron absorption (Chase *et al.*, 1952a), and copper deficiency in pigs resulted in impaired iron absorption and reduced iron utilization in hemoglobin synthesis (Gubler *et al.*, 1952) and caused iron deposits in the cells of the duodenal mucosa. These effects were reversed by administration of copper. Ceruloplasmin, a copper containing enzyme, is the agent through whose feroxidase activity copper affects iron metabolism.

In swine suffering from copper-deficiency anemia, there were low serum iron, decreased iron uptake for hemoglobin synthesis, low liver iron (Gubler *et al.*, 1952), and the erythrocyte life span was 20% that of controls (Jensen *et al.*, 1956; Bush *et al.*, 1956a). There was also an impaired ability to absorb iron from the gastrointestinal tract, and a slower rate of disappearance of radioactive iron from liver and bone marrow (Gubler *et al.*, 1952).

Since, in the rat, it is unlikely that copper exerts a direct effect on iron absorption (Cunningham, 1931; Houk *et al.*, 1946; Chase *et al.*, 1952a, 1952b), Matrone (1960) proposed the effect is indirect. A lack of copper induces a mucosal block which decreases iron absorption from the gastrointestinal tract during copper deficiency. This block is lifted during copper adequacy, thus allowing iron to enter hemoglobin synthesis; in this fashion absorption of iron is increased.

In ruminants grazing copper deficient pastures, extensive iron deposits in the liver were reported by some workers (Marston *et al.*, 1948; Underwood, 1956; Marston, 1952), and in tissues the iron was in the form of hemosiderin (Marston, 1952). Matrone and associates (1957), investigating copper deficiency in calves, reported that the level of

iron in serum and liver was not affected by dietary copper levels. These researchers did not find excessive iron deposits in the calves' livers. Available data seem to show that in ruminants dietary copper has slight influence on iron metabolism.

2. Molybdenum and Sulfate

Ingestion of high levels of molybdenum by cattle grazing pastures normal in copper but high in molybdenum was established to be the cause of teartness in England (Ferguson *et al.*, 1943); this condition was overcome by supplemental copper. It therefore became evident that an interaction exists between copper and molybdenum since high molybdenum levels produced copper deficiency in situations where the copper level was adequate. Dick and Bull (1945) described hypercuprosis in sheep grazing low molybdenum pastures, and the condition was treated with supplemental molybdenum. Comar and coworkers (1949) investigating molybdenum-induced copper deficiency, administered radioactive copper and molybdenum to cattle and rats and observed an interference in bone metabolism caused by molybdenum; this action was thought to be due to lowered liver copper, molybdenum interference and inhibition of bone enzyme systems, and molybdenum competition with phosphorus.

The role of inorganic sulfate in ameliorating the toxic effects of molybdenum was first noted by Dick (1953) who fed lucerne hay to sheep and observed that decreased blood molybdenum levels were due to high levels of inorganic sulfate in the hay. He also reported that as the sulfate concentration increased, with dietary molybdenum held constant, blood molybdenum was lowered. Similar observations were discussed in rats by Miller and associates (1956); in sheep, Miller and Engel (1960)

observed increased blood copper concentrations, and copper losses in liver and blood were reported in cattle (Mylrea, 1958). High dietary sulfate at constant dietary levels of molybdenum caused increases in blood copper of sheep, while in cattle molybdenum and sulfate favored copper losses (Miller and Engel, 1960). These authors reported that in rats increased dietary sulfate at constant molybdenum intake decreased blood copper; and in sheep induced molybdenum loss via urine, feces and tissues. Although dietary molybdenum and sulfate affected copper storage in ruminants, some workers reported that blood copper was not affected but there was tissue copper depletion in sheep (Suttle and Field, 1968; Marcilese *et al.*, 1969, 1970).

A copper-molybdenum-sulfate interaction has been shown to successfully prevent copper accumulation in the liver (Dick, 1953; Wynne and McClymont, 1955; Cunningham *et al.*, 1959). Such interaction if it exists should be able to eliminate excessive hepatic copper levels in swine. Experiments performed with pigs showed that such an interaction between copper, sulfate and molybdenum does exist, but differs from the one in ruminants since there was no reduction in tissue copper levels (Gipp *et al.*, 1967; Hays and Kline, 1969; Kline *et al.*, 1971, 1972, 1973; DeGoey *et al.*, 1971). Depressing effects of high dietary copper (500 ppm) however, were partially overcome in pigs by addition of sulfide to the diet with no apparent response due to molybdenum. Levels of 450 to 700 ppm sulfide were added to diets containing 250 ppm copper and 1800 ppm sulfide added to 500 ppm copper resulted in liver copper concentrations similar to those of pigs fed low copper levels (15 ppm) (Kline *et al.*, 1973). In studies with sheep, Dick (1954) showed that sulfide reduced absorption and retention of ingested copper, and Mills (1960)

reported that rats developed anemia when fed sulfide and molybdenum. Anemia was a result of sulfide-induced hypocuprosis causing a decrease in available copper needed for hemoglobin synthesis. Research done with pigs on corn-soy diets containing 11 to 18 ppm copper showed that addition of 0.4% sulfate significantly increased plasma copper concentrations while 50 to 1500 ppm molybdenum significantly depressed liver copper uptake and a combination of sulfate and molybdenum increased liver storage of copper (Dale *et al.*, 1973; Standish *et al.*, 1975).

Although the site of action and mechanism at which molybdenum and sulfate influence copper metabolism is not known (Dale *et al.*, 1973) it has been shown that in the gastrointestinal tract molybdenum forms a complex with copper in the ratio of 3 to 4 (Dowdy and Matrone, 1968a, 1968b) and 1 to 1 *in vitro* (Neilands *et al.*, 1948; Britton and German, 1931). Formation of this copper-molybdenum complex may render copper biologically unavailable and inactive in the sheep, chick, and pig.

3. <u>Calcium and Zinc</u>

Any interaction between copper and calcium must be indirect and has not been clearly defined. However Marston *et al.* (1948) and Macpherson and Hemingway (1968) reported that copper requirements of sheep grazing highly calcareous or limed soils were significantly increased. Copper absorption in sheep was greatly depressed by high intakes of dietary calcium carbonate (90g/day) which limited storage of copper in livers of adult sheep (Dick, 1954). It is thought that calcium carbonate depresses copper absorption by increasing the pH of the intestine (Underwood, 1971). In pigs, Hoefer and coworkers (1960) reported that at high calcium levels copper significantly increased growth rate, reduced incidence and severity of parakeratosis but was less effective

than zinc.

Antagonism between copper and zinc with respect to liver storage was reported earlier by some researchers (Van Reen, 1957; Allen *et al.*, 1958; Davis, 1958). Ritchie and associates (1963) showed that 100 ppm zinc added to a diet containing 250 ppm copper prevented parakeratosis, reduced liver copper concentrations and protected against copper toxicosis resulting from 250 ppm copper. Toxicity signs included anemia, internal hemorrhage, jaundice, yellow cirrhotic livers, weakness, gastric ulceration and incoordination. Similar signs were reported by Gordon and Luke (1957), O'Hara *et al.* (1960), Wallace *et al.* (1960), Buntain (1961), and Allen and Harding (1962). In pigs, 500 ppm zinc or 750 ppm iron in presence of 750 ppm copper was shown to eliminate jaundice, normalize serum copper and restore aspartate transaminase activity to normal; however only 750 ppm iron afforded protection against anemia induced by high copper concentrations. In the absence of zinc and iron, 425 ppm copper caused severe toxicosis in swine, the effects of which were eliminated by addition of zinc and iron; in zinc deficiency, 250 ppm copper caused toxicosis and exaggerated parakeratosis which resulted from the zinc deficiency (Suttle and Mills, 1966b). The mode of action by which zinc antagonizes copper is not clearly understood, however Starcher (1969) investigated the mechanism of copper absorption from the gastrointestinal tract of chicks and proposed a scheme: it appears that copper is bound to a duodenal protein with an approximate molecular weight of 10,000; this constitutes an important step in the process of copper absorption. Zinc inhibits copper absorption by binding to, and competing for, the same protein-binding sites in the duodenum required for copper.

4. Other Minerals

Cadmium at high dietary doses has been reported to adversely depress copper uptake from the gastrointestinal tract and influence tissue distribution of copper (Hill *et al.*, 1963; Van Campen, 1966). In chicks, silver exacerbated the effects of copper deficiency (Hill *et al.*, 1964), and in rats mercury slightly suppressed the uptake of copper ^{64}Cu) from the gastrointestinal tract (Van Campen, 1966). Whanger and Weswig (1970) reported that in rat diets containing 6 ppm copper, addition of 100 ppm cadmium, 200 ppm silver, 500 ppm molybdenum or 10,000 ppm sulfate showed silver to be the strongest antagonist of copper, followed by cadmium, molybdenum, zinc and sulfate in that order. Other elements such as nickel, tungsten, vanadium, chromium, rhenium, uranium and tantalum did not exert any observable influence on copper retention (Cunningham, 1950; Dick, 1954).

F. High Level Copper Feeding

Initial studies were conducted by Braude (1945, 1948) to show the growth-promoting effects of copper when used as a dietary supplement for pigs. The pigs showed a craving for copper, and a preference when given a choice, for diets supplemented with copper; these observations were confirmed by Mitchell (1953). Following reports by Barber and associates (1955) that addition of high levels of copper (250) ppm in the form of copper sulfate to diets of growing pigs resulted in improved growth rate, numerous workers conducted studies using various levels of copper: 100, 125, 150, 200, 250 ppm, and higher. These studies reported improved growth rate and overall performance with levels up to 250 ppm (Bowler *et al.*, 1955; Barber *et al.*, 1960; Allen *et al.*, 1961; Barber *et*

al., 1961; Lucas *et al.*, 1962a; Lucas and Calder, 1957a), and thereby verified the findings of Barber (1955). Most of these investigations were done by European workers.

Daily gains of growing pigs were significantly improved by addition of 250 ppm copper to the diets, and the increases were of the same magnitude as those obtained by Barber and coworkers (1955b) with antibiotics; further comparative effects of copper and antibiotics were confirmed subsequently by Bowler *et al.* (1955). Addition of 230 ppm copper by Schurch (1956) caused increased weight gains and improved feed utilization. Barber (1957) later confirmed previous findings in which copper exerted effects similar to those obtained from chlortetracycline and oxytetracycline.

The results obtained by Lucas and Calder (1957a), Bellis (1971) and Bunch *et al.* (1963) with high level copper supplementation indicated that growth response reached a peak and plateaued when pigs attained 50 to 60 kg bodyweight, and that a decline in response arose as a consequence of copper accumulation in the liver.

Workers in several countries have reported (Lucas *et al.*, 1962a; Bunch *et al.*, 1963; Wallace, 1967; Drouliscos *et al.*, 1970) that copper supplementation at high dietary levels exerts relatively more pronounced effects on growing pigs by increasing digestive and/or metabolic efficiency. Others (Mitchell, 1953; Barber *et al.*, 1962; Braude, 1965; Young *et al.*, 1970) attribute the effect of high level copper to a stimulatory effect on feed consumption especially in young pigs. Variation in responses to increased copper levels was indicated in reviews made by Braude (1965), and Wallace (1967). The extent of these responses seems to be influenced particularly by the level of protein in the diet (Wallace *et al.*, 1960;

Lucas *et al.*, 1962b; King, 1964; Combs *et al.*, 1966); while the source of dietary protein has been implicated (Barber *et al.*, 1962; Beames and Lloyd, 1965; Drouliscos *et al.*, 1970).

Levels of copper up to 250 ppm in the diet were shown to improve growth rate during the growing phase of pigs, but removal of copper after eight weeks was shown by Miller *et al.* (1969) and other workers (NCR-42 Committee on Swine Nutrition, 1974; Gipp *et al.*, 1973) not to affect rate of gain or feed efficiency. Gipp and associates (1973) reported that the addition of 250 ppm copper to corn-soy diets did not consistently affect rate of body weight gain during starter, grower, finisher, or overall weaning-to-market periods and carcass measurements.

While some researchers in Europe and the United States were observing improved performance as a result of high copper feeding, contradictory reports appeared in the literature. No beneficial growth response was observed in pigs fed 200 or 250 ppm copper (Lucas and Calder, 1957b; Teague and Grifo, 1966; Livingstone and Livingston, 1968; Parris and McDonald, 1969; Elliot and Bowland, 1970; Amer and Elliot, 1973). Ho and associates (1975) reported that the presence of high levels of dietary copper caused deterioration in both average daily gain and feed conversion. Canadian investigators failed to show consistent benefits similar to those reported by European workers. Mostly, high level copper feeding yielded no improvements (Castell and Bowland, 1968; Drouliscos, 1970; Young *et al.*, 1970; Castell *et al.*, 1973). Earlier studies by Bass *et al.* (1956) and Davis (1958) had shown irregular growth effects and possible toxicity when copper was fed to swine in high concentrations. Elliot and Amer (1973) obtained an improved growth rate but it was nonsignificant, and only up to 23, 46 or 69 kg live weight;

at 92 kg, growth was depressed significantly, and upon removal of supplemental copper from the diet at 23, 46 or 69 kg live weight, growth was depressed. Withdrawal of copper from the diet between 46 and 57 kg live weight did not affect performance but allowed liver concentration of copper to return to normal by slaughter weight (Teague and Grifo, 1966).

Some Canadian workers obtained beneficial results with 250 ppm copper in starter diets of pigs and only up to 50 kg live weight (Beames and Lloyd, 1965; Young and Jamieson, 1970). Recently Braude and Ryder (1973) re-confirmed the optimum level of copper supplementation to be 250 mg copper per kilogram of diet. However, the improved performance was slightly less than the 8.1% for average daily gain, and 5.4% for feed converstion efficiency obtained by Braude (1965) earlier.

Despite some reports to the contrary (Livingstone and Livingston, 1968; Elliot and Bowland, 1970; Amer and Elliot, 1973), many workers have shown overwhelmingly that copper is essential for growth and improvement of performance in pigs especially at a dietary level of 250 ppm (Braude, 1945; Barber *et al.*, 1955; Wallace, 1967; Braude and Ryder, 1973; Young and Jamieson, 1970; Ho and Elliot, 1973; Braude *et al.*, 1970). The effect on growth and performance produced when swine diets are supplemented by copper sulfate has been shown by researchers (Barber *et al.*, 1957; Bowland *et al.*, 1961; Hawbaker *et al.*, 1959) to be due to the copper radical and not the sulfate. The mechanism by which copper exerts its beneficial action at high dietary levels is not clearly understood. Hawbaker and coworkers (1961) postulated that the beneficial action of copper is probably due to its antibiotic-like action on the intestinal microflora. Work done by other researchers opposes this theory by showing that, as a resulf of fecal flora studies (Fuller *et al.*,

1960) and gastrointestinal tract flora count (Smith and Jones, 1963), copper causes a change in microflora from one type to another, but not a population decrease.

G. Copper Requirements

1. Pigs

Although numerous studies have been conducted using various levels of copper in pig diets, as yet the minimum copper requirements of pigs have not been precisely established. Ullrey and associates (1960) fed baby pigs diets containing 6, 16 and 106 ppm copper and observed no significant differences in treatments in growth rate, feed efficiency or hematology. Based upon this report the National Research Council (1973) recommended 6 mg of dietary copper per kg of diet, and stated that 0.1 to 0.15 mg per kg body weight is near the minimum requirement. British Agricultural Research Council (1967) estimated that 4 ppm copper is adequate for growing pigs up to 90 kg liveweight. The fact that most natural swine rations analyze higher than 4 to 6 ppm copper seems to render copper supplementation of normal rations unnecessary (Underwood, 1971).

As previously discussed, interaction of copper with other minerals (calcium, zinc, sulfate, molybdenum, cadmium and iron) and various dietary factors such as protein, affects its absorption and utilization, and therefore influences its requirement. According to Underwood (1966, 1971) the basic requirement for copper is one at which all the factors affecting its utilization are at optimal levels. Responses of pigs to high copper intakes (500 to 750 ppm) have been shown to be influenced by protein sources (Combs *et al.*, 1966; Suttle

and Mills, 1966a; Young *et al.*, 1970; Parris and McDonald, 1968), and dietary protein levels (Combs *et al.*, 1966; Wallace *et al.*, 1960). Suttle and Mills (1966a) conducted studies using soybean meal, dried skim milk and white-fish meal as protein sources and suggested that the influence of protein source is probably due to differences in calcium content of the various proteins.

2. Sheep and Cattle

Dick (1954) estimated that the copper requirement of sheep was 1.0 mg copper per day or less. As in pigs, the interaction between copper and other minerals influences the amount of copper required by ruminants. In South Australia, sheep grazing pastures containing 3 ppm copper and growing on calcareous soils were reported to develop signs of copper deficiency (Marston *et al.*, 1948a, 1948b). Administration of 8 ppm copper prevented copper deficiency but was insufficient for adequate wool keratinization and maintenance of normal blood copper levels. Intakes of high concentrations of calcium carbonate and moderate molybdenum and sulfate by wool-sheep place their copper requirement at 10 ppm; the minimum for cattle and cross bred sheep is 4 ppm; and for Merino sheep 6 ppm (Underwood, 1971). High intakes of sulfate and molybdenum at 0.5 mg per day affect retention of copper in wool-sheep (Dick, 1954). Based on the effects of interaction among molybdenum, sulfate, calcium and copper, and the findings of several workers (Spais *et al.*, 1968; Allcroft and Parker, 1949; Hunter *et al.*, 1945; Innes and Shearer, 1940), it is difficult to specifically define the copper requirements of ruminants. In Australia, field studies showed that application of 5 to 7 kg of copper sulfate per hectare to pasture increases its copper content and alleviates copper deficiency (Underwood,

1971).

3. Other Species

In rats fed milk diets fortified with iron and manganese, Schultze and coworkers (1934) found that supplements of 0.01 to 0.05 mg copper per day were optimal for promoting growth and synthesis of hemoglobin. Copper deficiency has been produced in rats by several researchers using diets which contained less than 1 ppm copper. Studies by Mills and Murray (1960) established the minimum copper requirements for 70 g rats as 1 ppm for hemoglobin formation, 3 ppm for growth, and 10 ppm for maintenance of normal melanin concentration in hair; reproduction and lactation would be maintained by 50 ppm.

Everson and associates (1967) kept female guinea pigs on diets containing 0.5 to 0.7 ppm copper and reported normal reproduction when compared to control females on 6 ppm copper. However, anemia and hair depigmentation later developed and growth of young was adversely depressed.

Poultry diets containing 4 to 5 ppm copper are generally sufficient, and all normal poultry rations contain more than 5 ppm copper (Underwood, 1971).

EXPERIMENTAL PROCEDURES

A. Introduction

Three trials involving 52 baby pigs were conducted to determine the minimum copper requirement of baby pigs on semi-purified diets. Baby pigs were obtained from Yorkshire, Hampshire and Yorkshire-Hampshire crossbred herds from the Michigan State University Swine Research Farm, where the experiments were performed. High-protein casein was used as the protein source and mineral and vitamin premixes were made from reagent grade compounds. Energy sources included glucose and fat. The room temperature was initially 30°C but was lowered to 25°C later in the course of the trials. All diets were stored at a temperature of 4°C.

B. Experiments

1. Experiment 1

Sixteen 7-day-old baby pigs were taken from sows on low-copper gestation and lactation diets (3.6 ppm copper, Table A-2) and raised individually in stainless steel cages. They were adjusted to the basal purified diet for 4 days during which period they were fed twice daily and given water *ad libitum*. At the end of the adjustment period the pigs were randomly alloted to four experimental diets as shown in Table 1. The diets were supplemented with anhydrous copper sulfate resulting in the following dietary copper levels: diet 1 (basal), 1.3 ppm; diet 2, 3.2 ppm; diet 3, 5.6 ppm; diet 4, 9.3 ppm.

TABLE 1. COMPOSITION OF DIETS USED IN EXPERIMENT 1

Ingredient	1	2	3	4
Casein[a]	2100	2100	2100	2100
Cerelose[b]	3500	3360	3220	2940
α-Cellulose[c]	350	350	350	350
Mineral premix[d]	420	420	420	420
Lard[e]	350	350	350	350
Fat soluble vitamins[f]	70	70	70	70
Water soluble vitamins[f]	140	140	140	140
Corn oil[g]	70	70	70	70
Copper premix[h]	0	140	280	560
	7000	7000	7000	7000
Copper conc. (analyzed, ppm)[i]	1.3	3.2	5.6	9.3

[a]High protein casein, General Biochemicals, Chagrin Falls, Ohio.

[b]Dextrose 2001, CPC International, Englewood Cliffs, New Jersey.

[c]Solka-Floc, Brown Co., Berlin, New Hampshire.

[d]Premix made from reagent grade compounds (See Table A-4).

[e]Armour Co., Detroit, Michigan.

[f]See Table A-4.

[g]Mazola, Best Foods, Englewood Cliffs, New Jersey.

[h]Copper premix in cerelose containing 100 ppm Cu from anhydrous $CuSO_4$.

[i]As fed basis.

Initial weights of pigs were taken and blood parameters including plasma copper levels were determined at this time. Subsequently they were weighed and blood samples taken bi-weekly from the anterior vena cava for hemoglobin, hematocrit, ceruloplasmin and plasma copper determinations. All feed was weighed each time and individual feed consumption recorded. Some pigs suffered from diarrhea, but addition of neomycin to the drinking water brought it under control. Three pigs died of undetermined causes, one from each of treatments 2, 3 and 4.

After 6 weeks a copper balance trial was conducted. Pigs were adjusted to being fed semi-liquid diets (feed was mixed with water to make a slurry) and to being reared in stainless steel metabolism cages and also to the frequent handling necessitated by transfer to and from the feeding cages for 3 days prior to a 5-day collection of feces and urine. During each feeding, pigs were removed from the metabolism (collection) cages and placed in the feeding cages. Their snouts were wiped clean after feeding to avoid contamination of feces and urine with feed. A fine wire screen was used to separate feces from urine, and the urine was collected in polyethylene buckets (acidified with 6N HCl) through a funnel stuffed with glass wool. Throughout the collection period, feed intake was kept constant and unconsumed feed was collected, air-dried and weighed. For each pig total feces output was oven-dried at $50^{o}C$ for 48 hours, weighed, ground and stored in airtight polyethylene sacs, and total urine output was recorded, sampled and stored in acid washed polyethylene bottles at $4^{o}C$.

At the conclusion of the trial, all pigs were killed by exsanguation after anesthesia using intravenous injection of sodium pentabarbital. Gross and microscopic pathological observations were made.

Various tissues, organs and glands were removed and weighed. They were then placed in polyethylene sacs and frozen immediately over dry ice, and later stored at -20°C until used for analysis. Hair samples were removed from the loin of each pig and the 7th rib was dissected out at the costochondral junction, fixed in formalin and submitted for histopathological examination.

2. Experiment 2

Fifteen 3-day-old baby pigs from sows on low-copper (3.6 ppm copper) gestation and lactation diets were adjusted to a basal semi-purified diet for 9 days in stainless steel cages. The casein used in this basal diet was washed with Na_2EDTA to remove as much of the copper as possible. After adjustment the pigs were allotted to 3 experimental diets (Table 2) with copper levels as follows: diet 1 (basal), 0.6 ppm; diet 2, 1.9 ppm; diet 3, 2.8 ppm.

Six pigs died, two from each of the treatments. A balance trial was conducted after 28 days, and at the end of the trials the pigs were killed, postmortem examinations performed and tissues, organs and glands collected, weighed and stored at -20°C. The tarsal and metatarsal bones were submitted for histopathological examination.

3. Experiment 3

The same procedure used in the previous trials was followed except:

a. Twenty week-old baby pigs were assigned to 4 dietary treatments analyzing 0.9 ppm; 2.0 ppm; 4.0 ppm and 4.9 ppm copper (Table 3).

b. Pigs were group fed and distilled deionized water was provided

TABLE 2. COMPOSITION OF DIETS USED IN EXPERIMENT 2

Ingredient	1	2	3
Casein[a]	2100[a]	2100	2100
Cerelose	3300	3265	3230
α-Cellulose	350	350	350
Cu-free mineral premix[b]	420	420	420
Lard	350	350	350
Fat soluble vitamin premix[c]	70	70	70
Water soluble vitamin premix[d]	140	140	140
Corn oil	70	70	70
Copper premix[e]	0	35	70
Selenium premix[f]	100	100	100
Chromium premix[g]	100	100	100
	7000	7000	7000
Copper conc. (analyzed, ppm)	0.6	1.9	2.8

[a]Casein treated with Na_2EDTA.

[b]See Appendix, A, Table A-3.

[c,d]See Appendix A, Table A-4.

[e]Copper premix in cerelose containing 100 ppm Cu from anhydrous $CuSO_4$.

[f]Selenium premix in cerelose containing 7 ppm Selenium from N_2SeO_3.

[g]Chromium premix in cerelose containing 70 ppm Chromium from $CrCl_3 \cdot 6H_2O$.

TABLE 3. COMPOSITION OF DIETS USED IN EXPERIMENT 3

Ingredient	1	2	3	4
Casein	2100	2100	2100	2100
Cerelose	3300	3230	3090	2950
α-Cellulose	350	350	350	350
Cu-free mineral premix[a]	420	420	420	420
Lard	350	350	350	350
Fat soluble vitamin premix[b]	70	70	70	70
Water soluble vitamin premix[c]	140	140	140	140
Corn oil	70	70	70	70
Copper premix[d]	0	70	210	350
Selenium premix[e]	100	100	100	100
Chromium premix[f]	100	100	100	100
	7000	7000	7000	7000
Copper conc. (analyzed, ppm)	0.9	2.0	4.0	4.9

[a]See Appendix A, Table A-3.

[b,c]See Appendix A, Table A-4.

[d]Copper premix in cerelose containing 100 ppm Cu from anhydrous $CuSO_4$.

[e]Selenium premix in cerelose containing 7 ppm selenium from Na_2SeO_3.

[f]Chromium premix in cerelose containing 70 ppm chromium from $CrCl_3 \cdot 6H_2O$.

as drinking water.

c. Casein was used untreated with Na_2EDTA.

d. A balance trial was conducted after 65 days for 5 days following 4 days of adjustment.

C. Analytical Methods

1. Hematology

a. Hemoglobin. Determination of hemoglobin was by the cyanmethemoglobin method of Crosby *et al.* (1954). Using a dry, clean and calibrated Sahli hemoglobin pipette, 0.02 ml whole blood was measured into 5 ml Drabkin's solution.[1] The blood and solution were mixed thoroughly on a Vortex-Genie[2] device and allowed to stand for 10 minutes at room temperature to allow formation of cyanmethemoglobin. Optical density (OD) of the sample was determined on a Coleman Junior II spectrophotometer set at a wavelength of 540 nanometers. Hemoglobin concentration was calculated as follows:

(OD_{540}) x (Standard hemoglobin factor of Drabkin's solution)
= hemoglobin concentration in g /100 ml.

b. Hematocrit. Hematocrit was determined using the method of McGovern *et al.* (1955). A capillary tube (75 mm long x 2 mm diameter)

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[1]Drabkin's solution was made up by dissolving 1.0 g sodium bicarbonate ($NaHCO_3$), 0.05 g potassium cyanide (KCN) and 0.2 g potassium ferricyanide ($K_3Fe^3[CN]_6$) in deionized distilled water and diluting it to 1 liter.

[2]Scientific Industries, Inc., Springfield, Mass.

containing dried heparin was filled by capillary attraction with blood to within 1 or 2 cm of the end. The unfilled end of the tube was heat-sealed over a micro-burner gas flame for 2 to 4 seconds. Constant rotation of the tube was maintained to ensure a flat inner base of the tube. After sealing, the tube was identified and placed with the sealed end in contact with the peripheral rim of the centrifuge head[1]. The flat removable centrifuge head cover was then screwed in place, the automatic timer set for 5 minutes and the motor turned on. At the end of 5 minutes of centrifugation at 10,000 rpm, the motor was automatically turned off and the hematocrit read promptly. Hematocrit was expressed as percentage of whole blood.

c. Centrifugation. About 10 to 12 ml of blood were withdrawn from the pig's vena cava and immediately placed in a heparinized and acid-washed centrifuge tube. The tube was then shaken thoroughly to mix the blood with heparin and to prevent clotting. The blood sample was next centrifuged at 2000 x G for 12 minutes. Cell-free plasma was carefully decanted, using disposable pipettes, into acid-washed vials. About 3 ml were kept on ice and used immediately for determination of ceruloplasmin, and the rest was stored at $-20^{\circ}C$ for subsequent mineral analysis. The red cells in experiment 3 were stored at $4^{\circ}C$ for assay of superoxide dismutase activity.

2. Physical Determinations

a. Treatment of casein. In order to produce very low-copper casein for use in the basal diets, as much copper as possible was

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[1]International Hemacrit Centrifuge, International Equipment Company, Boston, Massachusetts.

removed from high-protein casein using a modified method of Shanklin *et al.* (1968). High-protein casein was treated by suspending it in deionized distilled water (120 g casein per liter of water). The slurry was mechanically stirred in a polyethylene container while in a water-bath at 50°C. The pH of the slurry was adjusted to 4.6, the isoelectric point of casein, with 0.1 N hydrochloric acid. Disodium ethylenediaminetetraacetate (Na_2EDTA) was added at a level of 0.5% of the protein (0.52 g per 120 g casein), and the mixture was stirred for 45 minutes. The casein-Na_2EDTA mixture was then allowed to settle and the supernatant siphoned off. The casein was resuspended in deionized, distilled water and treated again with Na_2EDTA as previously described. This procedure was repeated a total of 5 times for each batch of casein. Disodium EDTA was removed from the casein by repeated washing with deionized distilled water until the absence of EDTA in the supernatant was indicated by formation of calcium oxalate precipitate when a drop of a saturated solution of calcium chloride was added to 10 ml of supernatant which had been combined previously with 5 ml of an ammonium oxalate solution and adjusted to pH 11.0 with sodium hydroxide solution. Finally as much water as possible was removed from the casein by filtering, after which the casein was dried in an oven at 50°C and finely ground before use in the purified diets.

Analysis of treated and untreated casein and the diets used in Experiment 2 yielded the copper concentration values presented in Table 4.

b. Bone mechanics. Left femurs were removed from pig carcasses and cleaned of all muscle, connective tissue and periosteum and stored in air-tight polyethylene sacs at -20°C. Before use, the bones were

TABLE 4. AVERAGE COPPER CONTENT OF TREATED AND UNTREATED CASEIN AND DIETS

Casein or diet	Copper (ppm)[1]
Untreated high-protein casein	1.6
Na_2EDTA treated high-protein casein	0.3
Untreated high-protein casein diet	1.3
Na_2EDTA treated high-protein casein diet	0.6

thawed at room temperature. Strength characteristics--bending moment, moment of inertia, maximal load, maximal stress, and Young's modulus of elasticity--were determined using an Instron Testing Instrument, Model TT CML[2]. The instrument was equipped with an FM-compression load cell having 250 kg full scale. Cross-head speed was 0.5 cm per minute and chart speed 1.0 cm per minute. A broad, flat porcelain plate was used as a base for the two fulcra supporting the femur. Deflection and maximum load were recorded automatically on the chart. In calculating strength characteristics of the femurs the formulas of Miller *et al.* (1962) were used and are as follows:

Maximal bending moment, M $= Wl/4$

Moment of inertia, I $= \pi/64\ (BD^3 - bd^3)$

Maximal stress, S $= MD/2I$

Young's modulus of elasticity, E $= Wl^3/48IY$

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[1]As fed basis.

[2]Instron Engineering Corporation, Canton, Massachusetts.

where

W = maximal load (kg)

l = distance between fulcra (cm)

B = outer horizontal diameter (cm)

b = inner horizontal diameter (cm)

D = outer vertical diameter (cm)

d = inner vertical diameter (cm)

Y = deflection at center of bone when load W is applied (cm)

3. Chemical Analyses

a. Plasma copper. Plasma stored at -20°C was thawed at room temperature and diluted 1:6 with deionized distilled water. Using artificially prepared serum as standards[1], copper concentration was determined with the aid of an atomic absorption spectrophotometer at a wavelength of 324.7 nm. Plasma copper was expressed in micrograms per 100 ml.

b. Tissue copper. Slices were made from frozen tissues and organs without thawing. Using a Polytron[2], homogenates were prepared by blending slices 1:2 with deionized distilled water while placing ice

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[1]Artificial serum standards were prepared to contain the following:
Std. 1: 2000 ppm Na, 100 ppm K, 1.0 ppm Cu, 0.5 ppm Zn, 50 ppm Ca, 20 ppm Mg, 25 ppm P, and 1.0 ppm Fe
Std. 2: 3000 ppm Na, 200 ppm K, 1.5 ppm Cu, 1.0 ppm Zn, 100 ppm Ca, 40 ppm Mg, 50 ppm P, and 2.0 ppm Fe.
Std. 3: 4000 ppm Na, 300 ppm K, 2.0 ppm Cu, 1.5 ppm Zn, 150 ppm Ca, 60 ppm Mg, 75 ppm P, and 3.0 ppm Fe.

[2]Brinkmann Instruments, Westbury, New York.

around the base of the blender beaker. The homogenates were weighed into 250 ml Phillips beakers in duplicate and wet ashed. Digestion consisted of the addition of 60 ml concentrated (12N) nitric acid to the homogenates followed by heating on a hot plate until almost dry. Upon cooling, 7 ml of 72% perchloric acid were added to each beaker which was then covered by a watch glass to prevent excessive evaporation while on the hot plate. Oxidation was continued until the reaction was completed. The watch glass was removed when dense white fumes appeared. Heating was continued until the volume was reduced to 2 to 3 ml. After cooling, each solution was made up to volume by addition of deionized distilled water.

Standards were prepared using the same procedure. Copper content of samples was determined by atomic absorption spectrophotometry at a wavelength of 324.7 nm and expressed as parts per million.

c. Feed and feces. Samples of feed and feces were finely ground in a Wiley mill[1], weighed into tared acid-washed 250 ml Phillips beakers in duplicate and digested using the wet ashing procedure already described for tissues. Analysis was done by atomic absorption spectrophotometry and copper concentration was expressed as parts per million (ppm).

d. Urine. Urine samples previously stored at 4°C were poured into beakers and shaken thoroughly for homogeneity. Ten milliliters of each sample were pipetted into 250 ml Phillips beakers in duplicate and digested according to the wet ashing procedure. Digested samples were

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[1]Arthur H. Thomas Co., Philadelphia, Pennsylvania.

diluted to volume with deionized distilled water and copper content was determined by atomic absorption spectrophotometry and expressed as ppm.

e. Hair. Samples of hair were soaked in deionized distilled water in 200 ml beakers for 45 minutes and drained on filter papers. They were then immersed in 95% ethanol for 45 minutes in order to remove adhering debris and foreign materials. Upon removal from ethanol the samples were air-dried at a temperature of 50°C and subjected to wet ashing. Copper content was determined by atomic absorption spectrophotometry and expressed as ppm.

f. Bone ash. Broken left femur samples from 2b above were cut longitudinally and laterally into approximately 8 pieces with a band saw. The pieces were weighed on tared filter paper, identified with pencil on a tag, and bone and tag were wrapped in cheesecloth. The wrapped pieces of bone were placed in a Soxhlet extractor and extracted with absolute ethanol for 24 hours[1] to remove water, and with anhydrous diethyl ether for 24 hours[2] to remove fat. The wrapped bones were removed, placed on a covered tray and allowed to dry in a hood until odor free. They were then placed in porcelain crucibles, oven dried, and weighed before being ashed in a muffle furnace at 600°C for 18 hours. Upon cooling the ash was weighed and percent ash was calculated as follows:

$$\%\text{ ash on fat free basis} = \frac{\text{Weight of ash}}{\text{Weight of dry fat free bone}}$$

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[1]Alcohol was poured over bones until extractor was filled 1½ to 2 times; 20 amp rheostat was set at 65.

[2]Anhydrous diethyl ether was poured as for alcohol; 20 amp rheostat was set at 35.

g. Bone copper. Ash from f above was finely ground using mortar and pestle, and approximately 300 mg of the well mixed powdered ash was dissolved in 5 ml of 6N hydrochloric acid. Two milliliter aliquots of the resulting ash solution were diluted 1:3 with deionized distilled water and copper content was determined by atomic absorption spectrophotometry at a wavelength of 324.7 nm and expressed as ppm on a dry fat free basis.

4. Enzymology

a. Plasma ceruloplasmin. Plasma ceruloplasmin activity was assayed on freshly obtained plasma using a modified method of Smith and Wright (1968). Buffer solution was prepared by dissolving 32.816 g sodium acetate and 1.55 g disodium ethylenediaminetetraacetate in deionized distilled water and diluting to 500 ml. The substrate, p-phenylenediamine dihydrochloride (PPD) was used without further purification. The working solution had a pH of 5.4 and was adjusted to a final pH of the reaction mixture of 6.3. Buffer and plasma were maintained at a temperature of 37°C in a water-bath before using, but the substrate (PPD) was kept at room temperature to minimize autooxidation. To 2.4 ml buffer and 0.1 ml substrate, 0.5 ml plasma was added and mixed on a Vortex-Genie device. The mixture was incubated at 37°C for 5 minutes. After incubation 1 ml of 0.3 mM sodium hydroxide solution, freshly prepared and kept at 0°C, was added to stop the enzyme reaction. The final mixture was thoroughly shaken and transferred into a colorimeter cell (1-cm). Optical density of the sample was read at a wavelength of 540 nm, and ceruloplasmin activity was expressed in terms of optical density per minute. Blanks were also measured for optical density, i.e., autooxidation in the absence of plasma enzyme, and actual enzyme activity

determined as follows:

$$\text{Ceruloplasmin activity in } OD_{540}/\text{min} = \left[OD_{540} \text{ of Sample}\right] - \left[OD_{540} \text{ of Blank}\right]$$

b. Brain and erythrocyte superoxide dismutase. A modified method of McCord and Fridovich (1969) was used to assay for superoxide dismutase activity in brain tissue and red blood cells as follows:

(1) Buffer. This consisted of 250 ml of 1.5 M KH_2PO_4 and 973 ml of 1.5 M K_2HPO_4 and 1.12 g of Na_2EDTA adjusted to a pH of 7.8.

(2) Xanthine. This solution was made by dissolving 130.5 mg sodium salt of xanthine and 16.7 mg NaCN in deionized distilled water and diluting to 500 ml.

(3) Cytochrome c. In 10 ml deionized distilled water, 37.2 mg ferricytochrome c (Sigma type III or equivalent) were dissolved and the solution stored in the dark.

(4) Xanthine oxidase. About 0.1 ml xanthine oxidase and 10 mg catalase were dissolved in 10 ml deionized distilled water and adjusted to give 0.250 A change per 10 minutes.

(5) Sample preparation.

(a) Red blood cells. About 0.1 ml of washed red blood cells was agitated in 1.0 ml deionized distilled water.

(b) Brain tissue. About 1.0 g tissue was homogenized in 10 ml deionized distilled water and the homogenate was spun at about 20,000 x G for 15 minutes. The supernatant was diluted 1:7 with deionized distilled water.

(6) Assay. A sample and control cuvette were analyzed simultaneously at 550 nm and 25°C. The control cuvette contained 2.6 ml deionized distilled water, 0.1 ml buffer solution, 0.1 ml cytochrome c solution, 0.1 ml xanthine solution; the sample cuvette contained 2.5 ml deionized distilled water, 0.1 ml buffer solution, 0.1 ml cytochrome c 0.1 ml xanthine solution and 0.1 ml sample. Initial absorbances were set and 0.05 ml xanthine oxidase solution rapidly added to control and sample cuvettes, mixed thoroughly and the reaction followed for 5 to 10 minutes. The recorder was set at 0.5 A full scale and travelled 0.2 in/min. The initial slopes of the resulting curves were used to calculate the change in Absorbance/10 minutes. The quantity of superoxide dismutase which gives 50% inhibition of the blank rate is defined as one unit.

$$\% \text{ Inhibition} - 1 - \frac{\text{Sample rate}}{\text{Control rate}} \times 100$$

$$\text{Units of Superoxide dismutase activity} = \% \text{ Inhibition/ } 50\% \text{ Inhibition/ Unit}$$

Superoxide dismutase activity was calculated per g protein for brain tissue and per ml red blood cells for blood.

D. Statistical Analyses

The data from Experiments 1, 2 and 3 were subjected to a one-way analysis of variance using the Unequal-1 format on a CDC[1] 6500 computer at the Michigan State University Computer Laboratory. Simple correlations were also calculated and the levels of significance of differences between means were determined using the Bonferroni t-statistics test with

[1]Control Data Corporation, Minneapolis, Minnesota.

the following formula:

$$t_B = \frac{\overline{Y}_1 - \overline{Y}_2}{\sqrt{MS_E \left(\frac{1}{r_1} + \frac{1}{r_2} \right)}}$$

where $\overline{Y}_1$ = mean for treatment 1

$\overline{Y}_2$ = mean for treatment 2

MS_E = error mean square

r_1 = number of observations in treatment 1

r_2 = number of observations in treatment 2

RESULTS AND DISCUSSION

A. <u>Experiment 1</u>: Copper requirement of baby pigs on semi-purified diets supplemented with varying levels of copper to yield upon analysis: 1.3 ppm, 3.2 ppm, 5.6 ppm and 9.3 ppm copper

Data for growth and food utilization are presented in Table 5. Feed intake, average daily gain and feed per gain were not significantly affected by dietary copper levels. Weight gain of pigs was at a normal rate. Ullrey and associates (1960) conducted baby pig studies with 6, 16 and 106 ppm copper and Gipp *et al.* (1973) with 2, 10 and 250 ppm copper and reported that average daily gain and feed per unit of gain were not significantly affected by treatment. Three weeks into the trial, two of the pigs on the basal diet exhibited signs of nervousness and developed leg problems and the inability to walk forward. These signs, however, disappeared before the fifth week. Similar observations were made by Teague and Carpenter (1951) and by Lahey *et al.* (1952) but these workers reported that the leg problems persisted through the trial.

Hematocrit, hemoglobin and mean corpuscular hemoglobin concentration values are summarized in Table 6. Although hematocrit values increased in the second, fourth and eighth weeks, except for values in diet one, and dropped in the sixth week (Figure 1.1), there was no consistent effect due to treatment. Hemoglobin, like the hematocrit, did not show any trend caused by dietary copper levels (Figure 1.2); there was

TABLE 5. THE EFFECT OF DIETARY COPPER LEVELS ON GROWTH (EXPT. 1)

Diet no.	1	2	3	4
Cu conc., ppm[1]	1.3	3.2	5.6	9.3
No. of pigs	4	3	3	3
Avg. init. wt., kg	1.28±0.09	1.20±0.11	1.42±0.11	1.23±0.11
Avg. final wt., kg	12.89±0.89	12.82±1.02	13.42±1.02	11.69±1.02
Avg. daily gain, g	276±0.01	282±0.02	273±0.02	269±0.02
Feed/gain	1.28	1.30	1.25	1.31

[1]Expressed on as fed basis.

TABLE 6. THE EFFECT OF DIETARY COPPER LEVELS ON HEMATOCRIT, HEMOGLOBIN AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (EXPT. 1)

Diet no.	1	2	3	4
Cu conc., ppm	1.3	3.2	5.6	9.3
No. of pigs	4	3	3	3
Hct.[1], %				
Initial	24.9±1.63	30.6±2.31	30.2±1.89	30.5±1.89
2 weeks	41.0±2.68	39.7±3.09	38.9±3.09	34.9±3.09
4 weeks	37.0±1.38	40.3±1.59	40.5±1.59	36.7±1.59
6 weeks	34.6±1.97	36.7±2.28	35.4±2.28	34.2±2.28
8 weeks	38.0±1.15	42.4±1.15	40.5±1.15	39.0±1.40
Hb[2], g/100 ml				
Initial	6.9±0.53	8.5±0.61	8.8±0.61	8.5±0.61
2 weeks	12.6±0.60	12.3±0.70	12.5±0.70	10.8±0.70
4 weeks	11.4±0.32	13.1±0.37[a]	13.8±0.37[b,c]	12.0±0.37[d]
6 weeks	11.3±0.70	11.7±0.81	13.0±0.81	12.8±0.81
8 weeks	11.1±0.60	13.6±0.60	12.6±0.60	12.9±0.73
MCHC[3], %				
Initial	27.7±0.59	27.3±0.83	29.4±0.68	27.6±0.68
2 weeks	20.8±2.22	30.9±2.56	32.7±2.56	31.6±2.56
4 weeks	31.1±1.63	32.7±1.89	34.2±1.89	32.8±1.89
6 weeks	41.6±2.22	32.0±2.57	36.9±2.57	38.0±2.57
8 weeks	29.4±1.43	32.2±1.43	31.1±1.43	32.9±1.75

[a]Significantly greater than least value ($P<0.05$).

[b]Significantly greater than least value ($P<0.01$).

[c,d]Significantly different ($P<0.05$).

[1]Hematocrit.

[2]Hemoglobin.

[3]Mean corpuscular hemoglobin concentration.

Figure 1.1. Influence of dietary copper levels on hematocrit (Expt. 1).

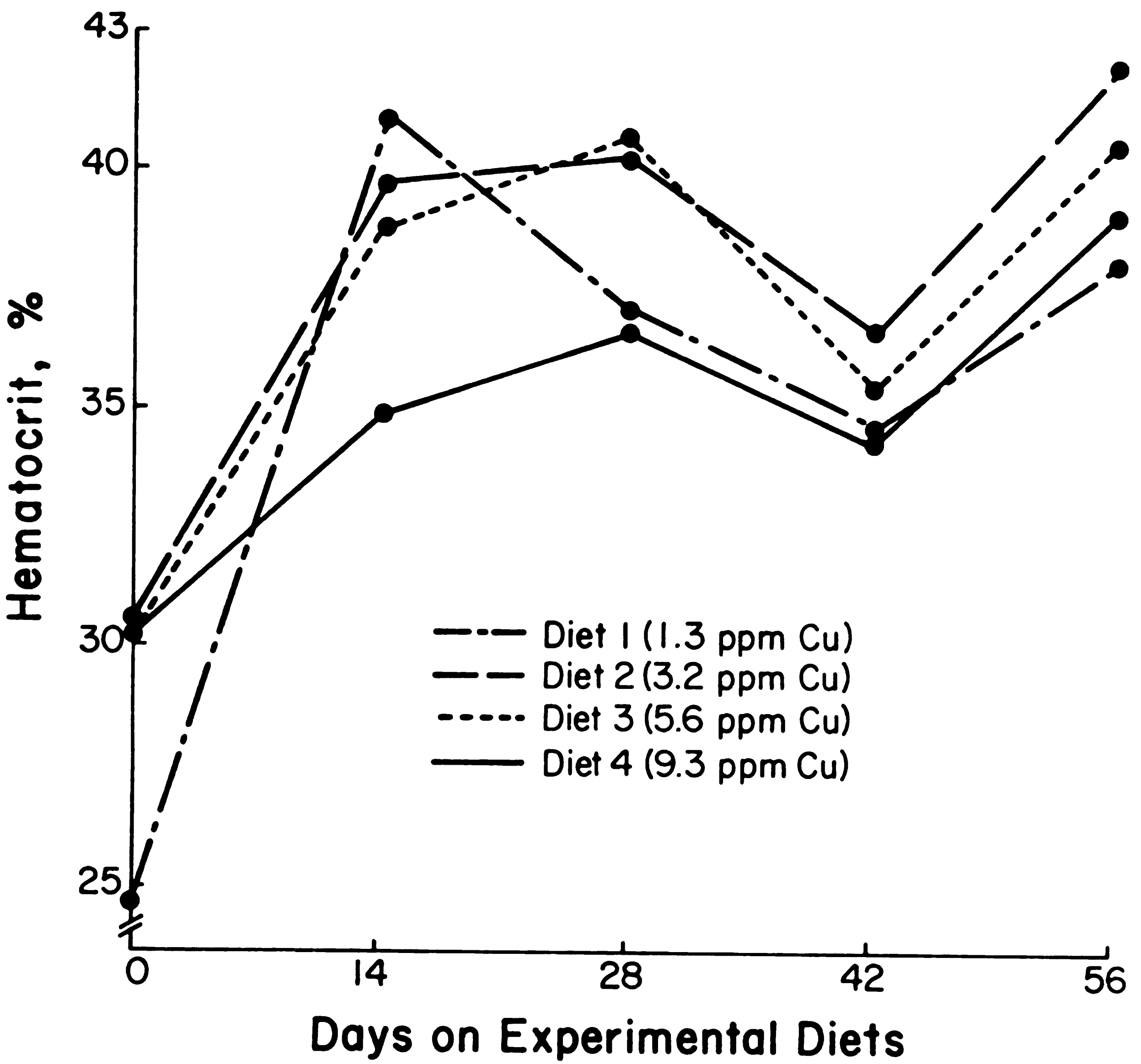


Figure 1.1.

Figure 1.2. Influence of copper intake on hemoglobin (Expt. 1).

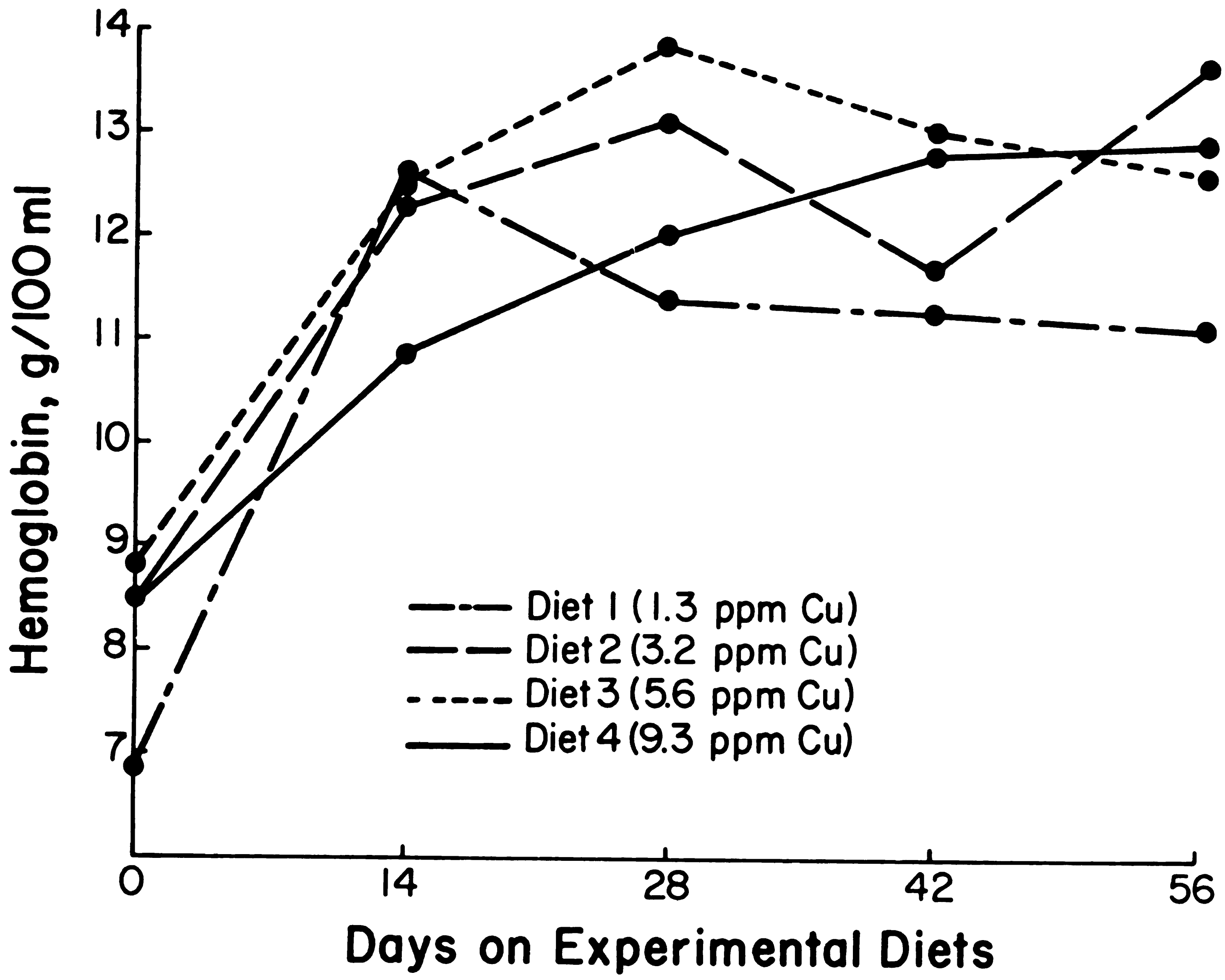


Figure 1.2.

however, a slight rise in the second and third weeks. In the fourth week hemoglobin values of pigs on the second and third treatments were significantly ($P<0.05$ and $P<0.01$, respectively) greater than corresponding values under the first treatment and there was a significant ($P<0.05$) difference between treatments three and four. Mean corpuscular hemoglobin concentration rose steadily after the second week and then dropped in the eighth week except for the second treatment (Figure 1.3). These observations are in agreement with the results of Ullrey and coworkers (1960) who reported that baby pig hematology was not significantly affected by 6, 16 and 106 ppm dietary copper. Gipp and associates (1973) observed no significant effects on hematocrit, hemoglobin and mean corpuscular hemoglobin concentration when young pigs were fed 2 ppm dietary copper.

Ceruloplasmin activity and plasma copper concentrations are shown in Table 7. Ceruloplasmin activity of pigs on the basal diet was significantly ($P<0.01$) depressed, dropping from an initial value of 0.17 OD/min to 0.02 OD/min in the sixth week and rising to 0.05 by the end of the trial (Figure 1.4). There was, however, no significant reduction within the rest of the treatments. Pigs on the basal diet exhibited ceruloplasmin values which were significantly ($P<0.01$) lower than corresponding values in treatments 2, 3 and 4, but there was no significant difference between treatments 2, 3 and 4. Identical results were reported by Gipp and coworkers (1973) who observed a significant ($P<0.01$) reduction in ceruloplasmin of pigs kept on diets containing 2 ppm copper. Williams and associates (1975) reported significantly ($P<0.01$) depressed ceruloplasmin activity down to 1% of control in pigs fed low-copper diets. Plasma copper concentrations were significantly

TABLE 7. THE EFFECT OF DIETARY COPPER LEVELS ON CERULOPLASMIN ACTIVITY AND PLASMA COPPER CONCENTRATION (EXPT. 1)

Diet no.	1	2	3	4
Cu conc., ppm	1.3	3.2	5.6	9.3
No. of pigs	4	3	3	3
Cp.[1], OD/min				
2 weeks	0.17±0.04	0.22±0.05	0.42±0.05[a]	0.23±0.05
4 weeks	0.03±0.02	0.20±0.03[b]	0.26±0.03[b]	0.21±0.03[b]
6 weeks	0.02±0.02	0.21±0.03[b]	0.23±0.03[b]	0.24±0.03[b]
8 weeks	0.05±0.01	0.28±0.01[b]	0.23±0.01[b]	0.24±0.02[b]
Pl. Cu[2], mcg/100 ml				
Initial	19.9±4.83	27.1±5.58	25.5±5.58	34.2±5.58
2 weeks	49.2±20.9	134.4±24.2	164.7±24.2[a]	198.4±24.2[b]
4 weeks	55.4±16.3	202.6±18.8[b,d]	256.2±18.8[b,c]	198.6±18.8[b,c]
6 weeks	51.3±6.78	188.3±7.83[b]	228.5±7.83[b]	201.6±7.83[b]
8 weeks	41.4±15.4	189.0±15.4[b]	191.3±15.4[b]	199.3±18.8[b]

[a]Significantly greater than least value ($P<0.05$).

[b]Significantly greater than least value ($P<0.01$).

[c,d]Significantly different ($P<0.05$).

[1]Ceruloplasmin activity.

[2]Plasma copper concentration.

Figure 1.3. Influence of dietary copper levels on mean corpuscular hemoglobin concentration (Expt. 1).

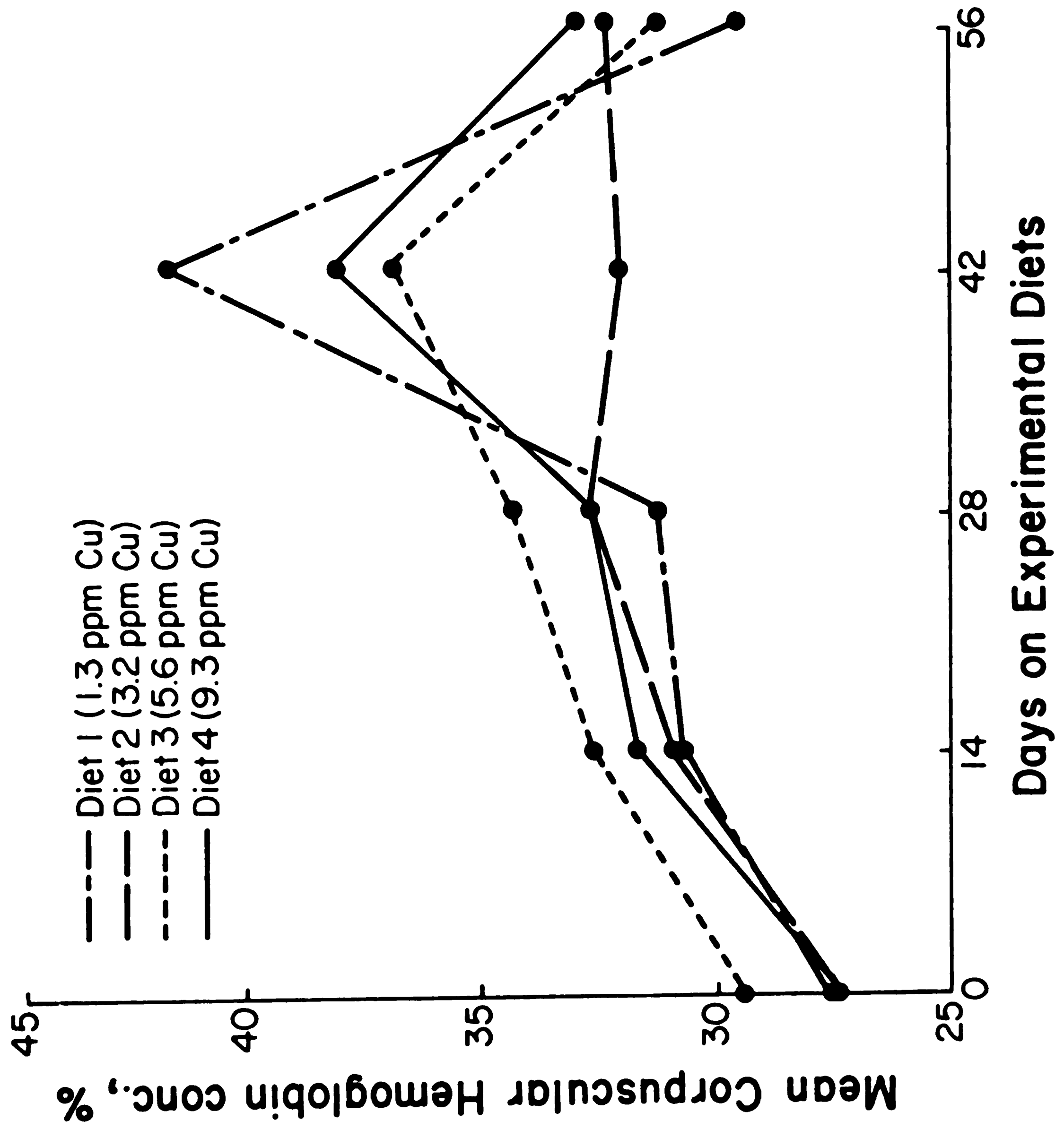


Figure 1.3.

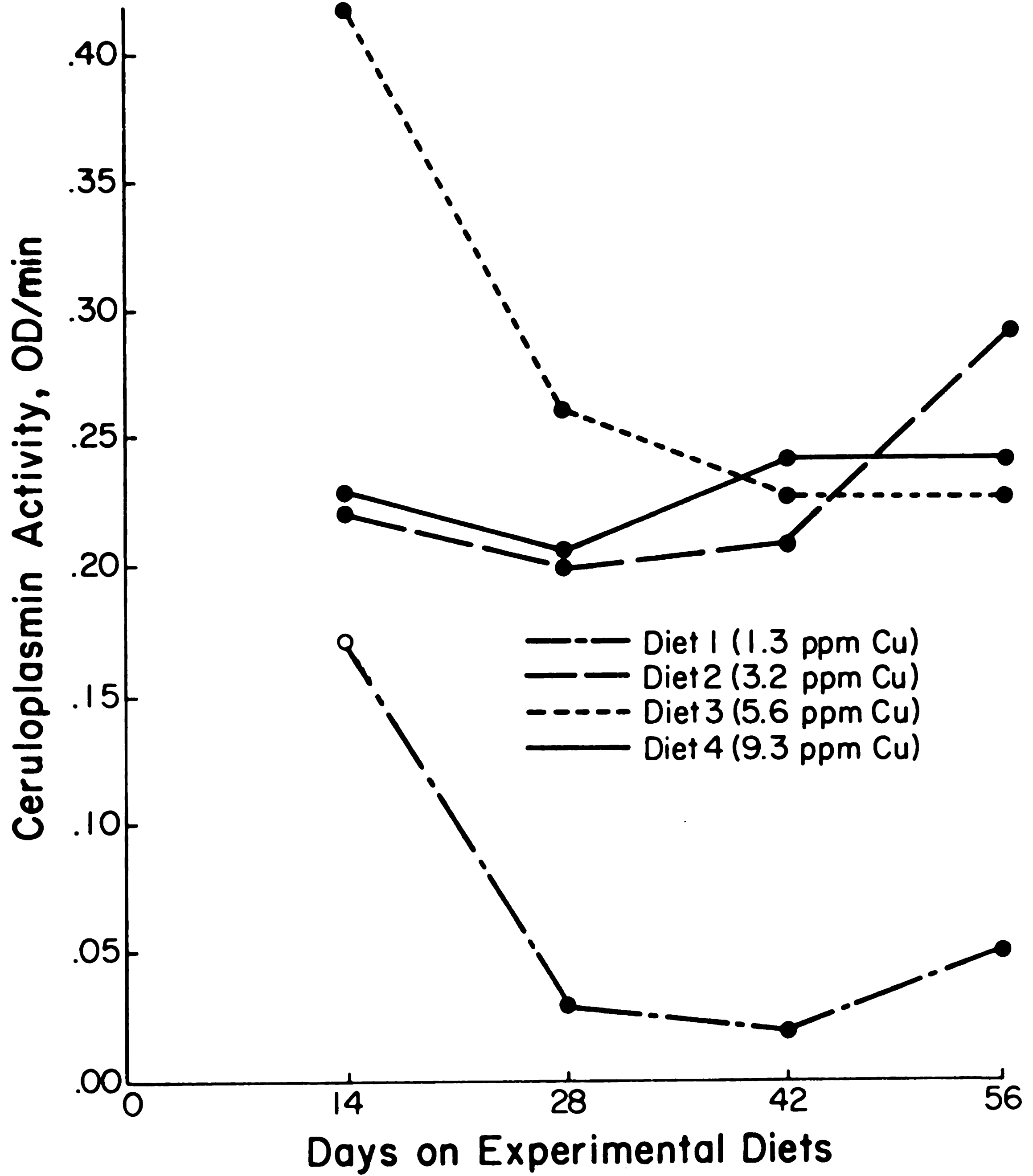
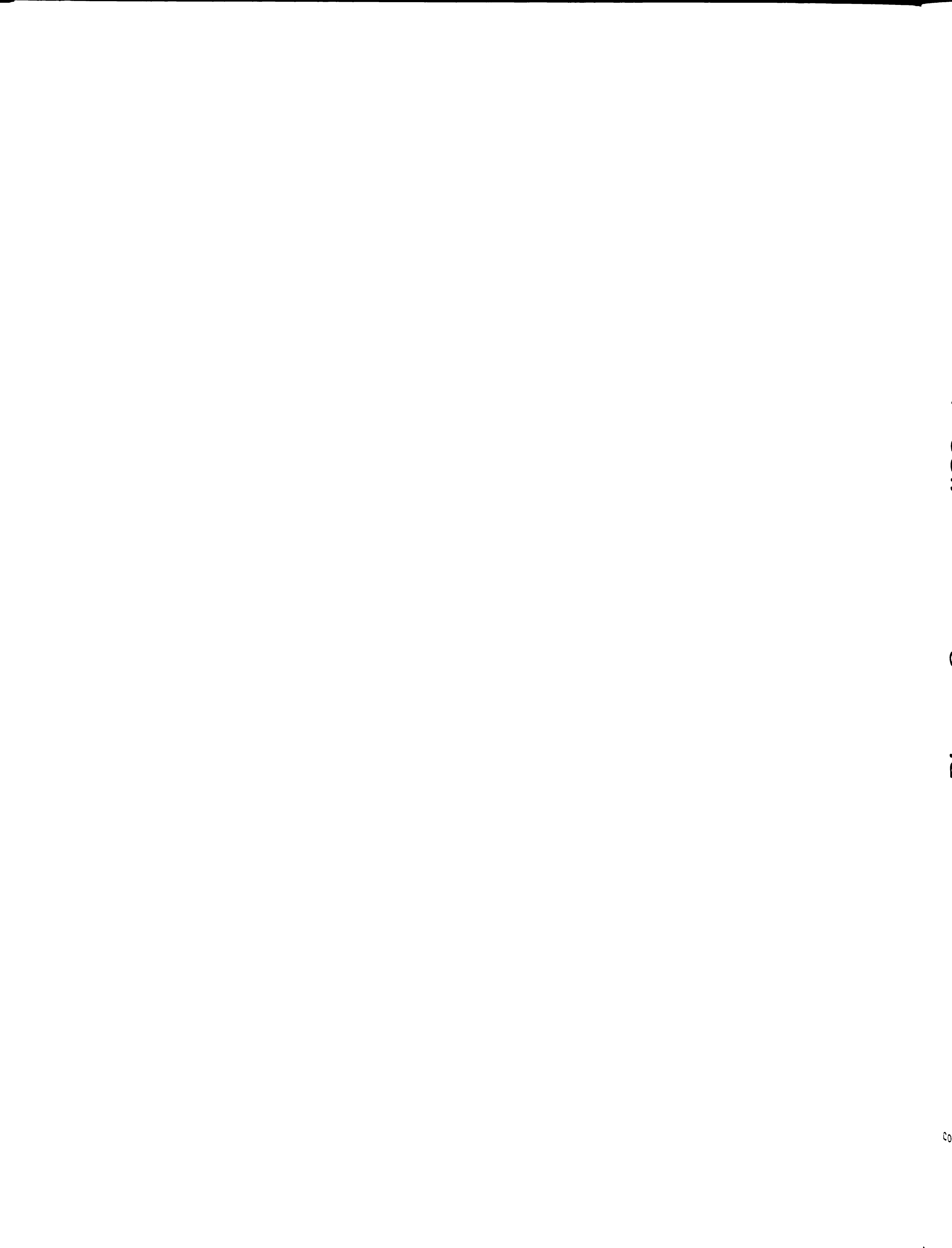


Figure 1.4. Influence of copper intake on ceruloplasmin (Expt. 1).

(P<0.01) increased by treatments 2 and 3 containing 3.2 and 5.6 ppm copper, respectively, when compared to treatment 1 up to the fourth week; there was a decline thereafter (Figure 1.5). Plasma copper of pigs on diet 4 reached a peak at the sixth week and subsequently declined slightly. There was a significant (P<0.01) difference between plasma copper levels of pigs on diet 1 and pigs on diets 2, 3 and 4, but no significant difference between plasma copper values of pigs on diets 2, 3 and 4. Depressed plasma copper concentrations in pigs have been reported by Gipp *et al.* (1973) and Williams *et al.* (1975) who showed that low dietary copper (2 ppm and less) significantly (P<0.01) reduced plasma copper. Dreosti and Quicke (1968) provided evidence to indicate that plasma copper was a sensitive index of copper status.

Tissue copper concentrations and brain superoxide dismutase activity are presented in Table 8. Liver, kidney and heart copper values were not significantly influenced by dietary copper. This is at variance with previous results which established that dietary copper strongly influences the levels of copper in the liver (Schultze *et al.*, 1936a; Lahey *et al.*, 1952; Wintrobe *et al.*, 1953; Dempsey *et al.*, 1958; Ullrey *et al.*, 1960). Spleen copper showed a significant (P<0.05) difference between treatment 1 and treatments 2 and 4. Brain copper concentration of pigs on the basal diet was significantly (P<0.05) lower than that of pigs on diet 4, but not on diets 2 and 3; brain copper of pigs on diets 3 and 4 were significantly (P<0.05) different. There was a significant (P<0.01) difference in hair copper between treatment 1 and treatments 2 and 3, and between treatment 1 and treatment 4 (P<0.05). Brain superoxide dismutase activity did not exhibit any significant treatment effects, an observation not in keeping with



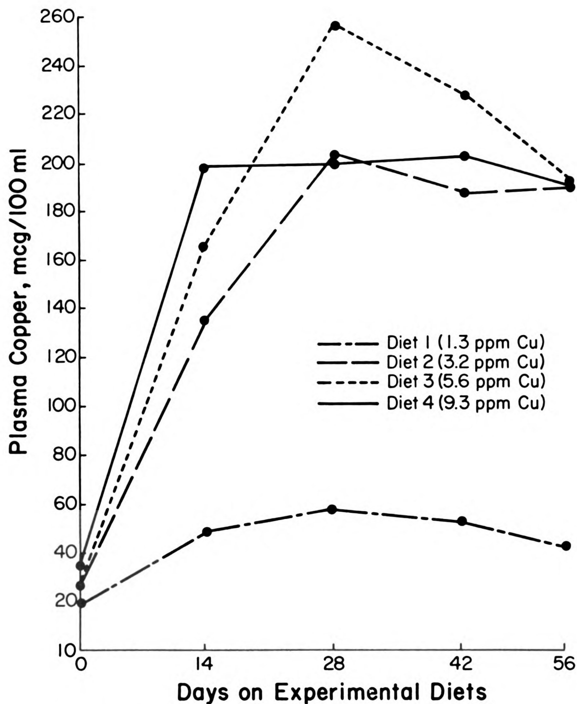


Figure 1.5. Influence of dietary copper levels on plasma copper concentrations (Expt. 1).

TABLE 8. THE EFFECT OF DIETARY COPPER LEVELS ON TISSUE COPPER CONCENTRATIONS AND BRAIN SUPEROXIDE DISMUTASE ACTIVITY (EXPT. 1)

Diet no.	1	2	3	4
Cu conc., ppm	1.3	3.2	5.6	9.3
No. of pigs	4	3	3	3
Tissue Cu, ppm[1]				
Liver	69.1±17.04	73.2±19.67	52.6±19.67	104.9±19.67
Kidney	64.8±9.55	46.7±11.03	64.1±11.03	57.2±11.03
Spleen	60.9±9.57	93.1±11.05[d]	76.2±11.05	88.8±11.05[d]
Heart	22.9±10.44	24.3±12.06	36.5±12.06	20.3±12.06
Brain	6.1±2.86[a]	8.8±3.30	8.3±3.30[a]	22.3±3.30[b]
Hair	10.2±0.65	14.5±0.76[c]	15.0±0.76[c]	13.8±0.76[d]
Brain SOD[2], units/mg protein	3.3±0.78	4.9±0.90	4.8±0.90	3.3±0.90

[1]Expressed on dry basis.

[a,b]Significantly different ($P<0.05$).

[c]Significantly greater than least value ($P<0.01$).

[d]Significantly greater than least value ($P<0.05$).

[2]Superoxide dismutase.

the results of Williams and associates (1975) who reported an 85% drop in hepatic and erythrocyte superoxide dismutase activity in copper deficient swine.

Results of the copper balance trial are given in Table 9. Copper intake, by virtue of increasing dietary copper concentration, was significantly (P<0.01) different between diet 1 and diets 3 and 4. Fecal copper of pigs on diet 4 was significantly (P<0.01) greater than that of pigs on diet 1. There was a significant (P<0.05) difference in urinary copper as percent of copper intake between diet 1 and diet 4; urinary copper (%) of pigs on diets 2 and 3 was greater, though not significantly, than corresponding values in diet 4. Absolute copper retention was influenced by dietary copper -- pigs on diets 3 and 4 retained significantly (P<0.05 and P<0.01, respectively) more copper than did pigs on diet 1. Percent copper retention was not significantly affected by treatments.

Strength characteristics of the left femur estimated by physical and chemical measurements are summarized in Table 10. Pigs on the basal diet had significantly (P<0.05) greater femur vertical and horizontal diameters[1] (D and B, respectively) than those of pigs on diet 3. Elasticity of femurs on treatments 2 and 3 was significantly (P<0.05) greater than elasticity values for treatment 4. Other physical characteristics, such as femur weight, maximum load and maximum stress, showed no significant treatment effects. Percent femur ash was not significantly different between treatments, but femur copper concentration of

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[1]Measured at mid-shaft of femur with the lateral and medial condyles facing downwards.

TABLE 9. THE EFFECT OF DIETARY COPPER LEVELS ON COPPER BALANCE (EXPT. 1)

Diet no.	1	2	3	4	SEM[1]
Cu conc., ppm	1.3	3.2	5.6	9.3	
No. of pigs	3	3	3	3	
Cu intake, mg/day	0.98	1.82	3.42[a]	5.84[b]	0.25
Cu excretion, mg/day					
Fecal	0.42	0.91	1.21	2.88[b]	0.24
Urinary	0.08	0.09	0.11	0.09	0.02
Cu excretion, % of intake					
Fecal	42.9	50.0	35.4	49.3	
Urinary	7.7[c]	5.1	3.3	1.6	
Cu retention, mg/day	0.48	0.82	2.10[c]	2.87[a]	0.37
Cu retention, % of intake	49.5	44.9	61.4	49.1	5.94

[1]Standard error of the mean (±).

[a]Significantly greater than least two values (P<0.01).

[b]Significantly greater than least three values (P<0.01).

[c]Significantly greater than least value (P<0.05).

TABLE 10. THE EFFECT OF DIETARY COPPER LEVELS ON PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE LEFT FEMUR (EXPT. 1)

Diet no.	1	2	3	4
Cu conc., ppm	1.3	3.2	5.6	9.3
No. of pigs	4	3	3	3
Physical characteristics				
Weight, fresh, g	61.2±2.81	61.3±3.24	53.5±3.24	52.4±3.24
External diameter[1], cm				
Horizontal (B)	1.48±0.02[a]	1.40±0.02	1.36±0.02	1.42±0.02
Vertical (D)	1.49±0.02[a]	1.40±0.03	1.36±0.03	1.42±0.03
Length (L), cm[b]	8.73±0.23	8.70±0.26	8.63±0.26	7.90±0.26
Maximum load, kg	57.0±4.45	67.3±5.14	57.7±5.14	46.7±5.14
Maximum bending moment, kg-cm	120.7±10.75	147.3±12.41	124.4±12.41	92.5±12.41
Moment of inertia, cm^4	0.20±0.01	0.17±0.01	0.14±0.01	0.18±0.01
Maximum stress, kg/cm^2	456±52.84	598±61.01	574±61.01	370±61.01
Modulus of elasticity, 1000 kg/cm^2	10.7±1.82	14.6±2.11[a]	14.1±2.11[a]	5.6±2.11
Chemical characteristics[c]				
Ash content, %	41.0±1.02	42.3±1.18	41.8±1.18	40.5±1.18
Cu, ppm	6.4±0.52	8.6±0.61	7.9±0.61	9.2±0.61[a]

[a]Significantly greater than least value ($P<0.05$).

[b]Distance from the mid-medial condyle to the fovea.

[c]Expressed on dry, fat-free basis.

[1]Measured at mid-shaft with medial and lateral condyles facing downwards.

pigs on diet 4 was significantly ($P<0.05$) greater than the value for pigs on the basal diet. Since clinical signs of copper deficiency were not apparent in pigs on the basal diet (1.3 ppm Cu), it seemed likely that depletion of body copper stores was not complete. Besides, there were no skeletal deformities observed towards the end of the trial as reported earlier in swine by Teague and Carpenter (1951), Lahey *et al.*, (1951, 1952) and Follis *et al.* (1955).

Correlations between copper balance, plasma copper, ceruloplasmin activity and average daily gain are presented in Table 11. There was a significant correlation ($P<0.01$) between plasma copper and ceruloplasmin on weeks 4, 6 and 8. Plasma copper was significantly ($P<0.01$) correlated to copper intake and fecal copper on week 4. Urinary copper as a percent of intake was significantly ($P<0.05$) correlated with plasma copper and ceruloplasmin on weeks 4 and 6. Average daily gain showed no significant correlation with copper balance, ceruloplasmin or plasma copper.

Table 11a shows correlations between liver and kidney copper and plasma copper and ceruloplasmin. Correlations between liver copper and plasma copper were generally low and non-significant except for initial plasma copper which showed a significant ($P<0.01$) correlation with liver copper. This observation does not agree with the report of Dempsey *et al.* (1958) who established a close correlation between plasma and liver copper. Correlations between liver copper and ceruloplasmin, and kidney copper and plasma copper and ceruloplasmin were low and non-significant.

TABLE 11. CORRELATIONS BETWEEN COPPER BALANCE, PLASMA COPPER, CERULOPLASMIN AND AVERAGE DAILY GAIN (EXPT. 1)

	Absolute copper balance				% of intake				Ceruloplasmin			
	Cu Intake	Fecal Cu	Urin. Cu	Cu Ret.	Cu Ret.	Fecal Cu	Urin. Cu	ADG	2 weeks	4 weeks	6 weeks	8 weeks
Pl. Cu, init.	.37	.38	-.10	.29	.006	.08	-.37	-.62	.15	.40	.33	.54
Pl. Cu, 2 weeks	.71[b]	.82[b]	.48	.48	-.18	.30	-.62	-.36	.59	.83[b]	.87[b]	.77[b]
Pl. Cu, 4 weeks	.51	.46	.23	.45	.05	.11	-.71[a]	-.07	.68[a]	.94[b]	.89[b]	.77[b]
Pl. Cu, 6 weeks	.62	.55	.34	.56	.10	.06	-.71[a]	-.32	.63	.90[b]	.90[b]	.89[b]
Pl. Cu, 8 weeks	.54	.55	.51	.50	.14	-.08	-.44	-.45	.28	.69[a]	.78[b]	.93[b]
Cp., 2 weeks	.20	.11	.26	.24	.31	-.22	-.44	-.13	---	.75[a]	.58	.30
Cp., 4 weeks	.50	.52	.22	.38	-.07	.22	-.67[a]	-.19		---	.90[b]	.75[a]
Cp., 6 weeks	.67[a]	.69[a]	.37	.51	-.15	.30	-.66[a]	-.17			---	.77[b]
Cp., 8 weeks	.38	.47	.52	.30	-.08	.13	-.27	-.25				---
ADG[c]	-.43	-.42	-.11	-.35	-.17	.12	.26	---				

[a]Significant ($P<0.05$).

[b]Significant ($P<0.01$).

[c]Average daily gain.

TABLE 11a. CORRELATIONS BETWEEN LIVER AND KIDNEY COPPER AND CERULOPLASMIN AND PLASMA COPPER (EXPT. 1)

	Plasma Copper					Ceruloplasmin Activity			
Item	Init.	2 weeks	4 weeks	6 weeks	8 weeks	2 weeks	4 weeks	6 weeks	8 weeks
Liver Cu	.82[a]	.27	.18	.34	.53	.04	.28	.20	.49
Kidney Cu	-.01	-.18	-.06	-.04	-.04	.01	-.03	-.17	-.08

[a]Significant ($P<0.01$).

1. Histopathology

a. Histopathologic examination.[1] The bone and costochondral junction of the eighth ribs were examined and the following parameters evaluated: cartilage vacuolation, cellularity near cartilage junction and active junction. The scores were averaged to give the most meaningful ranking, the overall ranking. Vacuolation of cartilage was scored on the amount of vacuoles present in the cartilage cells and the uniformity of alignment of cartilage cells. Cellularity near the cartilaginous junction refers to increased cellularity just below the cartilaginous junction at the point of bony spicule development, and this cellularity consisted of osteoblasts, osteoclasts and fibrous connective tissue stroma. Active bone marrow production of cells began at a rather uniform distance from the cartilaginous plate. Compact bone formation on the diaphysis was basically uniform in that most bones averaged 3 low power fields below the cartilaginous plate, and this measurement was primarily based upon the first appearance of continuous compact bone below the periosteum and distal from the cartilaginous plate. Results of examination, based upon the overall ranking, indicated that there were no significant treatment effects. The overall ranking[2] was: treatment 1, 1.5; treatment 2, 2.3; treatment 3, 1.3; and treatment 4, 2.7.

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[1]Examination was conducted and reported by Dr. K. K. Keahey, Department of Pathology, Michigan State University.

[2]1=normal, 2=very slight change, 3=slight change, 4=moderate change, 5-7=severe change.

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B. Experiment 2: Copper requirement of baby pigs on semi-purified diets (the casein in the basal diet was treated with Na_2EDTA) supplemented with copper sulfate to yield upon analysis: 0.6 ppm, 1.9 ppm and 2.8 ppm copper

During the period of adjustment to the basal diet, 2 pigs from each of the treatments died of causes unrelated to copper nutrition. The responses of average daily gain, feed efficiency and tissue copper concentration to dietary copper are presented in Table 12. Average daily gain and feed efficiency were not significantly affected by treatments, although there were slight increases in average daily gain with each increase in dietary copper. Liver, heart, brain and hair copper concentrations did not show any significant differences due to treatment. Pigs on diets 2 and 3 had kidney copper levels which were significantly ($P<0.05$ and $P<0.01$, respectively) greater than the values for pigs on diet 1. It is noteworthy that, although liver copper increased with dietary copper of diets 1 and 2, the change was not significant. Liver copper values for all treatments: 3.9, 7.5 and 9.1 ppm (fresh basis) for diets 1, 2 and 3, respectively, agree with the values reported for mature pigs on normal diets: 12 to 48 ppm (dry basis) (Cunningham, 1946); 4.5 ppm, 4.8 ppm (fresh basis) and 30.0 ppm (dry basis) (NRC-42 Committee, 1974). It has been established that dietary copper influences liver copper stores in many species including pigs (Schultze *et al.*, 1936a; Wiener and Field, 1970; Rucker *et al.*, 1969).

The response of hematocrit, hemoglobin and mean corpuscular hemoglobin concentrations to dietary copper levels are summarized in Table 13. Although there were slight increases in hematocrit on days 7 and 14 (Figure 2.1), there were no significant differences due to treatment. Hemoglobin and mean corpuscular hemoglobin concentration

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TABLE 12. THE EFFECT OF DIETARY COPPER LEVELS ON GROWTH AND TISSUE COPPER CONCENTRATIONS (EXPT. 2)

Diet no.	1	2	3	SEM[3]
Cu conc., ppm[1]	0.6	1.9	2.8	
No. of pigs	3	3	3	
Avg. init. wt., kg	1.49	1.54	1.59	0.10
Avg. final wt., kg	7.45	8.66	8.74	0.63
Avg. daily gain, g	164	196	212	19.97
Feed/gain	1.26	1.30	1.29	0.03
Tissue Cu conc., ppm[2]				
Liver	3.9	7.5[a]	9.1[b]	1.28
Kidney	1.1	2.2[a]	2.9[b]	0.26
Heart	2.9	3.0	3.5	0.28
Brain	2.3	2.7	1.5	0.52
Hair	10.5	9.7	10.9	0.49

[1]Expressed on as fed basis.

[a]Significantly greater than least value ($P<0.05$).

[b]Significantly greater than least value ($P<0.01$).

[2]Expressed on fresh basis.

[3]Standard error of the mean (±).

TABLE 13. THE EFFECT OF DIETARY COPPER LEVELS ON HEMATOCRIT, HEMOGLOBIN AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (EXPT. 2)

Diet no.	1	2	3	SEM
Cu conc., ppm	0.6	1.9	2.8	
No. of pigs	3	3	3	
Hct.[1], %				
Initial	33.8	34.5	37.3	1.24
7 days	40.2	36.0	36.7	2.00
14 days	37.6	39.2	39.0	1.71
22 days	34.7	36.0	35.9	1.27
30 days	33.9	36.9	36.7	1.66
Hb.[2], g/100 ml				
Initial	16.6	18.2	18.4	0.63
7 days	9.2	9.6	10.7	1.51
14 days	12.4	12.3	12.6	0.61
22 days	10.5	11.2	10.8	0.37
30 days	8.4	11.5	11.3	1.07
MCHC[3], %				
Initial	49.2	52.7	49.7	2.53
7 days	22.5	26.5	28.9	3.03
14 days	33.2	31.4	32.3	1.07
28 days	30.3	31.2	30.0	0.62
30 days	24.5	31.3	30.8	2.53

[1]Hematocrit.

[2]Hemoglobin.

[3]Mean corpuscular hemoglobin concentration.

Figure 2.1. Effect of copper intake on hematocrit (Expt. 2).

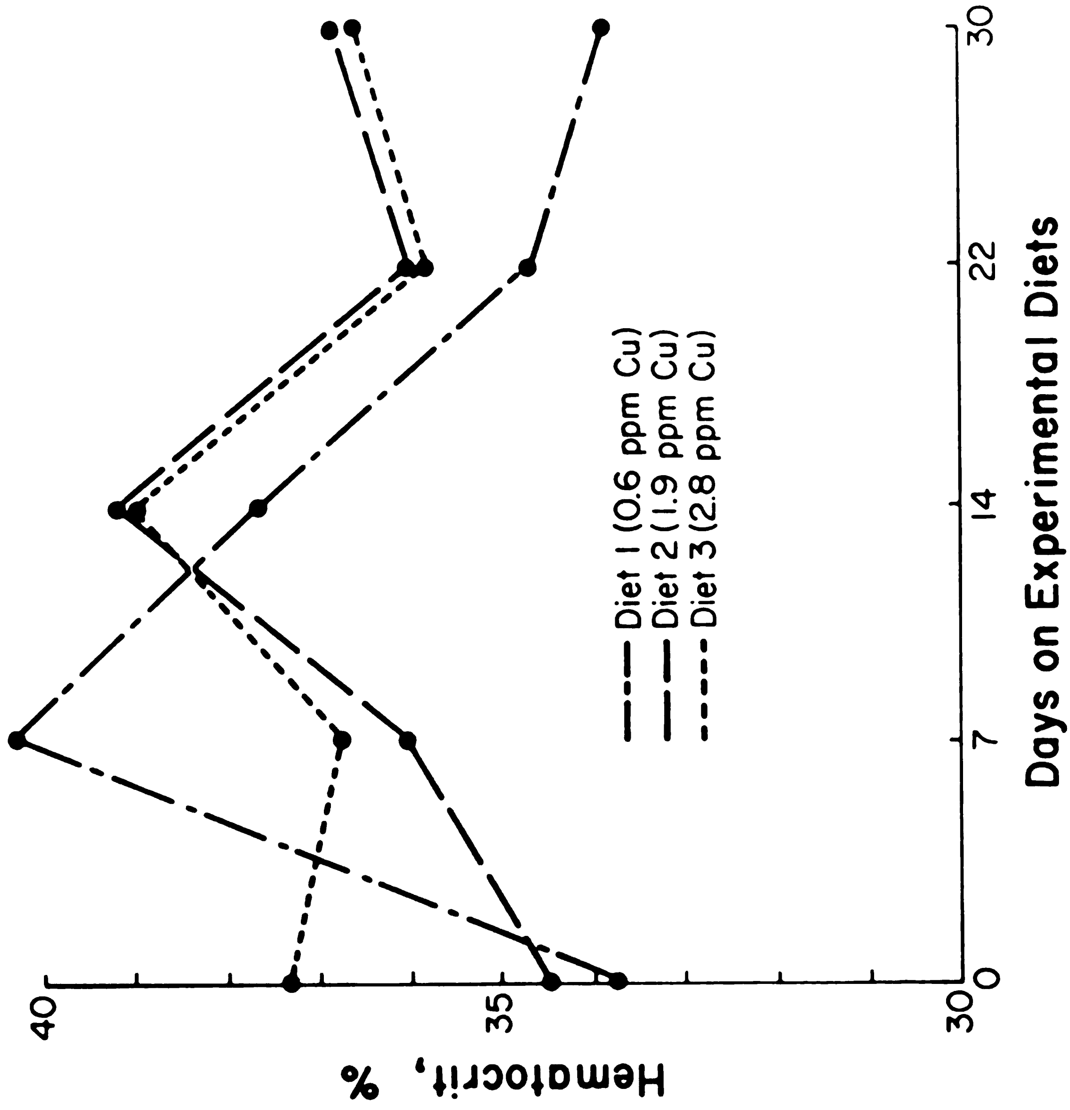

Figure 2.1.

(Figures 2.2 and 2.3) like the hematocrit, showed no significant treatment effects. Hemoglobin of pigs on the basal diet on day 30 was 8.4 g/100 ml and might have decreased further with time. Elvehjem and Hart (1929) reported anemia when hemoglobin level fell from 8.0 to 4.0 g/100 ml.

Ceruloplasmin activity and plasma copper levels are shown in Table 14. Ceruloplasmin of pigs on diet 1 reached a peak on day 7 and plateaued through day 14, declining toward the end of the trial, while that of pigs on diets 2 and 3 peaked at days 22 and 14, respectively (Figure 2.4). There was a significant ($P<0.01$) difference between ceruloplasmin of pigs on diets 2 and 3. The low ceruloplasmin activity of pigs on diet 1 indicates that 0.6 ppm copper was inadequate to maintain a steady activity as diet 3 (2.8 ppm copper) did. Ceruloplasmin activities of pigs on diet 1 agree with values obtained by Gipp *et al.* (1973) and Williams *et al.* (1975). Plasma copper levels for all pigs rose after the initial period and declined sharply in the cases of diets 1 and 2 before rising again on day 30 (Figure 2.5). After day 14, plasma copper showed significant ($P<0.01$) differences between treatments. On day 22 plasma copper of pigs on diet 1 reached a low value of 7.7 mcg/100 ml, much below the critical level of 20 mcg/100 ml necessary to support hematopoiesis (Lahey *et al.*, 1952) but anemia was not observed; corresponding hemoglobin and hematocrit values were 10.5 g/100 ml and 34.7%. Plasma copper levels for pigs on diets 2 and 3 for the entire trial period were well within, and in the case of diet 3, greater than the normal range of 50 to 150 mcg/100 ml reported by Beck (1961).

Results of the copper balance trial are given in Table 15. Copper intake was significantly ($P<0.05$) different between diets 2 and 3,

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TABLE 14. THE EFFECT OF DIETARY COPPER LEVELS ON CERULOPLASMIN ACTIVITY AND PLASMA COPPER CONCENTRATION (EXPT. 2)

Diet no.	1	2	3	SEM
Cu conc., ppm	0.6	1.9	2.8	
No. of pigs	3	3	3	
Cp.[1], OD/min				
Initial	0.02	0.02	0.03	0.003
7 days	0.06	0.17	0.22	0.05
14 days	0.06	0.24[a]	0.30[b]	0.01
22 days	0.05	0.29[a]	0.29[a]	0.02
30 days	0.04	0.27[a,c]	0.30[a,d]	0.01
Pl. Cu[2], mcg/100 ml				
Initial	55.6	58.2	59.6	4.73
7 days	190.0	151.0	170.0	21.90
14 days	32.3	108.8[a,c]	153.1[a,d]	13.64
22 days	7.7	73.4[a]	155.3[b]	7.82
30 days	33.4	93.2[a]	184.2[b]	5.45

[a]Significantly greater than least value (P<0.01).

[b]Significantly greater than least two values (P<0.01).

[c,d]Significantly different (P<0.05).

[1]Ceruloplasmin activity.

[2]Plasma copper concentration.

Figure 2.2. Effect of dietary copper levels on hemoglobin (Expt. 2).

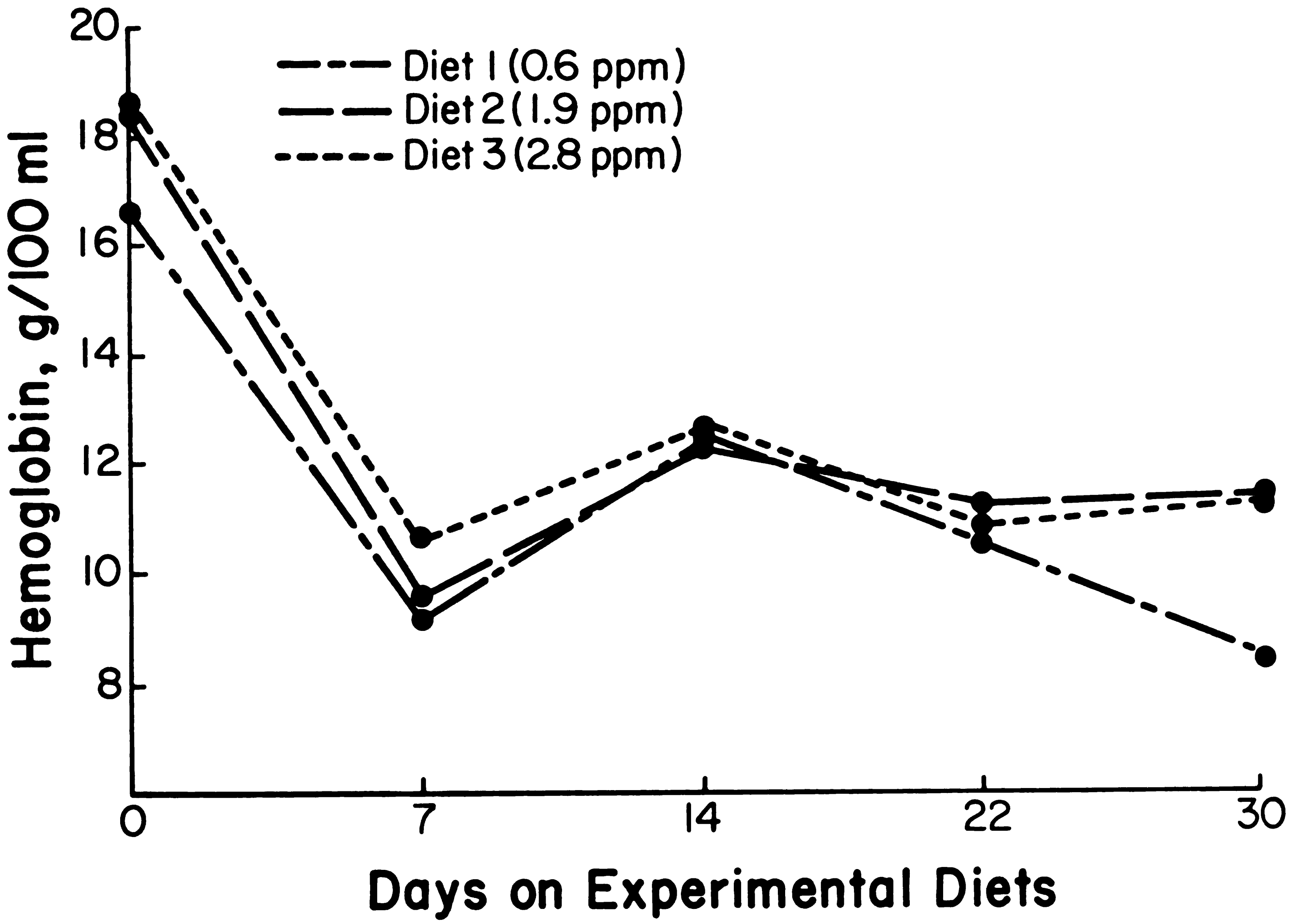

Figure 2.2.

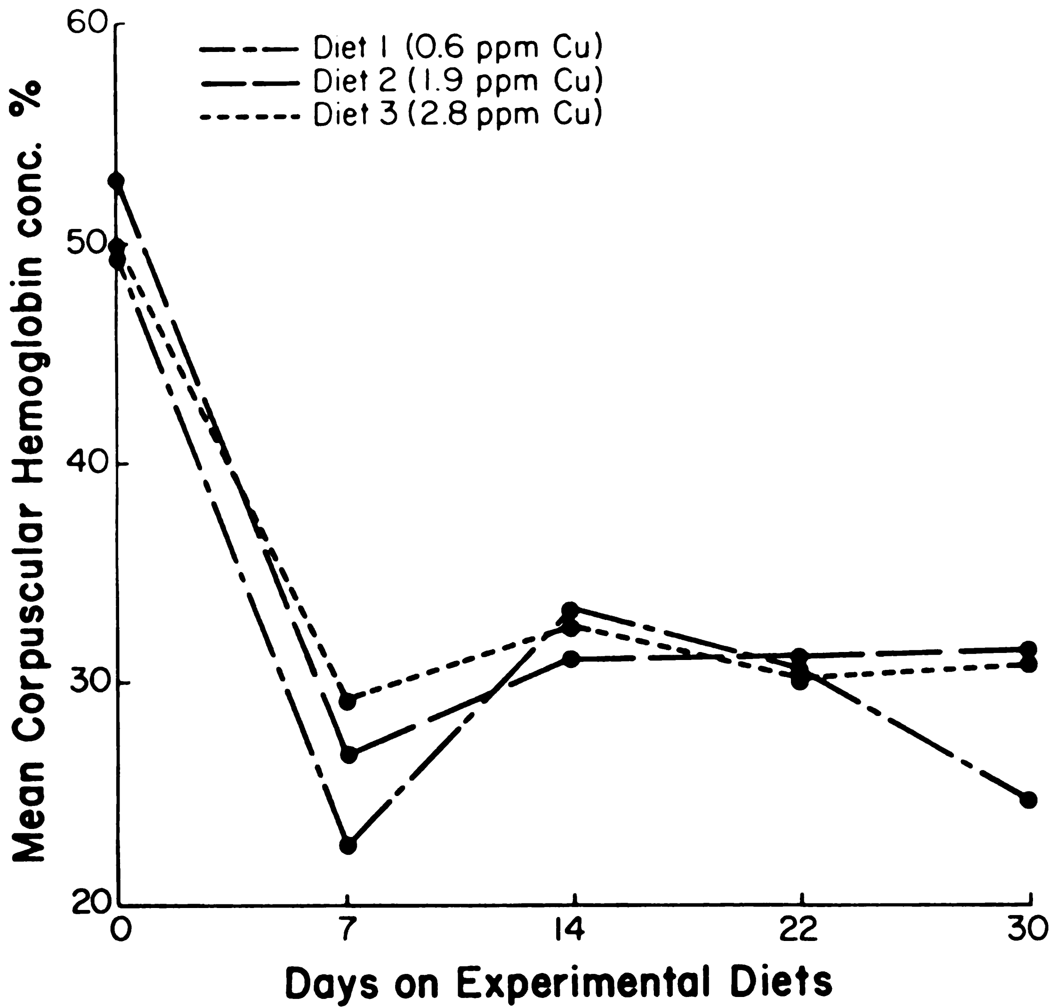

Figure 2.3. Effect of copper intake on mean corpuscular hemoglobin concentration (Expt. 2).

Figure 2.4. Effect of copper intake on ceruloplasmin activity (Expt. 2).

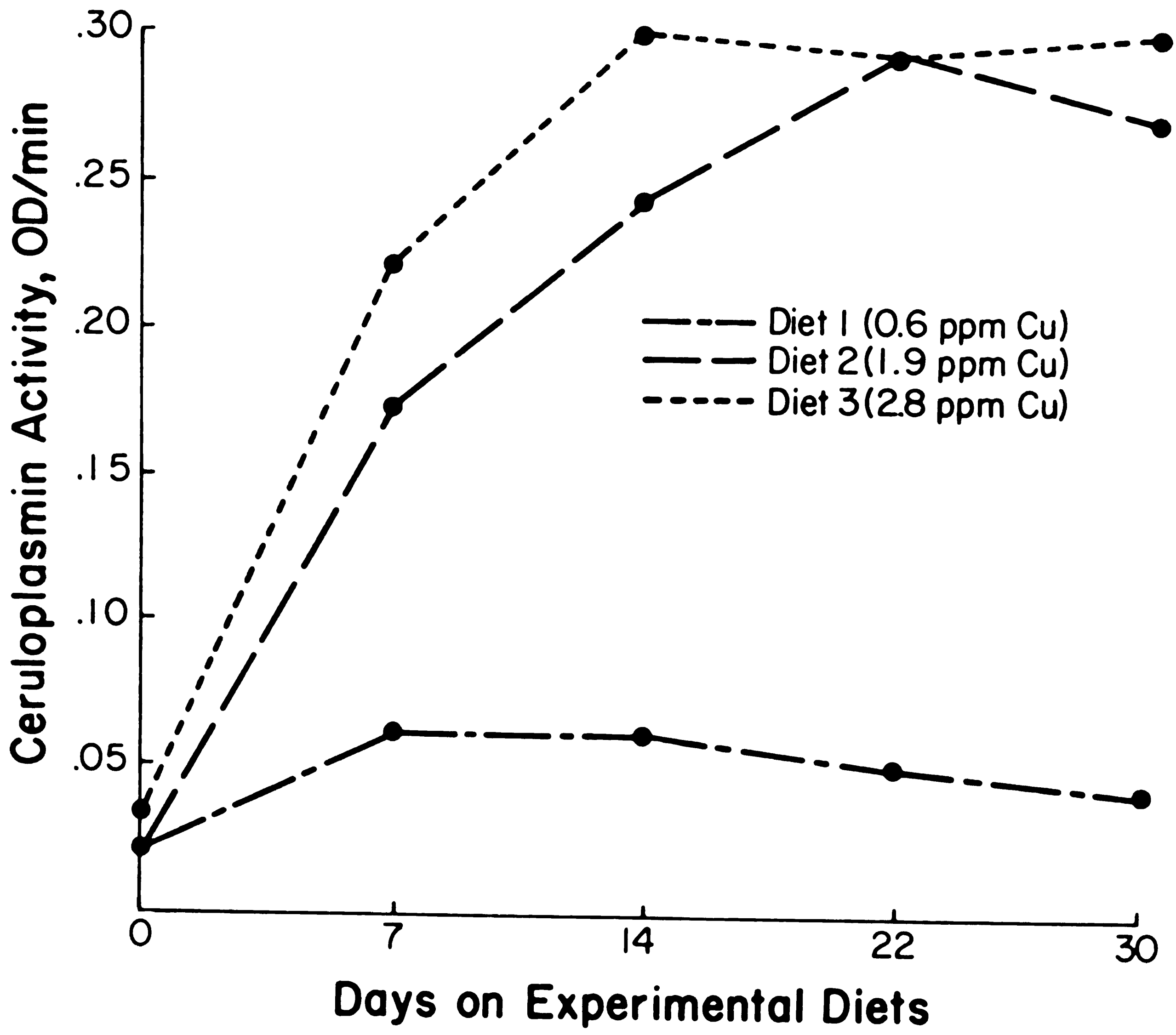

Figure 2.4.

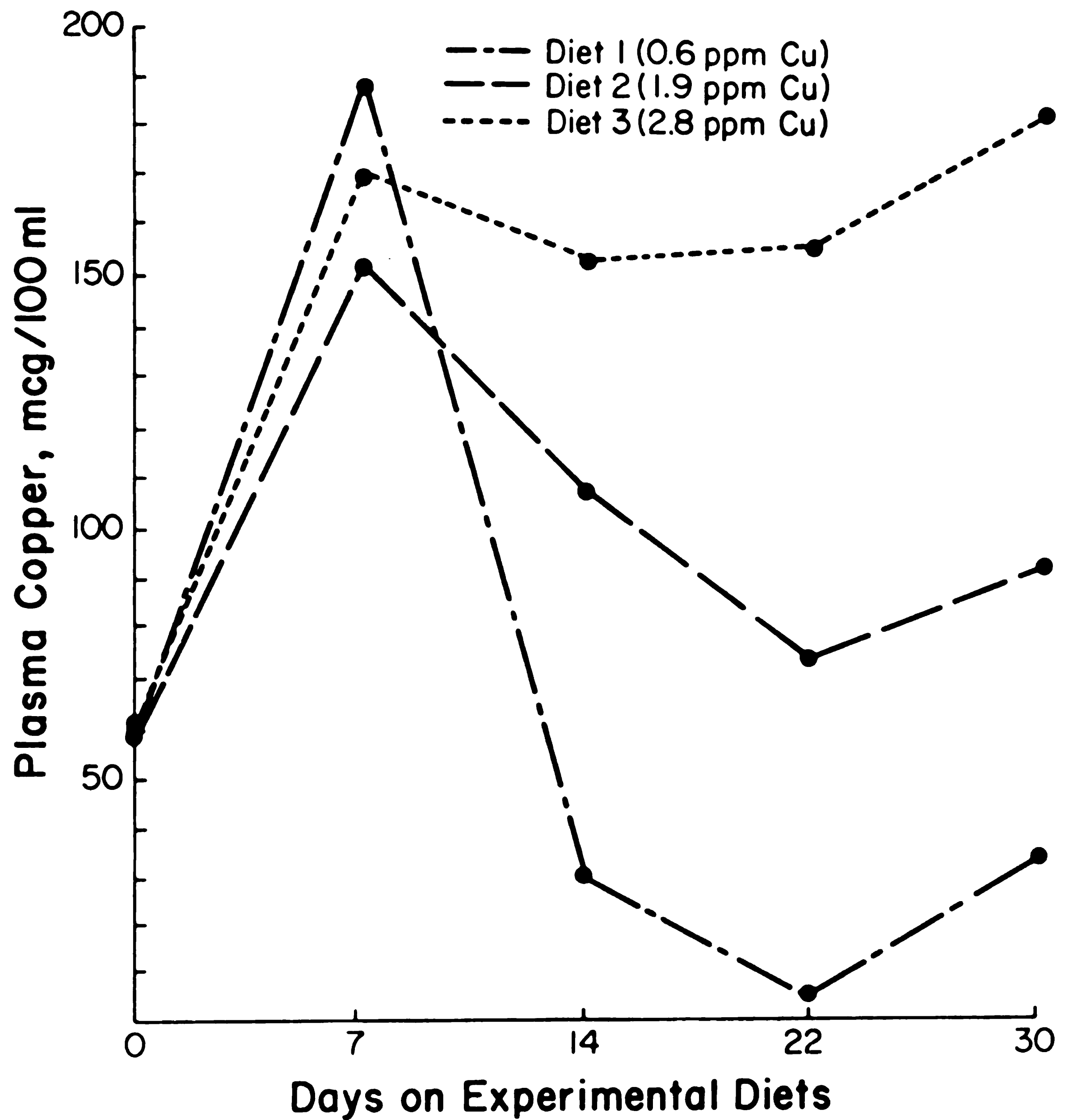

Figure 2.5. Effect of copper intake on plasma copper levels (Expt. 2)

TABLE 15. THE EFFECT OF DIETARY COPPER LEVELS ON COPPER BALANCE (EXPT. 2)

Diet no.	1	2	3	SEM
Cu conc., ppm	0.6	1.9	2.8	
No. of pigs	3	3	3	
Cu intake, mg/day	0.13	0.48[a,c]	0.77[a,d]	0.06
Cu excretion, mg/day				
Fecal	0.11	0.25	0.20	0.05
Urinary	0.02	0.02	0.03[b]	0.01
Cu excretion, % of intake				
Fecal	91.0	50.1	27.1	16.66
Urinary	12.7[e]	4.3	3.9	0.77
Cu retention	0.004	0.22[a]	0.54[a]	0.08
Cu retention, % of intake	3.7	45.6	68.5	16.94

[a]Significantly greater than least value (P<0.01).

[b]Significantly greater than least two values (P<0.05).

[c,d]Significantly different (P<0.05).

[e]Significantly greater than least two values (P<0.01).

and significantly ($P<0.01$) different between diet 1 and diets 2 and 3. Absolute urinary copper excretion of pigs on diet 3 was significantly ($P<0.05$) greater than corresponding values on diets 1 and 2, while urinary copper as a percent of dietary intake was significantly ($P<0.01$) higher for pigs on the basal diet than for pigs on diets 2 and 3. Pigs on diets 2 and 3 retained significantly ($P<0.01$) more copper than did pigs on the basal diet, whereas percent copper retention showed no significant treatment effects.

Left femur strength measurement data are presented in Table 16. Femur weight and elasticity did not show significant treatment effects. Both horizontal and vertical diameters of femurs from treatment 3 were significantly ($P<0.05$) greater than corresponding values from treatment 1. Femur length, maximum load and bending moment were significantly ($P<0.01$) different between treatment 1 and treatments 2 and 3; bending moment was also significantly ($P<0.05$) different between treatments 2 and 3. The inertia value for treatments 1 and 2 was 0.17 cm^4 and was significantly ($P<0.05$) less than the corresponding value, 0.23 cm^4 for treatment 3. There were significant ($P<0.05$ or $P<0.01$) treatment differences in the stress values between diets 1, 2 and 3. Although percent ash and copper concentration did not show any significant treatment effects, copper concentration increased with increases in dietary copper: 5.3, 7.5 and 10.5 ppm, which suggests that dietary copper exerted a slight effect on femur copper.

Correlation coefficients between copper balance, plasma copper, ceruloplasmin and average daily gain are given in Table 17. Ceruloplasmin activity from day 7 through day 30 was highly and significantly ($P<0.05$ or $P<0.01$) correlated with plasma copper. There were

TABLE 16. THE EFFECT OF DIETARY COPPER LEVELS ON PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE LEFT FEMUR (EXPT. 2)

Diet no.	1	2	3	SEM
Cu conc., ppm	0.6	1.9	2.8	
No. of pigs	3	3	3	
Physical characteristics				
Weight, fresh, g	44.3	49.3	48.5	2.39
External diameter[1], cm				
Horizontal (B)	1.42	1.39	1.52[a]	0.03
Vertical (D)	1.40	1.41	1.50[a]	0.02
Length (L), cm	7.5	7.9[b]	8.0[b]	0.06
Maximum load, kg	47.7	57.3[b]	62.0[b]	1.59
Maximum bending moment, kg-cm	89.4	113.2[b,c]	124.0[b,d]	2.81
Moment of inertia, cm^4	0.17	0.17	0.23[e]	0.01
Maximum stress, kg/cm^2	364	472[b]	405[a]	17.56
Modulus of elasticity, 1000 kg/cm^2	4.6	5.6	4.5	0.32
Chemical characteristics[f]				
Ash content, %	41.6	40.5	42.5	0.81
Cu, ppm	5.3	7.5	10.5	0.58

[a]Significantly greater than least value (P<0.05).

[b]Significantly greater than least value (P<0.01).

[c,d]Significantly different (P<0.05).

[e]Significantly greater than least two values (P<0.05).

[f]Expressed on dry, fat-free basis.

[1]Measured at mid-shaft with medial and lateral condyles facing downwards.

TABLE 17. CORRELATIONS BETWEEN COPPER BALANCE, PLASMA COPPER, CERULOPLASMIN AND AVERAGE DAILY GAIN (EXPT. 2)

	Absolute copper balance				% of intake				Ceruloplasmin				
	Cu intake	Fecal Cu	Urin. Cu	Cu ret.	Cu ret.	Fecal Cu	Urin. Cu	ADG	Initial	7 days	14 days	22 days	30 days
Pl. Cu													
Initial	.26	.33	.54	.16	.13	-.14	-.02	.51	.004	.38	.21	.24	.24
7 days	-.03	-.56	-.20	.18	.05	-.08	.20	.19	-.20	-.79[a]	-.31	-.32	-.34
14 days	.96[b]	.32	.78[a]	.93[b]	.87[b]	-.84[b]	-.91[b]	.67[a]	.22	.31	.92[b]	.85[b]	.90[b]
22 days	.90[b]	.33	.87[b]	.86[b]	.75[a]	-.73[a]	-.79[a]	.49	.37	.67[a]	.92[b]	.80[a]	.88[b]
30 days	.96[b]	.35	.91[b]	.92[b]	.76[a]	-.74[a]	-.80[a]	.61	.24	.49	.91[b]	.77[a]	.86[b]
Cp.													
Initial	.09	-.28	.33	.19	.52	-.56	-.15	.03	---	.33	.21	.06	.17
7 days	.40	.51	.53	.25	.34	-.32	-.48	.11		---	.59	.57	.60
14 days	.91[b]	.45	.79[a]	.83[b]	.78[a]	-.74[a]	-.92[b]	.52			---	.94[b]	.99[b]
22 days	.86[b]	.51	.65	.76[a]	.76[a]	-.72[a]	-.94[b]	.59				---	.98[b]
30 days	.90[b]	.52	.75[a]	.79[a]	.79[a]	-.74[a]	-.95[b]	.34					---
ADG	.74[a]	.29	.71[a]	.70[a]	.72[a]	-.71[a]	-.55	---					

[a]Significant ($P<0.05$).

[b]Significant ($P<0.01$).

significant (P<0.05 or P<0.01) correlations between plasma copper and copper balance; correlations between copper excretion as a percent of intake and plasma copper were generally high (P<0.05 or P<0.01) and negative. After day 7, ceruloplasmin activity was significantly (P<0.05 or P<0.01) correlated with copper balance parameters except for fecal copper; copper excretion as a percent of intake was negatively but significantly (P<0.05 or P<0.01) correlated with ceruloplasmin. Average daily gain was significantly (P<0.01) correlated with copper intake, urinary copper, copper retention (absolute and as a percent of intake) and fecal copper, but was not significantly correlated with ceruloplasmin activity and plasma copper.

Correlations between liver and kidney copper, ceruloplasmin and plasma copper are shown in Table 17a. Liver copper was significantly (P<0.05 or P<0.01) correlated with plasma copper and with ceruloplasmin activity on days 14 through 30. Dempsey and associates (1958) reported that plasma and liver copper were greatly influenced by dietary copper and that a close correlation exists between plasma and liver copper. Kidney copper was also significantly (P<0.05 or P<0.01) correlated with plasma copper and ceruloplasmin within the same period.

1. Histopathology

a. Histopathologic examination. The left tarsal and metatarsal bones were examined. Sections were taken from each bone, and the tarsal bone consisted of a long bone with an epiphyseal plate. The metatarsal bone was a small round bone with joint cartilage at each end. The

TABLE 17a. CORRELATIONS BETWEEN LIVER AND KIDNEY COPPER AND CERULOPLASMIN AND PLASMA COPPER (EXPT. 2)

	Plasma copper					Ceruloplasmin activity				
	Init.	7 days	14 days	22 days	30 days	Init.	7 days	14 days	22 days	30 days
Liver Cu	.07	-.36	.70[a]	.74[a]	.72[a]	.41	.54	.83[b]	.67[a]	.77[a]
Kidney Cu	.33	-.52	.74[a]	.89[b]	.82[b]	.41	.74[a]	.83[b]	.75[a]	.83[b]

[a]Significant (P<0.05).

[b]Significant (P<0.11).

parameters measured were: epiphyseal cartilaginous plate, 10 x 10^{1} diaphysis, epiphysis, diaphysis and cellularity. The scores on all parameters were averaged to give an overall ranking.[2]

Overall ranking of the tarsal bones indicated that there was no real difference histologically from one pig to another, and therefore no significant treatment effects.

The metatarsal bones were ranked on the basis of histologic conformation. At least one pig on each diet had metatarsal bones with persistent osteoid material in the region where there should be bone formation in the marrow cavity.

C. Experiment 3: Copper requirement of baby pigs maintained on distilled deionized water and fed semi-purified diets supplemented with copper sulfate to yield upon analysis: 0.9 ppm, 2.0 ppm, 4.0 ppm and 4.9 ppm copper

Results of the effect of dietary copper levels on average daily gain and organ weights as percentage of final body weight are shown in Table 18. Average daily gain and feed efficiency were not significantly different due to treatment effects. Although there was a slight increase in heart and brain as percentage of body weight for pigs on diet 1, there was generally no significant treatment effect. Gubler and associates (1957) reported that in copper-deficient swine there was a 200% increase in heart weight and a moderate increase in liver and

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[1]10 x 10 objective by 10x ocular field.

[2]See histopathology under Experiment 1.

TABLE 18. THE EFFECT OF DIETARY COPPER LEVELS ON GROWTH AND ORGAN WEIGHTS AS PERCENTAGE OF FINAL BODY WEIGHT (EXPT. 3)

Diet no.	1	2	3	4
Cu conc., ppm[1]	0.9	2.0	4.0	4.9
No. of pigs	5	5	5	5
Growth				
Avg. init. wt., kg	1.64±0.22	1.66±0.22	1.71±0.22	1.73±0.22
Avg. final wt., kg	13.18±5.19	17.67±5.19	16.23±3.67	16.01±3.67
Avg. daily gain, g	160±0.07	216±0.04	195±0.05	193±0.05
Feed/gain	1.30	1.27	1.32	1.31
Organ wts. (fresh), % of final body wt.				
Thyroid	0.10±0.002	0.01±0.001	0.01±0.001	0.01±0.001
Spleen	0.17±0.02	0.14±0.01	0.18±0.01	0.15±0.01
Adrenals	0.02±0.003	0.01±0.002	0.01±0.002	0.01±0.002
Liver	2.25±0.15	2.24±0.10	2.40±0.11	2.63±0.11
Heart	0.51±0.03	0.47±0.02	0.48±0.02	0.47±0.02
Pancreas	0.20±0.02	0.17±0.01	0.20±0.01	0.18±0.01
Brain	0.62±0.17	0.47±0.11	0.41±0.12	0.43±0.12
Kidneys	0.32±0.14	0.44±0.08	0.40±0.10	0.38±0.10

[1]Expressed on as fed basis.

kidney weights as percentage of body weight, while other organs remained unaffected. The failure of results of this trial to agree with the findings of Gubler *et al.* (1957) is probably due to the fact that copper deficiency was not produced even with the diet analyzing 0.9 ppm Cu. The kidney values were lower on diet 1 than on all other diets.

Hematocrit, hemoglobin and mean corpuscular hemoglobin concentration values are summarized in Table 19. Hematocrit values showed no consistent trend nor any significant differences due to treatment. There was, however, a slight drop during the fourth and sixth weeks (Figure 3.1). There was a nonsignificant increase in hemoglobin during the second week in all treatments followed in the fourth week by a significant ($P<0.01$) drop on the third diet. There was a significant ($P<0.05$) difference in hemoglobin due to treatment between diet 3 and diets 1 and 4 (Figure 3.2). On the eighth week, the basal diet hemoglobin value dropped to 7.3 g/100 ml and was significantly ($P<0.01$) different from the mean values in treatments 3 and 4; however this value increased to 9.6 g/100 ml at the end of the trial. Mean corpuscular hemoglobin concentration showed no significant treatment effects; however, in the fourth week MCHC values for diets 1, 2 and 4 were significantly ($P<0.01$) higher than the value for diet 3 (Figure 3.3). These hematocrit, hemoglobin and MCHC values produced only limited evidence of anemia, even though anemia as indicated by very low hematocrit, hemoglobin and mean corpuscular hemoglobin concentration has been observed in pigs fed diets containing higher copper levels than the basal diet (0.9 ppm Cu) used in this study.

Several workers have reported depressed hemoglobin, hematocrit and mean corpuscular hemoglobin concentration in animals placed on low

TABLE 19. THE EFFECT OF DIETARY COPPER LEVELS ON HEMATOCRIT, HEMOGLOBIN AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (EXPT. 3)

Diet no.	1	2	3	4	SEM[4]
Cu conc., ppm	0.9	2.0	4.0	4.9	
No. of pigs	5	5	5	5	
Hct.[1], %					
Initial	32.1	29.8	30.7	28.5	1.11
2 weeks	42.3	41.9	42.0	40.4	1.61
4 weeks	39.3	37.1	34.9	38.2	1.92
6 weeks	34.0	35.0	33.1	37.1	1.78
8 weeks	30.6±2.10	31.9±1.33	34.2±1.49	34.8±1.49	
10 weeks	32.6±2.80	36.0±1.77	33.6±1.98	34.6±1.98	
Hb.[2], g/100 ml					
Initial	9.8	9.1	9.0	8.9	0.38
2 weeks	12.5	12.3	13.0	12.0	0.51
4 weeks	11.6[a]	11.1	9.3	11.7[a]	0.57
6 weeks	10.8	11.0	10.7	11.8	0.56
8 weeks	7.3±0.42	8.3±0.27	9.1±0.30[b]	9.5±0.30[b]	
10 weeks	9.6±0.66	11.1±0.41	11.3±0.46	10.7±0.46	
MCHC[3], %					
Initial	30.5	30.5	29.3	31.4	1.11
2 weeks	29.8	29.3	30.9	29.5	0.84
4 weeks	29.6[b]	30.0[b]	26.5	30.8[b]	0.65
6 weeks	31.8	31.2	32.3	31.7	0.38
8 weeks	23.7±1.53	26.4±0.97	26.8±1.08	27.4±1.08	
10 weeks	29.6±1.70	31.0±1.08	34.0±1.20	30.9±1.20	

[a]Significantly greater than least value ($P<0.05$).

[b]Significantly greater than least value ($P<0.01$).

[1]Hematocrit.

[2]Hemoglobin.

[3]Mean corpuscular hemoglobin concentration.

[4]Standard error of the mean (±).

Figure 3.1. Response of hematocrit to varying levels of dietary copper (Expt. 3).

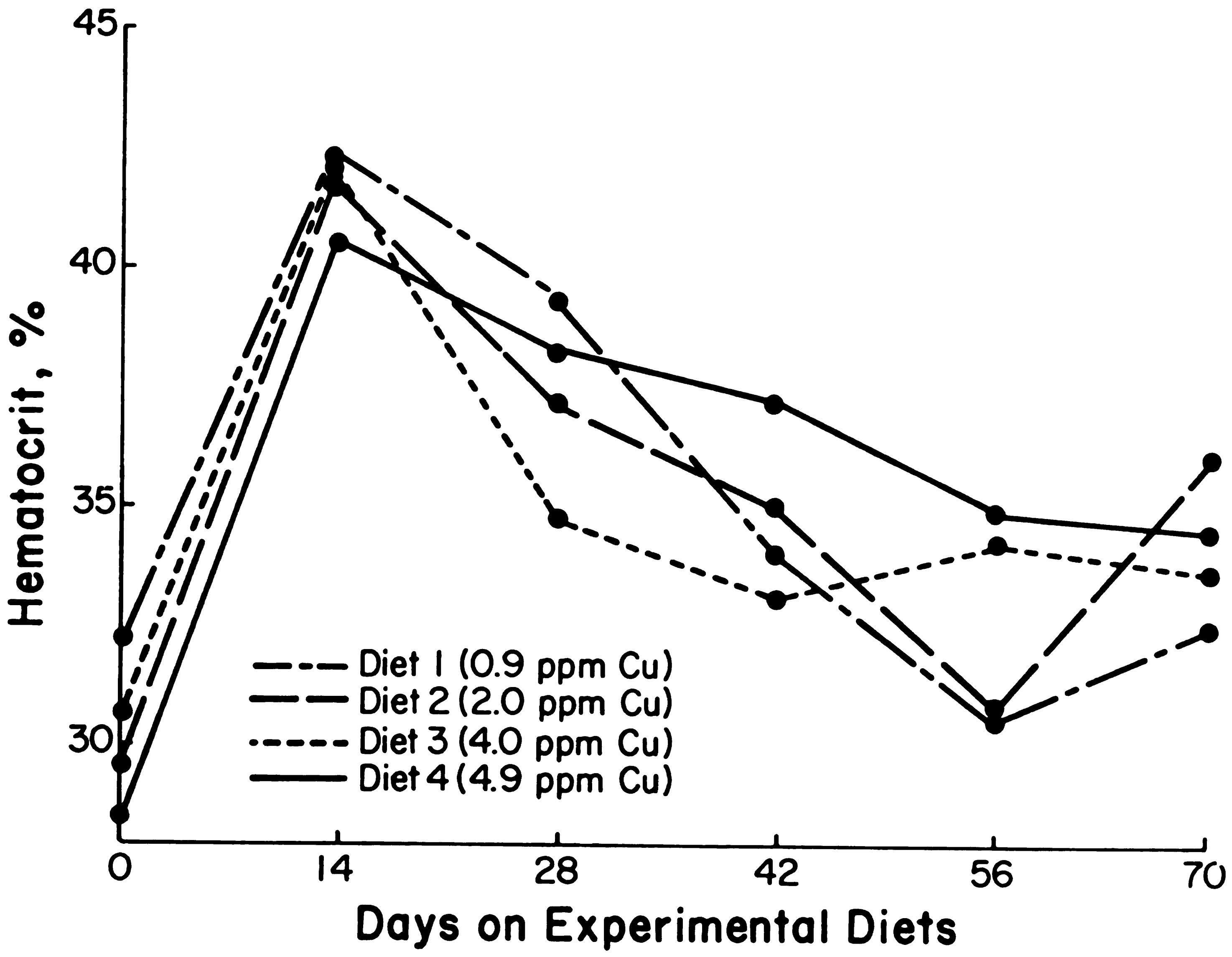

Figure 3.1.

Figure 3.2. Response of hemoglobin to dietary copper levels (Expt. 3).

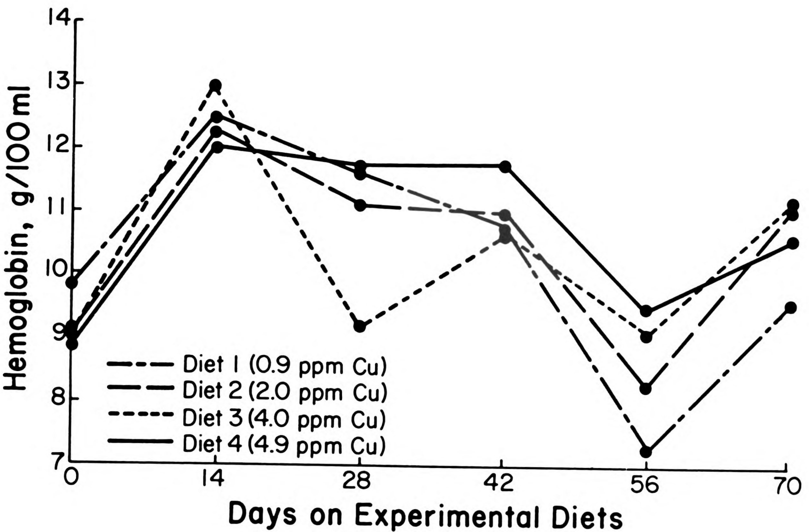

Figure 3.2.

Figure 3.3. Response of mean corpuscular hemoglobin concentration to copper intake (Expt. 3).

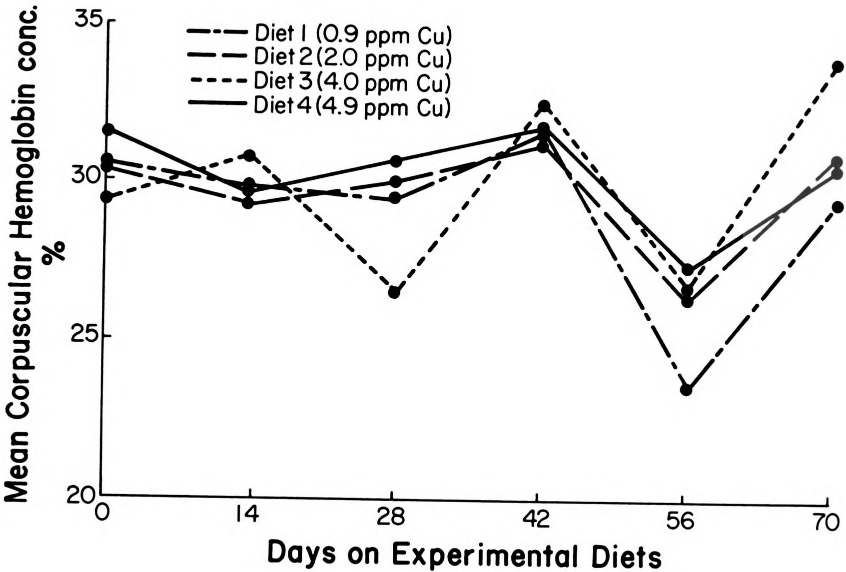

Figure 3.3.

copper diets (<1.0 ppm copper) (Elvehjem and Hart, 1929; Schultze *et al.*, 1936b; Smith *et al.*, 1944; Gipp *et al.*, 1973; Evans and Abraham, 1973); but the results obtained in Experiments 1, 2 and 3 suggest only subclinical anemia on the basal diet.

The effects of dietary copper levels on erythrocyte superoxide dismutase (SOD) activity are shown in Table 19a. SOD on the basal diet was initially higher than on the rest of the diets (155 units per ml rbc), then dropped to 114 on the 4th week, and rose slightly before decreasing to 62 units at the end of the trial. Activity of SOD on diet 4 showed a similar trend while enzyme activity of pigs on diet 2 decreased steadily from an initial value of 144 units/ml rbc to 74 units in the 8th week before rising slightly to 78 in the 10th week. SOD activity on diet 3 did not demonstrate any consistent trend. There were no significant differences between treatments in the 2nd, 4th, 8th and 10th weeks. In the 6th week, enzyme activity on diets 3 and 4 was significantly ($P<0.05$) greater than that on the basal diet. Williams and associates (1975) reported that erythrocyte superoxide dismutase depended on adequate levels of dietary copper for its activity. The authors presented data to show that, in copper-deficient swine, SOD activity of the red blood cells was depressed to 15% of the control values. SOD values obtained in this study indicate that 0.9 and 2.0 ppm copper were able to sustain as much enzyme activity as were 4.0 and 4.9 ppm copper since, with the exception of week 6, SOD values were not significantly different due to dietary copper levels.

Ceruloplasmin activity and plasma copper are presented in Table 20. Pigs on the basal diet showed a consistent drop in ceruloplasmin activity from the first week through the eighth week (Figure 3.4).

TABLE 19a. ACTIVITY OF ERYTHROCYTE SUPEROXIDE DISMUTASE (SOD) IN RESPONSE TO DIETARY COPPER LEVELS (EXPT. 3)

Diet no.	1	2	3	4	SEM
Cu conc., ppm	0.9	2.0	4.0	4.9	
No. of pigs	5	5	5	5	
SOD, Units/ml rbc[1]					
2 weeks	155	144	128	122	19.0
4 weeks	114	137	135	141	9.8
6 weeks	123	137	157[a]	164[a]	9.2
8 weeks	74	74	58	77	9.0
10 weeks	62	78	80	61	9.4

[1]Red blood cells.

[a]Significantly greater than least value ($P<0.05$).

TABLE 20. THE EFFECT OF DIETARY COPPER LEVELS ON CERULOPLASMIN ACTIVITY AND PLASMA COPPER CONCENTRATION (EXPT. 3)

Diet no.	1	2	3	4	SEM
Cu conc., ppm	0.9	2.0	4.0	4.9	
No. of pigs	5	5	5	5	
Cp.[1], OD/min					
Initial	0.05	0.05	0.06	0.06	0.008
2 weeks	0.04	0.05	0.05	0.04	0.004
4 weeks	0.02	0.04[a]	0.04[a]	0.05[a]	0.004
6 weeks	0.02	0.05[b]	0.07[a]	0.09[a]	0.007
8 weeks	0.01±0.005	0.01±0.003	0.05±0.004[c]	0.04±0.004[c]	
10 weeks	0.03±0.007	0.04±0.004	0.07±0.005[c]	0.07±0.005[c]	
Pl. Cu[2], mcg/100 ml					
Initial	98.0	106.2	97.4	109.3	17.67
2 weeks	125.2	224.5[b]	279.9[a]	274.1[a]	23.45
4 weeks	86.2	163.2[b]	193.7[a]	249.7[c]	17.47
6 weeks	41.9	118.3[e]	187.9[a,d]	249.7[c]	21.46
8 weeks	5.9±19.60	90.0±12.40[a]	197.8±13.86[c]	211.1±13.86[c]	
10 weeks	5.2±20.12	73.3±12.72[b]	194.5±14.23[c]	207.7±14.23[c]	

[a]Significantly greater than least value (P<0.01).

[b]Significantly greater than least value (P<0.05).

[c]Significantly greater than least two values (P<0.01).

TABLE 20 (CONT'D.)

[d,e]Significantly different ($P<0.05$).

[1]Ceruloplasmin activity.

[2]Plasma copper concentration.

In the fourth, sixth, eighth and tenth weeks there was a significant (P<0.01 or P<0.05) difference between ceruloplasmin values of pigs on the basal diet and diets 2, 3 and 4.

These data are in agreement with the work of Evans and Abraham (1973) and Gipp *et al.* (1973) who reported reduced values with diets containing 2 ppm copper or less. Plasma copper levels rose significantly (P<0.01) from the first week and peaked in the second week (Figure 3.5). For pigs on diet 1, plasma copper fell drastically from the fourth week from 86.2 mcg/100 ml to 5.2 mcg/100 ml. In the second week there was a significant (P<0.01) difference between plasma copper of pigs on diet 1, diet 2 and diets 3 and 4. Subsequent plasma copper levels showed highly significant (P<0.01 or P<0.05) differences between treatments. It has been suggested that 0.2 mcg/ml is the minimum level at which normal hematopoiesis can occur in pigs (Schultze, 1936b; Lahey *et al.*, 1952); but plasma copper levels of 5.9 to 5.2 mcg/100 ml observed in the last four weeks of the trial for pigs on the basal diet (0.9 ppm Cu) failed to produce signs of severe anemia. Dempsey *et al.* (1958), Williams *et al.* (1975), and others have shown that plasma copper responds dramatically to dietary copper. It is possible that pigs on the basal diet might have developed anemia had the trial been extended beyond 10 weeks and plasma copper maintained at such sub-optimal levels.

Tissue copper concentrations are given in Table 21. Liver and brain copper concentrations of pigs on diets 1 and 2 were significantly lower (P<0.01 or P<0.05) than those of pigs on diets 3 and 4. Kidney and heart copper concentrations were significantly (P<0.05 or P<0.01) different due to treatments. Spleen copper showed no significant difference between treatments. Dietary copper has been shown to

TABLE 21. THE EFFECT OF DIETARY COPPER LEVELS ON TISSUE COPPER CONCENTRATIONS (EXPT. 3)

Diet no.	1	2	3	4	SEM
Cu conc., ppm	0.9	2.0	4.0	4.9	
No. of pigs	5	5	5	5	
Tissue Cu, ppm[1]					
Liver	3.0	3.7[d]	8.3[a,c]	9.3[b]	1.16
Kidney	3.7	6.6	12.2[a]	14.4[b]	1.54
Brain	2.8	3.2[d]	4.3[a,c]	4.9[b]	0.24
Heart	1.8	2.5[e]	3.2[f]	3.2[f]	0.16
Spleen	1.0	1.1	1.2	1.3	0.09

[a]Significantly greater than least value (P<0.01).

[b]Significantly greater than least two values (P<0.01).

[c,d]Significantly different (P<0.05).

[e]Significantly greater than least value (P<0.05).

[f]Significantly greater than least two values (P<0.05).

[1]Expressed on a fresh basis.

Figure 3.4. Response of ceruloplasmin activity to dietary copper levels (Expt. 3).

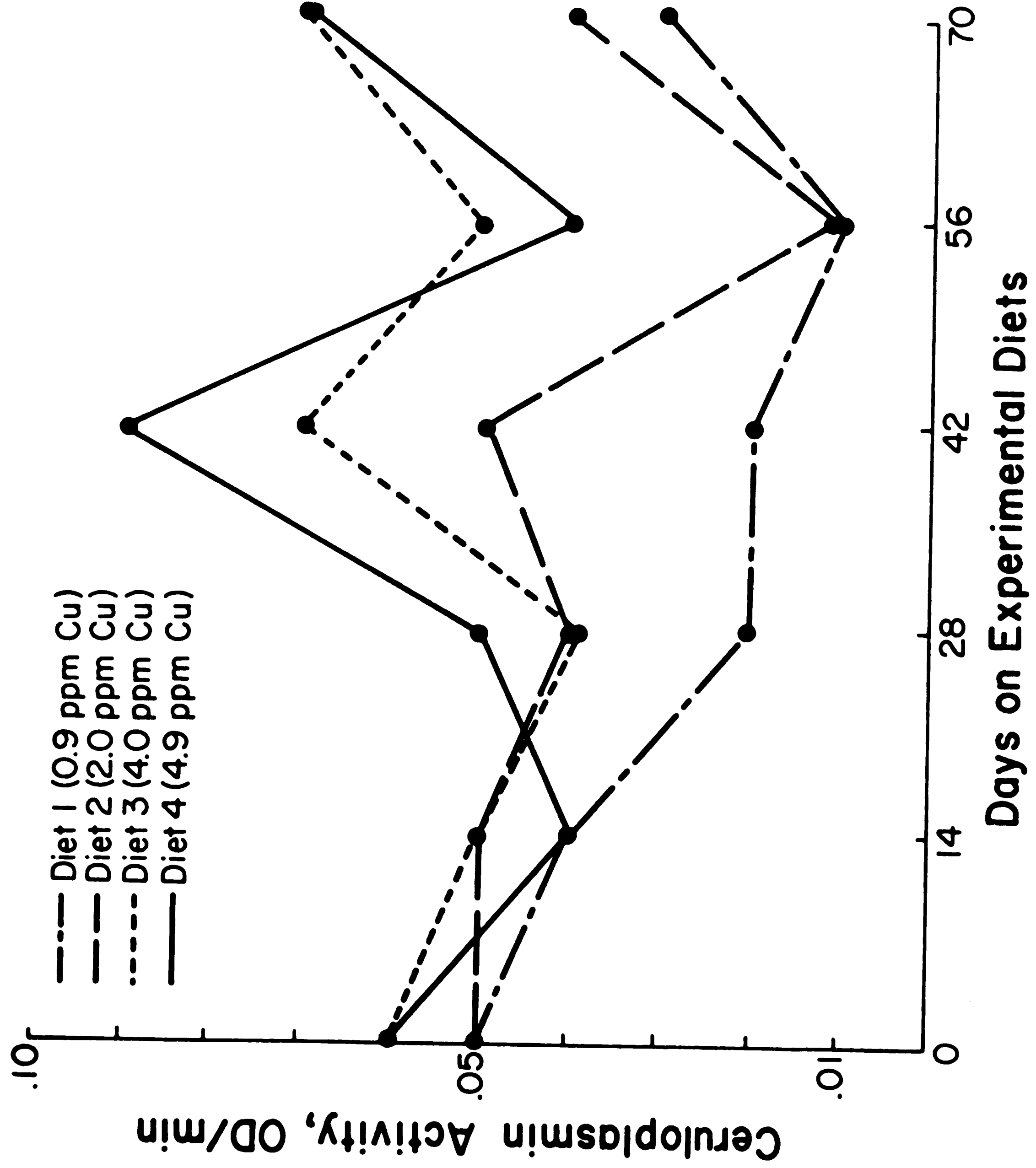

Figure 3.4.

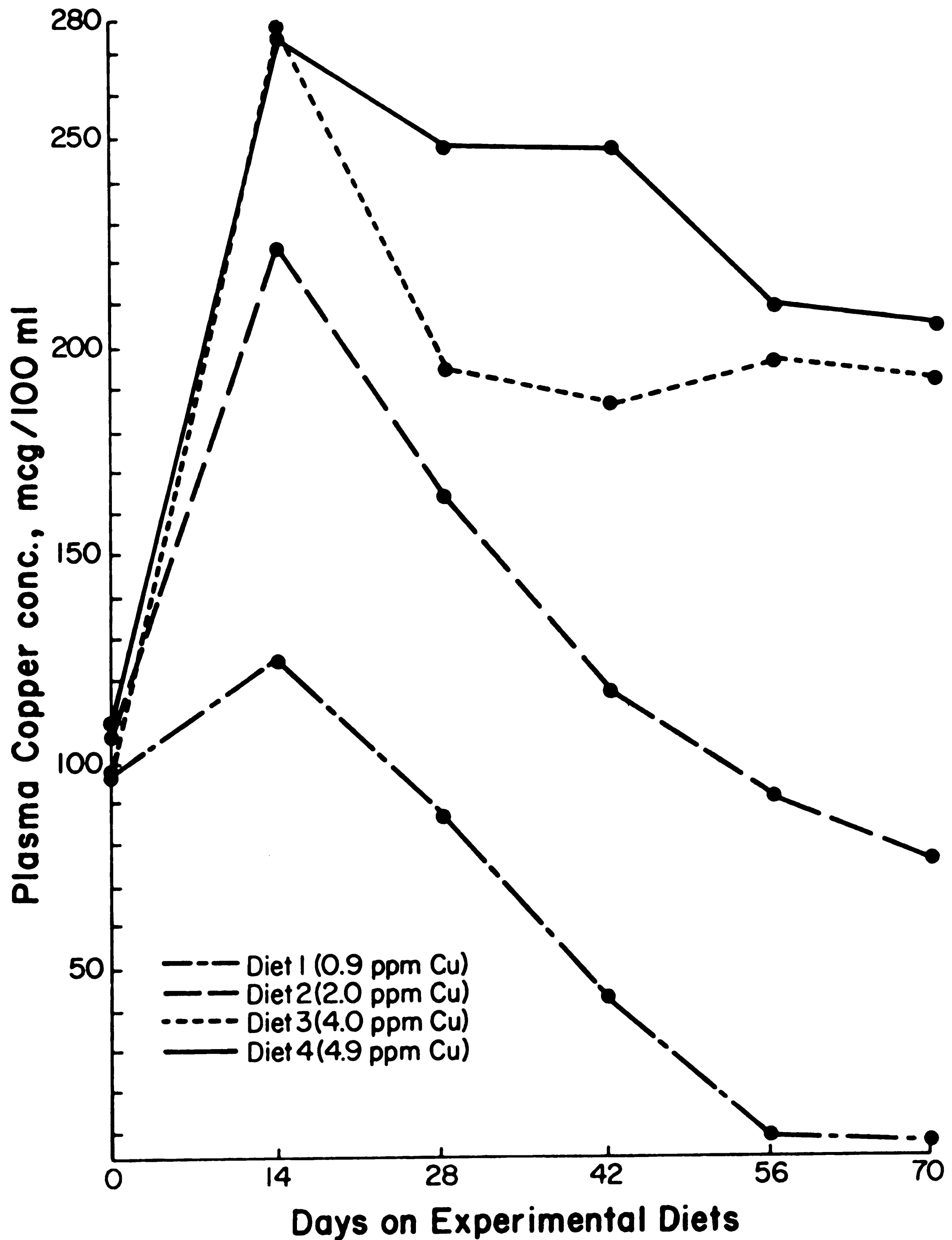

Figure 3.5. Response of plasma copper levels to copper intake (Expt. 3).

influence tissue copper concentrations, especially of liver (Dempsey *et al.*, 1958; Ullrey *et al.*, 1960; Sutter *et al.*, 1958).

Data from copper balance trials are given in Table 22. There was a significant (P<0.01) difference in copper intake due to treatment. Absolute copper excretion and copper excretion as percent of intake were not significantly different between diets. Absolute copper retention showed a significant (P<0.01) difference between diet 4 and diets 1 and 2 while copper retention as percent of intake did not differ significantly between treatments.

Physical and chemical measurements of the left femur are summarized in Table 23. None of the characteristics measured showed any significant effects due to treatment. Rucker *et al.* (1969a) reported that in copper deficient chicks bone copper content was 50% of controls; data obtained in this trial indicate that dietary copper had no influence on bone ash and copper content, or that the low copper basal diet was not fed long enough to deplete bone copper stores.

Table 24 shows correlations between copper balance, plasma copper, ceruloplasmin and average daily gain. Plasma copper levels after the second week are strongly and significantly (P<0.05 or P<0.01) correlated with ceruloplasmin activity. Copper intake and absolute copper retention were significantly (P<0.05 or P<0.01) correlated with plasma copper concentration. Average daily gain was significantly (P<0.01) correlated with initial ceruloplasmin, fecal copper and urinary copper, and negatively but significantly (P<0.05) correlated with percent copper retention. The correlation between copper intake and ceruloplasmin on weeks 6, 8 and 10 was highly significant (P<0.01); and ceruloplasmin activity on weeks 6, 8 and 10 was significantly correlated

TABLE 22. THE EFFECT OF DIETARY COPPER LEVELS ON COPPER BALANCE (EXPT. 3)

Diet no.	1	2	3	4
Cu conc., ppm	0.9	2.0	4.0	4.9
No. of pigs	2	3	3	3
Cu intake, mg/day	0.73±0.08	1.15±0.06[a]	2.25±0.06[b]	2.74±0.06[c]
Cu excretion, mg/day				
Fecal	0.26±0.23	0.59±0.19	1.03±0.19	0.71±0.19
Urinary	0.03±0.03	0.06±0.03	0.06±0.03	0.11±0.03
Cu excretion, % of intake				
Fecal	41.1±15.89	52.4±12.98	46.6±12.98	25.9±12.98
Urinary	4.3±1.83	4.9±1.49	2.8±1.49	4.0±1.49
Cu retention, mg/day	0.44±0.30	0.50±0.25	1.16±0.25	1.93±0.25[b]
Cu retention, % of intake	54.6±16.92	42.7±13.82	50.6±13.82	70.2±13.82

[a]Significantly greater than least value ($P<0.01$).

[b]Significantly greater than least two values ($P<0.01$).

[c]Significantly greater than least three values ($P<0.01$).

TABLE 23. THE EFFECT OF DIETARY COPPER LEVELS ON PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE LEFT FEMUR (EXPT. 3)

Diet no.	1	2	3	4	SEM
Cu conc., ppm	0.9	2.0	4.0	4.9	
No. of pigs	5	5	5	5	
Physical characteristics					
Weight, fresh, g	65.3	80.0	66.1	63.7	12.53
External diameter, cm					
Horizontal (B)	1.47	1.56	1.45	1.48	0.11
Vertical (D)	1.52	1.62	1.56	1.51	0.11
Length (L), cm	8.8	9.2	8.8	8.9	0.45
Maximum load, kg	66.6	88.6	63.6	85.2	20.02
Maximum bending moment, kg-cm	148.3	207.9	144.1	198.9	52.18
Moment of inertia, cm^4	0.25	0.31	0.28	0.24	0.07
Maximum stress, kg/cm^2	557	580	488	650	123.36
Modulus of elasticity, 1000 kg/cm^2	11.3	12.4	10.7	17.6	3.5
Chemical characteristics[a]					
Ash content, %	41.9	43.2	42.7	43.0	1.22
Cu, ppm	7.6	7.7	7.6	7.5	0.94

[a]Expressed on dry, fat-free basis.

TABLE 24. CORRELATIONS BETWEEN COPPER BALANCE, PLASMA COPPER, CERULOPLASMIN AND AVERAGE DAILY GAIN (EXPT.3)

	Absolute copper balance				% of intake			Growth	Ceruloplasmin					
	Cu intake	Fecal Cu	Urin. Cu	Cu ret.	Cu ret.	Fecal Cu	Urin. Cu	ADG	Initial	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Pl. Cu														
Initial	.32	.32	.41	.17	-.21	.19	.30	.59	.52	.10	.22	.06	.16	.41
2 weeks	.67[a]	.67[a]	.16	.39	-.02	.07	-.39	-.11	.18	.26	.61[a]	.58	.44	.54
4 weeks	.90[b]	.41	.55	.78[b]	.45	-.45	-.25	-.14	.34	.20	.80[b]	.78[b]	.66[a]	.69[a]
6 weeks	.91[b]	.60	.54	.69[a]	.21	-.20	-.18	.07	.50	.01	.68[a]	.90[b]	.82[b]	.82[b]
8 weeks	.93[b]	.54	.55	.74[a]	.28	-.28	-.19	.08	.41	-.11	.64	.86[b]	.92[b]	.92[b]
10 weeks	.94[b]	.52	.56	.76[b]	.29	-.29	-.18	.05	.40	-.11	.65	.86[b]	.92[b]	.94[b]
Cp.														
Initial	.19	.57	.59	-.13	-.51	.47	.63	.79[b]	---	-.14	.27	.43	.33	.46
2 weeks	-.07	.22	-.23	-.18	-.05	.08	-.18	-.16		---	.32	.43	-.22	.46
4 weeks	.70	.57	.71[a]	.45	-.03	.00	.29	.07			---	.67	.51	.58
6 weeks	.91[b]	.69[a]	.53	.64[a]	.09	-.09	-.13	.21				---	.79[b]	.84[b]
8 weeks	.86[b]	.43	.53	.72[a]	.31	-.31	-.16	-.03					---	.91[b]
10 weeks	.90[b]	.47	.60	.74[a]	.28	-.28	-.07	-.05						---
ADG	-.36	.13	.09	-.48	-.70[a]	.67[a]	.71[a]	---						

[a]Significant ($P<0.05$).

[b]Significant ($P<0.01$).

with absolute copper retention.

Correlations between tissue copper, plasma copper and ceruloplasmin activity are presented in Table 24a. Liver copper showed a significant ($P<0.05$ or $P<0.01$) correlation with plasma copper, and this is in agreement with the work of Dempsey *et al.* (1958). There was a significant ($P<0.05$ or $P<0.01$) correlation between kidney, brain and heart copper concentrations and plasma copper level and ceruloplasmin activity. Spleen copper correlation with plasma copper and ceruloplasmin was non-significant.

1. Histopathology

a. Histopathologic examination. Sections of brain, kidney, liver and aorta were fixed in 10% neutral formalin, sectioned at 6 microns and stained with hematoxylin and eosin for histologic study. Rib sections were made at the costochondral junction, and the metatarsals were taken in the region of the joint. Rib parameters examined were the same as in Experiment 1, and an overall ranking was obtained by averaging all rib parameters. The metatarsal bones were also given an overall ranking as described in Experiment 1 under "Histopathology".

The liver, kidney and aorta sections showed no changes. Overall ranking of the rib sections indicated that pigs on diets 1 and 4 had normal ribs, while one pig on diet 2 and three pigs on diet 3 had ribs with very slight changes. These changes, however, were non-significant, but are inexplicable since there were no gross skeletal malformations as would be manifested by lameness, deformed legs and a tendency to lie down all the time. Brain sections showed that at least one pig from each treatment had diffuse mild spongiosis which probably represents

TABLE 24a. CORRELATIONS BETWEEN TISSUE COPPER CONCENTRATIONS, PLASMA COPPER AND CERULOPLASMIN ACTIVITY (EXPT. 3)

	Plasma copper						Ceruloplasmin activity					
	Initial	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	Initial	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
Liver Cu	.35	.65[a]	.64	.69[a]	.81[b]	.82[b]	.50	-.18	.42	.54	.70[a]	.69[a]
Kidney Cu	-.04	.43	.68[a]	.80[b]	.78[a]	.80[b]	.37	-.13	.53	.91[b]	.83[b]	.82[b]
Brain Cu	.04	.64	.84[b]	.88[b]	.86[b]	.90[b]	.39	.04	.63	.82[b]	.84[b]	.91[b]
Heart Cu	-.03	.70[a]	.80[b]	.80[b]	.85[b]	.83[b]	.24	.05	.57	.78[a]	.73[a]	.72[a]
Spleen Du	.10	.39	.62	.48	.45	.50	.07	.23	.44	.39	.47	.52

[a]Significant ($P<0.05$).

[b]Significant ($P<0.01$).

demyelination of nerve fibers in the white matter. This spongiosis appeared in the white matter in various areas of the brain but was most prominent in the white matter of the cerebellum. Again this observation cannot be explained since incoordination and other nervous disorders were not grossly evident. The metatarsal of one pig on the basal diet demonstrated a severe change histologically - cartilaginous cells persisted throughout the marrow cavity and there was excess osteoid in the region of spicules; one pig on diet 3 and two on diet 4 showed very slight to slight changes.

CONCLUSIONS

In the light of the results obtained from these three experiments and within the limits of experimental error and the limits imposed by degree of adequacy and sensitivity of the parameters studied, the following conclusions have been drawn:

1. The levels of dietary copper used in these studies (0.6 to 9.3 ppm) did not significantly influence average daily gain and feed conversion efficiency, and hence did not significantly affect growth.

2. Although tissue copper concentrations were slightly affected by dietary copper levels, the tissues were not markedly depleted of their stores as a result of low dietary copper, nor did they exhibit any gross or microscopic lesions attributable to low copper intakes.

3. The morphological and histochemical integrity of the skeletal system, as shown by histopathological examination of the rib, tarsus and metatarsus and by the strength characteristics of the left femur, was not significantly impaired by low-copper diets, thereby indicating that bone copper reserves were not significantly affected.

4. Brain and erythrocyte superoxide dismutase activity did not respond significantly to dietary copper levels; it is likely that, despite the low copper intakes, there was an adequate amount of this element in the brain and red blood cells to sustain activity of this enzyme; or superoxide dismutase activity in brain and erythrocyte might not be as sensitive a measure of copper deficiency as was suggested by

others.

5. Liver copper was not severely depleted by low dietary copper since analysis showed that liver copper concentrations were within the range of normal adult levels, although young pigs may normally have somewhat higher liver copper concentrations than adults have.

6. Grossly, anemia was not evident, but there was a consistent lowering of hemoglobin levels by diets low in copper (0.6, 0.9, 1.3 ppm copper). Although these differences were judged as statistically nonsignificant, the effect of low copper was considerable since hemoglobin levels should be more or less constant if the supply of copper is adequate. Such low hemoglobin values can be construed as signs of subclinical anemia. In addition, plasma copper levels of pigs on low copper diets were significantly and consistently depressed and in one instance fell (5.2 mcg/100 ml) much below the minimum hemopoietic level of 20 mcg/100 ml. Based on these criteria -- hemoglobin and plasma copper -- anemia and copper deficiency were biochemically present. Such a subclinical condition was also indicated by a significant depression of ceruloplasmin activity, an observation which suggests that on low dietary copper, ceruloplasmin activity cannot be sustained.

7. In spite of the low-copper diets (0.6, 0.9, 1.3 ppm copper), pigs demonstrated positive absolute copper retention, showing that copper loss via urine and feces did not exceed copper absorption.

8. It is conceivable that the inconsistent responses of the parameters chosen were because some were not sensitive indicators of copper status at such low dietary levels, e.g., average daily gain, soft tissue and skeletal copper concentration, and erythrocyte and brain superoxide dismutase activity, while hemoglobin, ceruloplasmin, plasma copper and

copper balance were sensitive measures.

9. Based on these parameters, the minimum copper requirement for the baby pig is very low and is probably between 3.0 and 4.0 ppm on an as-fed basis, or between 3.4 and 4.6 ppm on a dry basis.

MANGANESE

INTRODUCTION

Manganese requirements of pigs for growth, skeletal development and fertility have been placed at conflicting levels and have not been defined precisely. Levels of 11 to 14 ppm manganese in corn-soybean diets promoted growth but caused leg stiffness and lameness while 50 ppm manganese (Keith *et al.*, 1942) and 60 pm (Miller *et al.*, 1940) prevented skeletal malformations without curing them. Experiments by Johnson (1940, 1944) indicated that 0.3 ppm manganese resulted in satisfactory growth but caused poor reproduction and depressed tissue manganese while 6 ppm promoted successful reproduction. He also reported that diets containing 7 to 10 ppm manganese maintained adequate performance of pigs from weaning to market. Diets containing 12 ppm manganese were reported to be inadequate for reproduction but maintained bone and somatic growth (Grummer *et al.*, 1950), and when the same diets were supplemented with 40, 80 and 160 ppm manganese, optimum performance was recorded with the 40 ppm level, indicating that higher levels did not improve gains. Different performance results were reported for growing pigs placed on dietary manganese levels ranging from 0.5 to 34 ppm (Plumlee *et al.*, 1956), and for pigs taken from sows on 70 to 90 ppm manganese (Speer *et al.*, 1952). For baby pigs, the manganese requirement for optimal growth was placed at 0.4 ppm by Leibholz *et al.* (1962). Normal reproduction and farrowing were reported for sows on 80 to 117 ppm manganese

(Leibholz *et al.*, 1962) and for sows on 6 to 100 ppm manganese (Newland *et al.*, 1961). Underwood (1971) has concluded that growth of pigs is maintained on very low dietary intakes of manganese at the expense of tissue manganese, but such low levels for prolonged periods cannot promote fertility and reproduction. A level of 20 ppm manganese has been recommended for normal growth of baby pigs by the NRC (1973).

The trial reported here was conducted to determine the manganese requirement of baby pigs as a follow-up to the work of Kayongo-Male (1974) who reported that this requirement is between 3 and 6 ppm. Semi-purified diets were used along with various manganese levels. Manganese balance, serum and tissue manganese concentrations, serum alkaline phosphatase and bone strength characteristics were used as indicators of manganese status.

EXPERIMENTAL PROCEDURES

A. Introduction

An experiment was conducted as a follow-up to "Experiment 4" previously conducted by Kayongo-Male (1974) to determine the manganese requirement of baby pigs from sows fed a low-manganese diet (13.9 ppm Mn, Table B-2). The Michigan State University swine farm furnished the pigs and facilities used in this trial. Diets were semi-purified, the protein being supplied by high-protein casein.

B. Experiment 1 (Mn)

Sixteen 8-day-old baby pigs were taken from sows on a low manganese diet, housed and fed as previously described for Experiment 1 in the copper trials. They were adapted to the basal diet shown in Table 25 for 5 days. After adaptation, the pigs were allotted at random to the four diets shown in Table 25 which contained the following manganese levels: diet 1 (basal), 0.9 ppm; diet 2, 2.2 ppm; diet 3, 3.8 ppm; diet 4, 7.4 ppm. The pigs were kept on these diets for a 42-day growth trial.

Blood samples were taken initially and on days 7, 14, 28 and 42 of the trial for determination of hemoglobin, hematocrit, alkaline phosphatase activity and serum manganese.

At the end of the growth trial, the pigs were placed in stainless steel metabolism cages for a 2-day adjustment period followed by a 3-day collection of feces and urine. The pigs were then killed, weights

TABLE 25. COMPOSITION OF DIETS USED IN EXPERIMENT 1 (Mn)

Ingredient	Basal	B + 1.5 ppm	B + 3 ppm	B + 6 ppm
Casein	2100	2100	2100	2100
Cerelose	3500	3360	3220	2940
α-Cellulose	350	350	350	350
Low Mn-Mineral premix[a]	420	420	420	420
Lard	350	350	350	350
Fat sol. vit. premix[b]	70	70	70	70
Water sol. vit. premix[c]	140	140	140	140
Corn oil	70	70	70	70
Mn premix[d]	0	140	280	560
	7000	7000	7000	7000
Mn Conc. (analyzed, ppm)[1]	0.9	2.2	3.8	7.4

[a]See Appendix B, Table B-1.

[b,c]See Appendix A, Table A-4.

[d]Manganese premix in cerelose containing 100 ppm Mn from $MnSO_4 \cdot H_2O$, J. T. Baker reagent grade.

[1]Expressed on as-fed basis.

of various organs, tissues and glands were obtained, and liver, kidney and muscle were saved for mineral analysis. The left femur was taken for measurement of bone strength characteristics, and rib 7, metacarpal and radiocarpal joints were fixed in formalin solution and submitted for histopathological examination.

C. Analytical Methods

Hemoglobin, hematocrit, bone strength characteristics, bone ash, tissue, feed, feces and urine manganese were determined according to procedures previously described for the copper trials.

1. Bone minerals

a. Manganese. Ashed bone was finely ground and approximately 300 mg of powdered ash were dissolved in 5 ml of 6N HCl. Two milliliter aliquots of the acid-ash solution were diluted 1:2 with strontium mixture A[1], and the manganese concentration was determined by atomic absorption spectrophotometry at 279.4 nm, and expressed as ppm on a dry, fat-free basis.

b. Calcium and magnesium. Aliquots of the acis-ash solution were diluted 1:20 with deionized, distilled water. The resulting solutions were further diluted 1:100 with strontium mixture B[2] and calcium and magnesium levels determined by atomic absorption spectro-

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[1]Dissolve 60.86 g $SrCl_2 \cdot 6H_2O$ and 10.0 g NaCl in 1 liter of deionized distilled water to yield 20,000 ppm Sr.

[2]Dissolve 30.5 g $SrCl_2 \cdot 6H_2O$ and 5.0 g NaCl in 1 liter of deionized distilled water to yield 10,000 ppm Sr.

photometry at 422.7 and 285.2 nm, respectively. Calcium and magnesium concentrations were expressed as a percentage of dry, fat-free bone.

c. Phosphorus. Aliquots of the acid-ash solution were diluted 1:200 with deionized, distilled water, and phosphorus was determined by the colorimetric method of Gomorri (1942). To 0.5 ml of the 1:200 diluted solution, 2.5 ml of MS solution[1] were added, followed by 0.25 ml of Elon solution[2]. A water blank was treated in the same manner as was the sample and both were incubated for 45 minutes and analyzed for phosphorus using a Coleman Junior II spectrophotometer. Optical density was recorded at 700 nm and phosphorus concentration was expressed as percentage of dry, fat-free bone.

2. Serum manganese

Determination of serum manganese was performed by flameless atomic absorption spectrophotometry on the Instrumentation Laboratories, Inc., Model 455 flameless atomizer connected to an IL Model 453 atomic absorption unit. Duplicates of 10 µl aliquots of undiluted serum were dried at 100 to 600° over a period of 3 minutes, pyrolized at 1200° for 20 seconds and analyzed at 2400° for 15 seconds. A clean cycle was automatically completed at the end of each analytical period. To each serum aliquot was added 10 µl deionized distilled water or 10 µl standard manganese solution[3]. Since the volume of serum used was the same as the

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[1]Dissolve 5.0 g ($Na_2MoO_4 \cdot H_2O$) in 500 ml d.d. water, add 14 ml of 12N H_2SO_4 and make up to 1 liter.

[2]Dissolve 1.0 g Elon (p-methyl-amino-phenosulfate) in 100 ml of 3% $NaHSO_3$ and filter. Keep refrigerated.

[3]Standards of 5, 10, 20 and 30 ppm Mn were made from a stock solution of 1000 ppm.

addition volume and the slope was 1.0, serum manganese concentration in parts per billion, was read directly from the signal print-out recorded on a Jahrmann SC 1200-R strip chart. The flameless atomizer was purged with argon (at 20 psi) at the end of each cycle.

3. Serum alkaline phosphatase

The activity of serum alkaline phosphatase was determined according to the procedure outlined in Sigma Technical Bulletin No. 194 (1963). Assays were done using freshly obtained serum. Solutions used were obtained from Sigma[1] or were freshly prepared as follows:

a. Sodium hydroxide. In 1 liter of deionized distilled water 0.8 g anhydrous sodium hydroxide was dissolved to give approximately a 0.02N solution.

b. Standard solution of p-nitrophenol. This was obtained as Sigma Stock No. 104-1 (10 mM/liter) and was kept in the dark at 4°C.

c. Working standard solution. Into a 100 ml volumetric flask 0.5 ml p-nitrophenol standard solution was pipetted followed by 0.02N sodium hydroxide solution to 100 ml; the flask was thoroughly shaken to mix the solutions.

d. Sigma 104 phosphatase substrate. The substrate, p-nitrophenyl phosphate, was obtained from Sigma and stored at 4°C.

e. Stock substrate solution. In 10 ml deionized distilled water, 0.04 g substrate was dissolved; this was enough for 20 determinations.

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[1]Sigma Chemical Company, St. Louis, Missouri.

f. Alkaline buffer solution. About 0.75 g glycine from Sigma was dissolved in approximately 25 ml deionized distilled water. Three to 5 ml 1N NaOH were added, followed by a solution of 0.0203 g $MgCl_2 \cdot 6H_2O$ in 5 ml deionized distilled water. The solution was made up to 90 ml with water and the pH adjusted to 10.5. Water was added to make a final volume of 100 ml, and the solution was stored at 4^oC.

g. Alkaline buffered substrate. The alkaline buffer solution was mixed 1:1 with the stock substrate solution.

h. Assay. Into a test tube, 0.05 ml serum and 1 ml buffer substrate were added, shaken on a vortex and immediately placed in a water bath at 38^oC. Reagent blanks were similarly treated using deionized distilled water in place of serum. Exactly 30 minutes later, 10 ml 0.02N NaOH solution were added and mixed on a vortex. This stopped the enzyme activity and allowed the color produced to be measured. The color was formed by liberation of p-nitrophenol by the enzyme alkaline phosphatase. Optical density of the samples was determined on the Beckman DU Spectrophotometer[1] at 410 nm using reagent blanks as reference. Units of alkaline phosphatase (AP) were determined by using the calculated standard factor. Addition of 0.1 ml HCl to each sample resulted in a colorless solution, and a second optical density and AP units were determined.

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[1]Beckman Instruments, Inc., Fullerton, California.

The true corrected AP activity was calculated as follows:

Alkaline Phosphatase activity (Sigma Units/ml)

$$= 2 \times \left[\begin{pmatrix} \text{AP units of} \\ \text{colored sample} \end{pmatrix} - \begin{pmatrix} \text{AP units of} \\ \text{colorless sample} \end{pmatrix} \right]$$

D. Statistical Analyses

The data were subjected to a one-way analysis of variance using the Unequal-1 format on a CDC 6500 computer at the Michigan State University Computer Laboratory. Simple correlations were also calculated and the levels of significance of differences between means were determined using the Bonferroni t-statistics test.

RESULTS AND DISCUSSION

A. Experiment 1: Manganese requirement of baby pigs on semi-purified diets supplemented with manganese sulfate to yield upon analysis: 0.9 ppm, 2.2ppm, 3.8 ppm and 7.4 ppm manganese

The effects of dietary manganese levels on growth are shown in Table 26. Average daily gain of pigs on the diet containing 3.8 ppm manganese was the highest (362 g) followed by the diet containing 7.4 ppm manganese (295 g). Pigs on the basal diet (0.9 ppm Mn) gained weight at the same rate as did those on diet 2 (2.2 ppm Mn). However, there were no significant differences in average daily gain and feed efficiency due to dietary manganese levels.

Hematocrit, hemoglobin and mean corpuscular hemoglobin concentration values are presented in Table 27. Hematocrit values of pigs on diets 1 and 3 rose steadily, the first peaking on the fourth week, the third on the sixth week; for diets 2 and 4, hematocrit fluctuated (Figure 4.1). These values do not represent any significant treatment effects. Hemoglobin was not significantly affected by dietary manganese levels up to week 4. In week 6, hemoglobin was significantly ($P<0.05$) higher on diet 4 than on diets 1 and 3 (Figure 4.2). Mean corpuscular hemoglobin concentration rose until the fourth week in pigs on diets 1 and 2, while the rise continued into week 6 for pigs on diet 4; for diet 3 the values fluctuated (Figure 4.3). MCHC value for pigs on diet 4 was significantly ($P<0.05$) higher than the corresponding values for pigs on diets 1 and 2, while MCHC value for diet 3 was significantly ($P<0.01$)

TABLE 26. THE EFFECT OF DIETARY MANGANESE LEVELS ON GROWTH (EXPT. 1)

Diet no.	1	2	3	4
Mn conc., ppm[1]	0.9	2.2	3.8	7.4
No. of pigs	4	4	3	4
Avg. init. wt., kg	2.71±0.14	2.43±0.14	2.69±0.16	2.45±0.14
Avg. final wt., kg	13.28±0.72	13.23±0.72	15.90±0.83	13.85±0.72
Avg. daily gain, g	288	285	362	295
Feed/gain	1.35	1.42	1.30	1.32

[1]Expressed on as-fed basis.

TABLE 27. THE EFFECT OF DIETARY MANGANESE LEVELS ON HEMATOCRIT, HEMOGLOBIN AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (EXPT. 1)

Diet no.	1	2	3	4
Mn conc., ppm	0.9	2.2	3.8	7.4
No. of pigs	4	4	3	4
Hct.[1], %				
Initial	36.0±1.28	37.3±1.28	37.1±1.48	37.9±1.28
7 days	37.3±1.18	38.3±1.18	37.8±1.36	39.1±1.18
14 days	39.4±1.54	36.6±1.54	38.1±1.78	37.3±1.54
28 days	44.4±1.47	41.8±1.47	40.8±1.69	40.5±1.47
42 days	39.9±0.72	40.4±0.72	41.5±0.83	39.1±0.72
Hb.[2], g/100 ml				
Initial	10.0±0.26	9.4±0.26	10.5±0.30	9.5±0.26
7 days	11.0±0.29	11.1±0.29	12.0±0.34	11.4±0.29
14 days	11.9±0.62	10.8±0.72	11.5±0.72	11.8±0.62
28 days	14.5±0.58	14.2±0.58	13.8±0.67	13.5±0.58
42 days	12.0±0.45	12.6±0.45	12.0±0.52	14.1±0.45[a]
MCHC[3], %				
Initial	27.8±1.04	25.2±1.04	28.3±1.20	25.2±1.04
7 days	29.7±1.40	29.0±1.40	31.8±1.62	29.2±1.40
14 days	30.1±0.73	29.6±0.84	30.1±0.84	31.5±0.73
28 days	32.7±0.75	33.9±0.75	33.8±0.87	33.2±0.75
42 days	30.1±1.17[c]	31.1±1.17[c]	28.9±1.35[e]	36.1±1.17[b,d]

[a]Significantly greater than least two values (P<0.05).

[b,c]Significantly different (P<0.05).

[d,e]Significantly different (P<0.01).

[1]Hematocrit.

[2]Hemoglobin.

[3]Mean corpuscular hemoglobin concentration.

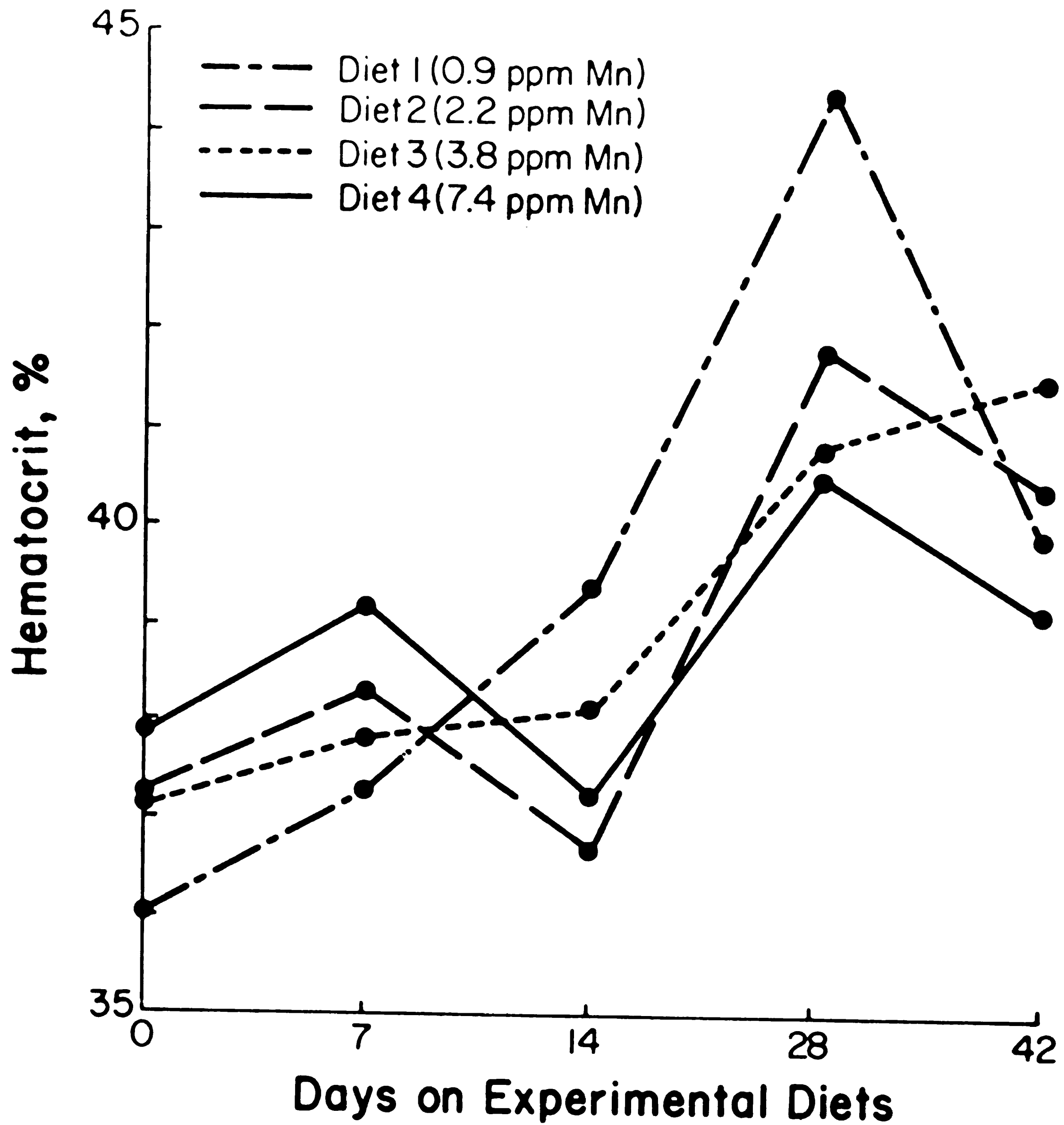

Figure 4.1. Effect of dietary manganese concentrations on hematocrit (Expt. 1).

Figure 4.2. Influence of manganese intake on hemoglobin (Expt. 1).

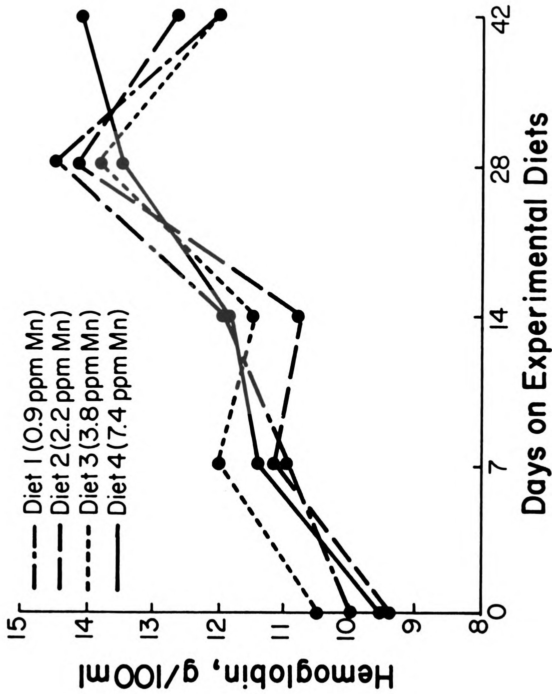

Figure 4.2.

Figure 4.3. Response of mean corpuscular hemoglobin concentration to dietary manganese levels (Expt. 1).

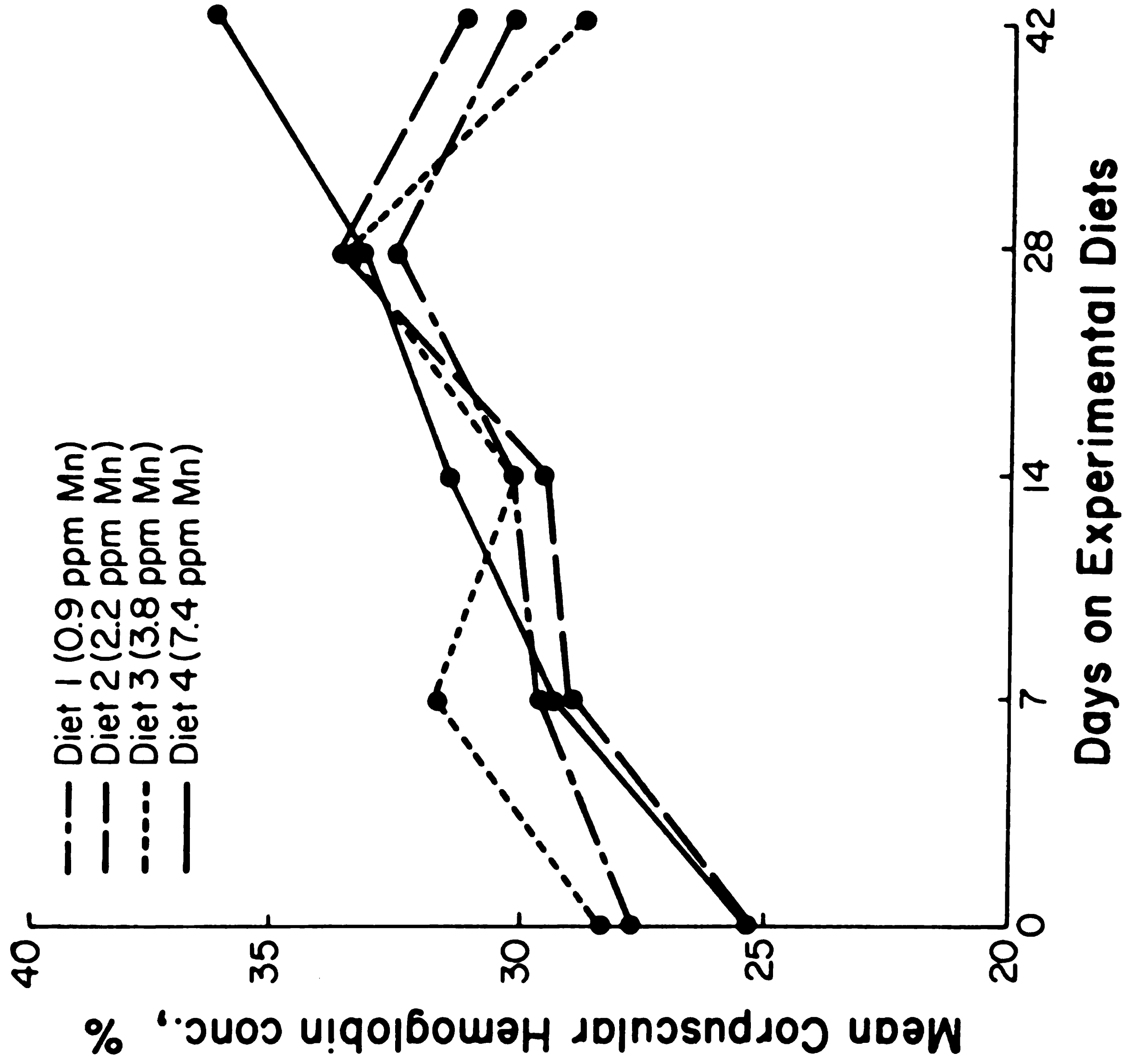

Figure 4.3.

lower than the value for diet 4.

The effects of dietary manganese levels on serum alkaline phosphatase activity and serum manganese are summarized in Table 28. Initial alkaline phosphatase activity was high -- 40.3, 59.7, 44.0 and 52.1 Sigma units for treatments 1, 2, 3 and 4, respectively -- but decreased dramatically until it reached 4.9, 6.0, 5.6 and 6.5 Sigma units, respectively (Figure 4.4). Lassiter *et al.* (1970) reported a rise in serum alkaline phosphatase activity in response to dietary manganese. There were, however, no significant differences between treatments. Serum manganese showed no consistent effects due to dietary levels of manganese. After a drop in the first week, all the treatments produced an increase in the second week which persisted in treatments 2 and 4. Treatments 1 and 3 caused fluctuations in serum manganese levels (Figure 4.5). However, on the sixth week serum manganese levels were lower on diet 1 than on all other diets -- 8.0, 10.0, 12.0 and 12.3 ppb for diets 1, 2, 3 and 4, respectively -- but this difference was not significant. Significant responses of serum manganese to dietary manganese were reported by Hawkins *et al.* (1955), Plumlee *et al.* (1956), Newland and Davis (1961) and Rojas *et al.* (1965) in swine and ruminants.

Table 29 shows the result of the manganese balance trial, and values of selected fecal and urinary minerals. Dietary manganese levels significantly ($P<0.01$) influenced manganese intake, fecal and urinary manganese excretion and manganese retention. Manganese intake was significantly ($P<0.05$ or $P<0.01$) different between the four diets. Absolute fecal manganese excretion was significantly ($P<0.05$) different between diets 1 and 2, and significantly ($P<0.01$) different between diets 1, 3 and 4. Absolute urinary manganese excretion was higher on

TABLE 28. THE EFFECT OF DIETARY MANGANESE LEVELS ON SERUM ALKALINE PHOSPHATASE ACTIVITY AND SERUM MANGANESE CONCENTRATION (EXPT. 1)

Diet no.	1	2	3	4
Mn conc., ppm	0.9	2.2	3.8	7.4
No. of pigs	4	4	3	4
SAP[1], Sigma units				
Initial	40.3±9.03	59.7±9.03	44.0±10.42	52.1±9.03
7 days	30.7±2.80	38.5±2.80	32.4±3.23	37.9±2.80
14 days	18.2±1.32	19.4±1.32	18.0±1.53	17.6±1.32
28 days	9.1±0.67	9.8±0.67	8.9±0.77	9.6±0.67
42 days	4.9±1.14	6.0±1.14	5.6±1.32	6.5±1.14
Serum Mn[2], ppb				
Initial	10.8±1.23	10.3±1.42	7.3±1.42	11.7±1.42
7 days	7.0±1.09	7.5±1.09	7.7±1.26	6.5±1.09
14 days	8.5±1.04	9.8±1.20	9.3±1.04	8.3±1.04
28 days	7.8±2.18	10.8±2.18	8.5±3.08	11.0±2.18
42 days	8.0±1.38	10.0±1.19	12.0±1.38	12.3±1.19

[1]Serum alkaline phosphatase.

[2]Serum manganese concentration.

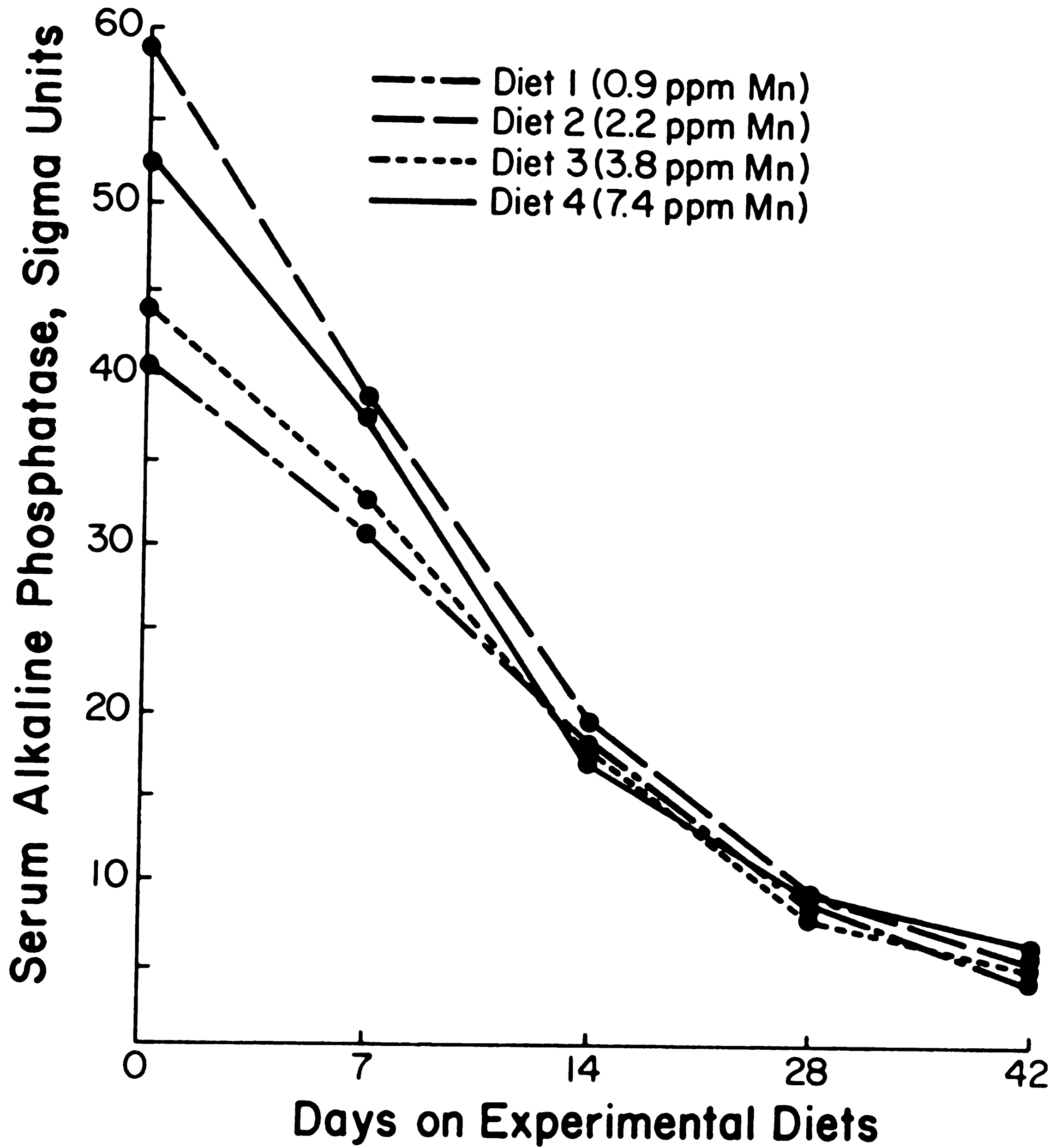

Figure 4.4. Effect of manganese intake on serum alkaline phosphatase activity (Expt. 1).

Figure 4.5. Response of serum manganese levels to dietary manganese (Expt. 1).

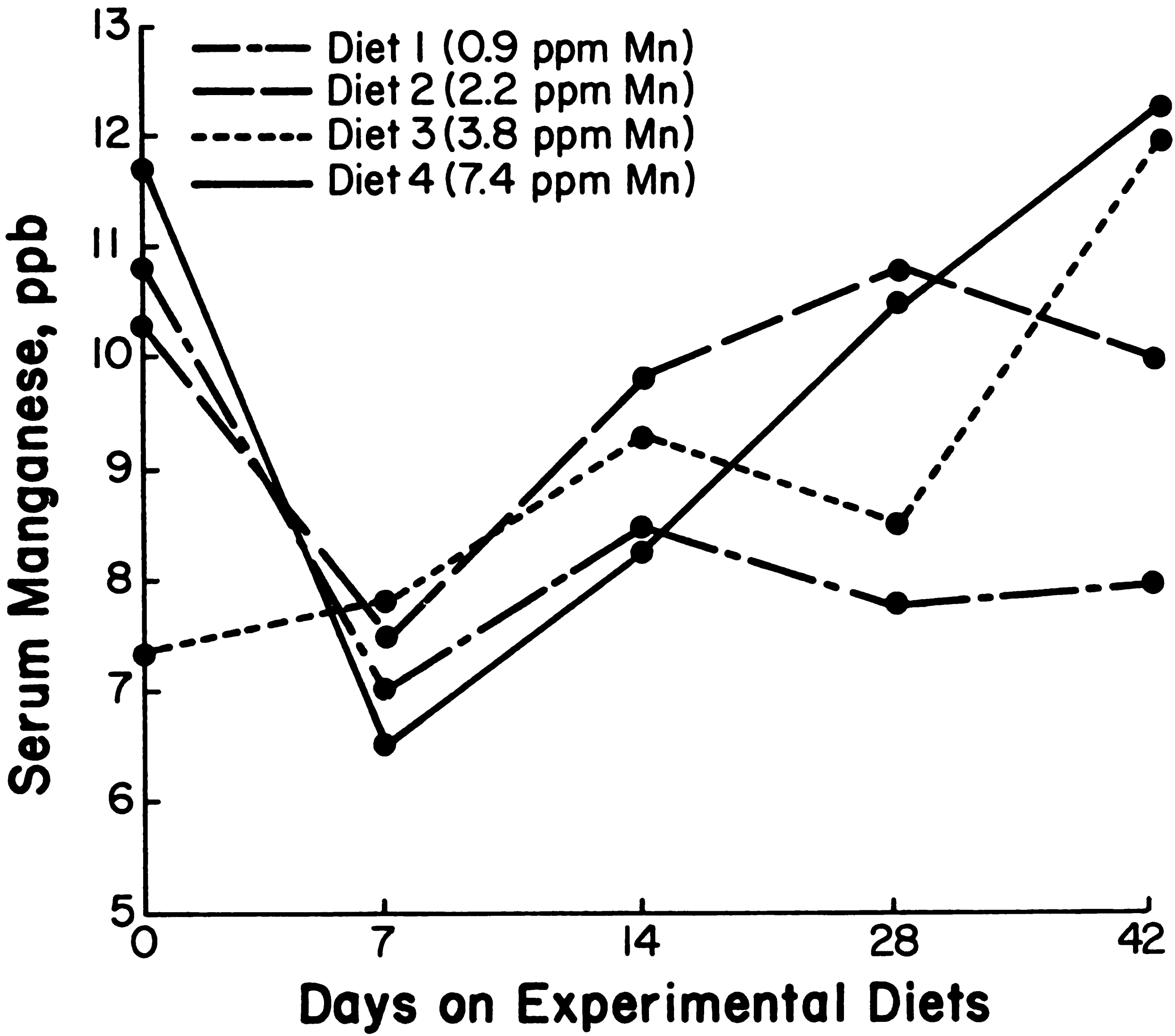

Figure 4.5.

TABLE 29. THE EFFECT OF DIETARY MANGANESE LEVELS ON MANGANESE BALANCE, FECAL AND URINARY ZINC, COPPER AND IRON (EXPT. 1)

Diet no.	1	2	3	4	SEM
Mn conc., ppm	0.9	2.2	3.8	7.4	
No. of pigs	3	3	3	3	
Mn intake, mg/day	0.31	0.85[a]	1.88[b]	3.02[c]	0.12
Mn excretion, mg/day					
Fecal	0.32	0.82[a]	1.66[b]	1.92[c]	0.07
Urinary	0.04	0.04	0.09	0.05	0.02
Mn excretion, % of intake					
Fecal	104.9[a]	95.9	88.7	64.6	8.57
Urinary	11.4[d]	4.3	4.6	1.5	1.73
Mn retention, mg/day	-0.05	-0.004	0.13	1.05[c]	0.15
Mn retention, % of intake	-16.4	-0.24	6.78	33.92[a]	8.59
Fecal Zn, mg/day	0.16	0.17	0.17	0.18	
Fecal Cu, mg/day	0.08	0.08	0.08	0.09	
Fecal Fe, mg/day	0.51	0.55	0.54	0.53	
Urinary Fe, mg/day	0.0008	0.0007	0.001	0.0007	
Urinary Cu, mg/day	0.00009	0.0002	0.0003	0.0001	
Urinary Fe, mg/day	0.02	0.02	0.02	0.02	

[a]Significantly greater than least value ($P<0.05$).

[b]Significantly greater than least two values ($P<0.01$).

[c]Significantly greater than least three values ($P<0.01$).

[d]Significantly greater than least value ($P<0.01$).

diet 3 than on diets 1, 2 and 4 but showed no statistical significance. Fecal manganese excretion as a percent of intake was very high on diet 1 (104.9%) and was significantly ($P<0.05$) greater than that on diet 4 (64.6%); urinary manganese excretion as a percent of intake was also significantly ($P<0.01$) higher on diet 1 (11.4%) than on diet 4 (1.5%). Absolute manganese retention was significantly ($P<0.01$) higher on diet 4 (1.05 mg/day) than on diets 1, 2 and 3 with -0.05, -0.004 and 0.13 mg/day, respectively, but there was no significant difference between diets 1, 2 and 3. Manganese retention as a percent of intake was significantly ($P<0.05$) different between diets 4 and 1. There were negative absolute manganese retention values on diets containing 0.9 and 2.2 ppm manganese (-0.05 and -0.004 mg/day, respectively), and negative manganese retention values as a percent of intake on the same diets (-16.4% and -0.24%, respectively). Obligatory manganese loss via fecal and urinary excretion by animals fed diets containing low levels of manganese has been reported by Zajcev (1959), Starodubova (1968) and more recently by Kayongo-Male (1974). Manganese retention seemed to be proportional to increased dietary manganese levels as previously shown by Gutowska and associates (1941). Dietary manganese levels did not exert any significant effect on daily fecal and urinary excretion of zinc, copper and iron.

The influence of dietary manganese levels on organ weights as percentage of final body weight is given in Table 30. Liver, pancreas, kidneys and adrenals did not show any significant differences between treatments. Although heart as percent of final body weight on diet 4 was not significantly different from values of diets 1 and 2, it was significantly ($P<0.01$) higher than on diet 3. Thyroid values on diets 1

TABLE 30. INFLUENCE OF DIETARY MANGANESE LEVELS ON ORGAN WEIGHTS AS PERCENTAGE OF FINAL BODY WEIGHT (EXPT. 1)

Diet no.	1	2	3	4
Mn conc., ppm	0.9	2.2	3.8	7.4
No. of pigs	4	4	3	4
Avg. final body wt., kg	13.28±0.72	13.23±0.72	15.90±0.83	13.85±0.72
Organ wts.[1], % final body wt.				
Liver	2.17±0.13	1.99±0.13	2.15±0.15	2.07±0.13
Pancreas	0.24±0.01	0.22±0.01	0.20±0.01	0.22±0.01
Kidneys	0.51±0.05	0.53±0.05	0.50±0.06	0.56±0.06
Heart	0.56±0.02	0.56±0.02	0.49±0.02	0.59±0.02[a]
Thyroid	0.05±0.001[b]	0.05±0.001[b]	0.04±0.002	0.04±0.001
Adrenals	0.05±0.004	0.05±0.004	0.04±0.004	0.04±0.004
Spleen	0.22±0.01[d]	0.22±0.01[d]	0.17±0.01	0.27±0.01[a,c]

[a]Significantly greater than least value ($P<0.01$).

[b]Significantly greater than least two values ($P<0.05$).

[c,d]Significantly different ($P<0.05$).

[1]Expressed on a fresh basis.

and 2 were significantly ($P<0.05$) higher than values on diets 3 and 4. Spleen was significantly ($P<0.01$) greater on diet 4 than on diet 3, and significantly ($P<0.05$) different between diet 4 and diets 1 and 2. Although these differences were significant, it has not been shown that manganese directly affects the weights of certain organs. Miller and associates (1967a) conducted trials using baby pigs reared on semi-purified diets made nutritionally complete or mononutrient-deficient in iron, calcium, phosphorus, vitamin D or magnesium and reported significant reduction of actual organ weights accompanied by increased relative weights of the organs except the heart and spleen in iron deficiency and the adrenals in iron, calcium and vitamin D deficiency.

Liver, kidney and muscle manganese concentrations in response to dietary manganese levels are presented in Table 31. Liver manganese tended to increase directly with increased dietary manganese, and showed a significant ($P<0.05$) difference between diets 1 and 2; diets 3 and 4 were significantly ($P<0.01$) greater than diets 1 and 2. Liver zinc was greater on diet 3 than on any other diet but the difference was not significant. Copper and iron concentrations of the liver did not show any significant effects due to dietary manganese. Kidney manganese was significantly ($P<0.01$) higher on diets 3 and 4 than on diet 1 and diet 2, and diet 2 was significantly ($P<0.01$) higher than diet 1. It is clear that dietary manganese influences liver and kidney manganese concentration, whereas zinc, copper and iron concentrations of the liver and kidney were not affected significantly by manganese intake. Muscle manganese was too low to be detected by flame atomic absorption spectrophotometry.

The effects of dietary manganese levels on physical and chemical

TABLE 31. THE EFFECT OF DIETARY MANGANESE LEVELS ON LIVER, KIDNEY AND MUSCLE MANGANESE, ZINC, COPPER AND IRON CONCENTRATIONS (IN PPM)[1] (EXPT. 1)

Diet no.	1	2	3	4
Mn conc., ppm	0.9	2.2	3.8	7.4
No. of pigs	4	4	3	4
Liver Mn	0.33±0.21	1.25±0.21[a]	2.51±0.24[b]	3.13±0.21[b]
Liver Zn	107.5±19.43	112.1±19.43	128.1±22.43	114.9±19.43
Liver Cu	16.0±5.56	16.5±5.56	14.7±6.42	22.4±5.56
Liver Fe	62.7±9.62	65.8±9.62	55.5±11.10	65.3±9.62
Kidney Mn	0.33±0.07	0.65±0.07[a]	1.00±0.08[b]	1.00±0.07[b]
Kidney Zn	24.4±1.37	24.4±1.37	26.2±1.58	25.1±1.37
Kidney Cu	24.4±2.57	28.2±2.57	32.2±2.96	28.4±2.57
Kidney Fe	51.7±9.83	51.1±9.83	67.9±11.35	60.3±9.83
Muscle Mn[2]	---	---	---	---

[1]Expressed on fresh basis.

[a]Significantly greater than least value ($P<0.05$).

[b]Significantly greater than least two values ($P<0.01$).

[2]Undetectable by flame atomic absorption spectrophotometry.

characteristics of the left femur are summarized in Table 32. Femur weight, external horizontal and vertical diameters[1], length and maximum load were greater on diets 2, 3 and 4 than on the basal diet, however the differences were not significant. Bending moment, inertia, stress and elasticity did not show any significant treatment differences. Ash content of the femur was significantly ($P<0.05$) higher on diet 2 (43%) than on either diet 1 or diet 4 (40.8% and 40.2%, respectively). Percent magnesium was significantly ($P<0.05$) greater on diets 2 and 3 than on diets 1 and 4, while manganese, percent calcium and phosphorus were not significantly influenced by dietary manganese levels. Smith *et al.* (1944c) and Ellis *et al.* (1974) have described abnormal bone development due to manganese deficiency in rabbits, characterized by reduction in size, manganese content and breaking strength. The results of the femur strength characteristics indicate that manganese deficiency was not produced or that these parameters are not sensitive indicators of manganese status since no significant treatment differences were ntoed in the bone parameters studied. Furthermore, the calcium and phosphorus contents of the femur were not significantly different between treatments, thereby supporting the conclusion of Parker *et al.* (1955) and Kayongo-Male (1974) that calcium and phosphorus content of bone is not affected by manganese intake. Lameness, crooked legs, enlarged hocks or bone deformities were not observed in any of the pigs on the experimental diets.

Correlations between manganese balance, serum manganese, serum

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[1]Horizontal and vertical diameters of the femur were measured at mid-shaft with the medial and lateral condyles facing downwards.

TABLE 32. THE EFFECT OF DIETARY MANGANESE LEVELS ON PHYSICAL AND CHEMICAL CHARACTERISTICS ON THE LEFT FEMUR (EXPT. 1)

Diet no.	1	2	3	4
Mn conc., ppm	0.9	2.2	3.8	7.4
No. of pigs	4	4	3	4
Physical characteristics				
Weight, fresh, g	48.4±5.14	65.7±5.14	64.8±5.94	61.8±5.14
External diameter[1], cm				
Horizontal (B)	1.31±0.07	1.53±0.07	1.52±0.08	1.48±0.07
Vertical (D)	1.47±0.05	1.50±0.05	1.65±0.06	1.65±0.05
Length (L), cm[a]	7.7±0.30	8.0±0.30	8.6±0.35	8.1±0.30
Maximum load, kg	53.0±5.73	57.0±5.73	68.7±6.62	57.3±5.73
Maximum bending moment, kg-cm	103.1±10.66	114.1±10.66	147.9±12.31	102.0±10.66
Moment of inertia, cm^4	0.17±0.05	0.17±0.05	0.26±0.06	0.21±0.05
Maximum stress, kg/cm^2	455±37.05	517±37.05	468±42.79	408±37.05
Modulus of elasticity, 1000 kg/cm^2	5.2±0.58	6.7±0.58	5.9±0.67	4.5±0.58
Chemical characteristics[b]				
Ash content, %	40.8±0.61	43.0±0.061[c]	42.2±0.71	40.2±0.61
Mn, ppm*	4.3±0.11	4.6±0.11	4.6±0.13	4.6±0.11
Mg, %*	0.61±0.05	0.77±0.05[d]	0.77±0.06[d]	0.66±0.05
Ca, %*	42.1±0.59	43.2±0.59	42.2±0.68	42.6±0.59
P, %*	16.8±0.31	17.4±0.31	16.7±0.36	16.5±0.31

[a]Measured from the mid-medial condyle to the fovea.

[b]Expressed on dry, fat-free basis.

TABLE 32 (CONT'D.)

[c]Significantly greater than least two values ($P<0.05$).

[d]Significantly greater than least value ($P<0.05$).

[1]Measured at mid-shaft with medial and lateral condyles facing downwards.

*Expressed on ash-content basis.

alkaline phosphatase and average daily gain are shown in Table 33. Serum manganese on 0 week and 1 week were significantly (P<0.05) correlated with urinary manganese; in the last week of the trial, serum manganese was significantly (P<0.05) correlated with manganese intake and fecal and urinary manganese excretion. Serum alkaline phosphatase on weeks 1 and 2 was significantly (P<0.05 or P<0.01) correlated with urinary manganese, absolute and percent manganese retention and percent fecal manganese retention. Average daily gain was significantly (P<0.05) and negatively correlated with alkaline phosphatase on the fourth week (r = -.57). Correlations between alkaline phosphatase and average daily gain on the 14th (r = -.45) and 42nd (r = -.42) days agree with the values reported by Miller and coworkers (1967b). In both studies the correlation coefficients were slightly high, negative and nonsignificant. Serum alkaline phosphatase activity decreased as the pigs matured. Serum alkaline phosphatase was poorly correlated with serum manganese throughout the trial, and in fact was mostly negatively correlated. Kayongo-Male (1974) reported a significant (P<0.05) correlation between serum manganese and alkaline phosphatase only towards the end of a 28-day trial. Serum manganese was nonsignificantly correlated with manganese intake throughout the trial period. This observation does not agree with the result of Kayongo-Male (1974) who reported a significant (P<0.01) correlation, but it supports the findings of Krieg (1966) who showed no correlation between dietary and serum manganese.

Correlations within parameters of manganese balance are presented in Table 34. Manganese intake was significantly (P<0.01) correlated with fecal manganese, absolute manganese retention and manganese retention as a percent of intake; fecal and urinary manganese as percent of

TABLE 33. CORRELATIONS BETWEEN MANGANESE BALANCE, SERUM MANGANESE, SERUM ALKALINE PHOSPHATASE AND AVERAGE DAILY GAIN (EXPT. 1)

	Absolute Mn balance				% of intake				Serum Alkaline Phosphatase				
Item[1]	Mn intake	Fecal Mn	Urin. Mn	Mn ret.	Mn ret.	Fecal Mn	Urin. Mn	ADG	Init.	7 days	14 days	28 days	42 days
SMn													
Init.	-.25	-.39	-.59[a]	.05	-.19	.22	-.01	-.30	.18	.41	.24	-.03	.26
7 days	-.11	-.07	.55[a]	-.18	-.10	.05	.29	.05	-.01	-.44	.15	.05	.12
14 days	-.13	-.11	-.13	-.11	-.19	.29	-.31	.36	.44	.45	-.38	-.02	-.51
28 days	.25	.29	-.02	.17	.39	-.41	-.13	-.22	-.17	-.12	-.03	.30	-.03
42 days	.52	.52	.68[a]	.36	.47	-.48	-.25	.04	.11	-.10	-.19	.06	.19
SAP													
Init.	.25	.10	-.15	.42	.46	-.41	-.50	.39	---	.50	-.59[a]	-.17	-.50
7 days	.22	.24	-.56[a]	.19	.14	.01	-.75[b]	.23		---	.03	.01	-.06
14 days	-.51	-.32	-.27	-.65[b]	-.58[a]	.60[a]	.23	-.45			---	.21	.80[b]
28 days	.06	.09	-.13	.01	.13	-.11	-.17	-.57[a]				---	.10
42 days	-.15	-.05	.07	-.27	-.23	.21	.24	-.42					---
ADG	.44	.50	.21	.27	.24	-.16	.45	---					

[a] Significant ($P<0.05$).

[b] Significant ($P<0.01$).

[1] Abbreviations are: SMn = Serum manganese.
SAP = Serum alkaline phosphatase.
ADG = Average daily gain.

TABLE 34. CORRELATIONS WITHIN MANGANESE BALANCE PARAMETERS (EXPT. 1)

Item[1]	Mn intake	Fecal Mn	Urin. Mn	Mn ret.	% Mn ret.	% Fecal Mn	% Urin. Mn
Mn intake	--	.94[b]	.27	.88[b]	.83[b]	-.79[b]	-.67[a]
Fecal Mn		--	.35	.65[a]	.68[a]	-.61[a]	-.69[a]
Urin. Mn			--	.04	.06	-.12	.25
Mn ret.				--	.87[b]	-.87[b]	-.54
% Mn ret.					--	-.98[b]	-.65[a]
% Fecal Mn						--	.51
% Urin. Mn							--

[1]Abbreviations are:
Fecal Mn = fecal manganese excretion
Urin. Mn = urinary manganese excretion
Mn ret. = absolute manganese retention
% Mn ret. = manganese retention as percent of intake
% Fecal Mn = fecal manganese as percent of intake
% Urin. Mn = urinary manganese as percent of intake

[a]Significant ($P<0.05$).

[b]Significant ($P<0.01$).

intake were negatively but significantly (P<0.01 and P<0.05, respectively) correlated with manganese intake. The retention correlation values obtained here agree with the findings of Woerpel and Balloun (1964), Hill and Holtkamp (1954) and Kayongo-Male (1974). Fecal but not urinary manganese was significantly (P<0.05) correlated with manganese retention (absolute and as a percent of intake), which agrees with the report of Mahoney and Small (1968) but not with that of Kayongo-Male (1974). Absolute fecal manganese was also significantly (P<0.05) but negatively correlated with fecal and urinary manganese as percent of intake. Absolute manganese retention was significantly (P<0.01) and positively related to retention as a percent of intake, and significantly (P<0.01) but negatively related to fecal manganese as percent of intake. In agreement with the findings of Kayongo-Male (1974), manganese retention as a percent of intake was highly negatively correlated (r = -.98) with fecal manganese as percent of intake, but contrary to his report, there was a highly significant (P<0.01) correlation of manganese intake with absolute manganese retention (r = .88), or with manganese retention as percent of intake (r = .83). These values are quite in agreement with the findings of Murty (1957) in sheep, and the report of Mathers and Hill (1967) in chickens. Urinary manganese as percent of intake was negatively correlated with absolute manganese retention, and significantly (P<0.05) but negatively related to manganese retention as percent of intake.

1. Histopathology

a. _Histopathologic examination._ Sections were made from the seventh rib at the costochondral junction, metacarpal bones including

the joint, and the radiocarpal bone and were examined histologically to evaluate the following parameters[1]: a) seventh rib: cartilage, percent 10 x 10^{2} diaphysis, b) metacarpal: joint and articular cartilage, spicules, c) radiocarpal bone: epiphyseal plate, thickness of epiphyseal plate, percent 10 x 10 diaphysis and thickness of diaphysis 10 x 20[3].

There were some variations in values for the cartilage at the junction of the cartilage with bone in the seventh rib, but these were not significant since they ranged from very slight to slight. All metacarpal bones and joint capsules appeared normal histologically. In the radiocarpal bones the configuration of the epiphyseal cartilaginous plate was normal in all bones except for pig 132-5 on diet 2 (2.2 ppm Mn) which had very slight change -- lack of uniformity of cartilaginous cells. The histologic appearance of the compact bone in the diaphysis of the radiocarpal bone appeared normal.

Since changes in the bones from one pig to another were very slight, there were no significant treatment effects.

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[1]Examination was conducted and reported by Dr. K. K. Keahey, Department of Pathology, Michigan State University.

[2]10x eye-piece and 10x objective.

[3]10x eye-piece and 20x objective.

CONCLUSIONS

Based on the data obtained in this study and those previously collected by Kayongo-Male (1974), the following conclusions are made:

1. Manganese intake was highly and positively correlated with fecal manganese, manganese retention (absolute and percent of intake), while negatively correlated with manganese excretion as percent of intake.

2. The major route of manganese excretion is via the feces; the urinary route handles very small amounts of manganese. Low dietary manganese induces an obligatory loss of body manganese through secretion into the gastro-intestinal tract; this results in a negative manganese balance.

3. Bone strength characteristics were not significantly influenced by dietary manganese levels.

4. Dietary manganese levels used in this trial did not significantly affect average daily gain, liver, kidney, fecal and urinary zinc, copper and iron concentrations.

5. Based on the balance trial and serum manganese concentrations, the dietary manganese requirement for the baby pig is approximately 3.8 ppm, on an as-fed basis, or 4.3 ppm on a dry basis.

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APPENDIX A

TABLE A-1. LOW-COPPER VITAMIN-TRACE MINERAL PREMIX USED IN GESTATION AND LACTATION RATIONS

Ingredient	Amount, g
Vitamin A[a]	300
Vitamin D_3[b]	200
Forbee mix[c]	2043
Vitamin B_{12}[d]	408.6
$ZnSO_4 \cdot H_2O$	254
$MnSO_4 \cdot H_2O$	310
$FeSO_4 \cdot 7H_2O$	810
KIO_3	12.5
Cerelose	1094.9
	5448.0

[a] 30,000 IU/gm.

[b] 3000 IU/gm.

[c] Dawe's Laboratories, Inc., Chicago Heights, Illinois. Analysis per pound:

i. Riboflavin 2000 mg
ii. D-Pantothenic acid 4000 mg (equiv. to more than 4300 mg calcium pantothenate)
iii. Niacin 9000 mg
iv. Choline chloride 10,000 mg

[d] 0.1% cyanocobalamin, Dawe's Laboratories, Inc., Chicago Heights, Illinois.

TABLE A-2. LOW-COPPER GESTATION AND LACTATION RATIONS[1]

Ingredient	Gestation kg	Lactation kg
Corn	775.5	716.4
Soybean meal (49%)	104.5	163.6
Defluorinated phosphate	11.4	11.4
Limestone ($CaCO_3$)	6.8	6.8
Salt (NaCl)	4.6	4.6
Vit. E-Selenium premix[2]	4.6	4.6
Low-copper VTM premix[3]	1.8	1.8
	909.2	909.2

[1]Analyzed 3.6 ppm copper (as fed basis).

[2]Containing 2200 IU of vitamin E and 20 mg Se per kg.

[3]See Table A-1.

TABLE A-3. LOW-COPPER MINERAL PREMIX USED IN SEMI-PURIFIED DIETS

Ingredient	Amount in 3 kg of premix (g)
KCl	300.00
KI	.06
$FeSO_4 \cdot 7H_2O$	31.50
$CuSO_4$[a]	0.00
$CoCO_3$	3.00
$MnSO_4 \cdot H_2O$	3.00
$ZnSO_4 \cdot H_2O$	12.00
$MgCO_3$	60.00
$NaHCO_3$	750.00
$CaHPO_4 \cdot 2H_2O$	1080.00
$CaCO_3$	375.00
Cerelose	385.44
	3000.00

[a]Copper premix was prepared separately in cerelose to contain 100 ppm copper.

TABLE A-4. VITAMIN MIXTURE USED IN COPPER AND MANGANESE SEMI-PURIFIED DIETS.

Ingredient	ppm in diet
Thiamine mononitrate	3
Riboflavin	6
Nicotinamide	40
D-calcium pantothenate	30
Pyridoxine hydrochloride	2
Para-amino benzoate	13
Ascorbic acid	80
D-α-tocopheryl acetate	10
Inositol	130
Choline chloride	1300
	ppb in diet
Pteroyl-glutamic acid	260
Biotin	50
Cyanocobalamin	100
2-methyl-1,4-naphthoquinone	40
Vitamin A palmitate	1500
Vitamin D_2	12.5

TABLE A-5. IL 453 ATOMIC ABSORPTION SPECTROPHOTOMETER SPECIFICATIONS FOR COPPER ANALYSIS

1. Instrumental parameters for IL MODEL 453	
Mode of operation	A-B
Hollow cathode	63041
Lamp current	5mA
Photomultiplier	20362-01 (R372)
P. M. voltage	530 V
Wavelength	324.7 nm
Burner	27019 Boling
Fuel	Acetylene
Fuel pressure	5.4 to 5.5 psig
Oxidant	Air
Flame	Stoichiometric
Burner height	11 mm
2. Standard conditions	
Optimal range	0.04 to 7 mcg/ml
Detection limit	0.001 mcg/ml
Sensitivity	0.03 mcg/ml

TABLE A-6. ANALYSIS OF VARIANCE FOR EFFECTS OF DIETARY COPPER LEVELS ON COPPER BALANCE, TISSUE COPPER CONCENTRATIONS AND GROWTH

Category	Mean	Min.	Max.	P-level of F-statistic
Copper balance				
Copper intake, mg/day	3.02	0.92	6.80	<0.0005
Copper excretion, mg/day				
Fecal	1.40	0.40	3.76	0.001
Urinary	0.09	0.04	0.14	
Copper retention, mg/day	1.52	0.43	3.40	0.005
Copper retention, % of intake	48.36	26.34	68.84	
Copper excretion, % of intake				
Fecal	47.27	27.47	68.73	
Urinary	4.37	0.72	11.73	<0.05
Tissue copper (fresh basis), ppm				
Liver	64.84	14.40	160.10	
Kidney	58.35	33.20	97.10	
Spleen	78.27	45.80	118.60	
Heart	32.62	5.50	75.50	
Brain	10.96	5.10	29.70	
Hair	13.13	8.50	15.70	<0.05
Femur	7.89	5.10	9.80	<0.05
Growth				
Average daily gain, g	275	260	287	

TABLE A-7. ANALYSIS OF VARIANCE FOR EFFECTS OF DIETARY COPPER LEVELS ON COPPER BALANCE, TISSUE COPPER CONCENTRATIONS AND GROWTH

Category	Mean	Min.	Max.	P-level of F-statistic
Copper balance				
Copper intake, mg/day	0.46	0.086	0.95	0.001
Copper excretion, mg/day				
Fecal	0.19	0.070	0.40	
Urinary	0.022	0.009	0.03	<0.05
Copper retention, mg/day	0.25	-0.033	0.75	<0.05
Copper retention, % of intake	36.8	-30.1	79.1	
Copper excretion, % of intake				
Fecal	56.2	17.8	118.6	
Urinary	7.0	3.2	15.1	<0.0005
Tissue copper (fresh basis), ppm				
Liver	6.8	3.7	12.8	
Kidney	2.1	0.9	3.5	<0.05
Heart	3.1	2.2	3.8	
Brain	2.2	1.3	4.0	
Hair	10.4	9.0	11.6	
Femur	7.8	4.6	11.4	
Growth				
Average daily gain, g	190.8	115.0	250.0	

TABLE A-8. ANALYSIS OF VARIANCE FOR EFFECTS OF DIETARY COPPER LEVELS ON COPPER BALANCE, TISSUE COPPER CONCENTRATIONS AND GROWTH

Category	Mean	Min.	Max.	P-level of F-statistic
Copper balance				
Copper intake, mg/day	1.81	0.58	2.83	<0.0005
Copper excretion, mg/day				
Fecal	0.68	0.14	1.52	
Urinary	0.07	0.008	0.20	
Copper retention, mg/day	1.06	0.15	2.11	<0.05
Copper retention, % of intake	54.5	19.0	82.6	
Copper excretion, % of intake				
Fecal	41.52	16.51	75.91	
Urinary	4.00	0.92	7.76	
Tissue copper (fresh basis), ppm				
Liver	6.10	1.30	15.70	<0.05
Kidney	9.23	3.00	21.50	<0.05
Brain	3.78	2.10	5.60	<0.005
Heart	2.66	1.00	3.40	<0.005
Spleen	1.15	0.70	1.70	
Femur	7.59	5.60	13.30	
Growth				
Average daily gain, g	197	50	341	

APPENDIX B

TABLE B-1. LOW-MANGANESE MINERAL PREMIX USED IN SEMI-PURIFIED DIETS

Ingredient	Amount in 3 kg of premix (g)
KCl	300.00
KI	31.50
$FeSO_4 \cdot 7H_2O$	31.50
$CuSO_4$	3.00
$CoCO_3$	3.00
$MnSO_4 \cdot H_2O$[a]	0.00
$ZnSO_4 \cdot H_2O$	12.00
$MgCO_3$	60.00
$NaHCO_3$	750.00
$CaHPO_4 \cdot 2H_2O$	1080.00
$CaCO_3$	375.00
Cerelose	385.44
	3000.00

[a]Manganese premix was prepared separately in cerelose to contain 100 ppm Mn.

TABLE B-2. LOW-MANGANESE GESTATION AND LACTATION RATIONS[1]

Ingredient	Amount, g	
	Gestation	Lactation
Yellow corn	785	685
Soybean meal (49%)	180	280
Dicalcium phosphate	15	15
Limestone ($CaCO_3$)	10	10
Salt (NaCl)	5	5
Low Mn-VTM premix[2]	5	5
	1000	1000

[1]Analyzed 13.9 ppm Mn (as fed basis).

[2]See Table B-3.

TABLE B-3. LOW-MANGANESE VITAMIN TRACE MINERAL PREMIX USED IN GESTATION AND LACTATION DIETS

Ingredient	Amount, g
Vitamin A[1]	100
Vitamin D_3[2]	67
Vitamin E[3]	36
Vitamin K[4]	4
Vitamin B_{12}[5]	18
Riboflavin	3
Niacin	16
D-calcium pantothenate	12
Choline chloride	168
$FeSO_4 \cdot 2H_2O$	180
$CuSO_4$	22.5
$ZnSO_4 \cdot H_2O$	180
Selenium premix[6]	200
Yellow corn	3984.5
	5000.0

[1] 30,000 IU/gm.

[2] 3000 IU/gm.

[3] D-α-tocopherylacetate.

[4] Menadione sodium bisulfite (2-methyl-1,4 naphthaquinone).

[5] Cyanocobalamin.

[6] Yields 0.044 ppm Se in diet.

TABLE B-4. IL 453 ATOMIC ABSORPTION/EMISSION SPECTROPHOTOMETER - IL MODEL 455 FLAMELESS SAMPLER SPECIFICATIONS FOR SERUM MANGANESE

1. Instrumental parameters for IL MODEL 453

Mode of operation	A-B
Hollow cathode	45472
Lamp current	5mA
Photomultiplier	R456
P. M. voltage	520 V
Slit width	320 μm
Wavelength	279.5 nm

Channel B, H_2-continuum, #IL 63490, 20 mA, 630 v

2. Recorder (full scale)

Response time	0.5 sec
Range	50 mV
Sensitivity	2.5

3. Parameters for IL MODEL 455 (flameless)

Mode	Automatic
Purge gas	Argon
Gas flow rate	20 SCFH
Cell pressure	20 psig
Dry setting	100 to 600°C
Pyrolyze setting	1000 to 1200°C
Analyze setting	2400 to 2600°C

TABLE B-5. ANALYSIS OF VARIANCE FOR EFFECTS OF DIETARY MANGANESE LEVELS ON MANGANESE BALANCE, TISSUE MANGANESE CONCENTRATIONS AND GROWTH

Category	Mean	Min.	Max.	P-level of F-statistic
Manganese balance				
Mn intake, mg/day	1.52	0.29	3.43	<0.0005
Mn excretion, mg/day				
Fecal	1.18	0.26	1.99	<0.0005
Urinary	0.05	0.02	0.16	
Mn retention, mg/day	0.28	-0.14	1.50	<0.05
Mn retention, % of intake	6.0	-25.2	43.9	<0.05
Mn excretion, % of intake				
Fecal	88.5	54.1	117.6	0.05
Urinary	5.5	1.2	13.7	<0.05
Tissue manganese, ppm (fresh basis)				
Liver	1.8	0.2	3.9	<0.0005
Kidney	0.7	0.3	1.2	<0.0005
Growth				
Average daily gain, g	307	280	372	

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