THE EFFECT OF SUPPLEMENTATION OF A BASAL PIG RATION WITH IRON AND COPPER ON THE COPPER LEVEL OF THE LIVER AND SPLEEN

by

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INTRODUCTION

Following the pioneer experiments that conclusively proved copper and iron to be essential nutrients in normally functioning biological systems, many new avenues of investigation have opened. One avenue is the study of the effects on the experimental animal of a diet containing increased amounts of one or more minerals.

Studies have been conducted to determine the effect of mineral supplementation on feed consumption, feed utilization and weight gain in swine. Other workers have investigated the theory that minerals fed in greater concentrations than normally consumed might have a beneficial effect on the quality of meat. Should high mineral levels in the animal's diet prove to be worthwhile in either of the areas of study (feed efficiency or meat quality), information as to quantity and distribution of such minerals in the animal's body would be advantageous in determining the feasibility of widespread practical application of mineral supplementation.

Determination of the correlation of iron or iron and copper ingestion to copper storage in the liver and spleen should, therefore, be beneficial in the evaluation of the effects of feeding quantities of mineral supplements considered to be above that of nutritional adequacy.

REVIEW OF LITERATURE

A review of the present knowledge concerning the function

of copper in mammalian biological systems and the relationship of this element to iron metabolism will aid in understanding and interpretation of the results of the experiment reported herein.

Role of Copper in Mammalian Biological Systems

A classic method for studying the essential nature and functions of a micronutrient is to produce a deficiency of the substance and then observe any changes in function and metabolism (Gubler, '56b). This, and an analysis of enzymes containing the element, have been the two chief methods used in studying the role of copper in metabolism (Glass, '50).

Experimentally Produced Copper Deficiency States. Rabbits were used in the original work by Hart et al. ('25). They developed anemia when placed on a diet of whole milk. Copper deficiency symptoms have been produced in other animals, i.e. in rats (Elvehjem and Sherman, '32) and in dogs (Baxter, '51).

The production of a copper deficiency state in swine was accomplished by Elvehjem and Hart ('32) after feeding young pigs a diet of raw cow's milk. When pure iron was added to the milk diet there was only a small, temporary improvement in the hemoglobin content of the blood but copper in addition to the iron effected a rapid and complete recovery. Although some of the pigs were maintained in an anemic condition for as long as 3 weeks, no other deficiency symptoms were observed. Schultze et al. ('36) reported a blood copper level as low as 20 mcg per 100 ml as sufficient for slow hemoglobin formation.

They stated that hemotopoiesis will cease if the level falls below 10 mcg%. These investigators suggested that a dietary copper level of from 2 to 4 mg per day was sufficient for hemoglobin formation.

Teague and Carpenter ('51) produced a copper deficiency in young pigs by feeding whole cow's milk. In addition to the previously reported anemia symptoms, they observed an unusual leg condition which developed between the fourth and sixth weeks of the experiment. Lack of rigidity in leg joints, excessively flexed hocks and crookedness in the forelegs causing difficulty in standing and walking were observed in these pigs. Administration of copper brought about a complete reversal of symptoms in some of the animals.

In an experiment by Lahey et al. ('52) evaporated cow's milk was fed, supplemented only by iron. The pigs developed a severe microcytic, hypochromic anemia and normoblastic hyperplasia of the bone marrow like that of iron-deficient pigs. In an extension of this experiment, Gubler et al. ('52) found a reduction of iron in the plasma and hemoglobin and a moderate reduction in the iron concentration of the liver, kidney and heart. There was also an increase in iron binding capacity of the plasma and impaired ability to absorb iron from the gastro-intestinal tract. Large quantities of iron administered orally or parenterally did not alleviate the anemia but administration of copper produced a rapid reduction of symptoms of anemia and restoration to a normal state. After studying the metabolism of iron in the copper-deficient, anemic

swine, Gubler et al. ('52) reported three major effects: (1) impaired ability to absorb iron from gastro-intestinal tract; (2) incomplete mobilization of iron from tissues; and (3) inability to utilize parenterally administered iron for hemoglobin synthesis. Cartwright et al. ('56) compared the morphology of erythrocytes from normal, copper-deficient and iron-deficient swine and concluded that copper- and iron-deficiency anemias are morphologically similar.

Naturally Occurring Copper Deficiency States. Marston ('50) described a copper deficiency state in lambs grazing in parts of Australia where the soil is deficient in this mineral. A stringiness in the appearance of the wool is the first symptom observed and occurs long before signs of ill health are exhibited by the animal. Black sheep follicles suffer a decrease in their capacity to crimp as well as their ability to synthesize melanin. Marston ('50) explained the role of copper in the formation of normal wool as a catalyst for the oxidative closure of the thiol residues of the prekeratin fibrous protein to the disulfide linkages of keratin. Copper has also been implicated with tyrosinase in the conversion of tyrosine to dehydroxyphenol-L-alanine (dopa) and in the conversion of dopa to melanin (Fitzpatrick et al., '50; Lerner et al., '50; Gubler et al., '57).

If the copper deficiency persisted in the Australian sheep, hypochromic anemia and nervous manifestations developed (Marston, '50). A slowly progressive ataxia has been described which is quite apparent in the lambs between 3 to 6 weeks of

age. If the "enzootic" ataxia is not relieved, their gait becomes stiff and there is increased difficulty in locomotion with the advancing deficiency state.

In New Zealand, deficiencies in sheep and cattle developed in those animals grazing on the reclaimed peat swamps deficient in copper (Cunningham, '50). The deficient cattle were reported to be anemic and unthrifty, exhibiting a poor growth rate and reduced reproductive capacity. They scoured profusely and their coats were bleached and rough. The calves demonstrated ataxia in some cases, their bones were fragile and quite susceptible to fracture, and they had a slowed growth rate. Adult sheep appeared healthy in spite of marked depletion in copper but the lambs may be affected by an acute osteoporosis predisposing to fractures or by an ataxic condition associated with regional degeneration of the myelin of the nervous system.

Human Pathological States. The first clearcut case of copper deficiency was observed in man by a group of scientists at the University of Southern California (Sturgeon and Brubaker, '56). An anemic condition in 5 young children was relieved when copper sulfate was administered.

Because copper is so widely distributed, it is extremely unlikely that a copper deficiency could occur in otherwise normal human beings even on suboptimal diets (Gubler, '56b). Conditioned copper deficiencies may be found in the neprotic syndrome (Cartwright et al., '54a). Hypocupremia is encountered in some pathological states such as heptolenticular degeneration or Wilson's disease (Bearn and Kunkel, '54;

Cartwright et al., '54b). However, copper deficiency is not present in this disease since the body stores are 10 to 30 times normal.

Hypercupremia was observed in leukemia, pernicious anemia, aplastic anemia, Hodgkin's disease, thalassemia, Banti's syndrome and "collagen diseases" (Cartwright et al., '48) as well as sickle cell anemia, polycythemia vera, malaria, arsenic poisoning, schizophrenia and various disorders of the central nervous system (Sachs et al., '35). Locke et al. ('32) listed Addison's disease and diabetes as diseases in which hypercupremia may be observed.

Principle Copper Compounds in Mammalian Tissue. According to Scheinberg and Sternlieb ('60), there are probably several forms in which copper in mammalial tissue may be found. It may exist as free cupric or cuprous ions or may be found in combination with amino acids, purines, pyrimidines, nucleotides, DNA, RNA and proteins. Copper-containing enzymes or enzymes associated with copper are believed to occur widely (Glass, '50).

Ceruloplasmin. This substance is a copper and alpha-globulin complex demonstrating oxidase activity toward certain polyphenols and polyamines, especially paraphenylenediamine (Scheinberg and Sternlieb, '60). A deficiency or absence of ceruloplasmin occurs in almost all patients with hepatolenticular degeneration. It has been classified as a true oxidase and has been implicated with the regulation of copper metabolism.

Tyrosinase. This copper-containing enzyme has oxidase activity toward poly- and monophenols. The enzyme has a role in the production of melanin (Fitzpatrick et al., '50).

Cytochrome c Oxidase. Wainio et al. ('59) have demonstrated the presence of copper in this enzyme, which has an important role in aerobic oxidation. Cytochrome c oxidase activity was markedly diminished in experimental copper deficiency (Gubler, '57).

Cerebrocuprein. A copper-protein complex isolated from bovine and human brain, this substance has a lower molecular weight than ceruloplasmin and lacks enzymatic activity toward paraphenylenediamine (Scheinberg and Sternlieb, '60).

Erythrocuprein. This copper-protein was isolated from human erythrocytes by Markowitz et al. ('59) and the protein was found to bind almost all the copper in the red blood cell.

Hepatocuprein. The copper-protein complex found in the liver is named hepatocuprein. Its function is unknown (Cartwright, '55).

Porcine hepatic uricase and butyryl coenzyme A dehydrogenase have been implicated as copper-containing compounds. Further investigation will be necessary before uricase can be accepted as a copper-containing enzyme (Scheinberg and Sternlieb, '60). Steyn-Parve and Beinert ('58) have shown that copper is not a constituent of the dehydrogenase.

Effects of Copper Supplementation

Fattening Swine. Consequences of copper deficiency have been studied since the early 1900's but not until more recent years have the effects of dietary copper supplementation been reported.

A series of experiments were conducted by investigators at the University of Reading in England. They reported studies on copper sulfate supplementation of basal pig rations showing a relationship between the copper supplement and improved weight gain and, perhaps, feed utilization. These studies were triggered by the experiments of Braude ('49) in which young pigs reared outdoors on pasture grew better than those reared indoors. These observations were confirmed by Barber et al. ('53; '55a,b). Pasture was determined to be the primary factor responsible for better growth when some soil placed indoors resulted in improvement of growth rate of the young pigs. Copper, as one of the components of the soil, was studied and the amount present in the soil was correlated to growth rate.

Mitchell ('53) noted that when given free choice of two creep meals, suckling pigs consumed the meal to which copper sulfate had been added (25 g $CuSO_4 \cdot 5H_2O$/ 100 lb of meal) in preference to the meal without supplementation. He suggested that if copper increased the palatability of the meal, encouraging pigs to eat more of it, then a faster weight gain should be expected and perhaps the pigs would start eating at an earlier age. Since the cost of copper sulfate is so small,

the addition of relatively large amounts of copper would be commercially feasible (Bowler et al., '55).

One of the first experiments reported on copper supplementation by Barber and other workers ('55a) at the University of Reading was conducted to study the effects of dietary supplements of a high-copper mineral mixture called XF on feed consumption and efficiency of feed utilization of fattening pigs. The mixture, XF, contained 250 ppm copper and increased the rate of gain, which was believed to be the direct result of increased feed consumption. Barber et al. ('55a) found no significant effect on the efficiency of feed utilization. However, when 0.1% $CuSO_4 \cdot 5H_2O$ was used as a copper supplement, a higher rate of growth was recognized and believed to be caused by significant improvement in the feed conversion ratio. The differences between control and experimental weight gains were established in the first 8 weeks of feeding.

Barber et al. ('55b) used a copper supplement of only 150 ppm (25 g/lb of meal) to study 300 suckling pigs raised indoors and found no substantial change in total consumption or weekly consumption from 3 to 8 weeks. The workers concluded that the increased palatability of the 150 ppm copper-supplemented meal was not of sufficient magnitude to induce increased consumption. Copper supplements for suckling pigs should, therefore, be at least as high as those used for fattening pigs (250 ppm).

Using 182 fattening pigs in 8 centers in England, Bowler

et al. ('55) fed 250 ppm copper-supplemented feed and found the mean growth rate to be significantly improved. The efficiency of feed conversion was not significantly improved, but there was an improvement of 0.09 lb meal/lb live•weight gain recorded for pigs with copper supplementation.

Results of experiments by Bass et al. ('56) at the University of Florida did not agree with those of the English workers. Bass et al. ('56) reported decreased weight gain and increased feed consumption per lb gain for pigs receiving feed containing 250 ppm copper. One pig died, and exhibited copper toxicity symptoms upon autopsy. In studying varying copper levels of 0, 100, 150 and 200 ppm, the 100 ppm was reported to have produced the best weight gain and most feed consumed per lb gain.

Barber et al. ('57) reported a decrease in feed intake in 8 pigs given 0.5% or 1% copper in their rations, which was easily remedied by the reduction of copper to 0.1% or less with immediate resumption of satisfactory growth and feed intake. In 70 pigs fed meals containing 0.1% copper sulfate, there was a significant increase in growth rate and rate of feed consumption. The improvement of 3.9% in feed conversion, though close, was not significant at the 5% level.

Results obtained from the above reports can be summarized generally in the following manner:

(1) Feed conversion is only slightly affected by copper sulfate supplementation.

(2) The effect of copper sulfate on growth rate and rate

of feed consumption is significant.

(3) For both fattening and suckling pigs, an addition of 0.1% (250 ppm) copper to the ration seems, at present, to be the concentration of choice.

(4) The faster growth of copper-supplemented pigs may be attributed to improved feed utilization as well as increased feed consumption (Barber et al., '57).

Quality of Meat. The effect of mineral supplementation of the diet on the quality of cooked muscle has been reported for veal (Hanning et al., '57; Bray et al., '59; Niedermeier et al., '59). Iron and copper supplements produced a more tender product and a more intense red color of the bones in a study by Hanning et al. ('57) and both minerals were factors that influenced meat color in studies by Niedermeier et al. ('59) and Bray et al. ('59).

Jacobson and Fenton ('56a,b) studied the quality of beef in relation to 3 levels of nutrition that included mineral supplementation. They concluded that if costs permitted, the palatability of roasts from some muscles may be improved by high levels of nutrition, particularly in young animals.

Harrison et al. ('62) studied the cooking time and losses, and the palatability of fresh ham slices, loin roasts and tenderloins from animals used for the present study of copper concentration in liver and spleen. The pigs were fed 3 diets: a basal ration, basal plus iron and basal plus a combination of copper and iron. The investigators reported that, in general, no significant differences attributable to ration were found

for cooking time and losses, flavor, juiciness or tenderness for any of the cuts studied.

Relationship of Copper to Iron Metabolism

Since the earliest experiments established copper as a micronutrient (Elvehjem, '35), a correlation between the concentration of copper available and normal iron function in biological systems has been recognized. Numerous studies since the 1930's have clarified some issues and introduced others in expanding the knowledge of the interrelationship of copper and iron.

Metabolism of Iron. The metabolism of any element depends on numerous factors in addition to dietary concentration of the element in question. Some of these factors are discussed below.

Absorption of iron. Divalent or ferrous iron is more readily absorbed than the trivalent or ferric form in experiments using dogs as well as human subjects (Hahn et al., '45). This fact is probably due to the greater solubility of ferrous complexes. The availability of reducing mechanisms, i.e. ascorbic acid and the sulfhydryl compounds, is probably related to the proportion of ferrous iron present. Assuming other factors to be equal, there is an inverse correlation between size of dose and percentage of dose absorbed but the total quantity of iron absorbed depends on the dose (Gubler, '56a). A greater efficiency of absorption will occur with a small dose but the total amount absorbed will be less than that

absorbed when a large dose is fed.

Other factors also affect absorption. These include an acid medium of below pH 5 in the stomach needed to convert ingested iron to a soluble form. Phosphate ions and phytic acid are factors that inhibit absorption of iron by forming insoluble complexes with the element. A pyridoxine deficiency produced an increased absorption of iron in rats (Gubler et al., '49). Copper deficiency lowers iron absorption (Wintrobe et al., '53).

Transportation of iron. In man and higher animals, absorbed iron is believed to pass through the mucosal cells directly into the blood stream. The plasma is the chief medium for iron transport (Gubler, '56a). Ferrous iron is oxidized to the ferric form by dissolved oxygen in the plasma and then forms a complex with a specific beta-1-globulin (transferrin, siderophillin). Iron is transported in the metal-globulin form.

Storage of iron. Little iron is excreted (from 0.5 to 1.5 mg per day) although 27 to 28 mg of iron is released in the human body per day from the breakdown of hemoglobin (Gubler, '56a). The released iron is reutilized in hemoglobin synthesis in preference to that stored or newly added by absorption (Hahn et al., '43). Also, newly absorbed iron is more readily utilized for hemoglobin synthesis than stored iron. The iron is stored in two forms, ferritin and hemosiderin. Liver contains the greater part of body ferritin but the spleen and bone marrow also contain considerable amounts (Gubler, '56a).

Biological role of iron. Iron is found in hemoglobin, myoglobin and the cytochrome system. Hemoglobin combines reversibly with oxygen in the lungs and gives up 70 to 90% of the oxygen in the tissues; its chief function is oxygen transport. Myglobin functions in oxygen transport and as an oxygen reservoir, delivering oxygen to the cytochrome system and other energy producing systems of the cell. The cytochrome system contains 4 iron-porphyrin-protein complexes referred to as cytochrome oxidase, cytochrome a, cytochrome b and cytochrome c which function in the transfer of electrons. Catalase and peroxidase are iron-porphyrin-protein enxymes present in nearly all the tissues, and are presumed to prevent toxic accumulation of peroxides in the cells (Gubler, '56a).

Metabolism of Copper. Copper has been recognized as a constituent of human blood since 1875 (Cartwright, '50) and as an essential micronutrient since the early 1930's (Elvehjem, '35).

Absorption of copper. Gubler ('56b) suggested that the absorption of copper in human beings is probably not regulated in any manner analogous to the regulation of iron absorption. In small doses, 30 to 40% of the ingested copper is absorbed but a very high intake produces excessive retention of copper. The excretion rate of copper is adjusted to accommodate the amount of copper absorbed. The liver is the chief organ of excretion of copper through the formation of bile; thus, 99% of copper excreted is by way of the stools.

Transportation of copper. There are two fractions of plasma copper (Gubler, '56b). About 5% is loosely bound to albumin or other plasma proteins; the other 95% is firmly bound to an alpha-2-globulin and is called ceruloplasmin.

Storage of copper. The liver is the chief storage organ of copper (Barber et al., '57; Bowland et al., '61). In man and higher animals, the high copper concentrations are in the spleen, muscles and bones (Gubler, '56b). The copper content of fetal liver is from 5 to 10 times greater than that of the normal adult.

Little is known of the form in which copper is stored. Such dietary factors as zinc and protein may influence the accumulation of copper in the livers of rats fed high levels of copper (McCall and Davis, '61). Data from experiments supervised by McCall and Davis ('61) showed that adequate or greater than adequate levels of protein inhibited the accumulation of copper in the liver where large amounts of copper were ingested. There was lack of evidence of a copper-zinc interaction when high levels of protein were available in the diet. The workers stated that the effect was the result of a complex interaction of the factors involved and was dependent upon their relative concentrations.

Biological role of copper. Copper was suggested as a component of porcine hepatic uricase, an enzyme required in purine metabolism (Gubler, '56a), and shown to be a part of the enzyme tyrosinase, which catalyzes the oxidation of L-tyrosine to dihydroxyphenyalanine (dopa) and the oxidation of dopa to

melanin pigments. This metal has been implicated in the oxidation of glutathione in tissues (Cartwright, '50) and is essential for the formation of normal wool by catalyzing the closure of the sulfhydryl groups in the keratin molecule to form disulfide bridges necessary for the normal kinky structure of the wool fibers (Marston, '50). Copper is essential for normal bone formation. In copper deficiency, bones become thin, crooked and show lack of normal trabeculation. This mineral has a role in the maintenance of normal myelin sheath of the central nervous system in ruminants (Cunningham, '50).

Interrelationship of Copper and Iron. Iron absorption from the gastro-intestinal tract of swine deficient in copper was markedly reduced (Gubler et al., '52; Wintrobe et al., '53) as indicated by the amount of iron in the blood, kidney, liver, spleen and heart of the animals tested. Chase ('52) used rats to study total body iron and found less absorption of iron from the gastro-intestinal tract of the copper deficient rats. Gubler ('56b) reported that the total iron of the body/kg body weight in copper deficiency is of the same order of magnitude as that found in iron deficiency although there is a high dietary intake of iron. The anemia of copper deficiency is attributed to a small extent to the poor iron absorption, but results mostly from failure to utilize iron for hemoglobin synthesis (Gubler, '56b). Bone marrow deficient in copper produces red blood cells with shortened survival time so that plasma iron turnover is increased with a normal rate of incorporation of iron into hemoglobin since copper deficient

marrow cannot increase its capacity for erythrocyte production (Gubler, '56b). Anemias of copper and iron deficiencies are both microcytic and hypochromic. Normoblastic hyperplasia of the bone marrow is observed in both deficiency states.

PROCEDURE

Specimens Examined in the Experiment

History of the Animals. The Kansas State University Department of Animal Husbandry raised the 26 crossbred York, Duroc and Poland China pigs to weaning period. At weaning time, they were divided at random into 3 lots according to breed and litter. Three barrows and 6 gilts were placed in Lots I and II; 3 barrows and 5 gilts were used in Lot III. The pigs were fed rations listed in Table 1. The feed was pelleted and both feed and water were available at all times from weaning at approximately 8 weeks of age to slaughter weight (approximately 210 lbs).

The pigs were housed on concrete, fed in wooden troughs and penned with galvanized wire. These precautions were taken to prevent ingestion of iron or copper by the pigs from sources other than the water and ration. Iron in the drinking water was negligible (0.03 ppm).

Processing and Preservation of Specimens. Normal slaughter procedures were used by the University meat laboratory. The carcasses were dressed packer style and chilled 24 hours in a cooler at about 34°F. Slaughter date,

TABLE 1

Composition of rations fed to pigs in each lot

Lot	Animal number	Constituents	Quantity
I	13-21	Basal ration[1]	
		Milo	79.0%
		Soybean oil meal	9.5%
		Alfalfa meal (dehydrated)	5.0%
		Meat scraps	5.0%
		"Aurofac"[2]	0.5%
		Iodized salt	0.5%
		Zinc oxide	2.0 oz per ton
II	22-30	Basal ration[1]	
		Ferrous sulfate[3] to make 300 mg iron/lb of ration (661.3 ppm)	
III	31-38	Basal ration[1]	
		Ferrous sulfate[3] to make 300 mg iron/lb of ration (661.3 ppm)	
		Cupric sulfate to make 30 mg copper/lb of ration (66.1 ppm)	

[1]contains 40.18 mg iron and 6.79 mg of copper/lb of ration.

[2]commercial Auromycin and vitamin B_{12} (1.8 gm chlortetracycline hydrochloride and 1.8 mg B_{12}/lb of ration).

[3]courtesy of Calcium Carbonate Company, Quincy, Illinois.

initial weight, slaughter weight and carcass grade were recorded (Appendix, Table 5). Samples of liver and spleen were removed using a stainless steel knife and sectioned on a wooden table top to minimize contamination by extraneous metals. The liver was placed on the table, gall bladder side up with the distal ends of the lobes toward the meat cutter. The lobes were designated 1, 2 and 3 from left to right as shown in figure 1. The meat cutter removed a one-half inch slice longitudinally from the center of each lobe. Each sample was wrapped separately in aluminum foil (0.0015 gauge), labeled with the animal and lobe number and immediately frozen on a plate freezer at -20°F.

A one-half inch slice was cut longitudinally from each spleen, avoiding the midline of the organ to prevent sampling of the adhering tissues. These samples were wrapped in aluminum foil (0.0015 gauge), labeled with the animal number and immediately frozen on a plate at -20°F. The samples of liver and spleen were stored at 0° to -10°F.

Procedure for Random Sampling

Selection of the order in which the samples were to be analyzed was accomplished by using Table 1.5.1 in Snedecor ('56). Beginning at the upper left-hand corner of the table, the numbers were read in groups of three from left to right going from line 00, column 45-49 to line 01, column 00-04 and so on as in the typical English reading style. Where 1 or 2 numbers were left on a line, they were carried to the next line

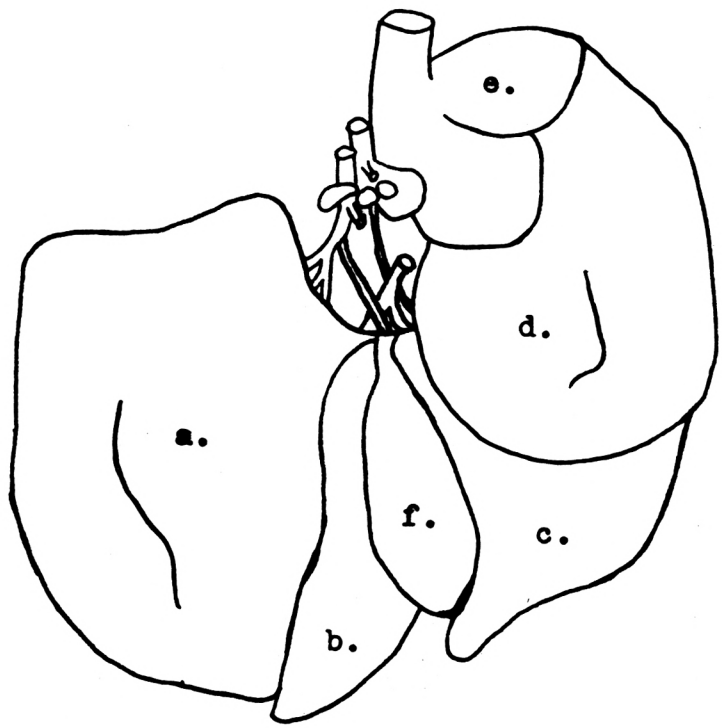

Fig. 1 Diagram of the pig liver showing the order in which the lobes were designated as 1, 2 and 3. a. Left lateral lobe, lobe 1; b.,c. Left and right central lobes, respectively, lobe 2; d. Right lateral lobe, lobe 3; e. Caudate lobe, not tested; f. Gall bladder.

and used to complete a group of three numbers. This procedure was continued until all samples were selected. There were 104 samples analyzed; 4 samples for each of the 26 pigs. The 4 samples, liver lobes 1, 2, 3 and the spleen, were listed in that order beginning with animal number 13 and progressing through number 38.

The first animal was given the numbers 000, 200, 400, 600 and 800 for lobe 1; numbers 001, 201, 401, 601 and 801 for lobe 2, etc. Each sample was given 5 numbers, the probability of finding a number associated with the sample was increased; thus abbreviating the time required to randomize the samples. Numbers 105-199, 305-399, 505-599, 705-799 and 905-999 were not used. The sample with the first acceptable number found in the table was given the number 1, meaning it would be analyzed first. The second number found was to be studied second, etc. until all were designated as to the order in which they were to be analyzed. Table 6 in the Appendix lists the specimens in the order of examination.

Method of Analysis

Sample Weights. A 2 X 2 cm square of glassine paper was weighed for each of the 4 copper standards, 4 reagent blanks, 1 reference blank and 6 samples to be used for each specimen. The 6 samples were cut at random from a frozen liver lobe or spleen to allow as unbiased and as complete sampling of the organ as possible. Each sample weighing between 0.12 and 0.16 g was placed on the previously weighed square of glassine paper

and weighed using an electric balance.

Chemical Analysis. All samples, blanks and standards were subjected to a wet ash digestion procedure. The analysis for copper was a modification of the method described by Rice and Grogan ('60) for the spectrophotometric determination of the metal using oxalyldihydrazide.

1. Digestion. One ml of copper standard was added to each of 4 calibrated 52 X 200 mm test tubes to which the weighed glassine paper had been added. One ml of deionized distilled water was added to each of the tubes containing the weighed tissue samples and to those designated as blanks. One ml of concentrated sulfuric acid followed by 1 ml of perchloric acid was pipetted into each tube. The tubes were placed in a sandbath where they were held at a high temperature until the solution was clear and colorless (2 1/2-3 hours). After clearing, the tubes were removed, covered with 2-inch squares of parafilm and allowed to cool.

2. Titration. The strongly acid solutions were diluted with 2 ml of deionized distilled water, allowed to cool and then a pinch of citric acid crystals and a 5 mm square of Congo Red indicator paper was placed in each tube. A pinch of ethylenediamine tetraacetic acid (EDTA) was added to the tube used as a reference blank. Each tube was titrated to the bright red of Congo Red indicator paper using concentrated ammonium hydroxide that had been saturated with oxalyldihydrazide.

3. Color production. One-half milliliter cold

acetaldehyde solution was added and the resulting solution was diluted with deionized distilled water to a 15 ml volume and mixed.

4. Determination of absorbancies. The tubes were allowed to stand at room temperature for at least 30 minutes and then the optical densities for each of the reagent blanks, copper standards and unknowns were read against the blank containing EDTA using a wave length of 542 mu. A Beckman DU spectrophotometer with 1 cm silica absorption cells was used for this purpose. The color is stable for at least several hours (Rice and Grogan, '60).

Maintenance of Glassware. With the exception of pipettes, glassware was washed with detergent and water, rinsed 8 times with tap water, rinsed 8 times with deionized distilled water and inverted to drain on a clean paper towel. The freshly cleaned glassware was covered with linen towels while drying. When dry, the glassware was covered with parafilm to protect it from contamination. After use, the pipettes were rinsed with tap water and placed in potassium dichromate cleaning solution for a few hours or overnight. They were removed, rinsed 8 times with tap water, 8 times with deionized distilled water and placed tip down on clean linen towels. A linen towel was used to protect them from contamination as they were allowed to drain.

Calculation. The following formula was used for the purpose of calculating the quantity of copper per gram of pork liver or spleen:

$$\frac{\dfrac{2 \text{ mcg (O.D. of unknown - mean O.D. of blank}}{\text{mean O.D. of standard - mean O.D. of blank}}}{\text{sample wt. (g)}} = \text{mcg/g tissue}$$

Statistical Analysis

All data were subjected to analysis of variance and least significant differences were calculated when appropriate. The analyses used were:

Source of variation	D/F
Liver:	
Lots	2
Lobes	2
Lots X lobes	4
Animals in the same lot	23
Samples	452
Total	483
Spleen:	
Lots	2
Animals in the same lot	22
Samples	127
Total	151

The F values were determined for the 3 lobes of the liver across the 3 lots.

RESULTS AND DISCUSSION

Liver Copper Levels

In studying the grand mean copper concentration levels in the livers (Table 2), it was determined that pigs receiving only the basal ration (Lot I) did not vary significantly from those fed copper and iron supplements in addition to the basal

TABLE 2

Mean values of copper concentrations in micrograms per gram and significance of F values for the livers of pigs on rations varying in iron and copper

Lot	Ration	Lobes			F[1]	Mean
		1	2	3		
		mcg	mcg	mcg		
I	Basal	8.041	8.603	8.434	ns	8.359
II	Basal plus iron	7.255	7.914	7.961	ns	7.710
III	Basal plus iron and copper	8.299	8.857	8.736	ns	8.631
F						***
Lsd						0.47-0.49[2]

[1] When correction was made for animal variation.

[2] Lot I 0.4682
Lot II 0.4682
Lot III 0.4890

ns Nonsignificant

*** Significant at the 0.1% level.

Lsd Least significant difference at the 5% level.

ration (Lot III). Copper concentration levels in the livers of pigs in both Lots I and III, however, were significantly different from those of pigs fed a basal ration plus an iron supplement (Lot II).

The basal ration fed to the pigs in Lot I contained 6.79 mg copper and 40.18 mg iron per lb of ration. This gave a ratio of copper to iron of 1:6. The pigs in Lot III received the basal ration plus copper and iron supplements which provided 30 mg copper and 300 mg iron per lb of ration, a 1:10 ratio. The copper:iron ratios of Lots I and III are similar, although the ration fed in Lot III contained 4.4 times more copper and 7.5 times more iron than the ration fed in Lot I. The pigs in Lot II received the basal diet plus an iron supplement, which provided 6.79 mg copper and 300 mg iron. The copper:iron ratio in this ration was 1:44.

In earlier analyses of the iron concentrations in the same organs analyzed in this experiment, the liver lobes of animals in Lot I showed significantly lower concentrations of iron than those in Lots II and III, whereas the latter lots did not differ significantly (Hunsader, unpublished data). Mean iron concentrations per gram tissue were: Lot I, 173.6 mcg; Lot II, 256.1 mcg; Lot III, 233.3 mcg. These data suggest that pigs receiving an iron supplement (661 ppm) show an overall increase in available tissue iron in the liver lobes, which may be attributed to increased absorption.

The absorption of copper and iron should be considered before attempting to relate the concentration of these dietary

elements to their concentrations in the liver and spleen. It is recognized that the amount of a mineral absorbed is not directly related to the quantity ingested. As mentioned by Gubler ('56b), there is an inverse correlation between the size of the dose and the percentage of the iron absorbed while absorption of copper is more directly proportional to the quantity ingested. However, total quantity of iron absorbed depends on the dose. These statements suggest that a definite increase can be expected in the quantity of either copper or iron absorbed where supplementation of the ration is accomplished using a relatively high concentration of the mineral.

In the experiment reported herein, rate of absorption is not known; however, the relation of the concentration of ingested mineral to its distribution and deposition in the animal tissue can be studied. When the ratios (Lot I, 1:6; Lot III, 1:10; Lot II, 1:44) were compared to the grand means of the copper concentrations of the livers in the 3 lots, it was concluded that where ingestion of iron alone is increased while copper ingestion remains at the previous lower level (Lot II), stored copper will be mobilized to meet the additional requirements for copper in the absorption, mobilization and utilization of the available iron, resulting in depletion of liver copper. If the ratio of copper to iron remains approximately constant as between rations fed in Lots I and III, then (at least within the limits of concentrations of copper and iron studied in this experiment) storage copper

levels in the liver will remain fairly constant.

Spleen Copper Levels

Unlike the concentrations of copper found in the liver, the group of pigs fed the basal diet (Lot I) differed significantly from those receiving a basal ration plus an iron supplement (Lot II) or a basal ration plus an iron and copper supplement (Lot III), Table 3. Levels of copper in the spleen (2-3 mcg/g) were much lower than those found in the liver lobes (7-9 mcg/g). The spleen is not considered a storage depot for copper (Gubler, '56a). It functions as a reservoir for erythrocytes, a storage depot for iron, a factor in hemolysis, and in some instances, functions as a center for blood cell formation (Cecil and Loeb, '56).

With an increase in dietary iron and, consequently, an increment in absorption, transportation and further metabolism of iron, an increase in copper requirement and copper concentration at the sites of active iron metabolism would be expected to occur. This may account for the higher levels of copper found in the spleens of pigs receiving either of the diets containing greater quantities of iron (Lots II and III).

The ingestion of the copper supplement by pigs in Lot III did not produce a significant variation in spleen copper concentration from that of Lot II. This fact suggests that copper at a level of approximately 2.9 mcg in the spleen is sufficient for the utilization of iron ingested at a concentration of 300 mg per day and absorbed at the rate

TABLE 3

Mean values of copper concentrations in micrograms per gram and significance of F values for spleens of pigs on rations varying in iron and copper

Lot	Ration	Mean
		mcg
I	Basal	2.345
II	Basal plus iron	2.915
III	Basal plus iron and copper	2.931
F		**
LSD		0.43-0.44[1]

[1] Lot I 0.4322
Lot II 0.4388
Lot III 0.4430

** Significant at the 1% level.

Lsd Least significant difference at the 5% level.

exhibited by the animals under the conditions of this experiment. Perhaps with increased dietary iron an increase in copper and iron concentrations in the spleen would be observed in further work since results obtained seem to indicate a direct relationship between copper and iron concentration in the spleen.

In analysis of this organ for iron levels by Hunsader (unpublished data), outstanding animal variations masked any differences between diet and concentration of iron in the spleen that might have existed.

Animal Variation

The wide variation that occurred among the animals throughout the experiment is shown by the high and low mean values for copper recorded for the liver lobes and spleens in each lot (Table 4) and for individual animals (Appendix, Table 7). In all lots, animal variation was shown to be very highly significant (Table 4). The fact that these pigs were crossbred York, Duroc and Poland China and included both sexes could account for some of the variation among the experimental animals.

Variation Among Liver Lobes

On preliminary examination, lobe 1 in each lot had a lower copper concentration than either of the other lobes in each of the 3 lots (Table 2). When correction for animal variation was made, however, no significance was found. The conclusion was

TABLE 4

High and low mean values within lots for copper levels in micrograms per gram of tissue and significance of F values for animals within lots for liver and spleen of pigs fed varying levels of iron and copper

Lot	Ration	Liver lobes			Spleen
		1	2	3	
I	Basal	5.4-12.2	5.0-13.9	6.4-12.4	1.4-3.5
II	Basal plus iron	5.8- 8.7	4.0-10.1	4.5- 9.9	1.6-4.7
III	Basal plus iron and copper	6.4-10.6	6.2-10.9	5.9-11.8	1.5-5.1
F			***		***

*** Significant at the 0.1% level.

drawn, therefore, that the copper stored in the liver was fairly evenly distributed among the 3 lobes.

SUMMARY

Twenty-six crossbred York, Duroc and Poland China pigs were divided into 3 lots at weaning time. Nine pigs in Lot I were fed a basal diet, whereas 9 pigs in Lot II received a basal diet supplemented with iron (661.3 ppm) and 8 pigs in Lot III were given a basal diet supplemented with iron (661.3 ppm) and copper (66.1 ppm). At a weight of approximately 210 lbs, the pigs were slaughtered and specimens removed from each of 3 liver lobes and the spleen for copper analysis.

Copper concentration levels in the livers of the pigs did not vary significantly between Lots I and III. However, copper concentration levels in Lot II were significantly lower from those of the other 2 lots. Ratios of copper:iron ingested for the 3 lots were: Lot I, 1:6; Lot II, 1:44; Lot III, 1:10. The average copper concentrations found for livers in the 3 lots were: Lot I, 8.4 mcg/g; Lot II, 7.7 mcg/g; Lot III, 8.6 mcg/g.

When the ratios of the metals ingested were compared to the levels demonstrated in the livers, it was concluded that, within limits, where the ratio of copper to iron remains relatively constant (approximately 1:10) copper storage in the liver will remain fairly constant. Increased ingestion of iron without an increase in copper in the diet as in Lot II resulted in a decrease in copper concentration in the liver tissue.

Spleen copper concentrations were similar for animals in

Lots II (2.915 mcg/g) and III (2.931 mcg/g). The levels in the spleens of animals in Lot I (2.3 mcg/g) were significantly different from those of pigs in Lots II and III. It was suggested that an increase in dietary iron in Lots II and III increased the copper requirement and, thus, the copper concentration in the spleen which is known to be a site of active iron metabolism. Mean levels of copper in the spleen in both Lots II and III suggest that a level of approximately 2.9 mcg/g in the tissue of the spleen is sufficient for the utilization of iron ingested at a concentration of 300 mg per day.

Copper levels in the liver lobes were found to be much higher than those of the spleen in all animals studied. Mean liver copper levels ranged from 7.7 mcg to 8.6 mcg per gram of tissue; copper levels in the spleen ranged from 2.3 mcg to 2.9 mcg per gram of tissue. When data were corrected for animal variation, there were no significant differences in copper concentrations among the lobes of the liver. These findings indicate a fairly even distribution of copper among the 3 liver lobes. A wide variation in copper concentration of both liver and spleen was observed among animals throughout the experiment.

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APPENDIX

TABLE 5

Feedlot and carcass data for each animal

Animal number	Sex[a]	Initial weight (lbs)	Slaughter weight (lbs)	Slaughter date	Days on feed	Average daily gain	US carcass grade
Lot I							
13	G	54.5	213	1/19	74	2.14	2
14	B	48.0	217	2/1	87	2.06	1
15	B	40.5	216	2/10	96	1.83	2
16	G	45.0	210	2/12	98	1.68	1
17	G	45.0	211	2/12	98	1.69	1
18	G	47.0	210	2/12	98	1.66	2
19	G	47.0	207	1/12	98	1.63	1
20	B	40.0	210	2/17	103	1.65	1
21	G	47.0	198	3/8	122	1.32	1
Average:		46.0	210		97	1.74 lbs/day	
Feed efficiency:		3.37 lbs feed/lb gain					
Lot II							
22	G	62.0	207	1/19	74	1.96	2
23	B	51.5	216	2/1	87	2.00	2

24	G	47.0	214	2/10	96	1.74	1
25	B	32.0	209	2/12	98	1.81	2
26	B	44.0	214	2/12	98	1.73	2
27	G	50.0	211	2/19	105	1.53	1
28	G	43.0	206	2/22	108	1.51	1
29	G	41.5	206	2/22	108	1.52	1
30	G	40.0	192	2/23	108	1.53	1
Average:		45.7	208			1.70 lbs/day	
Feed efficiency:	3.19 lbs feed/lb gain						
Lot III							
31	B	57.5	210	1/19	74	2.06	2
32	G	52.5	207	2/1	87	1.78	2
33	G	48.5	217	2/1	87	1.94	2
34	G	47.5	210	2/4	90	1.80	2
35	B	37.0	207	2/4	90	1.89	2
36	B	35.0	211	2/10	96	1.83	2
37	G	44.0	210	2/10	96	1.73	1
38	G	44.0	205	2/29	115	1.40	1
Average:		45.8	210		92	1.80 lbs/day	
Feed efficiency:	3.42 lbs feed/lb gain						

[a] G, gilts; B, barrows.

TABLE 6

Design for analysis of specimens

Number			Number			Number		
Random	Animal	Lobe **	Random	Animal	Lobe **	Random	Animal	Lobe **
1	21	1	36	25	1	71	32	1
2*	24	-	37	13	1	72	19	-
3	29	2	38	37	2	73	15	-
4	35	3	39	21	-	74	21	3
5	27	1	40	30	3	75	38	2
6	27	3	41	14	3	76	31	3
7	33	2	42	25	2	77	13	3
8	28	2	43	17	2	78	36	3
9	14	2	44*	31	1	79	37	3
10	38	-	45	29	-	80	23	2
11	23	3	46	33	1	81	27	2
12	37	-	47	33	-	82	28	1
13	34	3	48	35	-	83	20	1
14	36	2	49	24	3	84	16	1
15	27	-	50	22	-	85	28	-
16	16	3	51	19	1	86	36	-
17	31	-	52	15	2	87	23	-
18	33	3	53	20	2	88	21	2
19	18	1	54	32	2	89	15	1
20	32	3	55	24	1	90	14	-
21	20	3	56	15	3	91	22	3
22	37	1	57	35	2	92	34	2
23	13	2	58	19	2	93	30	2
24	29	3	59	35	1	94	26	-
25	30	1	60	31	2	95	38	1
26	18	2	61	16	2	96	18	3
27	22	1	62	14	1	97	26	3
28	17	1	63	26	2	98	24	2
29	28	3	64	29	1	99	19	3
30	17	-	65	13	-	100	30	-
31	32	-	66	20	-	101	25	-
32	34	1	67	18	-	102	26	1
33	38	3	68	16	-	103	22	2
34	34	-	69	17	3	104	23	1
35	25	3	70	36	1			

* Specimen was lost.
** Animal number without lobe number indicates the spleen.

Ranges and means of copper in micrograms per gram for the
of various levels

Lot and animal number	Liver					
	Lobe 1			Lobe 2		
	Determination	Range	Mean	Determination	Range	Mean
	no.	mcg	mcg	no.	mcg	mcg
Lot I						
13	6	5.0- 5.8	5.4	6	6.4- 8.7	7.1
14	12	7.3-11.8	9.4	6	7.1-17.4	10.3
15	6	6.8- 9.6	8.4	6	7.8-12.9	10.0
16	6	5.5- 9.2	7.4	6	8.1-11.3	9.8
17	6	5.2- 7.1	6.1	6	6.9- 9.8	8.0
18	5	4.8- 7.3	6.3	6	4.2- 7.2	5.0
19	6	8.2-10.5	9.7	6	11.7-16.1	13.9
20	6	6.3- 8.3	7.5	6	5.1-10.2	6.7
21	10	6.0-21.1	12.2	9	6.9- 9.2	6.7
Lot II						
22	6	4.8- 7.3	6.1	5	5.6- 7.7	6.9
23	5	7.0- 9.5	8.2	5	8.7-10.8	9.7
24	6	5.8- 7.4	6.8	6	6.3- 9.3	7.6
25	10	6.8-11.1	8.7	6	3.1- 5.6	4.0
26	6	4.9- 6.9	5.8	6	6.1- 9.2	7.6
27	5	5.6- 8.0	7.2	6	5.7- 8.8	7.3
28	9	7.3-10.9	8.6	6	6.3-18.9	10.1
29	5	5.9- 8.8	7.3	11	5.0-13.9	9.8
30	6	5.7- 7.8	6.6	6	7.1-10.4	8.3

LE 7

lobes of the liver and the spleen of individual pigs on diets of iron and copper

Liver				Spleen		
Lobe 3			Grand mean			
Determination	Range	Mean		Determination	Range	Mean
no.	mcg	mcg	mcg	no.	mcg	mcg
6	6.1- 7.9	6.7	6.4	6	0.4-5.9	2.6
6	4.9- 8.0	6.4	8.7	5	2.4-4.5	3.5
5	6.0- 9.2	8.2	8.9	6	0.9-2.4	1.4
5	9.5-12.5	9.2	8.8	5	3.0-4.0	3.5
5	6.1- 9.1	6.3	6.8	6	1.6-2.3	1.8
6	9.1-12.1	10.2	7.2	6	1.4-4.0	2.9
6	9.8-14.3	12.4	12.0	6	0.0-2.0	1.0
6	5.8- 7.1	6.4	6.9	5	2.5-3.2	2.8
5	7.0-14.2	10.0	9.6	6	0.3-3.0	1.5
6	7.1-13.5	10.0	7.7	6	1.5-3.5	2.9
5	8.2-11.1	9.6	9.2	6	2.4-3.7	2.9
6	6.3-10.0	8.7	7.7	-	---	--*
10	4.6- 9.2	7.1	6.6	6	0.9-3.1	1.6
5	5.0-11.4	8.8	7.4	6	2.6-7.0	4.7
6	6.4-12.0	8.8	7.8	6	1.8-2.9	2.3
6	5.6- 8.0	6.8	8.5	6	1.6-5.4	3.9
6	3.2- 7.0	4.5	7.2	6	2.4-3.4	2.9
5	7.0- 7.8	7.3	7.4	6	1.1-2.7	2.2

TABLE 7

Lot and animal number	Liver					
	Lobe 1			Lobe 2		
	Determination	Range	Mean	Determination	Range	Mean
	no.	mcg	mcg	no.	mcg	mcg
Lot III						
31	--	---	---*	5	8.0- 8.7	8.3
32	6	5.0- 7.6	6.4	6	7.4-10.3	9.2
33	11	6.1-10.7	7.2	6	7.8-19.3	10.8
34	6	6.9- 9.0	8.1	6	8.6-12.8	10.9
35	6	7.1- 8.8	7.8	6	6.1- 7.5	6.8
36	6	8.4-12.0	10.1	6	8.5-13.5	10.9
37	6	5.4- 9.4	7.8	6	4.9- 8.0	6.2
38	6	8.2-13.5	10.6	6	5.3-10.2	7.8

* Specimen was lost.

(concl.)

Liver			Grand mean	Spleen		
Lobe 3						
Determination	Range	Mean		Determination	Range	Mean
no.	mcg	mcg	mcg	no.	mcg	mcg
10	6.0-10.1	7.6	8.0	5	2.0-6.8	3.6
5	5.9- 7.0	6.5	7.4	6	0.6-2.4	1.6
6	7.8- 9.2	8.4	8.8	6	2.2-6.1	3.6
6	10.0-16.5	11.8	10.0	12	2.2-7.5	4.1
6	8.0-10.6	9.5	8.0	6	0.6-2.2	1.5
5	9.0-11.8	10.6	10.5	6	1.5-4.3	2.5
6	8.7-10.7	9.5	7.8	6	0.4-3.4	1.5
6	5.2- 6.5	5.9	8.1	6	3.8-7.1	5.1

THE EFFECT OF SUPPLEMENTATION OF A BASAL PIG RATION WITH IRON AND COPPER ON THE COPPER LEVEL OF THE LIVER AND SPLEEN

by

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Twenty-six crossbred York, Duroc and Poland China pigs were divided into 3 lots at weaning time. Nine pigs in Lot I were fed a basal diet, whereas 9 pigs in Lot II received a basal diet supplemented with iron (661.3 ppm) and 8 pigs in Lot III were given a basal diet supplemented with iron (661.3 ppm) and copper (66.1 ppm). At a weight of approximately 210 lbs, the pigs were slaughtered and specimens removed from each of 3 liver lobes and the spleen for copper analysis.

Copper concentration levels in the livers of the pigs did not vary significantly between Lots I and III. However, copper concentration levels in Lot II were significantly lower from those of the other 2 lots. Ratios of copper:iron ingested for the 3 lots were: Lot I, 1:6; Lot II, 1:44; Lot III, 1:10. The average copper concentrations found for livers in the 3 lots were: Lot I, 8.4 mcg/g; Lot II, 7.7 mcg/g; Lot III, 8.6 mcg/g.

When the ratios of the metals ingested were compared to the levels demonstrated in the livers, it was concluded that, within limits, where the ratio of copper to iron remains relatively constant (approximately 1:10) copper storage in the liver will remain fairly constant. Increased ingestion of iron without an increase in copper in the diet as in Lot II resulted in a decrease in copper concentration in the liver tissue.

Spleen copper concentrations were similar for animals in Lots II (2.915 mcg/g) and III (2.931 mcg/g). The levels in the spleens of animals in Lot I (2.3 mcg/g) were significantly different from those of pigs in Lots II and III. It was suggested that an increase in dietary iron in Lots II and III

increased the copper requirement and, thus, the copper concentration in the spleen which is known to be a site of active iron metabolism. Mean levels of copper in the spleen in both Lots II and III suggest that a level of approximately 2.9 mcg/g in the tissue of the spleen is sufficient for the utilization of iron ingested at a concentration of 300 mg per day.

Copper levels in the liver lobes were found to be much higher than those of the spleen in all animals studied. Mean liver copper levels ranged from 7.7 mcg to 8.6 mcg per gram of tissue; copper levels in the spleen ranged from 2.3 mcg to 2.9 mcg per gram of tissue. When data were corrected for animal variation, there were no significant differences in copper concentrations among the lobes of the liver. These findings indicate a fairly even distribution of copper among the 3 liver lobes. A wide variation in copper concentration of both liver and spleen was observed among animals throughout the experiment.