DIETARY COPPER: ITS RELATIONSHIP TO ZINC AND PARAKERATOSIS IN THE PIG

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Harlan D. Ritchie
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This is to certify that the

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DIETARY COPPER: ITS RELATIONSHIP TO ZINC AND PARAKERATOSIS IN THE PIG

presented by

Harlan D. Ritchie

has been accepted towards fulfillment
of the requirements for

Ph D degree in Animal Husbandry

J. A. Hoefer
Major professor

Date February 20, 1964

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ABSTRACT

DIETARY COPPER: ITS RELATIONSHIP
TO ZINC AND PARAKERATOSIS IN THE PIG

by Harlan D. Ritchie

Five experiments, involving a total of nine trials and 438 weanling pigs, were conducted to investigate relationships between porcine parakeratosis and various dietary trace elements, particularly copper and zinc. Interrelationships between these two elements and iron were also studied. Copper was added at two levels (125 and 250 ppm) and in two forms (sulfate and oxide) to diets which varied in calcium and zinc content. Copper was also compared with a broad-spectrum antibiotic as a dietary growth stimulant.

In the first experiment, parakeratosis occurred on low-zinc basal diets (28-39 ppm Zn) at three levels of calcium intake (0.55, 1.05, and 1.31% Ca). Compared to healthy pigs, parakeratotic animals were characterized in most instances by depressed growth rate, higher serum gamma globulin fraction, lower serum albumin fraction, and lower serum alkaline phosphatase activity, in addition to skin lesions typical of this syndrome. Lower hemoglobin and hematocrit, and higher total serum protein were also noted in several parakeratotic lots. Zinc supplementation (50 or 75 ppm) was completely effective in preventing parakeratosis; copper (125 ppm) was completely effective at 0.55 and 1.05% calcium levels and partially effective at 1.31% level; iron (100 ppm) was partially effective at lowest level but was ineffective at a higher calcium level. Copper-fed pigs exhibited significantly higher liver

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copper concentration than control pigs. When zinc was fed in combination with copper, storage of copper in the liver was reduced to that of the basal level.

In the second experiment, two trials were conducted to study effect of adding two levels of copper sulfate (125 and 250 ppm Cu), with or without supplemental zinc (100 ppm), to high-calcium, low-zinc basal diets. In each trial, the parakeratotic basal lot was divided after several weeks to study effects of copper therapy. In one trial, the higher copper level was almost completely effective in preventing parakeratosis, while in the other it was only partially effective; the lower level was partially effective in both trials. Copper toxicity occurred at the 250 ppm level, but there was little or no evidence of it at the lower copper level. Supplemental zinc profoundly reduced liver copper levels and appeared to furnish complete protection against copper toxicity. Supplemental copper significantly lowered liver iron storage, but this effect was overcome when zinc was added to the ration. Copper had little influence on liver zinc stores. Either 125 or 250 ppm of dietary copper therapy in the basal lots brought about a marked recovery from parakeratosis.

Copper sulfate (125 ppm Cu) and chlortetracycline (10 mg./lb.) were added to a normal-calcium (0.63%) basal diet in the third experiment. Neither supplement alone accomplished a significant increase in growth rate. However, joint supplementation resulted in a significant response ($P<.05$) in daily gain. There were no significant differences in hematology.

In the fourth experiment, high-level (250 ppm Cu) copper sulfate

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or copper oxide was added to a normal-calcium diet, with and without supplemental zinc. There were no significant differences in performance or hematology. No clinical symptoms of copper toxicity were noted, but there was a high incidence (90%) of cirrhotic livers in pigs fed copper sulfate with no added zinc; a much lower incidence (30%) was observed in those fed copper oxide alone. Copper oxide-fed pigs stored significantly less liver copper than those fed copper sulfate. Both forms significantly reduced liver iron storage, copper sulfate having a greater effect than copper oxide. Neither form significantly increased loin copper levels.

Two trials were conducted in the fifth experiment to test the hypothesis that parakeratosis-producing diets inhibit activity of phytase or phosphatase in the intestine of the pig. Results were highly variable; in only one instance was there a significant difference favoring acceptance of the hypothesis.

DIETARY COPPER: ITS RELATIONSHIP TO ZINC AND PARAKERATOSIS IN THE PIG

By

Harlan D. Ritchie

A THESIS

Submitted to
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in partial fulfillment of the requirements
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Department of Animal Husbandry

1964

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Haute, Ind., and American Cyanamid Company, Pearl River, N. Y., are acknowledged for their generous supply of certain ration components.

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Harlan D. Ritchie

candidate for the degree of

Doctor of Philosophy

DISSERTATION: Dietary Copper: Its Relationship to Zinc and Parakeratosis in the Pig

OUTLINE OF STUDIES:

Main Area: Animal Husbandry (Animal Nutrition)

Minor Areas: Biochemistry, Physiology

BIOGRAPHICAL ITEMS:

Born: August 3, 1935; Albert City, Iowa

Undergraduate studies: Iowa State University, 1953-1957

Graduate studies: Michigan State University, 1957-1964

EXPERIENCE:

Assistant Instructor, Michigan State University, 1957-1964

MEMBER:

American Society of Animal Science

Society of Sigma Xi

Alpha Zeta

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I. INTRODUCTION

Recent years have seen an expanding interest in the role of trace elements in animal nutrition, both in this country and abroad. Most of the trace element research has involved two approaches: (1) studies of the effects on animals of basal diets in which the element under investigation is absent or present in abnormal or (2) applied studies of naturally occurring animal diseases that were found to be caused in part or *in toto* by a deficiency or an excess of one or more trace elements.

Much of the attention in monogastric species, particularly rats and swine, has been focused on three elements--iron, copper, and zinc. This thesis comprises a study of these elements, especially the latter two, in the nutrition of the growing pig.

The Wisconsin research group was the first to demonstrate that copper and zinc are dietary essentials for the rat (Hart *et al.*, 1928; Todd *et al.*, 1934). These findings were subsequently extended to other species.

Teague and Carpenter (1951) demonstrated the need for a small amount of copper in the diet of the pig and described symptoms of copper deficiency. Tucker and Salmon (1955) were the first to show that porcine parakeratosis could be prevented or cured by adding zinc to the ration. It was also reported by these and many other investigators that high levels of dietary calcium aggravate the parakeratotic condition; however, the mechanism involved has not been satisfactorily elucidated.

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As a result of the work of Barber and his colleagues (Barber et al., 1955a,b,c, 1957, 1960; Bowler et al., 1955) and Lucas and Calder (1957a,b), feeding of copper salts to growing swine has become rather widespread throughout Great Britain. These investigators found that a high level of copper--equivalent to many times the published dietary requirement--added to pig rations, improved performance to much the same extent as an antibiotic. Conversely, in this country Wallace et al. (1960) reported that levels of copper sulfate similar to those used in the British studies depressed growth rate and produced copper toxicity. It was discovered in one trial, however, that supplemental copper was effective in preventing parakeratosis. This effect of copper on parakeratosis has also been reported by Hoefer et al. (1960). Other workers have found copper to have little or no effect in preventing this disease (Hennig, 1960; O'Hara et al., 1960; Priebe et al., 1961; Smith et al., 1962), although Bunch et al. (1963) have reported that zinc may be necessary for maximum response to high levels of copper.

In constrast to the negative results of the Florida station, Iowa investigators have found 250 ppm copper as copper sulfate or copper oxide to stimulate rate of gain and feed conversion during the growing period, with no gross symptoms of toxicity (Hawbaker et al., 1961; Bunch et al., 1961, 1963). However, copper sulfate gave no response after 125 lb. live weight, and, compared to copper oxide, resulted in lower hemoglobin values and a much higher storage of liver copper. Variable results were obtained with copper carbonate (Bunch et al., 1962).

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Coincident with the increased feeding of copper sulfate to pigs in the British Commonwealth, there have been several published reports of copper toxicity (Gordon and Luke, 1957; O'Hara et al., 1960; Buntain, 1961; Allcroft et al., 1961; Allen and Harding, 1962). The severity of the symptoms tends to be rather closely related to the level of copper in the tissues, especially the liver. In this respect, it is interesting to note that the liver copper values reported in these papers are considerably higher than those in the Iowa work, where no toxicity was found.

A number of experiments in this country have recently brought to light another possible causative factor in the parakeratosis syndrome, namely, phytic acid (Plumlee et al., 1960; Green et al., 1961; Oberleas et al., 1961, 1962a,b; Smith et al., 1962). It has been demonstrated that the phytic acid in soybean meal diets renders the zinc in these diets less available. Using the results of an in vitro study as evidence, Missouri researchers (Oberleas et al., 1962b) have postulated that the action of phytate on zinc availability takes place in the gastrointestinal tract, but this has not been confirmed elsewhere. These workers have also suggested that the deleterious effect of high dietary calcium on zinc utilization is mediated through the phytic acid content of the diet.

The work presented in this dissertation was initiated to: (1) determine the effects of supplemental copper fed at various levels and in different forms to growing swine and its interaction with other elements, namely, zinc, calcium, and iron; (2) elucidate the role of

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calcium and its relationship to zinc and copper in porcine parakeratosis. In order to attain these objectives, various physiological, biochemical, and pathological observations were made, in addition to collecting growth and dietary intake data. The reports of Hoefer et al. (1960) and Ritchie et al. (1961, 1962, 1963) comprise portion of this thesis.

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II. REVIEW OF LITERATURE

Biochemical Role and Metabolism of Copper

The first conclusive evidence that copper is an essential dietary nutrient for animals emerged from the classical studies of Hart et al. (1928). This group of investigators demonstrated that it was necessary to supplement highly purified iron salts with copper in order to restore normal levels of hemoglobin in the blood of anemic rats. The fact that copper is in some way essential for normal hematopoiesis in a number of species was subsequently verified by further work at Wisconsin and elsewhere (Elvehjem, 1935). This discovery stimulated research on the part of many workers who have attempted to define the biological role of copper at the cellular level. These studies have led to the isolation of several copper protein compounds some of which have been shown to be enzymes with oxidative activity.

Holmberg and Laurell (1948) isolated, crystallized, and characterized a plasma copper protein named ceruloplasmin which normally constitutes a high proportion of the plasma copper of swine, rats, dogs, and humans (Cartwright et al., 1950; Wintrobe et al., 1953). This compound is an α_2-globulin; it contains about 0.3% copper which corresponds to eight atoms of copper per molecule. Ceruloplasmin is a true oxidase but its physiological role as such has not been elucidated. The copper of ceruloplasmin is tightly bound to the globulin moiety. Scheinberg and Sternlieb (1960), in their compre-

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hensive review of copper metabolism, point out that most investigators agree that there is a small proportion of plasma copper which is not a part of ceruloplasmin. This is present as free cupric ion which is in equilibrium with copper loosely bound to albumin. When copper first enters the plasma from the gastrointestinal tract it is probably in this form. Thereafter, it is transferred progressively, presumably in the liver (Lang and Renschler, 1958), to ceruloplasmin (Bearn and Kunkel, 1954). The loosely bound copper is probably that portion in transport (Gubler, 1956), for, unlike ceruloplasmin copper, it can diffuse freely across semipermeable membranes.

Copper in red blood cells is found in at least two forms. A minute portion is in diffusion-equilibrium with plasma (Bush et al., 1956a). The major portion is not diffusible and is tightly bound to a copper protein of red cells, erythrocuprein (Markowitz et al., 1959) or hemocuprein (Mann and Keilin, 1938). Red cell copper is less labile than plasma copper, the latter being a more reliable indicator of changes in the copper status of an animal than whole blood copper (Underwood, 1962). In the copper-deficient pig, the fall in plasma copper is greater than the fall in red cell copper (Wintrobe et al., 1953). The physiological function of red cell copper remains unknown. Unlike ceruloplasmin it has no oxidase activity.

Cytochrome oxidase is a well-known component of the electron transfer system, which is concerned with cellular oxidation (Keilin and Hartree, 1939). Wainio et al. (1959) have rather definitely demonstrated that this protein is a copper-containing enzyme. Moreover, there is considerable evidence showing an early and marked loss of cytochrome

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oxidase activity in the tissues of the copper-deficient rat, pig, and chick (Schultze, 1939, 1941; Gubler et al., 1957; Gallagher et al., 1956a). The latter group has reported evidence which indicates that the loss of cytochrome oxidase activity results from a failure of synthesis of its prosthetic group, heme a, rather than of its protein component. Underwood (1962) believes that the synthesis of the heme group of cytochrome oxidase must be regarded as one of the basic functions of copper, unrelated to a general suppression of iron metabolism. There is no evidence that the role of copper in erythrogenesis is related to this particular function.

Tyrosinase is a mammalian copper-containing metalloenzyme which exhibits oxidase activity (Brown and Ward, 1959). It is thought to be involved in the conversion of tyrosine to melanin, a pigment of the integument. Albino humans, for example, possess no detectable tyrosinase activity (Harris, 1959). Furthermore, achromatrichia has been observed in the copper-deficient rat, rabbit, cat, dog, goat, sheep, and cattle, but not in the pig (Underwood, 1962).

Porter and Folch (1957) have isolated a copper protein named cerebrocuprein from bovine and human brains. Its physiologic function, if any, is unknown.

The functions of hepatocuprein (Mann and Keilin, 1938) and a copper protein isolated from horse liver (Mohamed and Greenberg, 1954) are unknown. It is highly possible that these are the same protein.

Uricase catalyzes the oxidation of uric acid to allantoin. A porcine hepatic uricase was isolated by Mahler et al. (1955), who reported that its activity appears to be related to the presence of copper

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in the enzyme. Nevertheless some doubt persists that uricase is a cuproenzyme.

Butyryl coenzyme A dehydrogenase and δ-aminolevulinic acid dehydrase had been previously reported as being copper proteins but subsequent work refuted the earlier observations (Scheinberg and Sternlieb, 1960). In this case, copper was apparently a contaminant.

The activity of catalase, the hemin-protein which catalyzes the decomposition of hydrogen peroxide, has been reported by Gubler et al. (1957) to be lowered in the liver of copper-deficient pigs; but other researchers have not found a consistent reduction in catalase activity.

The specific role of copper in hematopoiesis and erythrogenesis has not been satisfactorily resolved. Elvehjem (1935) concluded that copper is not concerned with assimilation of iron but with transformation of ingested iron into hemoglobin. Schultze (1940) also indicated that copper is not necessary for absorption and storage but is needed for utilization of iron by the blood-forming organs and for mobilization of iron from the tissues. However, in a more recent study (Bush et al., 1956b), it was observed that the transport and mobilization of iron was not impaired in copper-deficient pigs but that there was a reduced ability to absorb iron from the gastrointestinal tract. In his review, Matrone (1960) maintains that the effect of copper on iron absorption, in the rat at least, is largely indirect. He suggests that hemoglobin synthesis is blocked in copper deficiency so that absorbed iron can only enter the tissue iron pool, which eventually sets up a "mucosal block," decreasing iron absorption.

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formation has been extremely elusive. Iodice et al. (1958) suggested that the anemia of copper deficiency may be related to a reduction in the activity of δ-aminolevulinic acid dehydrase, which is involved in the biosynthesis of hemoglobin and which they showed to be a copper-containing enzyme. However, as mentioned previously, copper content was subsequently shown to be unrelated to the activity of this enzyme. Copper is apparently not necessary for protoporphyrin synthesis, since the free erythrocyte protoporphyrin of copper-deficient sheep is actually increased (Allen, 1956). Anderson and Tove (1958) reported that there is a lowered incorporation in vitro of radioglycine into heme in the blood of copper-deficient chicks; the addition of copper restores the incorporation of glycine toward normal. Conversely, copper deficiency in swine is associated with a decrease in the concentration of hemin-proteins only when an increased requirement is evident (Gubler et al., 1957). For example, hemoglobin levels may be reduced as a result of the inability of the hematopoietic tissues to keep up with the increase demanded by a shortened red cell survival time (Bush et al., 1956b). The latter workers suggest that copper is an essential component of adult red cells and that a certain minimum of copper must be available both for their production and for the maintenance of their integrity in the circulation.

Underwood (1962) states that "the evidence so far available is therefore inadequate to implicate copper unequivocally with any stage in the biosynthesis of hemoglobin and that the anemia of copper deficiency must be explained, for the time being, on the basis of impaired erythrocyte maturation and a reduced survival time of the

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Copper apparently plays a role in normal bone development but the mechanism is not understood. Teague and Carpenter (1951) and Follis et al. (1955) have reported that young pigs deprived of copper exhibit rather severe deformities of the limbs, characterized by excessively flexed hocks and crooked forelegs. Baxter and Van Wyk (1953) have noted bone deformities and fractures in young copper-deficient dogs. The bones show abnormally thin cortices, deficient trabeculae, and wide epiphyses, while serum calcium and phosphorus levels are normal. Spontaneous fractures and osteoporosis have been observed in copper-deficient cattle and sheep (Cunningham, 1950). It has been suggested, without proof, that the bone abnormalities in copper deficiency result from excessive resorption of bone or decreased deposition of bone matrix, or both (Adelstein and Vallee, 1962).

In studies designed to measure defects in synthetic processes occurring in copper deficiency, Gallagher et al. (1956b) demonstrated that a deficiency in phosphatidic acids in copper-deficient rats was probably due to a lowered capacity for coupling coenzyme A-activated fatty acids to glycerophosphate. They suggested that this discovery may be important in understanding the pathogenesis of the demyelinating disease, "swayback" (neonatal enzootic ataxia), which is seen in newborn lambs dropped by ewes which are copper-deficient (Cunningham, 1950). If this defect in phospholipid synthesis had occurred in utero at a time when myelin, which is rich in phospholipid, was being formed, demyelination might have resulted. In addition, deficiency in cytochrome oxidase activity may be involved in producing demyelination,

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which can be caused by agents, such as potassium cyanide, that are known to inhibit cytochrome oxidase. A disease similar to enzootic ataxia has been observed in pigs and calves (Cunningham, 1950).

In sheep suffering from naturally occurring copper deficiency, a defect in wool-keratin has been noted (Underwood, 1962). This has been thought to be related to an abnormality in the cross-linkages of keratin which normally occur through disulfide bridges.

Balance studies utilizing radiocopper have been valuable in tracing absorptive and metabolic movements of copper; but little is known regarding the biochemical mechanisms involved in these movements. The magnitude of copper absorption in the ruminant has been estimated by Comar et al. (1948). Within 5 days after administration, 75% of an oral dose of Cu^{64} was found in the feces and 5% in the urine, while 3% of an intravenous dose was found in the feces and 3% in the urine. In a reivew of all species by Underwood (1962), it was reported that under normal conditions 90% or more of ingested copper appears in the feces; although most of this consists of unabsorbed copper, active excretion via the bile occurs in all species. Likewise, with sheep, Lassiter and Bell (1960) found about 90% of the Cu^{64} eliminated through the feces and only 0.5-1.0% in the urine 96 hours following oral dosing. Studies with swine have been somewhat variable. In young growing pigs, Buescher et al. (1961) reported that within 96 hours after an oral dose of Cu^{64} labelled cupric sulfate, 64% of the dose was excreted via the feces, 1.4% by way of the urine, and 34% was retained. However, Bowland et al. (1961) found that only 5% of an oral dose of labelled cupric sulfate was absorbed in young pigs. It was calculated by these workers that the

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bile could account for up to 40% of the excretion; only a small fraction of the total excretion occurred in the urine. They observed that the liver was the major site of storage, containing 44-51% of the total dose of labelled copper 24 hours after intravenous injection. According to Saltman et al. (1959), it appears that there are specific copper-binding sites on or in the parenchymal cells of the liver, and the accumulation of copper is not directly coupled to metabolic energy or affected by sulfhydryl activators or inhibitors.

Biochemical Role and Metabolism of Zinc

Zinc was first demonstrated to be an essential nutrient for living organisms when Raulin (1869) showed that it is necessary for the growth of Aspergillus niger. It was not until 1934 that convincing evidence was reported concerning the need for zinc for the normal growth and development of mice (Bertrand and Bhattacherjee, 1934) and rats (Todd et al., 1934). With the improvement of the purified diet technique for studying relatively uncomplicated zinc deficiency, the importance of zinc for the normal development of these species became even more apparent. In 1955, Tucker and Salmon showed that zinc deficiency is the basis of the syndrome in swine known as parakeratosis. Following this significant discovery, O'Dell et al. (1958) reported that zinc is essential for growth and normal development of birds.

Keilin and Mann (1940) presented the first evidence of a fundamental role for zinc in metabolism. They showed that this element is a constituent of the enzyme carbonic anhydrase, which catalyzes the reversible reaction between carbon dioxide and water. Zinc was shown to

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make up 0.33% of the enzyme molecule and was found to be essential for its activity. Since that time, zinc has been found to exist in several other highly purified enzyme systems.

Vallee and Neurath (1954, 1955) have demonstrated that zinc is an integral, structural part of the pancreatic carboxypeptidase molecule; it appears that one atom of zinc is firmly bound to one molecule of enzyme protein.

Alcohol dehydrogenase of yeast (Vallee and Hoch, 1955a,b) and of equine liver (Theorell et al., 1955; Vallee and Hoch, 1956) was found to contain four and two moles of zinc per mole of protein, respectively. Furthermore, it is thought that each zinc atom serves as a locus of reversible attachment for a molecule of the coenzyme, DPN (Hayes and Velick, 1954).

Glutamic dehydrogenase, which catalyzes the reversible oxidative deamination of glutamate, was reported by Adelstein (1957) to be a zinc metalloenzyme. The average zinc content was 3.4 ± 1.0 atoms per molecule. Like alcohol dehydrogenase, it appears to bind one DPN molecule per atom of zinc.

Further studies by Vallee et al. (1956) indicated that other pyridine nucleotide dehydrogenases may prove to be zinc metalloenzymes.

Mathies (1958) has established that alkaline phosphatase from swine kidneys is a zinc enzyme. The best preparations contained 0.15% of firmly bound zinc. It was surmised that one molecule of the enzyme contains several atoms of zinc.

The isolation of a zinc-dependent hexokinase from Neurospora crassa led to the suggestion that this is a zinc metalloenzyme (Medina

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and Nicholas, 1957). However, the criteria for this conclusion were not identical with those employed to evaluate the zinc metalloenzymes described above.

In addition to the metalloenzymes, there are several other enzymes whose activity is increased by the addition of zinc ions (Vallee, 1959). Most of them lack specificity as evidenced by the fact that they are activated by other ions also. Some of these zinc enzyme complexes are: arginase, carnosinase, histidine deaminase, lecithinase, enolase, yeast aldolase, oxalacetic decarboxylase, serum and intestinal alkaline phosphatase, and several peptidases.

In blood serum, zinc exists in at least two fractions--firmly and loosely bound zinc. The former is reported to amount to about 34% and the latter to 66% of the total zinc content (Vikbladh, 1951). The loosely bound complex appears to be concerned primarily with zinc transport. Neither substance has been shown to exhibit enzymatic properties.

Determinations of zinc content and carbonic anhydrase activity of human erythrocytes have implied that they are mutually dependent variables. In a number of pathological conditions the two parameters vary in a parallel fashion, allowing the interpretation that a large percentage of erythrocyte zinc is an integral part of carbonic anhydrase (Vallee et al., 1949). Since erythrocytes are rich sources of pyridine nucleotide-dependent dehydrogenases, it is possible that the fraction of zinc that does not form part of the carbonic anhydrase may be associated with some of these enzymes (Vallee et al., 1956).

Human leucocytes have been reported to contain a zinc metalloprotein; however, no enzymatic activity in this complex nor any corre-

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lation between the zinc content of leucocytes and the activity of several zinc-containing enzymes has been demonstrated (Vallee, 1959).

Several studies have shown that large oral doses of zinc significantly increase whole blood and plasma zinc in rats, rabbits, and cats (Underwood, 1962). Miller and Miller (1962) recently reported that zinc-deficient calves exhibit a reduced blood zinc content.

On the other hand, zinc-deficient rats and chicks have not inevitably shown a reduction in plasma zinc concentration, nor has a reduction in blood carbonic anhydrase activity, and therefore in erythrocyte zinc, been detected in this condition (Hove et al., 1940b; O'Dell et al., 1958). In pigs, Hoekstra et al. (1956) showed that adding 50 ppm of zinc to a zinc-deficient diet increased the zinc concentration of blood plasma but had no effect on the zinc content of erythrocytes.

Since the report by Bertrand and Vladesco (1921), several groups of workers have confirmed the finding of high concentrations of zinc in the male sex organs and fluids of various species. The biochemical forms and functions of zinc in these glands and their secretions have not been determined. Zinc deficiency in rats (Millar et al., 1958, 1960) results in degeneration of the testes, hypoplasia of the coagulating glands, the seminal vesicles and prostate, and relative or complete decrease in the numbers of sperm in the epididymes. All changes produced by zinc deficiency, except the testicular atrophy, were reversed when supplemental zinc was added to the diet. It appears that the impaired development of the male accessory sex organs in rats may be a result of severe inanition which in turn causes reduced gonadotropin secretion from the pituitary and a consequent fall in androgen production.

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It is not clear why the inanition of zinc deficiency does not affect the female reproductive system in a similar manner. The evidence suggests that the testicular atrophy associated with zinc deficiency is due directly to the lack of this element. Recently, Prasad et al. (1963) presented some interesting evidence that zinc deficiency may have been responsible for the hypogonadism and dwarfism observed in several young male patients in Egypt.

The tissues of the eye, especially the choroid, are known to contain high concentrations of zinc in a wide range of animal species. However, the exact function of zinc in eye tissues remains unexplained (Underwood, 1962).

Since the early work of Lutz (1926), skin and hair have been known to contain high zinc levels compared to most soft tissues of the body. Alopecia and parakeratotic skin lesions are characteristic of dietary zinc deficiency in all species, but consistent and conclusive evidence is lacking that these deficiency symptoms are associated with a significant reduction in the levels of zinc in the integument (Underwood, 1962).

In addition to showing symptoms manifested in other species, zinc-deficient chicks exhibit an abnormal respiration and a shortening and thickening of the long bones (O'Dell et al., 1958). The abnormal bone condition appears to arise from a failure of cartilage cell development in the epiphyseal plate region of the long bones and decreased osteoblastic activity in the thin bony collar. Keinholz et al. (1961) altered the development of chicken embryos by withholding zinc from the maternal diet; the major defect was grossly impaired skeletal development. These authors noted that excess dietary calcium aggravated the zinc deficiency, which is in agreement with some other studies of

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calcium and zinc interactions in the nutrition of swine and poultry, as reviewed by Forbes (1960).

Enzyme studies with zinc-deficient animals have not been particularly fruitful in correlating deficiency symptoms with biochemical functions of zinc. Hove et al. (1940b) reported that carbonic anhydrase activity remains within normal limits in the blood of zinc-deficient rats. Similar findings have been reported in the blood of zinc-deficient chicks (O'Dell et al., 1958), but Miller and Miller (1962) demonstrated a reduced carbonic anhydrase activity in calves fed a low-zinc diet. Subnormal levels of intestinal and kidney phosphatase have been reported in zinc-deficient rats (Hove et al., 1940a) and of serum alkaline phosphatase in parakeratotic pigs (Luecke et al., 1957; Newland et al., 1958). Other workers have not been able to correlate levels of the latter enzyme with the incidence of parakeratosis in pigs (Stevenson and Earle, 1956) or with symptoms of zinc deficiency in chicks (Morrison and Sarett, 1958). More recently, however, Earle et al. (1961) have reported decreased alkaline phosphatase activity in several different tissues of pigs suffering from parakeratosis. Need for further studies on the activity of zinc enzymes in tissues of zinc-deficient animals would seem to be indicated.

Balance studies using radioactive Zn^{65} have been valuable in tracing the absorption, excretion, and metabolic pathways of zinc, although the results have been variable and frequently difficult to interpret. In steers, an average of 70% of an oral dose was recovered in the feces and only 0.3% in the urine, while 20% of an intravenous dose was found in the feces and 0.2% in the urine during a 6-day balance trial (Feaster

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et al., 1954). This pattern of excretion was followed on both normal- and high-zinc diets. Zinc that is absorbed and reexcreted into the intestine is done so chiefly via the pancreatic juice and only in minute amounts via the bile (Sheline et al., 1943).

In swine and rats, apparent absorption of zinc varies inversely with the zinc content of the diet. Whiting and Bezeau (1958) reported that in pigs receiving 34 ppm of zinc, 21% of the zinc was apparently absorbed, while Beardsley (1958) found that in pigs fed only 9 ppm, 68% of the zinc intake was absorbed. Qualitatively similar results were obtained by Forbes and Yohe (1960) with rats.

Degree of zinc absorption appears to vary with the chemical form or combination in which it is ingested. Zinc in the form of the sulfate, carbonate, oxide, or metal seems to be equally available to the chick, whereas the zinc in certain ores is poorly utilized (Edwards, 1959; Roberson and Schaible, 1960). Although similar comparative studies have not been conducted with swine, the carbonate, oxide, and sulfate forms have all been used successfully in the prevention and treatment of parakeratosis.

Different protein sources also vary in their capacity to supply zinc for the needs of chicks, rats, and pigs. Soybean protein, which contains substantial amounts of phytic acid, is a less effective source of zinc for these species than casein or egg white, which contain none of this compound (O'Dell and Savage, 1960; Forbes and Yohe, 1960; Oberleas et al., 1962a).

The soft tissues of the body constitute a large pool of exhangeable zinc which is in equilibrium with the zinc of plasma. Feaster et al. (1954) found that the most rapid accumulation and turnover rate

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of retained zinc occurs in the pancreas, liver, kidney, pituitary, and adrenals in steers. In swine, Hoekstra et al. (1956) showed that liver, kidney cortex, spleen, and small intestine all contained similar concentrations of zinc in pigs fed a low-zinc basal ration. When this diet was supplemented with zinc, the liver and kidney cortex exhibited a much greater increase in zinc concentration than the other organs studied.

High-Level Dietary Copper[1]

Evvard et al. (1928) were perhaps the first to report a beneficial response from copper supplementation of swine rations. The use of copper salts to treat various disorders of swine is probably quite old and at one time drenching with a solution of copper sulfate was popular therapy for enteric disturbances (Luecke et al., 1963).

Using various plates and salt bricks, Braude (1948) demonstrated that young pigs fed a normal diet exhibited a craving for metallic copper. Mitchell (1953) reported that nursing pigs given their choice of either a basal creep meal or one supplemented with 25 gm. copper sulfate per 100 lb. of ration (150 ppm copper) overwhelmingly preferred the one containing added copper. However, in a subsequent trial (Barber et al., 1955b), copper supplementation failed to have any effect on either growth rate or creep feed consumption during the suckling period.

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[1]The compounds--copper sulfate, copper sulfide, and copper carbonate--whenever used in this thesis, refer to the cupric form of copper (oxidation state of +2). However, the commercial copper oxide used in this thesis is a mixture of the cupric (+2) and cuprous (+1) forms.

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Widespread interest in copper supplementation was precipitated by the announcement of Barber et al. (1955c) that daily gains of growing pigs were improved by feeding 250 ppm of supplementary copper as copper sulfate; this improvement was established during the first 8 weeks of an 18-week trial. In a more extensive experiment involving eight feeding centers in England, Bowler et al. (1955) confirmed the growth promoting properties of 250 ppm of copper. Another report by Barber et al. (1955a) indicated that this level of copper was equally as effective as chlortetracycline in significantly increasing the growth rate of pigs, but they found no additive response from feeding the two supplements in combination.

In Germany, Schurch (1956) added 230 ppm of copper to the ration of pigs and noted an increased weight gain and an improved feed utilization. He suggested that the copper may have protected the pigs against an infection which affected the controls. In more recent work in that country, Hennig (1960) did not obtain a significant response from adding 265 ppm of copper as copper sulfate to a growing pig diet consisting of potato silage plus a grain-soybean meal supplement.

Barber et al. (1957) confirmed their previous work by demonstrating that the effect of 0.1% copper sulfate (250 ppm copper) was comparable to that obtained from oxytetracycline (10 gm./ton) or chlortetracycline (20 gm./ton) and that there was no additive effect from copper plus an antibiotic. Liver copper concentration of the pigs receiving copper-supplemented rations averaged 109 ppm, wet basis, which was eight times that of the controls. Smaller increases in other tissues from the supplemented pigs were observed. They also reported that the ad libitum feeding of rations containing 0.5% or 1.0% copper sulfate for

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a period of from 3 to 5 weeks caused a rapid reduction in feed intake and body weight gains. However, no mortality occurred and all pigs resumed a satisfactory growth rate and feed intake immediately following reduction or omission of supplemental copper from the diet.

In the studies cited up to this point, the pigs were fed in a way that allowed some expression of appetite. In several instances the increased rate of gain from the use of supplemental copper sulfate appeared to result from an increase in feed consumption, there being little or no effect on the efficiency of feed utilization. However, in an experiment in which pigs were fed according to a fixed scale based on live weight, Lucas and Calder (1957a) were still able to obtain growth responses to copper supplementation. They found that copper sulfate and penicillin together were no better than either one fed separately, but chlortetracycline added to control diets already supplemented with 250 ppm of copper significantly improved performance over that of the controls. Most of the response to copper supplementation was observed during the growing period (40 to 100 lb.) rather than during the finishing phase (100 to 200 lb.). These investigators also determined liver copper concentrations. On a dry matter basis, they found the controls to have 25 ppm; the pigs fed copper from 40 to 100 lb., 82 ppm; and those fed copper from 40 to 200 lb., 506 ppm of copper in the liver. Therefore it was possible to markedly reduce accumulation of liver copper by removing the copper supplement after 100 lb. live weight.

In a subsequent study, Lucas and Calder (1957b) fed five levels of copper sulfate, ranging from 0.012% (31 ppm copper) to 0.2% (500 ppm copper), to pigs from weaning to market weight. Before 100 lb., the highest levels of copper caused the greatest improvements in rate

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of gain and feed efficiency. Between 100 and 200 lbs., none of the copper levels improved performance, and reductions in rate of gain and feed efficiency with the 0.2% level suggested marginal toxicity. Over the entire experimental period, rate of gain and feed efficiency were highest at the 0.05% level, but overall treatment differences did not approach statistical significance. Up to the 0.025% level (62 ppm copper), there was no increase in liver copper over that of the controls. On a dry matter basis, pigs receiving 0.05% copper sulfate were found to have 163 ppm of liver copper; those fed 0.1%, 575 ppm; and those receiving the 0.2% level, 3,085 ppm. Thus, above a certain level, further increases in dietary copper appear to result in a geometric, rather than an arithmetic, increase in concentration of copper in the liver.

Lucas et al. (1961), in a continuation of their earlier work, observed that growing pigs fed rations supplemented with 250 ppm of copper gained more rapidly than those supplemented with 16, 62, or 125 ppm. As before, copper additions had no influence on performance from 100 to 200 lb. live weight. Comparing this trial with the previous study (Lucas and Calder, 1957b), it was concluded that in pigs fed according to a fixed scale the greatest consistent increases in growth rate and feed efficiency occurred at the 250 ppm level of copper supplementation.

In the Netherlands, Dammers et al. (1959) have observed an improved rate of gain and feed conversion from high levels of copper supplementation. They found the response was greater with 250 than with 125 ppm of copper as copper sulfate, but there was little difference between 250 and 187 ppm.

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that 250 ppm of copper in the sulfate form increased the growth rate of pigs in nine out of ten experiments but the magnitude of this increase was statistically significant in only one experiment. The authors indicated that under Tasmanian conditions of pig feeding the use of high-level copper supplementation is associated with some danger of copper toxicity. In one of their trials a pig died from perforation of the digestive tract, which they believed was due to the ingestion of a particle of copper sulfate as a result of inadequate mixing of the ration.

Barber et al. (1960) reexamined the effects of copper sulfate (250 ppm copper) and antibiotics fed separately or in combination. Pooled results from three experiments showed that copper sulfate alone significantly increased growth rate, feed consumption, and feed efficiency. Oxytetracycline or chlortetracycline, added to a diet already supplemented with copper sulfate, significantly increased growth rate and feed efficiency but not feed consumption. More recently, however, a coordinated trial carried out at 21 centers revealed that copper sulfate and oxytetracycline stimulated performance when fed alone but that there was no further improvement when both supplements were fed together (Braude et al., 1962). It is interesting to note that in the latter trial copper sulfate stimulated growth rate and feed efficiency significantly greater than did the antibiotic.

Bellis (1961) concluded from his study that the mode of action of copper and antibiotics is similar, and that in general farm practice in England there is unlikely to be any economic advantage in supplementing growing pig diets containing 250 ppm copper with chlortetra-

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In this country, effects of feeding high levels of copper to swine have been somewhat more variable and less conclusive than those reported by investigators in Great Britain. A study at the Arkansas station reported by Scott (1958), involving levels of 50 and 250 ppm of copper alone and in combination with chlortetracycline and/or brewers dried yeast, showed that only the antibiotic increased gains significantly.

Investigators at the Florida station (Wallace et al., 1960) observed that copper levels of 250 ppm or higher proved toxic and that consistent improvement in performance was not obtained at any level of copper supplementation. It was shown that elevating the protein content of the corn-soybean meal basal diet from 15 to 20 or 25% significantly reduced the toxic effects of 750 ppm of supplemental copper; this agrees with the observations of McCall and Davis (1961) in the rat.

Hawbaker et al. (1961), on the other hand, reported a significant improvement in growth rate of pigs fed supplemental copper sulfate, and no toxicity was observed. They found that 250 ppm copper was the optimum level for maximal response in growth rate and feed conversion. A combination of copper sulfate and antibiotic produced an additive effect with respect to rate of gain, but no such response was obtained when copper sulfate and an arsenical were fed jointly. Liver copper values for the copper-fed pigs were 10 times higher (293 ppm) than those of the untreated controls but there was no significant difference in loin copper values. In comparing the seemingly divergent results of the Iowa and Florida studies, it may be of some significance that in all

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but the instance the Iowa trials were of shorter duration (42-56 days) than those of the Florida workers (70-82 days). Hawbaker and his associates also made fecal flora counts and reported that copper sulfate had a significant effect on the numbers of certain types of microorganisms. They suggested that the alterations in the fecal microflora of copper-supplemented pigs might account for the improved performance of these animals.

In further work at the Iowa station, Bunch et al. (1961) compared the effects of copper sulfate, copper oxide, and chlortetracycline on baby pig performance. They again found 0.1% copper sulfate (250 ppm copper) to be the optimum supplemental level for this form of copper. Copper oxide compared favorably with similar levels of copper sulfate in improving performance. Moreover, copper oxide had the advantage of depositing much less copper in the tissues (liver and loin) than the sulfate form. Copper sulfate exhibited the further disadvantage of depressing hemoglobin concentrations when added to the diet at the rate of 250 ppm copper or higher. These results confirmed those of Hawbaker et al. (1959), who showed that the copper radical, and not the sulfate radical of copper sulfate, was producing the growth response.

Allen et al. (1961) studied various aspects of high-level copper supplementation and found that copper carbonate was equally as effective as copper sulfate in improving performance of growing pigs. Buescher et al. (1961) conducted a radiocopper balance experiment in which they found that the availability of copper whether as sulfate, carbonate, or oxide was similar for swine.

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(CuS), a very insoluble compound, and reported that the response of growing pigs to sulfide was much less than that to the sulfate. The sulfate form, but not sulfide, significantly increased liver and kidney stores of copper. It was suggested that the effectiveness of copper in promoting growth is related to the amount of soluble copper in the gut, but whether the site of action is systemic, enteric, or both, remains unknown.

Using labelled copper compounds in a balance study, Bowland et al. (1961) proved conclusively that a higher percentage of copper sulfate is absorbed from the gastrointestinal tract than copper sulfide.

In an attempt to resolve the discrepancies reported in the response of pigs to copper supplementation in the United Kingdom and the United States, Lucas et al. (1962a) compared the effect of adding copper sulfate to a barley-fish meal diet and a corn-soybean meal diet. The former ingredients are commonly used in the U. K. and other European countries, while the latter are basic components of U. S. swine rations. Both diets were hand-fed according to a fixed scale based on live weight. Copper sulfate fed at a rate of 250 ppm of supplemental copper similarly stimulated growth rate of pigs on either ration from 40 to 100 lb. live weight. From 100 lb. to market weight, copper sulfate added to either ration exerted no influence on rate of gain. The copper supplement slightly improved the efficiency of utilization of the barley-fish meal ration but had no effect on the corn-soy diet.

Barber et al. (1962) designed a similar experiment in which they compared copper supplementation of barley-fish meal and barley-soybean meal rations. In contrast to the results described above, these workers observed a significantly greater response when copper was added

to the diet containing fish meal than when added to the all-vegetable diet. In this trial, however, the pigs were not restricted but fed to appetite. Copper-supplemented pigs fed the fish meal diet consumed more feed than those on the soybean meal ration but this alone did not account for the difference in growth rate. This same research group had previously shown that the response to copper is slightly greater when pigs are fed ad libitum than when they are hand-fed (Allen et al., 1961).

King (1960) studied the effect of environmental temperature on the response to copper sulfate and antibiotics. He found the response to these growth stimulants was greater when pigs were maintained at 54° F. than when they were kept at 65° F. In a more recent experiment, King (1963) compared the response to copper sulfate of pigs fed two different levels of water mixed with their feed. He concluded that the level of added water had no significant influence on the response to supplemental copper.

The effect of 0.1% copper sulfate in the diet of early-weaned pigs was investigated by Lucas et al. (1962b). They found that the performance of pigs weaned at 10 days of age and fed to 45 lb. live weight was improved by the feeding of copper sulfate. When chlortetracycline was added to the copper-supplemented ration, there was a slight additive response. In this country, Meade et al. (1961) also reported a response from adding 50 to 200 ppm of copper as copper sulfate to the diet of early-weaned pigs. They found that pigs weaned at 23 days of age and fed a copper-supplemented ration for 46 days gained 10-15% faster than the controls; there was a further small improvement from including oxytetracycline in the diet.

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Bunch et al. (1963) recently confirmed their earlier work by demonstrating that copper oxide and copper sulfate are similar in their ability to stimulate weight gains of pigs from 14 to 125 lb. live weight. From 125 to 200 lb., however, copper sulfate slightly depressed gains while copper oxide increased daily gain compared to the control pigs. These investigators also confirmed their previous finding that pigs fed copper sulfate deposit significantly higher concentrations of copper in the liver than do those fed the oxide form; on the other hand, they found no difference in the copper content of the loin muscle.

In other work in the U. S., Miller and Barnhart (1961) reported that 0.1% copper sulfate resulted in a considerable improvement in daily gain and that it was comparable to various antibiotics in this respect.

A search of the literature indicates that Ogilvie (1942) was probably the first to describe what was suspected of being copper poisoning in pigs. Nothing further was reported for several years until Gordon and Luke (1957) found evidence of an outbreak in which it was known that the feed had been supplemented with copper sulfate, although it was not possible to ascertain the amount of copper that had been fed. Since then, several reports of copper toxicity have paralleled the increased use of copper sulfate as a growth stimulant in the United Kingdom and elsewhere.

Indications of marginal toxicity and liver copper levels up to 3,000 ppm were reported by Dammers et al. (1959) in pigs fed wet meal containing 0.3% copper sulfate. Two of these livers had a grayish-brown appearance and showed some connective tissue proliferation. The pigs on this ration grew slowly. At levels of supplementation of

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0.2% and below, no toxicity was apparent.

O'Hara et al. (1960) in Australia reported the occurrence of copper poisoning in young pigs receiving a diet supplemented with copper sulfate at the rate of 250 ppm copper for a period of 16 weeks.

Buntain (1961) gave a detailed account of the death of 23 fattening pigs and condemnation of several carcasses in a herd of 200 pigs fed a diet containing approximately 130 ppm of copper. Livers and kidneys had up to 2,200 and 950 ppm copper, respectively, compared with normal values of about 50 and 25 ppm. It was suggested that the dangerously high copper levels found in these organs from all pigs examined, together with some other unidentified toxic factor in the diet, may have caused the losses.

Allcroft et al. (1961) compared diets containing various levels of added copper sulfate fed ad libitum to pigs from weaning to market weight. No significant increase in growth rate was obtained on diets containing 0.06 to 0.16% supplemental copper sulfate; additions of 0.2 and 0.4% reduced weight gains significantly, caused jaundice and the death of three out of seven pigs. In spite of careful mixing, these workers found wide variations in the copper content of the feed at each level of supplementation. Liver copper accumulation increased sharply on diets containing more then 0.06% added copper sulfate but no toxic symptoms were produced up to the 0.16% level. The authors concluded from their results that while high liver copper levels per se do not necessarily produce toxic symptoms it appears that above a certain liver concentration, other factors may precipitate a syndrome attributable to copper toxicity.

Allen and Harding (1962) experimentally poisoned pigs by feeding

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diets containing up to 1,000 ppm of copper as copper sulfate, copper oxide, or commercial mineral mixture. The first indication of toxicity was a rise in blood copper concentration. The chief clinical signs were jaundice, dullness, weakness, anemia, trembling, and respiratory distress. The principal macroscopic lesions were jaundice, poorly clotted blood, yellow to orange liver, pulmonary edema, ulceration of the esophageal zone of the stomach, and blood-stained intestinal contents. Distinctive histological changes were found in the liver and kidneys. Similar findings have been reported in one or more of the studies referred to previously. Allen and Harding believed the cause of death in the poisoned pigs to have been the anemia brought about by intravascular hemolysis and loss of blood into the alimentary tract.

Hemingway (1962) and others have criticized Allen and Harding's paper because the work was based on only one pig per treatment.

In the first of two trials, Bunch et al. (1962) reported that copper carbonate added to the diet at levels of 250 and 500 ppm copper depressed gains and hemoglobin concentrations. In the second trial, pigs fed the 250 ppm copper gained faster and exhibited higher hemoglobin levels than either the controls or those receiving the 500 ppm of copper. Pigs fed the highest level of copper carbonate showed lower weight gains and depressed levels of hemoglobin, ceruloplasmin, and liver iron compared to the other two groups; they also had significantly higher concentrations of liver copper.

Zinc and Porcine Parakeratosis

According to Kernkamp and Ferrin (1953), the syndrome which they called parakeratosis was widespread among swine herds in this country.

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They described the gross symptoms and histopathology of this disease and suggested that it was of nutritional origin. The condition is characterized by a dermatosis in which the skin becomes thickened and heavily incrusted, with deep fissures breaking the incrusted areas; these incrustations begin on the ventral abdominal wall and become most severe in the regions of the legs, thighs, ears, and head. Depending upon the severity of the disease, it is often accompanied by diarrhea, anorexia, and retardation of growth. In extremely severe cases the disease occasionally terminates in death, but in mild or moderately affected pigs spontaneous recovery may eventually take place. Parakeratosis has been observed primarily in swine receiving rations composed of corn and a vegetable protein supplement. Such rations have often, but not always, been fortified with a mineral mixture to the extent that the total calcium content of the diet was in excess of the published requirement. This disease is most apt to occur in young rapidly growing pigs after they have been weaned.

Raper and Curtin (1953) reported that when supplemental cobalt and zinc were added to a corn-cottonseed meal ration no symptom of the disease was observed. Then, Tucker and Salmon (1955) made the important discovery that symptoms of parakeratosis could be prevented or cured by zinc supplementation. In addition, these investigators showed that high levels of calcium and/or phosphorus aggravated the condition. Their work prompted studies elsewhere concerning the influence of various nutritional factors, particularly zinc, on parakeratosis in swine.

Luecke et al. (1956) were among the first to confirm the fact that zinc supplementation (20 ppm as zinc carbonate) prevents parakeratosis and that raising the calcium level of the diet increases the incidence

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of the disease. Lewis et al. (1956) reported similar results when they added 50 ppm of zinc in sulfate form to corn-soy rations that ranged from 0.8 to 1.4% calcium. This level of zinc supplementation did not prevent the appearance of parakeratosis in all of the experimental pigs, but a level of 100 ppm was completely effective. The latter workers also demonstrated the therapeutic effect of zinc treatment on established cases of parakeratosis; pigs injected with zinc sulfate responded similarly to those fed this compound, but the injections caused severe local irritation. Phosphorus supplementation had no significant effect on weight gains but significantly decreased skin lesions when added to the 0.8% calcium basal ration. Bellis and Philp (1957) have reported similar findings. This would appear to vindicate phosphorus as a causative agent in the parakeratotic syndrome.

Hoekstra et al. (1956) observed that 50 ppm of supplemental zinc as zinc sulfate had no effect on the zinc content of erythrocytes, spleen, intestine, or pancreas of pigs fed either a normal (0.8%) or high (1.4%) calcium ration. But zinc additions did increase the concentration of zinc in blood plasma, liver, and kidney. As the authors point out, the latter results could represent either a return to "normal" concentration or simply an increase due to excess dietary zinc. They declined to speculate as to which of these alternatives may be the more plausible one. However, their plasma zinc values for 10 "normal" pigs would suggest that the parakeratotic pigs were subnormal in this respect. The high-calcium ration had little effect on tissue zinc levels when no additional zinc was fed, but when supplemental zinc was added the zinc concentations of liver and kidney were decreased compared with those from pigs fed the normal-calcium-plus-zinc diet. Excess

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calcium did not appear to influence the pH of the intestinal tract and thereby reduce the absorption of zinc.

Stevenson and Earle (1956) concluded from their studies on parakeratosis that in diets for growing pigs which contain up to 1.0% calcium, the minimum zinc content for prevention of this disease is between 44 and 80 ppm. They found depressed levels of hemoglobin, serum inorganic phosphorus and blood sugar, and a shift in serum albumin-globulin ratio in parakeratotic pigs. The depression of various blood components may be a reflection of the inanition which accompanies parakeratosis rather than a result of zinc deficiency *per se*. Serum alkaline phosphatase activity was more variable among parakeratotic than among normal pigs and did not appear to be related to gross symptoms of the disease. On the other hand, Luecke *et al.* (1957) and Newland *et al.* (1958) found that the activity of this enzyme was lowered in pigs afflicted with parakeratosis and elevated in zinc-supplemented pigs.

Luecke *et al.* (1957) were surprised to find that pigs receiving a ration which contained only 0.5% calcium, a level that is below N.R.C. (1959) recommendations for growing pigs, exhibited a 40% incidence of parakeratosis. In a previous trial, pigs fed a diet containing 0.65% calcium had shown an incidence of only 10%. The explanation for these somewhat incompatible observations may at least partially rest on the fact that the low-calcium ration contained less zinc than the normal-calcium diet (32 vs. 45 ppm). It was evident from this experiment that low calcium levels are no insurance against parakeratosis and that supplementation of the ration with zinc is a more effective means of prevention. Similar results were obtained by Lewis *et al.* (1957b). They concluded that zinc supplementation appears to be a much more

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effective method of controlling parakeratosis than limiting calcium intake below National Research Council recommendations. Their investigations also revealed that when the amount of calcium in rations is increased from 0.5% to 0.8% and then to 1.2%, the zinc concentrations in bone, hair, kidney, and liver declined significantly, but the zinc of plasma, pancreas, skin, and intestine did not show a similar change. They surmised that excessive amounts of calcium probably reduced the zinc content of tissues by hindering zinc absorption from the gastrointestinal tract. No significant increase in the zinc concentration of the skin, where parakeratotic lesions occur, was noted when the zinc content of the ration was increased to 128 or to 1,028 ppm. However, these amounts of supplemental zinc produced varying degrees of increase in the zinc concentration of pancreas, liver, hair, bone, blood plasma, kidney, and intestine. It was suggested that none of the body components analyzed represent the site at which zinc exerts its beneficial effect on parakeratosis.

In a subsequent trial, Lewis et al. (1957a) demonstrated that zinc was readily removed from an in vitro solution during the precipitation of calcium phosphates brought about by increasing from 3 to 6 the pH of solutions of calcium, phosphate, and zinc. These conditions were said to simulate somewhat the change in pH of ingesta during its passage from the pig's stomach to his small intestine. Increasing the Ca:P ratio of these solutions markedly increased the amount of zinc removed from solution. The authors suggested that this type of phenomenon may explain the detrimental effect of a high-calcium diet on parakeratosis. In this same experiment, they also observed that method of feeding influenced the severity of parakeratosis induced by feeding a high-

calcium ration. Ad libitum feeding of the dry diet was more detrimental than hand-feeding the same ration wet; however, the latter method by no means prevented parakeratosis.

In more recent work, the Wisconsin group (Smith et al., 1960a) autoclaved a high-calcium diet in order to rule out the possibility that any infectious agent in the feed might influence the development of parakeratosis. Pigs fed the autoclaved ration exhibited improved performance, a lower incidence of parakeratosis, a greater zinc retention, and a lowered zinc excretion per gm. of feces. The increase in zinc retention was not a result of an increase in zinc solubility due to autoclaving, nor did it result from an elevated level of water extractable phosphorus in the autoclaved feed.

Several studies of zinc balance and excretion at different levels of dietary calcium have been conducted in an attempt to reveal a relationship that would explain aggravation of symptoms of zinc deficiency by increasing calcium intake.

The data of Beardsley (1958) indicate that calcium supplementation of a diet extremely low in zinc (9 ppm) has no effect on zinc absorption. However, urinary excretion of zinc was increased in pigs fed 1.5% calcium, so that the retention of absorbed zinc amounted to 91 and 76%, respectively, for pigs fed low- and high-calcium diets. Since rate of growth and tissue concentrations of zinc were not lowered by calcium supplementation, the significance of the greater urinary excretion is not clear.

The balance study of Whiting and Bezeau (1958) failed to show that calcium has a significant effect on absorption of zinc or on retention of absorbed zinc. On the basal diet (34 ppm zinc), 21% of the zinc was

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apparently absorbed, irrespective of calcium level, and retention of absorbed zinc equalled 31 and 22% for low- and high-calcium. Qualitatively the data are in good agreement with those of Beardsley (1958), although the degree of zinc deficiency was much more severe in the latter's experiment.

Newland et al. (1958) found that when the calcium content of a ration (30 ppm zinc) was increased from 0.64 to 1.19%, the endogenous fecal zinc content was increased. They postulated that this, together with an increased specific activity of certain tissues, was an indication of a higher rate of zinc metabolism in pigs receiving high-calcium diets unsupplemented with zinc.

In a balance study with rats, Forbes and Yohe (1960) were unable to find a specific effect of calcium on zinc absorption or on the zinc requirement.

In a review of zinc and calcium interrelationships, Forbes (1960) concluded that on the basis of evidence presented up to that time interference in zinc function by calcium apparently occurs at the cellular level rather than in the gastrointestinal tract. In support of this conclusion, he cited the balance data of Beardsley (1958), Whiting and Bezeau (1958), and Forbes and Yohe (1960). The reports of Lewis et al. (1957a,b) indicated that calcium may act in the gut of the pig, but Forbes rejects these studies because the conditions employed have little in common with those present in the intestinal tract. However, in a recent study with rats, Heth and Hoekstra (1963) observed that as dietary calcium level was increased the retention of orally-administered Zn^{65} declined and the retention of injected Zn^{65} increased. The authors felt the results could best be explained by a decreased absorption of

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In connection with their studies on parakeratosis, Hvidsten et al. (1955) thought it necessary to investigate whether essential fatty acids might be involved in the disease. They found no significant difference in the level of essential fatty acids in the blood of normal and parakeratotic pigs and concluded that parakeratosis is not the result of a disturbance in the metabolism of these unsaturated acids.

Conversely, Hanson et al. (1958) indicated that parakeratosis may be linked with an essential fatty acid deficiency. They reported that supplementing a normal-calcium ration with a very high level of soybean oil (23% by weight) was more effective in preventing parakeratosis than 200 ppm of added zinc as zinc sulfate. However, in the treatment of parakeratotic pigs they found zinc to be comparable with soybean oil, which contained 54% linoleic acid. They concluded that zinc should still be considered the preferred treatment for parakeratosis, primarily because of its low cost.

Smith et al. (1960b) studied the serum protein patterns of parakeratotic pigs. They found a decrease in the albumin:globulin ratio in pigs with this disease, which confirmed the earlier work of Stevenson and Earle (1956). However, it was suggested that zinc deficiency, per se, is not directly involved in the altered serum protein pattern but that such alterations result instead from an increased susceptibility to invasion by infectious agents.

Missouri workers (Oberleas et al., 1962a) turned their attention to the pig following their report that the phytic acid in a soybean protein diet for chicks renders the zinc in the ration less available (O'Dell and Savage, 1960). Observations for the pig were similar to

those for the chick. Pigs fed a casein basal ration gained four times as rapidly as those fed a soybean basal diet even though the latter contained more zinc (25 ppm) than the former (14 ppm). When phytic acid was added to the casein ration, weight gains were reduced sharply and there were symptoms of parakeratosis. It is interesting to note that a high-calcium (1.5%) casein basal diet had no detrimental effect on growing pigs; in fact, gains were slightly higher than for pigs fed a normal-calcium (0.8%) casein diet. However, when these diets were supplemented with phytic acid, the normal-calcium ration supported a higher rate of gain than the high-calcium diet. From this it was suggested that the deleterious effect of calcium is mediated through the phytic acid content of the diet. These same workers observed similar results in rats (Oberleas et al., 1961).

Plumlee et al. (1960) and Smith et al. (1962) at the Purdue station have also reported that the utilization of zinc in various proteins is correlated with the level of phytic acid in the protein. Furthermore, they found an improvement in zinc utilization when EDTA (ethylene-diaminetetraäcetic acid) was added to a soybean protein ration. It was suggested that EDTA acts as a chelating agent to facilitate zinc absorption. In a radiozinc balance trial, Green et al. (1961) showed that the addition of EDTA to a soybean protein basal diet resulted in decreased fecal Zn^{65} and increased Zn^{65} in the urine and liver. Addition of phytic acid to a casein basal diet increased Zn^{65} in the feces.

Oberleas et al. (1962b) investigated interactions between calcium, zinc, and phytate by means of an in vitro technique. With an aqueous solution of calcium and phytate, there was a negligible quantity of

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precipitate formed; with zinc and phytate the quantity of precipitate increased. With calcium, phytate, and zinc the quantity of precipitate was more than additive and by doubling the calcium in this system, a marked increase of precipitate was obtained. The authors concluded that the action of phytate on zinc availability takes place in the gastro-intestinal tract rather than at the cell level.

Green et al. (1962), however, were unable to find an antagonism between phytate and zinc. They demonstrated that zinc phytate administered either in the diet or injected intraperitoneally was equally as effective for maintaining optimum performance and preventing parakeratosis as a dietary supplement of zinc oxide or EDTA.

Although parakeratosis usually has been observed in young growing pigs, Roberts et al. (1962) felt it expedient to examine the significance of zinc supplementation (100 ppm) of a high-calcium (1.6%), low-zinc (30 ppm) ration for breeding gilts. The experiment lasted from 5 or 6 months of age throughout gestation and lactation. Dietary zinc level had no effect on body weight gain up to parturition, nor was there any evidence of parakeratosis in the gilts. However, the unsupplemented diet had an adverse effect on birth weight, litter size, and 24-hour mortality of the newborn pigs. The pigs from unsupplemented gilts also exhibited considerably lower zinc concentrations in the liver and bones. At weaning age, 44% of the pigs in the unsupplemented group showed parakeratosis and lower serum alkaline phosphatase activity than pigs from the zinc-supplemented gilts. Thus, it appears that calcium and zinc levels in rations for reproducing gilts should be given somewhat more than passive consideration.

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Copper and Zinc Interrelationships

A number of observations concerning copper-zinc interactions have been made in connection with studies that have dealt with the effects of excessive dietary zinc. For example, Smith and Larson (1946) found that an anemia caused by 0.7% zinc in the diet of rats could be overcome by supplementation with copper sulfate. Later experiments in the same laboratory confirmed this finding and also demonstrated that 300 ppm of dietary copper as copper sulfate could alleviate the depressed heart cytochrome oxidase activity of rats fed 1.0% zinc (Gray and Ellis, 1950; Duncan et al., 1953). This work prompted Van Reen (1953) to investigate the effects of excessive dietary zinc on various enzymes in the liver of rats. He showed that a dietary level of 0.5 to 0.7% zinc resulted in a marked reduction in catalase and cytochrome oxidase activities, with little influence on diphosphopyridine nucleotidase, isocitric dehydrogenase and alkaline phosphastase. Supplementation of the zinc-toxic diet with small quantities of copper sulfate resulted in liver catalase and cytochrome oxidase activities of a magnitude equal to that of the normal controls. However, copper supplementation had no effect in correcting the growth inhibition produced by zinc.

Kulwich et al. (1953) studied the effects of adding 200 ppm of copper (sulfate) or 1,000 ppm of zinc (sulfate) to diets of weanling rats and pigs for 14 and 27 weeks, respectively. These supplements exerted no influence on growth rate or feed conversion in either species. Neither supplement appeared to have a significant effect upon the accumulation of the other metal in various tissues. Furthermore, neither supplement had an appreciable influence on the uptake of

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Cu^{64} by certain tissues, except for the reduced uptake by the blood and red bone marrow of rats fed the high level of zinc.

Allen et al. (1958) observed that a combination of 250 ppm copper and an equivalent amount of zinc failed to produce any better growth response in pigs than copper alone. The amount of copper stored in the liver of the pigs that received the high-copper diet was, however, reduced when zinc was added to the ration. These results were later confirmed by Barber et al. (1960), who reported that the addition of 250 ppm zinc to a diet supplemented with either copper (250 ppm) alone or with copper and oxytetracycline had no significant effect on performance but resulted in a reduction in the copper stores in the liver.

Davis (1958) reported that in studies at the Florida station, copper was demonstrated as having a marked and inverse relationship to zinc, at least within the liver of animals. It was noted that when copper levels in the liver rose to values above 3,000 ppm, there was a reduction of zinc from a normal value of 300 ppm to levels approaching the vanishing point of 1 ppm. Conversely, increasing the level of zinc in the diet caused a depression of copper values in the liver, but only when copper levels in the diet approached those of borderline or normal of around 5 to 10 ppm.

Grant-Frost and Underwood (1958) concluded from their experiment dealing with excessive dietary zinc for rats that: the depressing effect of high-zinc on growth is caused largely but not entirely by reduced food consumption, probably due to the unpalatability of the zinc-containing diet; the anemia is caused by a zinc-induced copper deficiency in the animals; and zinc not only profoundly reduces copper concentrations in the blood, liver, kidney, and whole body but probably

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Wallace et al. (1960) found that 200 ppm of copper as copper sulfate markedly reduced the hemoglobin level of growing pigs; this adverse effect was not alleviated by the addition of 500 ppm zinc to the ration. Copper or zinc alone or in combination had little influence on daily gain. However, in one trial, 150 ppm of copper completely prevented parakeratosis, while three out of four pigs receiving the unsupplemented basal ration developed severe parakeratotic symptoms. Wisconsin workers (Priebe et al., 1961), on the other hand, have concluded from their tests that copper does not exert a protective influence against parakeratosis. They were unable to find any evidence that copper can substitute for all or part of the zinc in a ration under Wisconsin conditions.

Cox and Harris (1960) reported that 0.4 to 0.6% zinc in the diet of rats resulted in a reduction of iron and copper in the liver. The loss of iron occurred preferentially to the loss of copper. From their data, they concluded that the lowered liver copper may be a result of the decreased liver iron rather than a direct effect of the high zinc. They suggested that copper probably acts in counteracting the anemia and depressed activity of iron-containing enzymes in zinc toxicity by further mobilizing iron in the liver.

Magee and Matrone (1960) also found a disturbance of copper and iron metabolism in rats fed high levels of zinc. Results of an isotope trial suggested that zinc interferes with copper metabolism by decreasing the utilization and increasing the urinary excretion of copper in the rat, but apparently has little effect on the absorption of copper. Another isotope experiment indicated that zinc does not interfere with the absorption of iron, but interferes in some manner with the utilization

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McCall and Davis (1961) found that 1,000 ppm of added zinc were effective in lowering the concentration of liver copper in rats on a 10% protein diet but had little effect on 17.5% protein. McCall et al. (1961) proposed that specific protein compounds in soybean meal form stable chelates with zinc which decrease the accumulation of zinc in the liver when otherwise toxic levels are ingested and eliminate the adverse interaction with copper and iron.

In a study with swine, Cox and Hale (1962) were unable to produce symptoms of a zinc toxicosis by feeding zinc levels of 0.2 or 0.4%. Neither of these levels caused a significant reduction of liver copper, but the higher level resulted in a lowering of liver iron.

Smith et al. (1962) showed that when zinc-deficient, parakeratotic pigs were fed an isolated soybean protein ration plus 125 ppm copper, no improvement in performance was observed. This agrees with the report of Priebe et al. (1961), but is not in line with the observations of Wallace et al. (1960). In an Australian study, O'Hara et al. (1960) observed the occurrence of parakeratosis after 10 weeks in pigs receiving a diet supplemented with 250 ppm of copper. Likewise, in Germany, Hennig (1960) found that 265 ppm of copper were not effective in preventing parakeratosis.

Bunch et al. (1963) recently reported that the addition of zinc may be required for maximum response from feeding high levels of copper supplements.

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III. EXPERIMENTAL PROCEDURE

General

Weanling pigs, ranging from 6 to 8 weeks of age, were used in each of the five experiments described in this thesis. The general procedure for lotting, management, and record keeping was essentially the same in every trial. All lots were distributed as evenly as possible for weight, sex, breed, litter, and thriftiness. The animals were maintained in concrete-floor pens and had free access to feed and water at all times. They were vaccinated for hog cholera, sprayed for mange, and wormed during the early stages of each trial.

Feed and growth data were collected at 2-week and, in some cases, 1-week intervals. Corn and soybean meal were the basic ingredients used in all diets. Protein level up to 125 lb. live weight was approximately 16%. Thereafter, protein content of the ration was reduced to about 13% by eliminating an appropriate quantity of soybean meal and replacing it with corn. Calcium levels were controlled primarily by varying the amount of limestone added to the ration. All diets were adequately fortified with vitamins in accordance with N.R.C. (1959) recommendations. With the exception of Experiment III, all basal diets were supplemented with antibiotics. Chemical analyses for protein, calcium, phosphorus, zinc, copper, and iron were performed on most rations throughout the study. A.O.A.C. (1955) methods were used for protein, calcium, and phosphorus. Methods for determining zinc, copper, and iron were essentially the same as those used for

tissue assays (see p. 47).

All data were treated statistically by analysis of variance (Snedecor, 1956). Treatment means were compared by the multiple range test of Duncan (1955).

Biochemical Determinations

Collection of Blood

Blood samples were obtained from the anterior vena cava according to the technique described by Carle and Dewhirst (1942). Two milliliters of blood were placed in a heparinized vial when hematological values were determined; all vials were tightly corked except when they were being sampled. Approximately 10 ml. of blood were obtained for serological determinations. Separation of serum from cells was completed in an International centrifuge, size 2, model V, at 2,000 x g. for 20 min.

Hematological Measurements

Hemoglobin. -- The cyanmethemoglobin method of Crosby et al. (1954) was used to determine hemoglobin. A 0.02 ml. sample of blood was collected with a Sahli pipet and diluted in 5 ml. of Drabkin's solution, which was prepared by dissolving 1 gm. $NaHCO_3$, 50 gm. KCN, and 200 gm. $K_3Fe(CN)_6$ in 1 liter of double distilled water. The light absorbance of the sample was read at 540 millimicrons on a Bausch and Lomb "Spectronic 20."

Hematocrit. -- The micro-method outlined by McGovern et al. (1955) was utilized for hematocrit measurements. Blood was taken up in capillary tubes, sealed with flame, and centrifuged at a speed of 11,000 rpm for 5 min. in an International "Hemacrit" centrifuge.

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The hematocrit readings were taken on an International micro-capillary reader.

Erythrocyte count. -- Blood was drawn into a "Zero Error Hellige Tru-Count" pipet and diluted with physiological saline. Counts were made on a Neubauer counting chamber by the method of Ham (1956).

Serum Protein and Electrophoresis

Total serum protein was determined according to the biuret procedure accepted by the American Association of Clinical Chemists (Reiner, 1953). The serum protein fractions were separated on a Spinco Model R paper electrophoresis system (Spinco Technical Bulletin 6050 A) at room temperature. The relative intensities of the separated proteins were determined by scanning with a Spinco Model RB Analytrol equipped with two 500 millimicron filters and a B-5 cam.

Serum Akaline Phosphatase Assay

Substrate. -- The method of Bessey et al. (1946), which calls for p-nitrophenyl phosphate as the substrate, was used in this assay. One-hundred milligrams of Sigma "104" phosphatase substrate were dissolved in 25 ml. water and 25 ml. alkaline buffer solution (see below).

Alkaline buffer. -- The buffer was prepared by dissolving 3.75 gm. glycine and 0.1015 gm. $MgCl_2 \cdot 6H_2O$ in 375 ml. water. Approximately 42 ml. of 1 N NaOH were added until the pH was 10.5. This solution was made to 500 ml. with water.

Assay procedure. -- A 1 ml. aliquot of substrate solution was pipetted into a test tube, which was then warmed for 5 min. in a water bath at 38° C. A 0.05 ml. aliquot of serum was added, the tube shaken gently, and incubated in the water bath for exactly 30 min.

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Another tube which contained water in place of serum was included to serve as a reagent blank. Following incubation, 10 ml. of 0.02 N NaOH (cold) were pipetted into each tube to stop activity, and the tubes were placed in ice water for 1 min. Optical density was read against the blank at a wavelength of 410 millimicrons on a Beckman Model DU spectrophotometer. Two drops (0.1 ml.) of concentrated HCl were added to each tube in order to remove the color due to p-nitrophenol, leaving the optical density due to the serum itself. Tubes were then read again on the spectrophotometer. Corrected optical density was obtained by subtracting the second reading from the first, and units of activity were derived from a standard calibration curve. One unit of enzyme activity was defined as that amount of phosphatase which would liberate 1 millimole of p-nitrophenol per hour. The results were expressed in units per liter of serum.

Trace Element Assay of Tissues

Wet ashing of tissues. -- Copper, zinc, and iron analyses of liver and/or loin were made on single digests which had been prepared from each tissue sample. Liver samples included specimens from each lobe; this sampling procedure was deemed necessary from the work of Cassidy and Eva (1958a), who found considerable variation between liver lobes in copper and iron concentrations. Samples were also taken for dry matter determination. Reagents utilized in wet ashing were HNO_3 and $HClO_4$. The procedure followed was the same as that outlined by Johnson et al. (1959), except that the weight of the fresh tissue sample was 25 to 30 gm. After digestion was complete and the residue had been evaporated to dryness, it was taken up in deionized water and transferred to a 100 ml. volumetric flask. The pH was adjusted

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Copper. -- The procedure used in analyzing for tissue copper was adapted from the methods of the A.O.A.C. (1955). An aliquot of the digest, estimated to contain 15 to 30 mcg. copper, was pipetted into a 125 ml. separatory funnel. Deionized water was added until the volume was at the 50 ml. capacity of the funnel. Five milliliters of 15% citric acid were added and the contents mixed. Concentrated NH_4OH was added slowly, drop-wise, until the contents of the separatory funnel were just basic to litmus; then two drops were added in excess. Ten milliliters of 0.1% sodium diethyldithiocarbamate were added and mixed thoroughly. Four milliliters of CCl_4 were added, the funnel stoppered, and the mixture shaken vigorously for 1 to 2 min. The layers were allowed to separate, and the yellow CCl_4 layer was drawn off into a dry 25 ml. Erlenmeyer flask. The extraction steps were repeated a total of four times -- until the CCl_4 layer was colorless. The CCl_4 extract was filtered through anhydrous Na_2SO_4 into a 25 ml. volumetric flask. Contents of the flask were made to volume with CCl_4 and stoppered immediately to prevent evaporation. The copper-containing extract was read in a Beckman Model DU spectrophotometer at 430 millimicrons against a curvette filled with CCl_4, set at 100% transmission. Tissue copper values were expressed as mcg. copper per gm. dry matter (ppm, dry-weight basis).

Zinc. -- The method of Johnson et al. (1959) was adapted for the assay of tissue zinc. Diphenylthiocarbazone (dithizone) was the complexing agent used in this procedure. A 1 to 2 ml. aliquot of the original digest (containing 10-30 mcg. zinc) was pipetted into a 1 X 8 in. glass-stoppered Pyrex test tube. This was evaporated to dryness

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and then 25 ml. of 0.01 N HCl were added to the tube. Ten milliliters of dithizone solution I (0.0375 gm. dithizone in 500 ml. CCl_4) were added and the contents of the stoppered tube were shaken vigorously for 1 min. to remove any interfering ions (Cu, Cd, etc.) from the aqueous phase. If the CCl_4 layer was still red or purple after shaking, it was removed with a pipet and the dithizone treatment of the aqueous phase repeated until the CCl_4 layer was definitely green, which indicated that the interfering ions had been eliminated. An aliquot of the aqueous solution, containing 1 to 3 mcg. zinc, was pipetted into a 125 ml. glass-stoppered Erlenmeyer flask of red low-actinic glass. Then the following reagents were added: 5 ml. 0.125% sodium diethyldithiocarbamate, 50 ml. ammonium citrate solution,[1] and 25 ml. dithizone solution II (solution I diluted 1:4 with CCl_4). The flask was stoppered and shaken vigorously for 1 min. The layers were allowed to separate and as much of the aqueous phase as possible was discarded without loss of the CCl_4 solution, which contained the zinc dithizonate. Fifty milliliters of 0.01 N NH_4OH were added and shaken with the contents of the flask in order to remove excess dithizone. The layers were allowed to separate and again as much of the aqueous phase as possible was discarded by decanting. If the color of the CCl_4 layer indicated excess dithizone was still present, the NH_4OH extraction was repeated. The optical density was then

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[1] Ammonium citrate solution: 226 gm. ammonium citrate were dissolved in 2 liters deionized water. Concentrated NH_4OH (80-85 ml.) was added until pH of solution was 8.5-8.7. Seventy ml. 1 N NH_4OH were added to 500 ml. of this solution and this was then diluted to 2 liters.

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measured in the same manner as described above for copper; wavelength setting was 535 millimicrons. Zinc concentration also was expressed in ppm, dry-weight basis.

Iron. -- A procedure outlined by Bandemer and Schaible (1944) was selected for the determination of tissue iron. However, 2,2'-bipyridyl, a compound very similar to o-phenanthroline, was used in place of the latter color-producing reagent. An aliquot, estimated to contain 10 to 30 mcg. iron, was pipetted into a 25 ml. volumetric flask. Two milliliters of 1% hydroquinone, 3 ml. of 0.1% 2,2'-bipyridyl, and 1 ml. of 25% sodium citrate were added and made up to volume with deionized water. This solution was allowed to stand for at least 1 hour at a temperature above 20° C. to assure complete reduction of iron and maximum color development. Optical density was read on a Beckman Model DU spectrophotometer against a reagent blank at a wavelength of 525 millimicrons. Iron concentration was expressed in ppm, dry-weight basis.

Intestinal Phytase Assay

Substrate. -- A 2% solution of sodium phytate was used as substrate in this assay, which is a modification of the one developed by Roberts and Yudkin (1961) for the measurement of intestinal phytase activity in the rat. Our substrate was prepared from commercial sodium phytate which had been purified in the laboratory of Dr. G. Kilgour, Department of Biochemistry, Michigan State University. The commercial phytate was found to contain 3.31% inorganic phosphorus; after purification its inorganic phosphorus concentration was 0.31%.

Veronal buffer. -- An alkaline buffer (pH 8.2) was prepared as follows: 50 ml. 1/7 M sodium veronal, 20 ml. 8.3% NaCl, 20 ml. 0.1

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Preparation of tissue. -- Pigs were fasted 18 hours prior to slaughter, as is customary at the Michigan State University meat laboratory. Following removal of viscera during the slaughter process, the first 12 in. of small intestine distal to the pylorus were excised, placed in a polyethylene bag, packed in a container of crushed ice, and taken without delay to the laboratory for further preparation. The sample of intestine was cleaned by flushing the lumen with cold water, stripped of adhering fat and as much of the serous coat as would come off easily, slit lengthwise, washed again in ice-cold distilled water, and then cut up into fine pieces with scissors. Fifteen grams were weighed out and homogenized in 90 ml. of water for 2 min. at high speed in a Servall "Omni-Mixer." The homogenate was centrifuged in a refrigerated centrifuge for 10 min. at 5,000 rpm. The centrifugate was decanted into a plastic jar with a snug fitting screw cap and held at + 2° C. until assayed. One milliliter of extract was taken for the determination of protein by the micro-Kjeldahl technique.

Assay procedure. -- One milliliter of tissue extract was diluted to 5 ml. with water. One milliliter of the diluted extract was then incubated for 16 hours at 37° C. with 1 ml. substrate, 5 ml. veronal buffer, 1 ml. $MgSO_4 \cdot 7H_2O$ solution (0.012 M), and 2 ml. water. An extract blank and a substrate blank were also included. All tubes were run in duplicate. Following incubation, 5 ml. of 30% trichloroacetic acid were added to each tube. After 30 min., the tubes were centrifuged at high speed for 10 min. in a Servall angle centrifuge. Inorganic phosphorus was determined on 1 ml. from each tube

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by adding 1 ml. molybdate II reagent (2.5% ammonium molybdate in 3 N H_2SO_4) and 1 ml. Elon[2] reagent (1 gm. Elon and 3 gm. $NaHSO_3$ in 100 ml. water). Tubes were diluted to 10 ml. with water and allowed to set for 45 min. for color development. Optical densities were read on a Beckman Model B spectrophotometer at 660 millimicrons against a reagent blank. Net optical density was calculated by subtracting the values for the extract and substrate blanks from that for the reaction tube. This was referred to a standard curve to obtain net mcg. inorganic phosphorus. Enzyme activity was expressed as mcg. inorganic phosphorus per mg. protein.

Intestinal Phosphatase Assay

Substrate. -- Twenty grams of sodium-beta-glycerophosphate were made to 100 ml. with veronal buffer (pH 9.8) in accordance with an assay of swine intestinal phosphatase described by Earle (1962).

Veronal buffer. -- An alkaline buffer (pH 9.8) was prepared as follows: 14.714 gm. sodium veronal, 9.714 gm. sodium acetate, and 3.4 gm. sodium chloride were made to 500 ml. with distilled water. When 10 ml. of buffer were included in 20 ml. of incubate, pH readings were consistently 9.4 to 9.5.

Preparation of tissue. -- The procedure for sampling and preparing the intestinal tissue for assay was identical to that outlined above for intestinal phytase.

Assay procedure. -- One milliliter of tissue extract was incubated for 2 hours at 37.5° C. with 1 ml. substrate, 10 ml. veronal buffer, 2 ml. $MgSO_4 \cdot 7H_2O$ solution (0.04 M), and 6 ml. water. A blank tube

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containing 7 ml. water but no substrate was included to correct for inorganic phosphorus present in the extract. It was not necessary to include a substrate blank since it was impossible to detect any trace of phosphorus in the substrate solution either prior to or following incubation. At the end of the 2 hours, 5 ml. of 30% trichloroacetic acid were added to the incubate. From thereon the assay proceeded in exactly the same manner as described previously for the determination of intestinal phytase.

Experiment I

Supplementation of Low- and High-Calcium Diets With Copper, Zinc, and Iron

The purpose of this work was to determine the effect of certain trace elements on the performance of growing pigs and the possible relationship between these elements and the incidence of parakeratosis. This experiment consisted of three separate trials involving a total of 158 pigs. The composition of the basal rations used in each trial is shown in table 1. The major variant between these rations was the calcium level, which varied from 0.55 to 1.31%.

Trial A

Fifty weanling pigs were allotted into the following ration treatments: lot 31, basal (0.55% Ca); lot 32, basal + zinc carbonate (50 ppm Zn); lot 33, basal + ferrous sulfate (100 ppm Fe); lot 34, basal + zinc (50 ppm) and iron (100 ppm); and lot 35, basal + copper sulfate (125 ppm Cu).

Hemoglobin and hematocrit values were determined at the beginning of the trial, during the seventh week, and at the end of the trial (13 weeks). Serum alkaline phosphatase activities were measured at

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Trial B

Forty-eight pigs were allotted into six treatment groups. In this trial the calcium level was increased to 1.05%, a level which in the absence of added zinc had consistently produced parakeratosis in previous studies (Luecke et al., 1956, 1957). The ration treatments

TABLE 1

Exp. I. -- Composition of basal diets

Ingredient	Percentage of diet, Trial A	Trial B	Trial C	Trial C
Corn	77.425	75.475	77.375	75.375
Soybean meal (44% protein)	16.00	16.00	16.00	16.00
Meat and bone scraps	2.00	2.00	2.00	2.00
Fish meal	1.00	1.00	1.00	1.00
Alfalfa meal	2.00	2.00	2.00	2.00
Limestone	0.50	2.50	0.70	2.70
Dicalcium phosphate	0.30	0.30	0.20	0.20
Iodized salt	0.50	0.50	0.50	0.50
B-vitamin supplement[a]	0.10	0.10	0.10	0.10
Vitamin B_{12} supplement[b]	0.05	0.05	0.05	0.05
Vitamin A and D supplement[c]	0.025	0.025	0.025	0.025
Antibiotic supplement[d]	0.10	0.05	0.05	0.05
Composition by chemical analysis				
Protein, %	17.3[e]	16.6[e]	16.4[e]	16.4[e]
Calcium, %	0.55	1.05	0.55	1.31
Phosphorus, %	0.55	0.55	0.50	0.51
Zinc, ppm	28	36	39	34
Copper, ppm	8	12	9	23
Iron, ppm	157	122	--	--

[a]Contained 2 gm. riboflavin, 4 gm. d-pantothenic acid, 9 gm. niacin, and 90 gm. choline chloride per lb. of supplement.

[b]Contained 9 mg. B_{12} per lb. of supplement.

[c]Contained 4,000,000 I.U. of A and 1,200,000 I.U. of D per lb. of supplement.

[d]Contained 10 gm. chlortetracycline or oxytetracycline per lb. of supplement.

[e]Protein reduced to 13.0% when pigs reached 125 lb.

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were as follows: lot 39, basal (1.05% Ca); lot 40, basal + zinc carbonate (50 ppm Zn); lot 41, basal + ferrous sulfate (100 ppm Fe); lot 42, basal + zinc (50 ppm) and iron (100 ppm); lot 43, basal + copper sulfate (125 ppm Cu); and lot 44, basal + zinc (50 ppm) and copper (125 ppm).

Hemoglobin, hematocrit, serum alkaline phosphatase, and serum protein measurements were taken three times during the trial--at the beginning, during the eighth week, and when the pigs were slaughtered (14 weeks). Livers were also obtained for trace element analyses.

Trial C

This trial consisted of six treatments (60 pigs) involving two calcium levels with either supplemental zinc carbonate or copper sulfate, but no combinations of trace element supplementation. They were as follows: lot 45, low-calcium basal (0.55% Ca); lot 46, low-calcium basal + zinc (75 ppm); lot 47, low-calcium basal + copper (125 ppm); lot 48, high-calcium basal (1.31%); lot 49, high-calcium basal + zinc (75 ppm); and lot 50, high-calcium basal + copper (125 ppm).

Hemoglobin, hematocrit, serum alkaline phosphatase, and serum protein determinations were made at the conclusion of the trial (12 weeks).

Experiment II

Addition of Copper Sulfate and Zinc Oxide to a High-Calcium Diet

The purpose of this experiment was to examine further the effect of adding various levels and combinations of copper and zinc to a high-calcium diet. It was designed to study a higher level of supplemental copper sulfate than had been fed in the previous investigation. Com-

position of the basal rations is given in appendix table 1. They were very similar to the high-calcium diets fed in Experiment I, except for the deletion of ingredients of animal origin from the basal ration fed in trial B.

Trial A

Sixty weanling pigs were divided into six lots and fed for a 15-week experimental period. Design of the ration treatments was as follows: lot 1, basal (1.3% Ca); lot 2, basal + 0.05% copper sulfate (125 ppm Cu); lot 3, basal + 0.1% copper sulfate (250 ppm Cu); lot 4, basal + 0.0125% zinc oxide (100 ppm Zn); lot 5, basal + zinc (100 ppm) and copper (125 ppm); and lot 6, basal + zinc (100 ppm) and copper (250 ppm).

When symptoms of parakeratosis in lot 1 (basal) appeared to be in their most severe stage, the pigs in this lot were divided equally according to weight and degree of dermatosis into two lots, which were referred to as lots 1A and 1B. Division of the control lot occurred approximately 10 weeks (71 days) after the trial had started. Lot 1A continued to receive the unsupplemented basal ration while lot 1B was given dietary therapy in the form of copper sulfate at a level of 250 ppm of copper.

The lots fed the copper supplement were observed closely for symptoms of copper toxicity; autopsies were performed by the Department of Veterinary Pathology, Michigan State University.

After the 15th week, the pigs were slaughtered and their livers obtained for analyses of copper, zinc, and iron. Just prior to slaughter, a number of pigs on each treatment were bled for hemoglobin and hematocrit determinations.

Trial B

The purpose of this investigation was to confirm, or to refute, the results obtained in the preceding trial. With that objective in mind, trial B was, with but a few exceptions, essentially a repeat of trial A.

Sixty pigs were divided evenly into six lots, 30 through 35 inclusive, for a 14-week trial. Design of the ration treatments was identical with that of trial A. After 8 weeks, when symptoms of parakeratosis became very severe in lot 30 (basal), pigs in this lot were divided as before into two lots, 30A and 30B. Both of these lots were then switched to a normal-calcium basal diet (0.65% Cu). Furthermore, the ration of lot 30B was supplemented with 125 ppm of copper as copper sulfate. This was a lower therapeutic level than had been fed in trial A.

Hematological determinations were made at 8 weeks and at the time of slaughter livers were examined grossly by the inspecting veterinarian for evidence of cirrhosis and were then taken for subsequent analysis of copper, zinc, and iron. In addition, specimens of the liver, kidney, heart, lung, and spleen were obtained for histological examination.

Experiment III

Addition of Copper Sulfate and Chlortetracycline to a Normal-Calcium Diet

This experiment was conducted to compare the effects of supplementing a growing-finishing ration with either copper sulfate or an antibiotic and to determine whether there is an additive response from joint supplementation of these two chemotherapeutic agents. The

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investigation was prompted by work in Great Britain which had shown copper sulfate to be equivalent to broad-spectrum antibiotics in stimulating pig growth and efficiency of feed conversion.

The basal ration (appendix table 2) was similar in composition to those fed in previous experiments except that it was designed to conform as nearly as possible to the N.R.C. (1959) recommended calcium level for the growing pig (0.65%). It was also supplemented with zinc oxide so as to eliminate the possibility of a zinc deficiency.

The design of the experiment was simply a 2 X 2 factorial arrangement: lot 2, basal (0.65% Ca); lot 3, basal + 10 mg. chlortetracycline/lb.; lot 4, basal + 0.05% copper sulfate (125 ppm Cu); and lot 5, basal + chlortetracycline (10 mg./lb.) and copper (125 ppm). Eight pigs were allotted to each ration treatment.

The experiment was terminated after 14 weeks, when the pigs in the heaviest lot averaged 200 lb. in weight. During the final week, all pigs were bled for the determination of hematocrit and hemoglobin.

Experiment IV

Addition of Copper Sulfate, Copper Oxide, and Zinc Oxide to a Normal-Calcium Diet

The purpose of this study was to compare the effects of supplementing a growing-finishing ration with either copper sulfate or copper oxide, alone or in combination with zinc. The impetus for this work was the report from the Iowa station (Bunch *et al.*, 1961) that the feeding of supplemental copper oxide resulted in much smaller increases in copper content of the tissues than the feeding of copper sulfate, with no appreciable difference in stimulation of growth rate or feed conversion. Assuming that high tissue copper levels are one

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indication of toxicity, it appeared that copper oxide may show promise of improving performance with less danger of toxicity than copper sulfate. It was planned in this experiment to compare the effectiveness of these two forms of copper in preventing parakeratosis, if in fact the disease should occur.

The composition of the normal-calcium, low-zinc basal ration (appendix table 3) was essentially the same as that of Experiment III, except for the omission of supplemental zinc and the inclusion of antibiotics.

Design of the experiment was an incomplete factorial arrangement in which copper sulfate and copper oxide[3] were compared alone and in combination with zinc oxide as follows: lot 10, basal (0.66% Ca); lot 11, basal + copper sulfate (250 ppm Cu); lot 12, basal + copper oxide (250 ppm Cu); lot 13, basal + zinc oxide (100 ppm Zn); lot 14, basal + copper sulfate and zinc oxide; and lot 15, basal + copper oxide and zinc oxide.

In the event of the development of parakeratosis in the basal pigs, it was planned to divide that lot and compare the effects of copper sulfate and copper oxide therapy.

After approximately 13 weeks (90 days), the trial was terminated. Blood was drawn for hematocrit and hemoglobin determinations, and the pigs were subsequently slaughtered. Livers were examined grossly for cirrhosis and taken for assay of copper, zinc, and iron. Samples were taken from the longissimus dorsi at the 10th rib region for copper assay.

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[3]Copper oxide was purchased from Calumet & Hecla, Inc., Calumet, Mich. It contained a mixture of cupric and cuprous oxides.

Experiment V

Intestinal Phytase and Phosphatase Activity of Pigs Fed Various Levels of Calcium, With and Without Supplemental Zinc and Copper

Concurrent research at Missouri (Oberleas et al., 1961, 1962a,b) and Purdue (Plumlee et al., 1960; Smith et al., 1962) has implicated phytic acid as a variable in the complex interrelationships that apparently prevail in the parakeratotic syndrome. Workers at these two stations found that dietary phytate reduced zinc availability and predisposed the pig to parakeratosis; when supplemental zinc was fed, this inhibitory effect was overcome.

As a means of providing a basis for investigating this phenomenon, the following hypothesis was made: perhaps the role of high-level dietary calcium in producing parakeratosis is to lower the activity of intestinal phosphatase (or more specifically, phytase), which would in turn make available more intact phytate to interact with dietary zinc. It was the purpose of the present experiment to test this hypothesis. A further objective was to determine what effect, if any, high levels of zinc or copper have on the activity of these intestinal enzymes.

Trial A

The ration treatments in this trial were arranged as follows: lot 26, normal-calcium basal (0.61% Ca); lot 27, high-calcium basal (1.26% Ca); lot 28, high-calcium basal + zinc oxide (100 ppm Zn); lot 29, high-calcium basal + copper sulfate (125 ppm Cu); lot 30, high-calcium basal + zinc (100 ppm) and copper (125 ppm); and lot 31, normal-calcium basal + zinc (100 ppm). Each lot consisted of nine pigs.

Composition of the basal rations is shown in appendix table 4. The normal-calcium basal diet was composed of the same ingredients in almost indentical proportions as the one fed in Experiment IV. The high-calcium basal was the same except for the replacement of 1.7% of corn with an equivalent amount of limestone.

The trial was terminated after 8 weeks at which time a majority of the pigs in the high-calcium basal lot exhibited severe parakeratotic lesions. Hemoglobin and hematocrit determinations were made, and the pigs were slaughtered. A 12-inch section from the proximal end of the small intestine was removed from each pig at slaughter for the assay of phytase and phosphatase activity.

Trial B

The second trial involved two treatments: lot 20, low-calcium (0.53%); and lot 21, high-calcium (1.42%). Composition of these rations may be seen in appendix table 4.

Supplemental zinc was not added at the outset of the trial since it had been intended originally to test only one variable, namely, calcium. However, 3 weeks from the start of the experiment the pigs receiving the low-calcium ration, as well as those in lot 21, developed definite symptoms of parakeratosis. Because it was desired to maintain the health of the pigs in lot 20, 100 ppm of zinc were added to their diet. From that point on, the parakeratotic lesions disappeared from these pigs and they grew and performed optimally.

All pigs were slaughtered at the end of the seventh week, when parakeratosis reached a very severe stage in the high-calcium lot. Sections of the small intestine were obtained for assay of phytase and phosphatase.

IV. RESULTS AND DISCUSSION

Experiment I

Trial A

The results of trial A are summarized in table 2. Differences in growth rate resulting from the addition of the various trace elements to the basal diet were highly significant ($P<.01$) in all cases. The most rapid gains were made by the pigs fed either supplemental zinc (50 ppm) or copper (125 ppm), with somewhat poorer gains being made by the pigs receiving either supplemental iron (100 ppm) or a combination of zinc and iron. There were no material differences between any of the treatments in feed efficiency.

The fact that joint supplementation with zinc and iron was somewhat less beneficial than zinc alone suggests an antagonism between these two elements. Cox and Harris (1960), Magee and Matrone (1960), McCall *et al*. (1961), and Cox and Hale (1962) have reported an interaction between these two elements in rats and swine. One pig in lot 34 died on the 47th day for reasons which appeared to be unrelated to the experimental treatment. Later in the trial three other pigs which had been performing poorly were removed from this lot and autopsied. No conclusions could be drawn from the autopsy report; livers from two of these animals were enlarged and cirrhotic.

The poor growth of the pigs on the basal ration was accompanied by a high incidence of parakeratosis, which was severe in four of the six pigs developing the skin lesions. It should be noted that the basal ration contained only 0.55% calcium, a level below that recommended by N.R.C. (1959). Others have also found parakeratosis in pigs fed low-

calcium rations with no added zinc (Stevenson and Earle, 1956; Luecke et al., 1957; Lewis et al., 1957b).

TABLE 2

Exp. 1, Trial A. -- Response of pigs on low-calcium diet to supplemental zinc, iron, and copper (13 weeks)

Item	Lot no. and treatment[a] 31 Basal (0.55% Ca)	32 + Zn	33 + Fe	34 +Zn+Fe	35 + Cu	Std. error of means[b]
No. pigs[c]	10	10	10	6	9	--
Initial wt., lb.	28.5	28.4	28.3	28.5	28.3	--
Final wt., lb.	120.1	163.9	148.5	150.7	163.7	--
Daily gain, lb.[d]	1.01	1.49	1.32	1.34	1.49	0.076
Feed/gain	3.03	3.07	3.04	3.03	3.07	--
Parakeratosis, %	60	0	10	0	0	--
Hemoglobin, gm./100 ml.						
Initial	11.1	10.9	10.2	11.1	11.8	0.44
47 days	11.1	12.0	11.2	11.9	12.0	0.34
Final	11.4	12.0	12.0	12.0	11.4	0.49
Hematocrit, %						
Initial	37.5	36.7	35.8	37.1	39.0	1.06
47 days	36.3	38.6	36.7	37.6	38.1	0.91
Final	36.6	37.0	38.3	36.5	36.3	1.48
S.A.P.[e], units/liter[f]	0.91	3.10	1.21	3.21	1.97	0.228

[a]Zinc added at rate of 50 ppm; iron, 100 ppm; and copper, 125 ppm.
[b]Based on 40 degrees of freedom.
[c]One pig in each of lots 34 and 35 died. Three other pigs in lot 34 were removed because of unthriftiness, which appeared to be unrelated to experiment.
[d]Lot 31 significantly less than all other lots ($P < .01$).
[e]Serum alkaline phosphatase.
[f]Lots 32 and 34 significantly greater than all other lots ($P < .01$). Lot 35 significantly greater than 31 ($P < .01$) and 33 ($P < .05$).

The fact that no parakeratosis occurred in the zinc-fed pigs was not unexpected. However, the occurrence of only one case in the group fed iron and none in the copper-fed pigs suggests that these elements may also be related in some manner to the disease. The pigs in lot 35 (copper) were especially thrifty in appearance and displayed excellent skin and hair coat condition. Fagan et al. (1961) likewise have noted a "glossier" hair coat on pigs fed either 125 or 250 ppm of copper as copper sulfate. It is not known whether this is related to melanin formation. Wallace et al. (1960), on the other hand, have reported a pronounced fading of the hair coat on Duroc pigs fed 0.1% copper sulfate (250 ppm Cu).

There were no significant differences in either hemoglobin or hematocrit as related to the various treatments.

Serum alkaline phosphatase activity was significantly higher ($P < .01$) in the pigs receiving supplemental zinc as compared with the other three lots. Furthermore, the copper-supplemented pigs showed significantly higher enzyme activity than either the basal lot ($P < .01$) or the pigs fed iron alone ($P < .05$). The difference between the latter two lots was not significant.

Trial B

The results of trial B are reported in tables 3, 4, and 5. The hemoglobin, hematocrit, and serum protein values obtained at the initial bleeding are not included in the tables, as there were no significant differences between treatments at that time. Because most of the evidence reported in the literature supports the thesis that high-calcium rations predispose the pig to parakeratosis, it was decided in this

TABLE 3

Exp. I, Trial B. -- Response of pigs on high-calcium diet to supplemental zinc, iron, and copper (14 weeks)

Item	Lot no. and treatment[a]						Std. error of means[b]
	39 Basal (1.05% Ca)	40 +Zn	41 +Fe	42 +Zn+Fe	43 +Cu	44 +Zn+Cu	
No. pigs[c]	7	7	7	7	8	8	--
Initial wt., lb.	24.1	23.7	25.1	25.0	24.5	24.2	--
Final wt., lb.	75.6	156.7	90.4	146.1	161.6	157.8	--
Daily gain, lb.[d]	0.52	1.36	0.67	1.24	1.40	1.36	0.093
Feed/gain	4.53	3.48	4.12	3.65	3.56	3.52	--
Parakeratosis, %	100	0	100	0	0	0	--
Hemoglobin, gm./100 ml.							
53 days[e]	11.8	12.2	11.9	12.3	13.1	13.4	0.34
Final[f]	12.5	13.7	12.0	13.9	13.8	14.5	0.36
Hematocrit, %							
53 days[g]	38.2	40.1	39.0	39.0	40.8	42.5	0.98
Final[f]	39.0	42.2	38.2	43.0	42.4	44.1	0.95
S.A.P.[h], units/liter							
53 days[i]	0.67	4.02	1.47	4.29	2.73	5.15	0.261
Final[j]	1.39	3.96	2.27	3.76	2.08	3.49	0.287

[a]Zinc added at rate of 50 ppm; iron, 100 ppm; and copper, 125 ppm.

[b]Based on 38 degrees of freedom.

[c]One pig removed from lots 39 through 42 for reasons unrelated to experiment.

[d]Lots 39 and 41 significantly less than all other lots ($P < .01$).

[e]Lot 44 significantly greater than 39, 41 ($P < .01$) and 40, 42 ($P < .05$). Lot 43 significantly greater than 39 and 41 ($P < .05$).

[f]Lot 41 significantly less than all other lots except 39 ($P < .01$). Lot 39 significantly less than 42, 44 ($P < .01$), and 40, 43 ($P < .05$).

[g]Lot 44 significantly greater than lot 39 ($P < .01$), and 41, 42 ($P < .05$).

[h]Serum alkaline phosphatase.

[i]Lot 39 significantly less than all other lots ($P < .01$). Lot 41 significantly less than 40, 42, 43, and 44 ($P < .01$). Lot 43 significantly less than 40, 42, and 44 ($P < .01$). Lot 44 significantly greater than 40 ($P < .01$) and 42 ($P < .05$).

[j]Lots 40, 42, and 44 significantly greater than other three lots ($P < .01$). Lot 41 significantly greater than 39 ($P < .05$).

trial to increase the calcium content of the basal ration to 1.05% so that the possible relationship of copper and iron to parakeratosis could be more critically tested.

Again, supplemental zinc (50 ppm) and copper (125 ppm) exerted a highly significant effect on growth rate, and no parakeratosis developed in either treatment. All the pigs on the basal diet developed dermatoses, six of which were very severe. In contrast to trial A, supplemental iron (100 ppm) was completely ineffective in preventing the disease.

In trial B both zinc and iron, and zinc and copper were combined as treatments. As in trial A, joint supplementation of zinc and iron produced somewhat poorer results than zinc alone. In the case of zinc and copper (lot 44), the two elements together were no more effective than either one alone.

Table 3 shows that hematocrit and hemoglobin values were increased in a significant manner at both the 53-day and final bleedings by certain of the trace element treatments. The combination of copper and zinc was particularly effective in this respect. These two elements alone and the zinc-iron combination were moderately effective. Iron alone did not elicit a hematopoietic response.

Serum alkaline phosphatase values were markedly increased at the second bleeding by all trace element additions. As in trial A, the order of effectiveness was as follows: Zn>Cu>Fe. At the final bleeding, the treatment differences were not as great which may have been a reflection of the general improvement in the condition of the parakeratotic pigs. In general, the skin lesions were most severe at about 8 weeks, followed by some recovery even though no therapeutic measures were taken.

Total serum protein and the albumin and gamma globulin fractions were influenced in a significant manner by all trace element additions

TABLE 4

Exp. I, Trial B. -- Effect of supplemental zinc, iron, and copper on serum protein values

Item	Lot no. and treatment[a] 39 Basal (1.05% Ca)	40 +Zn	41 +Fe	42 +Zn+Fe	43 +Cu	44 +Zn+Cu	Std. error of means[b]
53-day values							
Total serum protein, gm./100 ml.[c]	7.42	6.99	6.88	6.62	6.79	6.71	0.194
Albumin, %[d]	32.0	40.9	39.1	40.4	43.2	42.0	2.28
α globulin, %	24.5	23.9	23.6	25.5	22.6	22.4	1.22
β globulin, %	12.1	10.6	11.3	12.5	12.1	10.9	0.69
γ globulin, %[e]	31.4	24.6	25.9	22.8	22.1	24.7	1.74
Final (98-day) values							
Total serum protein, gm./100 ml.[f]	7.46	7.00	7.11	7.05	7.09	7.03	0.142
Albumin, %[g]	27.0	48.0	37.7	46.8	44.2	48.2	2.24
α globulin, %	21.3	17.3	21.5	19.4	19.7	18.3	1.30
β globulin, %	13.0	12.9	12.3	12.4	12.0	12.6	0.71
γ globulin, %[h]	38.6	21.8	28.5	21.4	24.0	21.0	2.00

[a] Zinc added at rate of 50 ppm; iron, 100 ppm; and copper, 125 ppm.
[b] Based on 38 degrees of freedom.
[c] Lot 39 significantly greater than 42, 43, and 44 ($P < .05$).
[d] Lot 39 significantly less than 43, 44 ($P < .01$), and 40, 41, 42 ($P < .05$).
[e] Lot 39 significantly greater than all other lots: 42, 43 ($P < .01$), and 40, 41, 44 ($P < .05$).
[f] Lot 39 significantly greater than 40 ($P < .05$).
[g] Lot 39 significantly less than all other lots ($P < .01$). Lot 41 significantly less than 40, 42, 44 ($P < .01$), and 43 ($P < .05$).
[h] Lot 39 significantly greater than all other lots ($P < .01$). Lot 41 significantly greater than 40, 42, and 44 ($P < .05$).

as indicated in table 4. At 53 days all supplemented lots exhibited a lower percentage of gamma globulins and a higher percentage of albumin

than the basal pigs. The net result was a significantly higher total serum protein in the basal lot. Final values revealed a further increase in the gamma globulins and a decrease in the albumin fraction in the basal and iron-fed (lot 41) pigs, whereas the reverse was true in the remainder of the lots. Stevenson and Earle (1956) and Smith et al. (1960b) likewise have noted a reduction in the albumin:globulin ratio of parakeratotic pigs as compared to animals fed an adequate level of zinc. Except for the iron lot (41), all of the supplemented lots in the present trial exhibited serum protein values that compare favorably with those reported by Miller et al. (1961) for healthy pigs of comparable ages.

None of the experimental treatments had an appreciable influence on either the beta or alpha globulin fractions. This too is in agreement with Smith et al. (1960b).

Table 5 shows the results of the trace element analyses of the livers. Also included are values obtained from the livers of 10 healthy, presumably "normal," pigs fed low-calcium diets (mean, 0.44% Ca) with no trace element supplementation. The pigs fed 125 ppm of copper alone (lot 43) exhibited a significantly higher ($P < .01$) concentration of copper in the liver than those in the remaining lots. There was apparently an interaction between copper and zinc, for the mean liver copper concentration of lot 44 was not significantly higher than that of lots receiving no additional copper. It was not surprising to find that 50 ppm of additional zinc significantly increased ($P < .01$) the liver zinc concentrations in all lots that received the zinc supplement. However, neither copper nor iron supplementation had a significant effect on liver zinc levels. Thus it appears that the role of copper in preventing parakeratosis may not be explained on the basis of an ability

TABLE 5

Exp. 1, Trial B. -- Effect of supplemental zinc, iron, and copper on trace element content of pigs' livers[a]

Item	Lot no. and treatment[b]						Std. error of means[c]	Mean ± SE of "normal" pigs[d]
	39 Basal (1.05% Ca)	40 +Zn	41 +Fe	42 +Zn+Fe	43 +Cu	44 +Zn+Cu		
No. pigs	7	7	7	7	8	8	--	10
Liver wt., gm.[e]	914	1321	1192	1320	1449	1341	95.2	--
Copper, ppm[f]	12	17	17	18	156	32	17.3	22±1
Zinc, ppm[g]	106	257	115	250	103	243	11.2	165±11
Iron, ppm[h]	315	592	535	662	452	769	53.6	666±47

[a]Expressed in ppm, dry-weight basis.
[b]Zinc added at rate of 50 ppm; iron, 100 ppm; and copper, 125 ppm.
[c]Based on 38 degrees of freedom.
[d]Fed diets which averaged 0.44% calcium.
[e]No significant differences when corrected for body weight.
[f]Lot 43 significantly greater than all other lots ($P < .01$).
[g]Lots 40, 42, and 44 significantly greater than other three lots ($P < .01$).
[h]Lot 39 significantly less than all other lots except 43 ($P < .01$). Lot 43 significantly less than 44 ($P < .01$) and 42 ($P < .05$). Lot 44 significantly greater than 41 ($P < .01$) and 40 ($P < .05$).

to influence zinc storage, at least within the liver. However, in a later trial (Exp. II, trial B) it will be seen that in one instance, copper supplementation resulted in a significant increase in liver zinc (see table 13).

In light of reports published in recent years, the liver iron values in this trial are rather difficult to interpret. As mentioned in the REVIEW OF LITERATURE section, studies in this country have indicated there is a loss of liver iron when additional zinc is fed; however, it should be noted that the levels of supplemental zinc were much higher than in the present investigation. In Great Britain, Cassidy and Eva (1958b) have observed a reduction in liver iron as dietary copper level is increased. Neither of these phenomena occurred in this trial. There was in fact a significant increase ($P < .01$) in liver iron concentration over that of the basal pigs in all supplemented lots with the exception of the pigs fed only additional copper (lot 43). The increase was especially marked in the lots fed zinc in conjunction with copper or iron. One may surmise that this was merely a reflection of a decreased ability on the part of the debilitated basal pigs to assimilate dietary iron. This conjecture is supported by the fact that the mean liver iron level of the basal lot was less than half that of the "normal" pigs. On the other hand, it may be argued that when iron was added to the basal diet in lot 41, the "subnormal" parakeratotic pigs in that lot were able to absorb and store the supplemental iron to a considerable extent.

Trial C

Results for trial C are reported in table 6. At the low-calcium level there was a nonsignificant improvement in rate of gain when either

zinc (lot 46) or copper (lot 47) was added to the ration. Only one mild case of parakeratosis developed in the basal lot (45) and none in the zinc- or copper-supplemented groups. This is in contrast to the high incidence of severe parakeratosis that developed in lot 31 (trial A) on a very similar basal ration. Extreme variation in the incidence of parakeratosis and performance of pigs fed seemingly identical rations has been reported by Hoefer and Pond (1961).

Pigs receiving the high-calcium basal ration (lot 48) made very poor growth and all but one pig in this lot were afflicted with severe parakeratosis. Supplementation of this diet with 75 ppm of zinc (lot 49) resulted in improved growth rate ($P < .01$) and complete suppression of the dermatosis. When 125 ppm of copper were added to the high-calcium basal ration (lot 50), the effect on growth rate was less than that of supplemental zinc but still highly significant ($P < .01$). An important finding in this trial was the development of moderately severe skin lesions in 5 of the 10 pigs receiving the high-calcium diet supplemented with copper. The lesions were neither as extensive nor as severe as those in lot 48; nevertheless, parakeratosis was definitely present in the copper-supplemented pigs. These results are in conflict with those obtained in the first two trials where 125 ppm of copper was 100% effective in the prevention of parakeratosis. It should be noted, however, that the calcium content of the high-calcium basal ration used in trial C was approximately 140% and 25% higher than that fed in trials A and B, respectively.

All three of the low-calcium lots gained significantly faster than the high-calcium lots. It was apparent that the high-calcium diet placed a stress on the pigs which could not be overcome by either of the

TABLE 6

Exp. I, Trial C. -- Effect of supplemental copper and zinc at varying levels of dietary calcium (12 weeks)

	Lot no. and treatment[a]						Std.
	45	46	47	48	49	50	error
Item	Basal	+Zn	+Cu	Basal	+Zn	+Cu	of means[b]
Dietary Ca, %	0.55	0.55	0.55	1.31	1.31	1.31	--
No. pigs	10	10	10	10	10	10	--
Initial wt.,lb.	32.3	32.3	32.5	32.4	32.8	32.3	--
Final wt.,lb.	163.2	165.7	169.5	70.0	141.4	123.2	--
Da. gn.,lb.[c]	1.56	1.59	1.63	0.45	1.29	1.08	0.085
Feed/gain	3.13	3.11	3.19	4.23	3.58	3.53	--
Parakeratosis, %	10	0	0	90	0	50	--
Hemoglobin, gm./100 ml.[d]	14.0	14.4	13.8	12.4	14.8	13.3	0.36
Hematocrit, %[e]	42.4	43.4	41.7	38.7	44.5	41.9	0.90
S.A.P., units/l.[f]	1.38	3.54	1.66	1.38	3.94	1.54	0.201
Total serum protein, gm./100 ml.	6.63	6.80	6.70	6.60	6.73	6.74	0.128
Albumin, %[g]	50.3	51.1	48.0	38.7	46.3	41.7	1.92
αglobulin, %[h]	18.2	18.3	20.2	21.3	20.5	21.4	0.67
βglobulin, %[i]	11.7	12.1	14.0	13.8	13.5	12.7	0.56
γglobulin, %[j]	19.8	18.5	17.8	26.2	19.7	24.2	1.67

[a]Zinc added at rate of 75 ppm; copper at 125 ppm.

[b]Based on 54 degrees of freedom.

[c]Lot 48 significantly less than all other lots ($P<.01$). Lots 45, 46, and 47 significantly greater than 50 ($P<.01$) and 49 ($P<.05$).

[d]Lot 48 significantly less than 45, 46, 49 ($P<.01$), and 47 ($P<.05$). Lot 50 significantly less than 49 ($P<.01$).

[e]Lot 48 significantly less than all other lots: 45, 46, 49 ($P<.01$), and 47, 50 ($P<.05$).

[f]Lots 46 and 49 significantly greater than all other lots ($P<.01$).

[g]Lots 48 significantly less than all other lots except 50 ($P<.01$). Lot 50 significantly less than 45, 46 ($P<.01$), and 47 ($P<.05$).

[h]Lots 45 and 46 significantly less than 48, 50 ($P<.01$), and 49 ($P<.05$).

[i]Lot 45 significantly less than 47, 48, and 49 ($P<.05$). Lot 46 significantly less than 47 and 48 ($P<.05$).

[j]Lot 48 significantly greater than 46, 47 ($P<.01$), and 45, 49 ($P<.05$). Lot 50 significantly greater than 46 and 47 ($P<.05$).

trace element additions.

All three lots on the low-calcium diet and the high-calcium-plus-zinc group (lot 49) exhibited significantly higher hemoglobin levels than the pigs receiving the high-calcium basal ration (lot 48). There was also a significant difference ($P < .05$) between lots 49 and 50. Differences in hematocrit values were much the same as for hemoglobin except that lot 50 had a significantly higher ($P < .05$) hematocrit than lot 48, whereas the hemoglobin difference between these two lots was not significant.

Serum alkaline phosphatase activities were much higher ($P < .01$) for the zinc-fed pigs regardless of calcium level. Copper had a slight but not significant effect on the activity of this enzyme. Although there was no difference between the basal lots, there was considerably more variation in the high-calcium group. Had serum alkaline phosphatase been measured when symptoms of parakeratosis reached their peak (9 weeks), as was done in trial B, the differences in activity might have shown up greater at that time.

An examination of the serum protein data reveals that the lots with a high incidence of parakeratosis (lots 48 and 50) had a significantly higher gamma globulin fraction and a significantly lower albumin fraction than most of the other lots. These lots also exhibited a significantly higher alpha globulin fraction than lots 45 and 46 ($P < .01$).

It is highly possible that the alterations in the albumin and gamma globulin fractions observed in this trial and the previous one are the result of an increased susceptibility of the parakeratotic pig to invasion by infectious organisms, as concluded by Smith et al. (1960b). In other words, the increase in gamma globulins may be a

reflection of an immunological response. Lever et al. (1951) have reported that the serum electrophoretic patterns of humans suffering from cutaneous diseases are reflections of the patient's reaction to the disease.

Experiment II

In view of the results obtained in Experiment I, it was decided to examine further the relationship between copper and parakeratosis. In order to ensure the development of severe parakeratosis in Experiment II, the calcium levels of the low-zinc, basal rations were placed at well above 1.0%. Two levels of copper sulfate were compared in this experiment--125 and 250 ppm.

Trial A

Table 7 compares the performance of the six treatments during the first stage of this trial, from 0 to 10 weeks. It was necessary to remove one or two pigs from each lot shortly after the trial began as a result of pneumonia. During the first 10 weeks, growth of the basal pigs was subnormal, and they were significantly lower than all five experiment lots. However, lots 2 through 6 were not significantly different from one another. The separate effects of copper and zinc were analyzed statistically and found to be highly significant ($P < .01$), as was their interaction. The pigs fed the basal ration required somewhat more feed per pound of gain than pigs in the supplemented lots, but there were no appreciable differences between the latter in feed conversion.

From 6 to 8 weeks after the experiment started, the pigs in lot 1 began showing skin lesions typical of parakeratosis, as a result of the

high-calcium basal ration (1.3% Ca) they were consuming. Two of these

TABLE 7

Exp. II, Trial A. -- Response of pigs fed a high-calcium diet to supplemental copper and zinc, 0-10 weeks

Item	Lot no. and treatment[a] 1 Basal (1.3% Ca)	2 +Cu 125	3 +Cu 250	4 +Zn 100	5 +Zn 100 +Cu 125	6 +Zn 100 +Cu 250	Std. error of means[b]
No. pigs[c]	8	8	8	9	9	9	--
Initial wt.,lb.	24.8	24.1	24.1	24.2	23.9	24.0	--
10-week wt.,lb.	59.0	101.6	109.7	107.7	113.1	112.3	--
Daily gn.,lb.[d]	0.48	1.09	1.21	1.18	1.26	1.24	0.062
Feed/gain	3.33	2.87	2.66	2.76	2.82	2.79	--
Parakeratosis, 10 weeks, %	88	0	0	0	0	0	--

[a]All supplemental additions of copper and zinc are in ppm.
[b]Based on 45 degrees of freedom.
[c]Two pigs removed from lots 1, 2, and 3, and one from lots 4, 5, and 6 as a result of pneumonia.
[d]Lot 1 significantly less than all other lots ($P < .01$).

were considered quite severe and the other five ranged from mild to severe. None of the pigs in the supplemented lots showed any symptoms of parakeratosis during the first 10 weeks. These observations are in good agreement with those of Wallace et al. (1960) who reported that parakeratosis was prevented in pigs receiving a corn-soybean meal diet supplemented with 150 ppm of copper for a 70-day experimental period. However, they are in direct contrast with the work of O'Hara et al. (1960) who observed the unexpected development of parakeratosis in pigs fed 0.1% copper sulfate (250 ppm Cu) for 10 weeks but found no occurrence

of this condition in pigs fed a basal ration containing wheat and meat meal.

Table 8 shows the results of the period from 10 to 15 weeks and summarizes the entire trial, from 0 to 15 weeks. The basal pigs, represented by lot 1A, showed some spontaneous dermal improvement during the final 5 weeks but failed to recover completely; their average growth rate was considerably below normal. Although their progress was not as rapid or spectacular as is usually the case with zinc therapy, the pigs in lot 1B experienced a marked recovery from the parakeratotic symptoms, and at the end of 15 weeks only one of the four pigs exhibited any skin lesions. On the other hand, all three of the pigs in lot 1A that initially showed lesions still showed some evidence of them at the end of the trial.

Average daily gains of lots 1B through 6 were all significantly different from lot 1A but not from one another due to considerable variation within treatments.

The 125 ppm level of copper used in lot 2 was successful in delaying the onset of parakeratosis until the 14th week, when six out of seven, or 86%, of the pigs in that lot suddenly developed a dermatosis; two of these cases were quite severe. The growth rate of lot 2 declined sharply during the final 2 weeks of the trial. The soybean meal content of the lot 2 ration had been reduced after the 12th week. Because the low-protein ration contained less zinc than its high-protein counterpart (29 vs. 41), this change in diet may have been a factor influencing the development of parakeratosis in lot 2.

The higher copper level (250 ppm) was apparently more effective in preventing parakeratosis since only one pig in lot 3 developed any

TABLE 8

Exp. II, Trial A. -- Effect of supplemental copper and zinc on pig performance for last 5 weeks (10th-15th week) and for entire trial (0-15th week)

Item	Lot no. and treatment[a] 1A[b] Basal (1.3% Ca)	1B[b] + Cu 250	2 + Cu 125	3 + Cu 250	4 + Zn 100	5 +Zn 100 +Cu 125	6 +Zn 100 +Cu 250	Std. error of means[c]
No. pigs	4	4	7[d]	6[e]	9	9	9	--
10-week wt., lb.[f]	60.3	57.8	98.4	110.2	107.7	113.1	112.3	--
15-week wt., lb.	86.3	102.0	140.9	152.8	158.1	160.7	164.4	--
Daily gain, lb.[g]	0.74	1.26	1.21	1.22	1.44	1.36	1.49	0.134
Feed/gain	3.78	2.74	3.90	4.52	4.27	3.89	3.90	--
Parakeratosis, %								
10 weeks	75	100	0	0	0	0	0	--
15 weeks	75	25	86	17	0	0	0	--
Performance, 0-15 wks.								
Daily gain, lb.[h]	0.58	--	1.10	1.21	1.26	1.29	1.32	0.064
Feed/gain	--	--	3.21	3.21	3.33	3.19	3.20	--

[a]All supplemental additions of copper and zinc are in ppm.

[b]Lot 1 (basal) was divided after 10 weeks into lots 1A and 1B.

[c]Based on 42 degrees of freedom.

[d]One pig removed for reasons unrelated to treatment. Escherichia coli isolated from kidney, spleen, and bladder.

[e]Two pigs died as a result of copper toxicosis. A third pig died from same cause after trial was terminated.

[f]Differences in 10-week weights were result of prior treatment differences.

[g]Lot 1A significantly less than all other lots: 1B, 2, 3 ($P<.05$), and 4, 5, 6 ($P<.01$).

[h]Lot 1A significantly less than all other lots ($P<.01$). Lot 6 significantly greater than lot 2 ($P<.05$).

skin lesions and they were very mild. However, several pigs in this lot exhibited symptoms of copper toxicity--severe anemia, internal hemorrhages, jaundice, yellow cirrhotic livers, gastric ulceration, loss of weight, weakness, and incoordination. These symptoms resemble those reported by other workers (see REVIEW OF LITERATURE section). Two pigs in lot 3 died suddenly during the 13th week and another died after the 15th week. Although accurate diagnoses were difficult because of extensive post-mortem changes, it appeared likely that these three pigs died as a result of copper toxicosis. Two other pigs in this lot were close to death when they were submitted for autopsy after the 15th week. There was little doubt that these two pigs were suffering from the toxic effects of copper. Dyspnea was observed in one of these pigs after being driven a short distance. A similar observation was made by O'Hara et al. (1960) who reported respiratory distress in pigs suffering from copper toxicity as a result of feeding 250 ppm of copper. In the latter study, the authors noted a sudden liberation of copper into the blood and proposed that this was associated with the development of a severe anemia which in turn resulted in anoxia, circulatory failure, and death.

One of the three remaining pigs in lot 3 had an enlarged cirrhotic liver, and the carcass was condemmed by the inspecting veterinarian due to a pronounced, generalized icterus. The other two pigs appeared normal in every respect except for the "muddy" color of their livers.

Studies at the Florida station have demonstrated that copper toxicity in rats (McCall and Davis, 1961) and swine (Wallace et al., 1960) may be alleviated by increasing the protein content of the ration to relatively high levels. Perhaps some degree of protection against copper toxicity was removed from lot 3 when their dietary protein was

reduced to 12.5% after the 12th week.

It was apparent in this trial that zinc offered considerable protection against copper toxicity since none of the pigs in lot 6 showed any signs of toxic effects. Furthermore, there was no evidence of toxicity in pigs receiving 125 ppm of copper, with or without zinc (lots 2 and 5). Had the trial continued for a longer period of time, it would have been enlightening to observe if and when the pigs in lot 1B (250 ppm Cu) might have shown symptoms of copper poisoning. Results of this trial would seem to confirm the data of Wallace et al. (1960) which suggest that the margin between safe and toxic copper levels is relatively narrow. It is also apparent that effects of feeding high-copper levels should not be evaluated on the basis of short-term trials. For example, copper toxicity in lot 3 and the onset of parakeratosis in lot 2 would not have been observed had the present trial been terminated after the first period (10 weeks).

A summary of the entire trial in table 8 shows there was a trend for improved growth rate as the dietary copper level was increased and as zinc was added to the ration. The animals in lot 6 gained significantly faster ($P < .05$) than those in lot 2, and the difference in gain between lots 2 and 5 approached significance. Feed conversion ratios for the supplemented lots were virtually identical.

The effect of copper therapy on parakeratosis is illustrated in figure 1. These two pigs were typical of their respective lots, both at 10 and at 15 weeks.

Hematological data of lots 1A through 6 may be compared in table 9. Hemoglobin and hematocrit values of pigs in lot 3 were significantly lower than those of the other treatments, which was probably a reflection

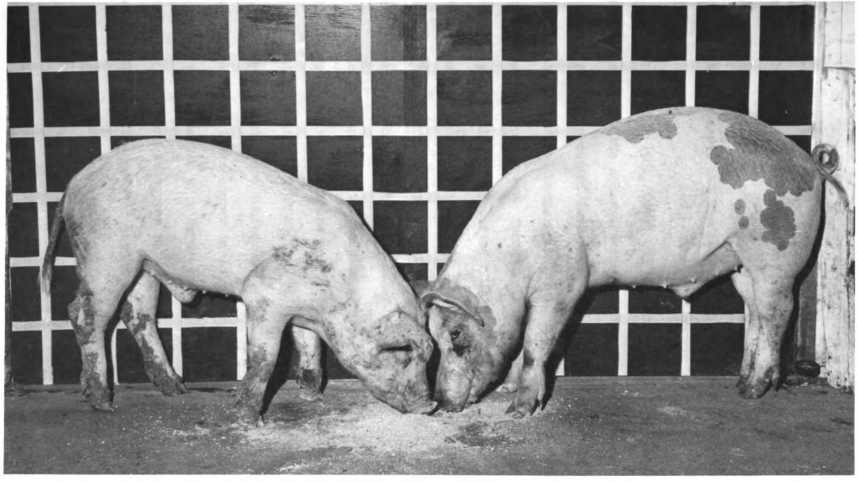

FIGURE 1. Exp. II, Trial A. -- Typical effect of copper therapy on parakeratosis. Both pigs weighed the same (60 lb.) after receiving high-calcium, low-zinc basal diet for 10 weeks. Pig on left continued to be fed basal ration, while pig on right was fed 250 ppm of supplemental copper for 5 weeks; difference in weight then was 38 lb.

of the copper toxicity prevalent in that lot. Although the differences between the other lots were not statistically significant, there was a tendency for hemoglobin and hematocrit values to decline as dietary copper was increased, and to rise when the ration was supplemented with zinc.

Bunch et al. (1961, 1963) and Wallace et al. (1960) have also shown that hemoglobin concentrations are depressed by high-copper diets. However, neither group of investigators found zinc to be of benefit in altering the subnormal hemoglobin levels found in pigs fed 200 or 250 ppm of supplemental copper even though weight gains were stimulated slightly. Simek et al. (1961) have reported that pigs fed high levels of copper sulfate exhibited lower hemoglobin concentrations but higher hematocrits. This is contradictory to the present data except for lots 1A and 1B, where such a relationship was found.

Table 10 summarizes the results of the trace element analyses of the livers. Individual values are given in appendix table 5. Table 10 shows that by adding 125 or 250 ppm of copper to the basal ration, liver copper levels were increased 11- and 60-fold, respectively. Concentration of liver copper in lot 3 was comparable to levels (684 to 1,800 ppm) observed by O'Hara et al. (1960) in the livers of pigs suffering from chronic copper poisoning. Buntain (1961) and Gordon and Luke (1957) have found liver copper levels of 710 to 2,200 ppm and 2,160 to 2,500 ppm, respectively, in pigs that apparently died as a result of copper toxicity. Allcroft et al. (1961) reported a mean liver copper level of 1,725 ppm in pigs fed a diet containing 0.1% copper sulfate for approximately 5 months. On the other hand, Iowa investigators (Hawbaker et al., 1961; Bunch et al., 1961, 1963) have

TABLE 9

Exp. II, Trial A. -- Hematology of pigs fed high levels of copper and zinc

Item	Lot no. and treatment[a] 1A[b] Basal (1.3% Ca)	1B[b] + Cu 250	2 + Cu 125	3 + Cu 250	4 + Zn 100	5 +Zn 100 +Cu 125	6 +Zn 100 +Cu 250	Std. error of means[c]
No. pigs	4	4	7	3	7	6	6	--
Hemoglobin, gm./100 ml.[d]	11.8	10.8	12.4	8.4	13.2	12.8	12.3	0.89
Hematocrit, %[e]	35.5	37.5	39.0	27.7	40.7	39.5	37.6	2.45

[a]All supplemental additions of copper and zinc are in ppm.
[b]Lot 1 (basal) was divided after 10 weeks into lots 1A and 1B.
[c]Based on 30 degrees of freedom.
[d]Lot 3 significantly lower than lot 1A ($P<.05$) and 2, 4, 5, 6 ($P<.01$).
[e]Lot 3 significantly lower than all other lots: 1A, 1B, 6 ($P<.05$), and 2, 4, 5 ($P<.01$).

TABLE 10

Exp. II, Trial A. -- Trace element content of livers from pigs fed high-calcium diet supplemented with copper and zinc[a]

Item	Lot no. and treatment[b] 1A[c] Basal (1.3% Ca)	1B[c] + Cu 250	2 + Cu 125	3 + Cu 250	4 + Zn 100	5 +Zn 100 +Cu 125	6 +Zn 100 +Cu 250	Std. error of means[d]
No. pigs	4	4	7	5	9	9	9	--
Liver wt., gm.[e]	1080	1122	1227	1315	1293	1239	1197	71.1
Copper, ppm[f]	25	437	276	1448	19	70	419	67.6
Zinc, ppm[g]	83	88	103	126	207	272	288	27.9
Iron, ppm[h]	299	125	325	96	537	420	225	41.4

[a]Expressed in ppm, dry-weight basis.

[b]All supplemental additions of copper and zinc are in ppm.

[c]Lot 1 (basal) was divided after 10 weeks into lots 1A and 1B.

[d]Based on 40 degrees of freedom.

[e]No significant differences when corrected for body weight.

[f]Lot 3 significantly greater than all other lots ($P < .01$). Lots 1B, 2, and 6 significantly greater than 1A, 4, and 5 ($P < .01$).

[g]Lots 4, 5, and 6 significantly greater than other lots ($P < .01$).

[h]Lot 4 significantly greater than 1A, 1B, 2, 3, and 6 ($P < .01$). Lot 5 significantly greater than 1B, 3, and 6 ($P < .01$). Lots 1A and 2 significantly greater than 1B and 3 ($P < .01$). Lot 6 significantly greater than lot 3 ($P < .05$).

found no evidence of toxicity in pigs fed 0.1% copper sulfate. The liver copper values reported in their work have accordingly been somewhat lower than those in table 10 and in the Australian and British studies cited above. It appears, therefore, that copper concentration of the liver may be a reasonably good diagnostic measure of copper toxicity. At any rate, the data of Allcroft et al. (1961) and Dammers et al. (1959) indicate that it is a more accurate measure of the copper status of the animal than either kidney or blood copper values. Allcroft et al. (1961) found that blood copper levels did not show a significant rise until the supplemental intake of copper sulfate reached 0.2% of the diet. Their studies also demonstrated that kidney copper concentrations did not exhibit a significant increase until the diet was supplemented with 0.12% copper sulfate.

Liver copper levels were dramatically reduced when zinc was added to the copper-supplemented rations. This is compatible with the observation that pigs receiving 250 ppm of copper with no added zinc developed symptoms of copper toxicity, whereas those receiving the same level of copper with added zinc showed no evidence of toxicity. The fact that the basal diets fed in the Iowa experiments, mentioned above, were well fortified with supplemental zinc (81.6 or 163.2 ppm) may be the explanation behind their reports of low liver copper levels and no toxicity in pigs fed 0.1% copper sulfate. Molybdenum content of the diet has also been found to influence tissue copper levels (Dick, 1956). Perhaps the Iowa rations were higher than normal in molybdenum content, which may have caused a reduction in liver copper storage. However, this is purely conjecture, as there were no molybdenum analyses reported for the rations fed in any of the studies

reviewed in this thesis.

Davis (1958) has reported that a high level of copper in the liver, such as may occur with copper toxicity, will result in an almost complete elimination of zinc from liver tissue. However, in this trial, increased levels of copper resulted in no depression of liver zinc; in fact there was a slight but not significant tendency for the storage of liver zinc to increase as copper increased.

As in Experiment I (trial B), the lower level of copper sulfate (125 ppm Cu) did not reduce the concentration of liver iron below that of the controls. However, the higher level (250 ppm Cu), both with and without supplemental zinc, did bring about a significant reduction ($P < .01$) in liver iron.

Trial B

It was decided that the results obtained in trial A merited further investigation. Trial B was essentially a repeat of the preceding one, with four exceptions: (1) ingredients of animal origin were deleted from the basal diet; (2) when the basal lot (30) was divided after 8 weeks, lots 30A and 30B were switched from the high-calcium basal diet to one which was lower (1.16% vs. 0.65) in calcium content, but the diets of the remaining lots were not altered; (3) the level of supplemental copper sulfate (125 ppm Cu) used as therapy in lot 30B was half the level fed to lot 1B in trial A; and (4) in addition to the values obtained at the time of slaughter, hematology was also studied midway through the experimental period.

Table 11 shows that the basal pigs (lot 30) performed very poorly during the first 8 weeks. These pigs began showing symptoms of parakeratosis at the third week, and by the sixth week, six out of the

nine cases were quite severe.

In contrast to trial A, parakeratotic lesions appeared in the copper-supplemented lots (31 and 32) at the end of the fourth week. By the eighth week, two pigs in lot 31 and three in lot 32 were considered to have severe cases of parakeratosis. Their reduced rates of growth in comparison to lots 33 through 35 ($P < .01$) were a reflection of their condition. It is apparent from the data that supplemental copper was at least partially effective in preventing parakeratosis. However, its influence was not as profound as in trial A, where the 250 ppm level furnished almost complete protection and 125 ppm delayed the disease for 14 weeks. Perhaps the slightly higher level of zinc in the basal diet in trial A (see appendix table 1) furnished the copper-supplemented pigs in that trial with some additional protection against parakeratosis.

At 8 weeks the hemoglobin values of the basal pigs were significantly lower than those of the other groups, with the exception of the pigs receiving 250 ppm of copper alone (lot 32). The hemoglobin level of the latter group was significantly lower than that of the zinc-fed lot (33). The hematocrit results were similar to those for hemoglobin. Qualitatively, these data resemble the 15-week values of the previous trial, in that there was a slight but not significant tendency for hematological values to regress with increasing copper levels and to improve with zinc supplementation.

TABLE 11

Exp. II, Trial B. -- Effect of supplemental copper and zinc on pig growth and hematology, 0-8 weeks

Item	Lot no. and treatment[a] 30 Basal (1.16% Ca)	31 + Cu 125	32 + Cu 250	33 + Zn 100	34 +Zn 100 +Cu 125	35 +Zn 100 + Cu 250	Std. error of means[b]
No. pigs	10	10	10	9[c]	10	10	--
Initial wt., lb.	30.3	30.2	30.3	30.8	30.3	30.4	--
8-week wt., lb.	44.6	77.5	77.3	105.3	103.9	104.4	--
Daily gain, lb.[d]	0.26	0.84	0.84	1.33	1.31	1.32	0.107
Feed/gain	6.69	3.50	3.04	2.83	2.87	2.72	--
Parakeratosis, 8 weeks, %	90	60	50	0	0	0	--
Hemoglobin, gm./100 ml.[e]	11.0	12.9	11.4	13.4	12.8	12.6	0.52
Hematocrit, %[f]	35.8	38.1	35.5	40.8	39.6	38.4	1.37

[a]All supplemental additions of copper and zinc are in ppm.

[b]Based on 53 degrees of freedom.

[c]One pig removed because of unthriftiness which was unrelated to treatment.

[d]Lot 30 significantly less than all other lots ($P < .01$). Lots 31 and 32 significantly less than 33, 34, and 35 ($P < .01$).

[e]Lot 30 significantly less than 33 ($P < .01$) and 31, 34, 35 ($P < .05$). Lot 32 significantly less than 33 ($P < .05$).

[f]Lots 30 and 32 significantly less than 33 ($P < .05$).

Table 12 shows the results of the period from 8 to 14 weeks and summarizes the entire trial, from 0 to 14 weeks. The pigs receiving the basal ration (lot 30A) made some improvement in rate of gain during this period, but their parakeratotic condition showed little or no improvement in spite of the fact that the calcium level of their diet had been reduced from 1.16% to 0.65%. The pigs receiving copper therapy (lot 30B) made an even more dramatic recovery than in the preceding trial. After 6 weeks on 125 ppm of copper, their mean body weight was equal to that of lots 31 and 32. When therapy was initiated, all five of the pigs in lot 30B carried skin lesions, most of them severe. By the end of the trial, three of these pigs had experienced a complete recovery and the other two exhibited only minor lesions.

Lots 33, 34, and 35 continued to gain significantly faster than lots 31 and 32 throughout the remainder of the trial. The growth rate and skin condition of the latter groups showed no appreciable change during the final 6 weeks.

At the final bleedings, there were no significant differences between treatments with respect to hematocrit, although the zinc-supplemented lot (33) was slightly higher than the copper-supplemented groups. Lots 30A and 32 had significantly lower ($P < .05$) hemoglobin values than lot 33. It is interesting to note that the hematological picture of lot 32 is not nearly as depressed as that of its counterpart in trial A (lot 3). This is consistent with the fact that there were no outward symptoms of copper toxicity in trial B, except for the death of one pig in lot 32 shortly after the trial was terminated. Since this pig had not yet been slaughtered, the final hematological measures could not be obtained. However, data obtained at the eighth week did not

TABLE 12

Exp. II, Trial B. -- Effect of supplemental copper and zinc during last 6 weeks (8th-14th week) and during entire trial (0-14th week)

Item	Lot no. and treatment[a] 30A[b] Basal (0.65% Ca)	30B[b] + Cu 125	31 + Cu 125	32 + Cu 250	33 + Zn 100	34 +Zn 100 +Cu 125	35 +Zn 100 +Cu 250	Std. error of means[c]
No. pigs	5	5	10	10	9	10	10	--
8-week wt., lb.[d]	44.6	44.6	77.5	77.3	105.3	103.9	104.4	--
14-week wt., lb.	78.2	114.4	114.8	115.7	172.7	172.2	170.9	--
Daily gain, lb.[e]	0.80	1.66	0.89	0.91	1.60	1.63	1.58	0.147
Feed/gain	4.32	2.58	4.29	4.08	4.00	3.66	3.89	--
Parakeratosis, %								
8 weeks	80	100	60	50	0	0	0	--
14 weeks	80	40	60	50	0	0	0	--
Hemoglobin, gm./100 ml.[f]	12.7	13.0	13.2	12.3	14.4	13.5	13.8	0.51
Hematocrit, %	39.0	40.2	40.4	39.3	43.6	41.3	41.6	1.46
No. cirrhotic livers	0	1	2	4	0	0	0	--
Performance, 0-14 wks.								
Daily gain, lb.[g]	0.50	--	0.86	0.87	1.45	1.45	1.43	0.120
Feed/gain	--	--	3.85	3.51	3.39	3.25	3.28	--

[a]All supplemental additions of copper and zinc are in ppm.

[b]Lot 30 (basal) was divided after 8 weeks into lots 30A and 30B. Calcium level of their diet was then reduced to 0.65%; other lots continued on high-calcium (1.16%) basal ration.

[c]Based on 52 degrees of freedom.

[d]Differences in 8-week weights were result of prior treatment differences.

[e]Lots 30A, 31, and 32 significantly less than all other lots ($P < .01$).

[f]Lots 30A and 32 significantly less than 33 ($P < .05$).

[g]Lots 33, 34, and 35 significantly greater than all other lots ($P < .01$). Lots 31 and 32 significantly greater than 30A ($P < .05$).

indicate any anemic condition. Furthermore, the liver copper concentration found in this animal--696 ppm--was below the average of the lot (see table 13). Nevertheless, post-mortem findings were highly suggestive of copper toxicosis. In the other pigs, there were no indications of copper toxicity until they were slaughtered. At that time, four of the pigs fed 250 ppm of copper alone (lot 32) showed cirrhotic livers, as indicated in table 12. One of these carcasses was condemmed due to a generalized icterus. One liver in lot 30B and two livers in lot 31 exhibited cirrhosis. None of the remaining lots showed any gross abnormalities of the liver.

Growth curves of several representative treatments for the entire trial are portrayed in figure 2. The marked effect of copper therapy in stimulating the growth rate of parakeratotic pigs is evident in this illustration.

It may be seen in table 13 that 250 ppm of copper, with or without zinc supplementation, significantly increased ($P < .01$) liver copper stores over those of all other lots. Contrary to previous results, addition of zinc oxide to the copper-supplemented rations did not cause a significant reduction in liver copper concentration owing to a great amount of variation within lots, but the difference between lots 32 and 35 approached statistical significance. Nine of the ten pigs in lot 32 had a liver copper concentration of 500 ppm or higher, whereas only three of the animals in lot 35 exhibited values of over 500 ppm. However, the highest liver copper level in the entire trial (1,819 ppm) was found in one of the pigs in lot 35. Individual values for liver copper, zinc, and iron are given in appendix table 6.

The lots receiving additional dietary zinc showed a significantly

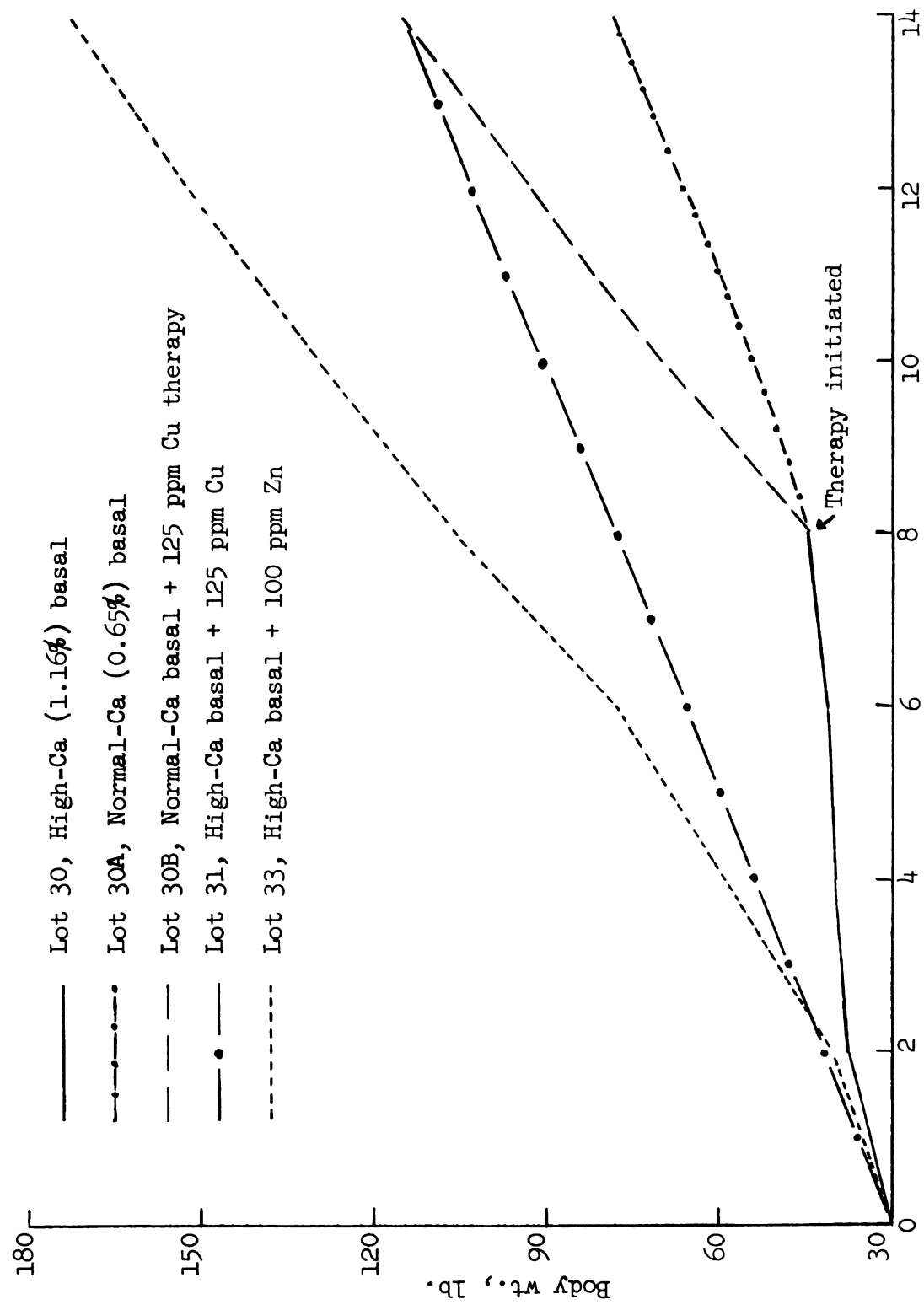

FIGURE 2. Exp. II, Trial B. -- Growth curves of pigs fed high- or normal-calcium diets supplemented with either copper or zinc.

TABLE 13

Exp. II, Trial B. -- Trace element content of livers from pigs fed supplemental copper and zinc[a]

Item	Lot no. and treatment[b]							Std. error of means[d]
	30A[c] Basal (0.65% Ca)	30B[c] + Cu 125	31 + Cu 125	32 + Cu 250	33 + Zn 100	34 +Zn 100 +Cu 125	35 +Zn 100 +Cu 250	
No. pigs	5	5	10	10	9	8[e]	10	--
Liver wt., gm.[f]	976	1135	1052	1228	1272	1226	1208	82.2
Copper, ppm[g]	20	64	58	869	24	34	571	109.4
Zinc, ppm[h]	104	222	103	105	239	260	256	27.0
Iron, ppm[i]	334	364	243	203	490	548	247	46.6

[a]Expressed in ppm, dry-weight basis.

[b]All supplemental additions of copper and zinc are in ppm.

[c]Lot 30 (basal) was divided after 8 weeks into lots 30A and 30B. Calcium level of their diet was then reduced to 0.65%; other lots continued on high-calcium (1.16%) basal ration.

[d]Based on 50 degrees of freedom.

[e]Two livers from lot 34 lost during slaughter.

[f]No significant differences when corrected for body weight.

[g]Lots 32 and 35 significantly greater than all other lots ($P < .01$).

[h]Lots 30B, 33, 34, and 35 significantly greater than other three lots ($P < .01$).

[i]Lot 34 significantly greater than 30A, 31, 32, 35 ($P < .01$), and 30B ($P < .05$). Lot 33 significantly greater than 31, 32, 35 ($P < .01$), and 30A ($P < .05$). Lot 30B significantly greater than 32 ($P < .05$).

higher ($P < .01$) liver zinc storage, which is consistent with previous results. However, lot 30B (125 ppm Cu therapy) also exhibited a significantly higher ($P < .01$) concentration of liver zinc than the other lots receiving no added zinc. In fact, the mean value for lot 30B was nearly as high as for the lots fed supplemental zinc. Because of this unexpected finding, the livers from this lot were assayed again for zinc; the repeat values were virtually the same as the originals. The higher feed consumption of the pigs in lot 30B may have partially, but not entirely, accounted for the difference. If indeed the therapeutic copper fed to this lot was responsible for an increased utilization of dietary zinc, then this fact could at least in part account for the marked recovery from parakeratosis noted in these pigs. The small number of pigs involved and the great amount of variation in the liver zinc values in this lot preclude such a conclusion.

In general, the liver iron data resemble those of the previous trial. Except for lot 34, all of the copper-supplemented lots were lower in liver iron than the pigs supplemented only with zinc (lot 33); some of these differences were significant, while others were not.

Tissues from 57 pigs were examined histopathologically in this trial. In general, there were few, if any, changes in the spleen, heart, lung, or kidney that could be attributed to the experimental treatments. In the liver, however, some histological changes were noted in those pigs fed supplemental copper sulfate. These observations are summarized in the following paragraphs, and the individual pig data are compiled in appendix table 7.

Lot 30A (Basal). -- The livers of five animals were examined microscopically. All were essentially normal except for the occurrence

of small foci of dark-staining lymphocyte-like cells. These were interpreted to be hemopoietic centers.

Lots 30B and 31 (125 ppm Cu). -- Fifteen livers from pigs fed 125 ppm of supplemental copper were examined. Nine of these were essentially normal. In five of the other six, there was a questionable but slight increase in connective tissue in the periphery of the lobules (interlobular septa). In the remaining liver, it was judged that there was a definite slight increase in the amount of interlobular connective tissue.

Lot 32 (250 ppm Cu). -- Seven livers from this lot underwent examination. One liver was essentially normal, but in the other six there was an increase in connective tissue peripherolobularly. Furthermore, there were focal areas of fibrosis in two of these livers. Eosinophils were present in these fibrotic areas.

Lot 33 (100 ppm Zn). -- Nine livers were examined and all were essentially normal except for the presence of hemopoietic centers in several of them.

Lot 34 (100 ppm Zn + 125 ppm Cu). -- Seven liver specimens were examined and six were essentially normal. There was an increased amount of connective tissue in the liver of one animal.

Lot 35 (100 ppm Zn + 250 ppm Cu). -- Eight livers were examined and six were essentially normal. In one liver, a slight increase in the amount of connective tissue in the interlobular septa was noted. In another, a marked degree of fibrosis was observed; the connective tissue was not only increased in the interlobular septa but also tended to replace entire lobules.

General. -- All seven of the livers that were grossly cirrhotic (see table 12) were likewise among those that showed an increase in

fibrous connective tissue when examined microscopically. Moreover, it was apparent upon histological examination that the lots having the highest incidence of fibrotic livers were those which had the highest accumulation of liver copper (see table 13).

Experiment III

The results of Experiment III are summarized in table 14. One pig in lot 3 became unthrifty and died 22 days after the start of the trial. This pig was submitted for autopsy, and liver changes suggestive of an acute toxicity snydrome were observed. It is possible that the copper-supplemented diet the pig had been consuming could have contributed to his death; however, it seems improbable that a toxic condition would develop so early in the experiment. On the other hand, Fagan et al. (1961) attributed the death of a single pig in a lot of 20 pigs receiving 0.1% copper sulfate to the ingestion of a particle of copper sulfate in feed which apparently had not been thoroughly mixed. They cautioned against the dangers of improper mixing of feeds supplemented with copper sulfate. In the present experiment, perhaps the pig in question consumed a portion of feed which contained an unusually high concentration of copper sulfate. Unfortunately, the liver from this animal was not obtained for copper assay.

Values for average daily gain indicate there was a small additive response to the combination of copper sulfate and chlortetracycline. All three treated lots out-gained the basal pigs, but only the difference between lots 2 and 5 was statistically significant ($P < .05$). Iowa workers have observed an additive response with respect to growth rate from a copper sulfate-antibiotic combination (see REVIEW OF

LITERATURE section). British reports have varied on this point, but fairly recent work by Bellis (1961) and by Braude et al. (1962) indicates there is little or no advantage in supplementing British pig diets, containing 250 ppm of copper, with a broad-spectrum antibiotic.

There were no appreciable differences in feed efficiency except for lot 4, which required approximately 7% more feed per pound of gain than the other groups.

None of the differences in hemoglobin or hematocrit were statistically significant.

TABLE 14

Exp. III.-- Response of pigs to copper sulfate and chlortetracycline, alone and in combination (14 weeks)

Item	Lot no. and treatment 2 Basal (0.63% Ca)	3 + CTC[a] 10 mg./lb.	4 + Cu 125 ppm	5 + CTC + Cu	Std. error of means[b]
No. pigs	8	8	7[c]	8	--
Initial wt., lb.	36.5	36.0	38.0	36.0	--
Final wt., lb.	184.4	196.9	195.0	200.0	--
Daily gain, lb.[d]	1.51	1.64	1.60	1.67	0.052
Feed/gain	3.04	3.06	3.26	3.03	--
Hemoglobin, mg./100 ml.[e]	13.0	13.4	13.7	12.9	0.48
Hematocrit, %[e]	39.1	39.6	40.5	38.6	1.48

[a]Chlortetracycline.
[b]Based on 27 degrees of freedom.
[c]One pig died suddenly after 22 days. Upon autopsy, it was concluded: "the liver changes suggest an acute toxicity syndrome."
[d]Lot 5 significantly greater than lot 2 ($P < .05$).
[e]No significant differences between lots.

Experiment IV

Experiment IV was designed to compare two common inorganic forms of copper, namely, the sulfate and oxide salts. It is evident in table 15 that the treatment differences in daily gain were small and nonsignificant. Pigs in the basal lot grew normally and displayed no symptoms of parakeratosis in spite of the fact that their diet contained only 30 ppm of zinc. It is noteworthy that in Experiment I (trial A), a high incidence of parakeratosis occurred in pigs fed a basal ration similar in calcium and zinc content to the present one. This again suggests that there are other factors involved in the etiology of the parakeratotic syndrome.

Neither copper sulfate nor copper oxide caused a significant reduction in hemoglobin or hematocrit. This is consistent with the fact that there were no clinical symptoms of copper toxicity at any time during the experiment. When the pigs were slaughtered, however, gross examination of the livers revealed that there were varying degrees of hepatic damage resulting from the copper-supplemented diets. Lot 11 ($CuSO_4$ alone) displayed by far the highest incidence of cirrhotic livers--90%. The pigs fed copper oxide alone (lot 12) showed considerably less cirrhosis (30%), followed by those lots which were fed zinc in combination with copper (lots 14 and 15).

The liver copper values (table 16 and appendix table 8) appeared to be directly related to the incidence of cirrhosis. Lot 11 exhibited a significantly higher ($P < .01$) concentration of liver copper than all the other groups. Lots 12 and 14 were also significantly greater ($P < .01$) than the lots ranking below them. The addition of 100 ppm of zinc to

TABLE 15

Exp. IV. -- Comparison of two forms of copper added to a normal-calcium diet with and without supplemental zinc (13 weeks)

Item	Lot no. and treatment[a] 10 Basal (0.66% Ca)	11 + $CuSO_4$	12 + CuO	13 + Zn	14 + Zn + $CuSO_4$	15 + Zn + CuO	Std. error of means[b]
No. pigs	9[c]	10	10	10	10	10	--
Initial wt., lb.	31.3	30.0	30.8	31.6	30.8	30.9	--
Final wt., lb.	177.6	169.1	175.5	178.4	170.1	181.4	--
Daily gain, lb.[d]	1.62	1.55	1.61	1.63	1.55	1.67	0.051
Feed/gain	2.88	2.78	2.70	2.85	2.68	2.75	--
Hemoglobin, mg./100 ml.[d]	12.0	11.6	12.0	12.1	12.1	12.4	0.32
Hematocrit, %[d]	38.3	37.4	36.9	37.6	37.9	37.7	0.95
No. of cirrhotic livers	0	9	3	0	1	1	--

[a]Copper was added at the rate of 250 ppm; zinc at the rate of 100 ppm.
[b]Based on 53 degrees of freedom.
[c]One pig removed from lot 10 due to chronic pneumonia.
[d]No significant differences between lots.

TABLE 16

Exp. IV. -- Trace element analyses of liver and loin from pigs fed copper sulfate and copper oxide with and without zinc[a]

Item	Lot no. and treatment[b] 10 Basal (0.66% Ca)	11 + $CuSO_4$	12 + CuO	13 + Zn	14 + Zn + $CuSO_4$	15 + Zn + CuO	Std. error of means[c]
Liver analyses							
No. pigs	9	10	10	10	10	10	--
Liver wt., gm.[d]	1346	1368	1291	1165	1385	1311	40.9
Copper, ppm[e]	39	1572	861	30	835	246	103.4
Zinc, ppm[f]	123	106	111	258	225	214	15.0
Iron, ppm[g]	558	118	157	637	181	421	35.8
Loin analyses							
No. pigs	3	4	4	3	3	3	--
Copper, ppm[h]	2.10	2.55	2.45	2.26	2.17	2.85	0.228

[a]Expressed in ppm, dry-weight basis.

[b]Copper was added at the rate of 250 ppm; zinc at the rate of 100 ppm.

[c]Based on 53 and 14 degrees of freedom for liver and loin values, respectively.

[d]Lot 13 significantly less than all other lots: 10, 11, 14 ($P < .01$), and 12, 15 ($P < .01$).

[e]Lot 11 significantly greater than all other lots ($P < .01$). Lots 12 and 14 significantly greater than 10, 13, and 15 ($P < .01$).

[f]Lots 13, 14, and 15 significantly greater than other three lots ($P < .01$).

[g]Lots 10 and 13 significantly greater than all other lots ($P < .01$). Lot 15 significantly greater than 11, 12, and 14 ($P < .01$).

[h]No significant differences between lots. There was no correlation between loin and liver copper concentrations ($r = 0.02$).

the diets of pigs fed either form of copper resulted in a significant reduction in the accumulation of liver copper. It is worthy of note that the oxide-fed pigs stored 70% and 45% less liver copper than those fed sulfate, with and without zinc, respectively. This is in agreement with the work of Bunch et al. (1961, 1963), who showed that pigs fed supplemental copper sulfate accumulated more than twice as much copper in their livers as those fed copper oxide. These workers also reported that supplemental zinc was necessary for maximum growth response to copper supplementation. This was not observed in the present experiment but was evident in previous trials.

It is somewhat surprising that no clinical symptoms of copper toxicity were observed in lot 11 when it is considered that their mean liver copper concentration was even slightly higher than that of pigs fed the same level of copper sulfate in Experiment II, trial A (1,572 vs. 1,448 ppm); the latter pigs showed definite clinical symptoms of a toxicosis. However, there is undoubtedly a considerable amount of variation among animals in their ability to tolerate high levels of dietary copper. Wallace et al. (1960) have reached this conclusion from their studies on high-level copper feeding.

As was anticipated, the zinc-supplemented lots--13, 14, and 15--had significantly higher liver storage levels of this element than did the unsupplemented lots--10, 11, 12. Neither copper oxide nor copper sulfate had a significant influence on liver zinc values.

Both forms of copper significantly depressed the storage of liver iron. Copper sulfate had a greater effect in this respect than copper oxide, especially when supplemental zinc was fed (lot 14 vs. lot 15). When zinc was introduced into the diet, the depressant effect of dietary

copper on liver iron storage was partially overcome, which agrees with Experiment II.

From the data compiled to date, it appears that within the liver of the pig there is competition between these three elements for storage sites. When a sufficient amount of copper is absorbed, iron storage appears to be inhibited. When zinc is added to a copper-supplemented diet, uptake of copper by the liver is depressed. This in turn could be the explanation for the higher iron levels observed in such livers, compared to livers from pigs fed copper alone. The data indicate that the interaction between copper and zinc is not reciprocal; that is, the copper levels employed in these experiments have not resulted in a lowered uptake of zinc. It cannot be said whether the interaction between copper and iron is reciprocal because high levels of these elements were not fed jointly; however, there was no depression of liver copper when 100 ppm of iron was added to the basal ration in Experiment I (table 5). There is no evidence in the literature that high levels of dietary iron interfere with liver copper storage. These competitive interactions may be considerably more complex than this writer has just indicated. Furthermore, any conclusions drawn from the results of this work cannot be justifiably applied to situations where the supplemental trace element levels differ from those employed here.

It was decided to assay loin samples from 20 of the pigs in this experiment in order to learn whether high levels of dietary copper result in an increased deposition of copper in skeletal muscle tissue. Each lot was randomly sampled. It is obvious from the data in table 16 that neither form of supplemental copper significantly increased the concentration of copper in the longissimus dorsi. Compared to the liver,

the loin values are extremely low, the highest individual value being 3.69 ppm (see appendix table 8). Bunch et al. (1963) have reported that levels of loin copper were increased significantly in pigs fed 250 ppm of supplemental copper from either the sulfate or oxide salts. Their mean values were somewhat higher than those reported here, ranging from 5.7 ppm for their basal lot to 10.3 ppm for pigs receiving copper oxide; however, the latter concentration could hardly be considered dangerous for human consumption. Barber et al. (1957) have pointed out that calf liver, containing 150 ppm of copper, is not considered unfit for human consumption.

In the present trial, there was no correlation between loin and liver copper concentrations ($r = 0.02$).

Experiment V

Trial A

The results of trial A are summarized in table 17. The high-calcium basal pigs (lot 27) grew significantly slower than the other lots and all but one pig in this lot exhibited symptoms of parakeratosis. Skin lesions began to appear after 3 weeks and became very severe after 8 weeks, when the trial was terminated and the pigs slaughtered.

The normal-calcium basal pigs (lot 26) performed as well as those fed the same diet supplemented with zinc (lot 31), even though two pigs in the former lot developed a mild dermatosis. Supplementing the high-calcium ration with 125 ppm of copper enabled the pigs in lot 29 to grow almost as rapidly as those in the other supplemented groups. By the time they were slaughtered, four of these pigs exhibited mild skin lesions which were perceptible only upon close examination. Thus, the

TABLE 17

Exp. V, Trial A. -- Effect of supplemental copper and zinc with varying calcium levels on growth, hematology, and intestinal phytase and phosphatase (8 weeks)

Item	Lot no. and treatment[a] 26 Normal Ca (0.61%)	27 High Ca (1.26%)	28 High Ca + Zn	29 High Ca + Cu	30 High Ca + Zn + Cu	31 Normal Ca + Zn	Std. error of means[b]
No. pigs[c]	8	9	8	9	8	9	--
Initial wt., lb.	33.5	33.1	33.1	33.0	32.9	32.7	--
Final wt., lb.	101.3	60.0	103.6	97.8	108.4	100.3	--
Daily gain, lb.[d]	1.21	0.48	1.26	1.16	1.34	1.21	0.069
Feed/gain	2.81	4.20	2.84	2.88	2.72	2.81	--
Parakeratosis, %	25	89	0	44	0	0	--
Hemoglobin, gm./100 ml.[e]	12.0	11.4	11.9	11.8	12.2	11.2	0.33
Hematocrit, %[f]	39.5	36.2	38.7	38.2	40.2	38.0	0.87
Intestinal phytase[e,g]	33.7	34.3	34.7	31.8	33.4	35.7	4.50
Intestinal phosphatase[g,h]	69.7	51.2	--	--	--	--	5.29

[a]Copper was added at the rate of 125 ppm; zinc at the rate of 100 ppm.

[b]Growth and hematology based on 45 degrees of freedom, phytase on 32, and phosphatase on 13.

[c]One pig removed from each of lots 26, 28, and 30 due to unthriftiness which appeared to be unrelated to the experiment.

[d]Lot 27 significantly less than all other lots ($P < .01$).

[e]No significant differences between lots.

[f]Lot 27 significantly less than 26 ($P < .05$) and 30 ($P < .01$).

[g]Activity expressed as mcg. inorganic phosphorus per mg. protein.

[h]Lot 27 significantly less than lot 26 ($P < .01$).

ability of supplemental copper to partially alleviate the symptoms of parakeratosis was reconfirmed (see tables 2, 3, 6, 7, 8, 11, and 12).

It is interesting to note that the high-calcium-plus-zinc lot (28) gained just as rapidly as its normal-calcium counterpart (lot 31). There was a slight additive response in growth rate when copper and zinc were combined in lot 30, but it was not statistically significant.

None of the differences in hemoglobin were significant. The mean hematocrit value of the high-calcium basal lot was significantly lower than that of lot 26 ($P < .05$) and lot 30 ($P < .01$). The blood picture of the copper-supplemented pigs was not depressed, as it was in Experiment II.

Thirteen of the intestinal tissue extracts were not assayed immediately and became contaminated with microbial growth which meant that it was necessary to discard them. Fortunately, each lot was about equally represented in this loss. There were no significant differences between lots with respect to intestinal phytase activity. The results of the assay were highly variable, which is probably indicative of inaccuracies inherent in the method.

In this trial, intestinal phosphatase assays were performed only on the normal- and high-calcium basal lots (26 and 27). The high-calcium pigs exhibited a significantly lower intestinal phosphatase activity than those fed the normal-calcium diet.

Trial B

This trial was conducted to further test the hypothesis that high-calcium, parakeratotic diets inhibit the activity of phytase or phosphatase in the intestine of the pig. The data are summarized in table 18.

As mentioned in the EXPERIMENTAL PROCEDURE section, it was necessary to add zinc to the low-calcium diet after 3 weeks to prevent

TABLE 18

Exp. V, Trial B. -- Effect of normal and parakeratotic diets on intestinal phosphatase activity (7 weeks)

Item	Lot no. and treatment: 20[a] Low Ca (0.53% Ca)	21 High Ca (1.42% Ca)	Std. error of means[b]
No. pigs	7	7	--
Initial wt., lb.	23.0	23.0	--
Final wt., lb.	79.0	47.7	--
Daily gain, lb.[c]	1.14	0.50	0.058
Feed/gain	2.44	3.43	--
Parakeratosis, %	0	100	--
Intestinal phosphatase[d]	53.2	61.0	3.36

[a]It was necessary to add 100 ppm of zinc to the lot 20 ration after 21 days in order to prevent parakeratosis completely.
[b]Based on 12 degrees of freedom.
[c]Lot 20 significantly greater than lot 21 ($P < .01$).
[d]Activity expressed as mcg. inorganic phosphorus per mg. protein.

the development of parakeratosis in lot 20. After 7 weeks, the weight gains of the high-calcium pigs (lot 21) had come to a virtual standstill and all of them showed symptoms of parakeratosis. At this time, both lots were slaughtered, and assays for phytase and phosphatase were performed on a section of small intestine from each pig.

Unfortunately, none of the intestinal samples exhibited phytase activity. The sodium phytate substrate was apparently unstable during

storage, for its inorganic phosphorus content was found to be three times higher than it had been originally (0.93% vs. 0.31%). Perhaps all of the hydrolyzable phosphate groups on the sodium phytate underwent a non-enzymatic hydrolysis during storage; or rather, something may have occurred during the preparation of the tissue extracts to destroy their phytase activity. Conversely, sodium-beta-glycerophosphate, used as substrate in the phosphatase assay, is apparently a very stable compound; no trace of inorganic phosphorus was detected in the substrate blanks during the assay of this enzyme.

Contrary to trial A, the high-calcium pigs in this trial showed a higher intestinal phosphatase activity than the low-calcium pigs. However, the difference was not statistically significant.

V. SUMMARY

Experiment I

Three trials, involving a total of 158 weanling pigs, were conducted to study the effect of adding copper (125 ppm), zinc (50 or 75 ppm), or iron (100 ppm) to basal diets varying in calcium content (0.55, 1.05, and 1.31%). Zinc-copper and zinc-iron combinations were employed in two of these trials.

Parakeratosis occurred in the basal pigs at all three levels of calcium intake. Compared to healthy pigs, the parakeratotic animals were characterized in most instances by depressed growth rate, higher serum gamma globulin fraction, lower serum albumin fraction, and lower serum alkaline phosphatase activity, in addition to the skin lesions typical of this syndrome. Lower hemoglobin and hematocrit, and higher total serum protein values were also noted in several lots in which parakeratosis was present.

Zinc supplementation was completely effective in preventing parakeratosis. Supplemental copper was completely effective at the 0.55 and 1.05% calcium levels and partially effective at the 1.31% level. Iron partially prevented the disease at the lowest level of calcium intake but had no effect when it was added to a ration having a higher calcium content. Besides eliciting an improvement in rate of gain and skin condition, these trace elements produced highly significant responses in certain of the biochemical observations. The albumin and gamma globulin fractions and alkaline phosphatase activities of the serum were particularly sensitive to zinc and to copper supplementation.

A combination of zinc and iron was not as beneficial in improving

growth rate as zinc alone, which suggests an interaction between these two elements. A zinc-copper combination proved no more effective in stimulating body weight gains than zinc alone.

Pigs fed 125 ppm of supplemental copper for 14 weeks exhibited a significantly higher ($P < .01$) concentration of copper in their livers. Including zinc in the copper-supplemented ration significantly reduced the concentration of liver copper, but the reverse situation was not observed; that is, supplemental copper had no effect on liver zinc storage. Pigs fed the 1.05% Ca basal diet showed less than half the concentration of liver iron found in "normal" pigs fed a low-calcium (0.44%) ration. Liver iron values were significantly increased by all trace element additions except copper alone.

Experiment II

Two trials were conducted to investigate the effect of adding two levels of copper sulfate (125 and 250 ppm), with or without supplemental zinc (100 ppm), to a high-calcium, low-zinc, parakeratosis-producing diet. In each trial, the parakeratotic basal lot was divided after several weeks to study the effect of copper therapy (125 or 250 ppm Cu). Each trial initially involved 60 pigs.

The following results were obtained in the first trial:

Copper sulfate, when added to the basal diet (1.3% Ca; 42 ppm Zn) at either supplemental level, improved pig growth and prevented parakeratosis during all but the last week of a 15-week trial. The 250 ppm level of copper was much more effective in preventing the disease than the lower level. Symptoms of copper toxicity were observed after 12 weeks in pigs receiving 250 ppm of copper without added zinc. These pigs exhibited significant reductions in hemoglobin and hematocrit,

and their livers were found to contain extremely high levels of copper. When zinc was added to the copper-supplemented rations, there was a slight increase in growth rate over those lots fed copper alone, but no improvement over the pigs fed zinc alone. As in the previous experiment, zinc completely prevented parakeratosis; it also profoundly reduced liver copper levels and appeared to furnish complete protection against copper toxicity. Copper significantly lowered the concentration of iron in the liver, but this effect was overcome when zinc was added to the ration. Copper had no significant influence on liver zinc. After 10 weeks, half of the pigs fed the basal diet began receiving the same ration plus 250 ppm of copper. A slow but marked recovery from parakeratosis occurred during the ensuing 5 weeks; there was no evidence of a toxicosis resulting from the copper therapy.

The second trial was initially composed of the same ration treatments as the first. The following observations were made:

Pigs fed either level of copper sulfate for 14 weeks gained significantly faster than those fed the basal diet (1.16% Ca; 30 ppm Zn). Supplemental copper reduced the incidence and severity of parakeratosis but was much less effective in this trial than in the previous one. The higher level of copper sulfate was no more effective in preventing the syndrome than the lower level, which is contrary to the first trial. Differences in hematology were small, although the pigs fed 250 ppm of copper alone tended to have lower hemoglobin and hematocrit values than those receiving zinc. Except for the death of one pig in the lot fed the higher copper level, there were no clinical symptoms of copper toxicity in this trial. Upon slaughter, however, four of these pigs exhibited cirrhotic livers and one carcass

was condemmed because of icterus. Other results obtained in this trial verified the findings of the first trial.

Experiment III

Thirty-two pigs were allotted into four treatment groups to compare the separate and combined effects of chlortetracycline (10 mg./lb.) and copper sulfate (125 ppm Cu) for 14 weeks. The basal diet contained a normal level of calcium (0.63%) and an adequate amount of zinc (175 ppm).

Either of these chemotherapeutics fed alone caused a small, non-significant increase in daily gain. When they were fed together, there was a significant ($P<.05$) additive response in growth rate. There were no significant differences in hematology.

Experiment IV

Sixty pigs were divided into six lots to compare the effect of high-level (250 ppm Cu) copper sulfate or copper oxide added to a normal-calcium (0.66%) diet, with and without supplemental zinc (100 ppm).

All lots grew rapidly and there were essentially no differences in performance throughout the 13-week experimental period. There was no evidence of parakeratosis. Differences in hematology were small and nonsignificant.

There were no clinical symptoms of copper toxicity, but when the pigs were slaughtered there was a high incidence (90%) of cirrhotic livers in the lot fed copper sulfate. The incidence was much lower (30%) in the lot receiving copper oxide.

The copper oxide-fed pigs stored significantly less copper in the liver than those fed copper sulfate; this was true either with or without added zinc. Both forms of copper significantly reduced liver iron

storage, copper sulfate having a greater effect than copper oxide.

Neither form of copper had a significant influence on the concentration of copper found in the longissimus dorsi muscle. None of the loins sampled contained as much as 4 ppm of copper.

Experiment V

Two trials were conducted to test the hypothesis that parakeratosis-producing diets inhibit the activity of phytase or phosphatase in the intestine of the pig. The study involved 68 weanling pigs.

The results were highly variable. In only one instance was there a significant difference favoring acceptance of the hypothesis.

VI. CONCLUSIONS

Within the confines of the experimental methods employed, the results of this study have led the author to make the following conclusions:

1. Supplemental copper, fed at the rate of either 125 or 250 ppm, is partially effective in reducing the incidence and severity of parakeratosis in growing-finishing swine. It is not, however, as effective as zinc, nor can it be considered a satisfactory substitute for zinc in the prevention or treatment of this disease.

2. Supplemental iron (100 ppm), when added to a low-calcium (0.55%) diet, has a positive but less pronounced effect than zinc or copper in preventing parakeratosis. When added to a high-calcium (1.05%) ration, iron is ineffective in the prevention of parakeratosis.

3. The growth rate of pigs fed a low-zinc diet is improved significantly when copper is added to the ration, especially during the period from 30 to 100 lb. live weight. However, there is little or no growth response when copper is added to a well-balanced diet which has been adequately fortified with zinc.

4. Copper sulfate, fed at a level of 125 ppm of copper, is no more effective in stimulating the growth rate of pigs than a broad-spectrum antibiotic (10 mg./lb.). However, there may be a slight additive response when the diet is supplemented jointly with these two additives.

5. Liver copper concentrations are significantly increased by high dietary levels of this element. Copper sulfate has a significantly greater effect in this respect than copper oxide. Neither form of

supplemental copper has an appreciable influence on the accumulation of copper in the longissimus dorsi muscle.

6. When 250 ppm of copper as copper sulfate is fed continually in the diet for more than 10 weeks, there is considerable risk of copper toxicity. However, 100 ppm of supplemental zinc will significantly lower the concentration of liver copper and reduce the probability of copper toxicosis. The accumulation of copper in the liver is significantly reduced when 125 ppm of copper is fed, and there is accordingly less danger of toxicity.

7. High levels of dietary copper significantly reduce the storage of liver iron, but this effect may be partially overcome by feeding additional zinc. The interaction between copper and zinc does not appear to be reciprocal; that is, high levels of copper have little or no influence on the storage of liver zinc.

8. A high level of calcium in the ration does not appear to inhibit intestinal phytase or phosphatase activity. The activity of intestinal phytase is not increased by supplemental zinc or copper.

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APPENDIX TABLE 1

Exp. II. -- Composition of basal diets

Ingredient	Percentage of diet, Trial A	Percentage of diet, Trial B
Corn	75.425	73.975
Soybean meal (44% protein)	16.00	19.60
Meat and bone scraps	2.00	--
Fish meal	1.00	--
Alfalfa meal	2.00	2.00
Limestone	2.50	2.75
Dicalcium phosphate	0.30	0.90
Iodized salt	0.50	0.50
B-vitamin supplement[a]	0.10	0.10
Vitamin B_{12} supplement[b]	0.05	0.05
Vitamin A and D supplement[c]	0.025	0.025
Antibiotic supplement[d]	0.10	0.10
Composition by chemical analysis		
Protein, %	15.6[e]	17.1[e]
Calcium, %	1.30	1.16[f]
Phosphorus, %	0.49	0.50
Zinc, ppm	42	30
Copper, ppm	13	19
Iron, ppm	--	88

[a]Contained 2 gm. riboflavin, 4 gm. d-pantothenic acid, 9 gm. niacin, and 90 gm. choline chloride per lb. of supplement.

[b]Contained 9 mg. B_{12} per lb. of supplement.

[c]Contained 3,632,000 I.U. of A and 800,000 I.U. of D per lb. of supplement.

[d]Contained 5 gm. procaine penicillin and 15 gm. streptomycin per lb. of supplement.

[e]Protein reduced to 12.5% when pigs reached 125 lb.

[f]Reduced to 0.65% for basal pigs (lots 30A and 30B) during last 6 weeks of trial; other lots continued on high-calcium ration.

APPENDIX TABLE 2

Exp. III. -- Composition of basal diet

Ingredient	Percent
Corn	78.4625
Soybean meal (50% protein)	19.00
Limestone	1.00
Dicalcium phosphate	0.80
Trace mineral salt[a]	0.50
Zinc oxide	0.0125
B-vitamin supplement[b]	0.10
Vitamin B_{12} supplement[c]	0.10
Vitamin A and D supplement[d]	0.025
Composition by chemical analysis	
Protein, %	17.9[e]
Calcium, %	0.63
Phosphorus, %	0.49
Zinc, ppm	175
Copper, ppm	18
Iron, ppm	142

[a]Contained 0.8% zinc, 1.2% manganese, 0.15% iron, 0.007% iodine, 0.01% cobalt, 0.005% copper, and 97% sodium chloride.

[b]Contained 2 gm. riboflavin, 4 gm. d-pantothenic acid, 9 gm. niacin, and 90 gm. choline chloride per lb. of supplement.

[c]Contained 6 mg. B_{12} per lb. of supplement.

[d]Contained 3,632,000 I.U. of A and 800,000 I.U. of D per lb. of supplement.

[e]Protein reduced to 13.0% when pigs reached 125 lb.

APPENDIX TABLE 3

Exp. IV. -- Composition of basal diet

Ingredient	Percent
Corn	78.125
Soybean meal (50% protein)	17.20
Alfalfa meal	2.00
Limestone	1.00
Dicalcium phosphate	0.90
Iodized salt	0.50
B-vitamin supplement[a]	0.10
Vitamin B_{12} supplement[b]	0.05
Vitamin A and D supplement[c]	0.025
Antibiotic supplement[d]	0.10
Composition by chemical analysis	
Protein, %	16.8[e]
Calciu, %	0.66
Phosphorus, %	0.51
Zinc, ppm	30
Copper, ppm	17
Iron, ppm	148

[a]Contained 2 gm. riboflavin, 4 gm. d-pantothenic acid, 9 gm. niacin, and 90 gm. choline chloride per lb. of supplement.

[b]Contained 9 mg. B_{12} per lb. of supplement.

[c]Contained 3,632,000 I.U. of A and 800,000 I.U. of D per lb. of supplement.

[d]Contained 5 gm. procaine penicillin and 15 gm. streptomycin per lb. of supplement.

[e]Protein reduced to 13.0% when pigs reached 125 lb.

APPENDIX TABLE 4

Exp. V. -- Composition of basal diets

Ingredient	Percentage of diet			
	Trial			
	A	A	B	B
Corn	78.075	76.375	78.425	75.825
Soybean meal (50% protein)	17.20	17.20	17.20	17.20
Alfalfa meal	2.00	2.00	2.00	2.00
Limestone	1.00	2.70	0.60	3.20
Dicalcium phosphate	0.90	0.90	0.90	0.90
Iodized salt	0.50	0.50	0.50	0.50
B-vitamin supplement[a]	0.10	0.10	0.10	0.10
Vitamin B_{12} supplement[b]	0.10	0.10	0.15	0.15
Vitamin A and D supplement[c]	0.025	0.025	0.025	0.025
Antibiotic supplement[d]	0.10	0.10	0.10	0.10
Composition by chemical analysis				
Protein, %	17.2	17.2	16.2	15.9
Calcium, %	0.61	1.26	0.53	1.42
Phosphorus, %	--	--	0.56	0.58
Zinc, ppm	28	29	23	23
Copper, ppm	10	16	--	--

[a]Contained 2 gm. riboflavin, 4 gm. d-pantothenic acid, 9 gm. niacin, and 90 gm. choline chloride per lb. of supplement.

[b]Contained 6 mg. B_{12} per lb. of supplement.

[c]Contained 3,632,000 I.U. of A and 800,000 I.U. of D per lb. of supplement.

[d]Contained 5 gm. procaine penicillin and 15 gm. streptomycin per lb. of supplement.

APPENDIX TABLE 5

Exp. II, Trial A. -- Copper, zinc, and iron content of livers

Pig No.	Cu, ppm	Zn, ppm	Fe, ppm
Lot 1A, Basal			
48-6	11	58	573
58-1	12	98	175
66-9	65	108	208
67-3	11	69	242
Mean	(25)	(83)	(299)
SE	13	12	92
Lot 2, 125 ppm Cu			
51-8	334	63	297
52-6	332	104	367
56-1	45	180	321
58-8	438	120	350
62-3	199	86	222
66-12	440	70	246
67-2	140	96	475
Mean	(276)	(103)	(325)
SE	57	15	32
Lot 4, 100 ppm Zn			
49-1	20	261	652
50-5	20	225	480
51-2	21	203	411
53-3	15	133	565
56-5	18	216	426
58-2	20	227	597
65-3	17	154	500
66-1	21	188	782
67-5	21	252	417
Mean	(19)	(207)	(537)
SE	1	14	42
Lot 6, 100 Zn + 250 Cu			
48-4	402	374	169
51-3	620	202	--
56-8	218	433	134
58-3	86	406	404
59-11	299	217	139
61-6	809	292	--
62-4	466	229	230
66-2	522	181	161
67-10	352	258	335
Mean	(419)	(288)	(225)
SE	86	36	40

Pig No.	Cu, ppm	Zn, ppm	Fe, ppm
Lot 1B, 250 ppm Cu			
52-2	457	84	47
53-1	328	99	161
54-8	199	71	133
58-7	764	98	159
Mean	(437)	(88)	(125)
SE	121	11	27
Lot 3, 250 ppm Cu			
53-2[a]	1236	83	103
62-9[b]	1435	165	77
65-2	1456	135	96
66-4[b]	1816	160	89
67-4	1294	90	117
Mean	(1448)	(126)	(96)
SE	101	17	7
Lot 5, 100 Zn + 125 Cu			
51-5	47	213	383
53-5	236	178	519
55-8	82	230	356
56-6	48	469	450
58-9	35	226	416
58-10	42	248	493
62-2	55	333	330
63-2	65	311	382
67-7	21	240	448
Mean	(70)	(272)	(420)
SE	22	29	21

[a]Carcass was condemned due to icterus.
[b]Exhibited symptoms of copper toxicity.

APPENDIX TABLE 6

Exp. II, Trial B. -- Copper, zinc, and iron content of livers

Pig No.	Cu, ppm	Zn, ppm	Fe, ppm
Lot 30A, Basal			
108-5	28	166	606
109-1	16	81	174
113-1	18	91	282
113-8	20	121	456
118-4	15	62	150
Mean	(20)	(104)	(334)
SE	2	18	87
Lot 31, 125 ppm Cu			
100-6	99	103	156
102-5	53	107	480
107-10	192	115	249
108-2	28	122	340
109-2	32	104	237
109-13	44	86	118
113-2	36	97	219
113-7	28	103	311
117-9	36	92	165
118-1	35	98	149
Mean	(58)	(103)	(243)
SE	16	3	35
Lot 33, 100 ppm Zn			
105-10	35	401	315
108-8	23	224	623
109-3	24	228	699
109-10	25	241	573
113-5	21	223	584
113-10	20	219	229
117-6	20	176	466
117-11	19	171	391
118-3	25	265	532
Mean	(24)	(239)	(490)
SE	2	23	51
Lot 35, 100 Zn + 250 Cu			
100-4	400	281	287
103-10	359	244	191
108-3	128	296	268
109-8	186	228	244
109-9	1303	487	188
111-3	519	203	180

Pig No.	Cu, ppm	Zn, ppm	Fe, ppm
Lot 30B, 125 ppm Cu			
100-8	32	107	302
103-2	88	468	319
109-14	98	148	454
116-2	47	286	307
117-10	56	104	437
Mean	(64)	(222)	(364)
SE	12	70	34
Lot 32, 250 ppm Cu			
97-5	616	113	113
98-9	1183	82	151
102-3	678	152	399
108-9	913	90	147
109-7	99	85	195
111-2	1716	143	108
113-6[a]	1224	80	99
116-4[b]	644	99	165
117-7[b]	696	111	--
119-4	918	92	454
Mean	(869)	(105)	(203)
SE	138	8	44
Lot 34, 100 Zn + 125 Cu			
98-11[c]	29	134	396
101-2[c]	--	--	--
102-6	36	323	772
103-7	30	189	481
108-6	41	380	446
109-11	24	213	506
113-4	39	269	733
116-1[c]	40	356	598
117-8[c]	--	--	--
118-2	34	214	449
Mean	(34)	(260)	(548)
SE	2	31	49
Lot 35 contd.			
113-3	260	195	167
117-5	1819	228	265
118-6	240	223	401
119-11	494	172	281
Mean	(571)	(256)	(247)
SE	174	28	22

[a]Carcass was condemned due to icterus

[b]117-7 died after 14 weeks; post-mortem findings were highly suggestive of copper toxicosis. Iron value was invalid because liver was gorged with clotted blood, resulting in extremely high concentration of iron.

[c]Liver was lost at time of slaughter.

APPENDIX TABLE 7

Exp. II, Trial B. -- Histopathological examination of livers from pigs fed two levels of copper sulfate with and without supplemental zinc

Pig no.	Degree of fibrosis[a]
Lot 30A, Basal	
108-5	-
109-1	-
113-1	-
113-8	-
118-4	-
Lot 30B, 125 Cu	
100-8	-
103-2	+
109-14	+
116-2	-
117-10	+
Lot 31, 125 Cu	
100-6	+
102-5	-
107-10	-
108-2	-
109-2	-
109-13	-
113-2	-
113-7	++
117-9	-
118-1	+
Lot 32, 250 Cu	
97-5	-
98-9	+++
102-3	++
108-9	b
109-7	b
111-2	+++
113-6	++
116-4	++
117-7	b
119-4	++
Lot 33, 100 Zn	
105-10	-
108-8	-
109-3	-
109-10	-
113-5	-
117-6	-
117-11	-
118-3	-
Lot 34, Zn + 125 Cu	
103-7	-
108-6	++
109-11	-
113-4	-
116-1	-
117-8	-
118-2	-
Lot 35, Zn + 250 Cu	
100-4	-
103-10	-
108-3	b
109-8	b
109-9	++
111-3	-
113-3	-
117-5	-
118-6	-
119-11	+++

[a]Degree of fibrosis was judged as follows:
- = negative ++ = slight
+ = questionable +++ = considerable

[b]Liver specimens were lost at time of slaughter.

APPENDIX TABLE 8

Exp. IV. -- Trace element analyses of livers and loins

Pig no.	Liver Cu, ppm	Liver Zn, ppm	Liver Fe, ppm	Loin Cu, ppm
Lot 10, Basal				
50-4	81	126	359	2.41
51-2	22	122	671	2.10
51-3	21	131	872	
52-1	21	112	606	
53-1	43	117	550	
54-2	33	111	562	
55-10	34	119	484	1.78
56-11	36	155	493	
57-8	63	116	428	
Mean	(39)	(123)	(558)	(2.10)
SE	7	4	33	0.18
Lot 12, CuO (250 ppm Cu)				
50-2	1193	102	124	
50-7	776	118	122	
51-4	378	117	210	
52-4	1214	101	124	
53-5	1338	103	147	
54-1	778	115	123	2.94
55-8	932	123	132	
56-10	429	108	143	2.11
56-12	575	123	296	2.32
57-13	998	97	147	2.45
Mean	(861)	(111)	(157)	(2.45)
SE	106	3	9	0.18
Lot 14, Zn + $CuSO_4$				
48-3	735	178	129	
50-6	169	379	281	2.04
51-11	554	323	258	2.64
52-6	741	195	111	
53-4	272	171	116	
53-12	1076	271	332	
54-4	448	175	132	
55-2	1492	213	133	
56-3	963	181	170	
57-5	1900	163	151	1.83
Mean	(835)	(225)	(181)	(2.17)
SE	172	23	25	0.24

Pig no.	Liver Cu, ppm	Liver Zn, ppm	Liver Fe, ppm	Loin Cu, ppm
Lot 11, $CuSO_4$ (250 ppm Cu)				
49-2	1388	100	170	
50-1	1488	88	93	2.31
51-10	754	113	111	
53-10	1630	109	169	2.79
55-6	1885	116	95	2.45
55-9	1449	108	107	
56-1	1752	118	119	
56-6	2279	120	107	
57-6	1701	95	101	
57-9	1398	88	105	2.64
Mean	(1572)	(106)	(118)	(2.55)
SE	125	4	9	0.11
Lot 13, 100 ppm Zn				
49-1	31	311	380	
50-5	38	267	585	
51-5	28	439	635	2.23
52-3	25	251	564	1.96
53-2	28	249	815	2.61
54-5	27	248	761	
55-3	26	152	851	
56-4	37	251	509	
56-13	27	223	601	
57-4	31	194	670	
Mean	(30)	(258)	(637)	(2.26)
SE	1	24	54	0.19
Lot 15, Zn + CuO				
47-4	524	153	322	
50-3	89	215	495	2.63
51-1	38	249	375	
51-8	52	245	648	
52-5	211	260	330	
53-13	82	193	308	3.69
55-1	326	228	340	
56-5	397	226	607	2.25
57-2	28	207	423	
57-11	709	163	364	
Mean	(246)	(214)	(421)	(2.85)
SE	75	11	39	0.43