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Studies of the toxicity of copper to pigs

1. Effects of oral supplements of zinc and iron salts on the development of copper toxicosis

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1. Six groups of four litter-mate female Large White pigs of about 17 kg live weight were allocated according to a 2 × 2 factorial design in each of two experiments in which rations containing 0 or 750 ppm copper were offered with either 0 and 500 ppm zinc, or 0 and 750 ppm iron. 2. Severity of toxicosis was assessed by determining aspartate transaminase activity in serum, observing the incidence of jaundice and determining the haemoglobin concentration in whole blood and Cu concentration in serum and liver. 3. Addition of 750 ppm Cu to the diet caused toxicity in nine out of twelve animals; it was most severe after about 4 weeks when two- to five-fold increases in serum Cu and aspartate transaminase levels were found and seven pigs were jaundiced. Serum Cu and aspartate transaminase concentrations and degrees of jaundice were apparently interrelated and returned to normal levels after 6 weeks, suggesting adaptation to the high Cu intake. Growth depression and a microcytic hypochromic anaemia persisted. 4. Addition of 500 ppm Zn or 750 ppm Fe in the presence of 750 ppm Cu eliminated jaundice and produced serum Cu and aspartate transaminase concentrations similar to control values after 4 weeks. Only supplementary Fe afforded protection against anaemia. 5. Variability in the response of the pig to Cu supplements could be partly due to variations in the intake of Fe and Zn. The addition of Fe and Zn supplements to pig diets supplemented with Cu would probably reduce the small risk of causing Cu poisoning.

The addition of copper salts to pig diets has been shown to produce increases in growth rate, food consumption and food conversion efficiency under a variety of experimental conditions (cf. Barber, Braude & Mitchell, 1955; Barber, Braude, Mitchell, Rook & Rowell, 1957; Lucas & Calder, 1957*a*, *b*; Dammers & Stolk, 1959; Bellis, 1961; Fagan, Iles, Slowitsky & Brocksopp, 1961; Hawbaker, Speer, Hays & Catron, 1961; King, 1960, 1963). In two co-ordinated trials in the UK, involving experiments at eight (Bowler, Braude, Campbell, Craddock-Turnbull, Fieldsend, Griffiths, Lucas, Mitchell, Nickalls & Taylor, 1955) and twenty-one locations (Braude, Townsend, Harrington & Rowell, 1962), it was found that the addition of 250 ppm Cu to the air-dry feed resulted in increases of 5·7 and 9·7% respectively in growth rate and 0·3 and 7·9% respectively in food conversion efficiency.

Similar quantities of supplementary Cu have occasionally caused Cu toxicosis in the USA (Wallace, McCall, Bass & Combs, 1960; Ritchie, Luecke, Baltzer, Miller, Ullrey & Hoefer, 1963), in Australia (O'Hara, Newman & Jackson, 1960), in Germany (R. Burke, 1963 personal communication) and in the UK (Buntain, 1961; Anonymous, 1963). The risk of toxicosis has restricted the use of Cu as a feed additive by foodstuff manufacturers and farmers. The object of our studies has been to study the influence of ration composition on the toxicity of Cu to the pig.

The addition of large quantities of Cu to the diet of the pig could conceivably create

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an imbalance of minerals within the animal and so impair body function. The variable responses of pigs to Cu supplements might therefore be partly due to differences in the content or availability of dietary minerals or to the availability of adventitious sources of minerals in the water supply, the soil and metallic pen structures. In this paper we record the protection from Cu toxicosis achieved by adding zinc and iron supplements to a diet containing 750 ppm Cu. The work has been briefly reported elsewhere (Suttle & Mills, 1963).

EXPERIMENTAL

Animals. Six groups of four female litter-mate weanling Large White pigs, of about 17 kg live weight, were used in both Expts 1 and 2. They were randomly allocated in a 2×2 factorial design on the basis of live weight and with a litter as the experimental block. Expts 1 and 2 ended after 49 and 46 days respectively when the animals were slaughtered.

Treatments. The treatments were: two levels of supplementary Zn (0 and 500 ppm as $ZnCO_3$) and two levels of supplementary Cu (0 and 750 ppm as $CuCO_3 . Cu(OH)_2 . H_2O$) in Expt 1; two levels of supplementary Fe (0 and 750 ppm as $FeSO_4 . 7H_2O$) and two levels of Cu (0 and 750 ppm as $CuCO_3 . Cu(OH)_2 . H_2O$) in Expt 2.

The composition of the basal diet (Table 1) was calculated to resemble that used by Wallace *et al.* (1960) in experiments in which only 250 ppm Cu produced toxicosis.

Table 1. *Composition (kg/100 kg) of basal pig diet*

Maize meal	80
Extracted soya-bean meal	17
Sterilized bone meal	1
Ground limestone	1
Vitamin supplement*	0·5
Mineral supplement†	0·5

* Provided 132 mg vitamin A, 11 mg ergocalciferol, 880 mg DL-α-tocopheryl acetate, 0·55 mg cyanocobalamin, 198 mg riboflavine, 1·45 g pyridoxine, 0·66 g calcium pantothenate, 88 g choline chloride, 2·2 g nicotinic acid, 0·22 g thiamine, 0·11 g folic acid.

† Provided 466 g NaCl, 18·9 g $MnSO_4 . H_2O$, 13·4 g $FeSO_4 . 7H_2O$, 0·207 g $CoCO_3$, 5·78 g $ZnSO_4 . 7H_2O$.

The basal diet contained (ppm) 5 Cu, 34 Zn and 144 Fe in Expt 1, and 5 Cu, 51 Zn and 118 Fe in Expt 2. It contained 14·5% crude protein in both experiments.

Management. After weaning at 8 weeks of age, the experimental animals were housed in insulated wooden huts with an outside concrete run. They were treated for internal parasites with a piperazine derivative and accustomed to individual feeding before the experiments began. Food allowances during the experiments were based on individual appetites by giving as much food as could be consumed in 30 min at each of the two daily feeds. Water was added to the food in the feeding trough to form a slurry, and a supply of water was continuously available in the run. Two replicates were housed in each pen, and no attempt was made to prevent coprophagy. Cu is excreted in pig's faeces largely in the form of the sulphide (Dammers & Stolk, 1959). The relative insolubility of this compound (Bowland, Braude, Chamberlain,

Glascock & Mitchell, 1961) suggests that coprophagy could produce only small increases in the amount of Cu available for absorption. The galvanized Fe feeding troughs were coated with a polyurethane paint to avoid contamination of the moist food with Zn.

Female pigs only were used in these experiments as preliminary work (Suttle, 1964) had shown that they were more susceptible to Cu toxicosis than males.

Collection of samples. Blood was obtained by removing the tip of the tail with a scalpel. The skin was first coated with a solution of collodion in acetone which, on drying, effectively prevented contamination of the blood with faecal material that had been adhering to the skin. Samples were taken about 3 h after the morning feed on one day in each week. Wintrobe's oxalate mixture (6 mg $(COONH_4)_2.H_2O$ and 4 mg $(COOK)_2.H_2O$/5 ml blood) was used as an anticoagulant in work with whole blood. When serum samples were required, the blood was allowed to clot and was then centrifuged at $2750 \times g$ for 15 min. Samples were stored at $+1°$ and were surrounded by ice immediately after collection if enzyme assays were to be performed.

Samples of fresh liver were removed after slaughter as slices 1 cm wide through the depth of each lobe in a median position. The slices were washed with deionized water to remove superficial blood and loose connective tissue, freeze-dried to a moisture content of about 2 % and finally ground in a carefully cleaned laboratory hammer mill with a 1·8 mm screen. Negligible amounts of Cu and Zn were found to contaminate samples of filter-paper similarly milled.

Analytical methods. Duplicate samples of diet or tissue were oxidized by heating them with a mixture of concentrated acids containing 2–4 ml nitric, 1 ml perchloric and 1 ml sulphuric acid. Cu was determined by a method derived from that of Eden & Green (1940) and Zn by a method derived from those of Malmström (1956) and Wolff (1954), based on the reaction of Zn with dithizone. Maximum sensitivity was obtained by reading 0–9·0 μg quantities of Zn at 535 nm as the Zn dithizonate formed and reading 9·0–20·0 μg quantities at 620 nm as the amount of free dithizone remaining unused. The thiocyanate method of Kennedy (1927) was used to determine Fe. For each method, all glassware was washed with concentrated nitric acid or a proprietary detergent and rinsed with glass-distilled or deionized water. In preparing for Zn assays, glassware was finally rinsed with a solution of dithizone in CCl_4.

Aspartate transaminase (AST) activity in serum samples was determined within 8 h of sampling by the method of Karmen, Wróblewski & LaDue (1955) and with a proprietary enzyme assay outfit (Boehringer; Mannheim). The unit of activity was taken as a change in optical density of 0·001/min at $23 \pm 1·5°$ at 340 nm with an assay volume of 3 ml and a 1 cm light path. This unit can be converted into the international enzyme unit by multiplying with a factor of 0·482.

Haematological studies involved the use of standard laboratory procedures for determining erythrocyte count and packed cell volume (cf. Whitby & Britton, 1950). Haemoglobin was determined spectrophotometrically according to the method of Nicholas (1951).

Statistical analysis. Treatment effects were assessed by conventional analysis of variance procedures for randomized block designs. Logarithmic transformations were

employed when necessary to give the values a more normal distribution. The number of replicates constituting the mean has only been stated when it was less than the scheduled six.

RESULTS

Expt 1

Live-weight gain, food consumption and food conversion efficiency. Although the results given in Table 2 show that by the the end of the 7-week period of the experiment there were no significant treatment effects on performance, during the first 3 weeks the addition of 750 ppm Cu to the diet increased live-weight gain and total food consumption, probably by eliminating the effects of diarrhoea affecting the groups not receiving Cu. In the 4 subsequent weeks, however, the groups showed the following mean daily live-weight gains: unsupplemented, 0·603 kg; 500 ppm Zn, 0·556 kg; 750 ppm Cu, 0·300 kg; 750 ppm Cu + 500 ppm Zn, 0·349 kg. Food consumption by the group receiving 750 ppm Cu remained at about 9 kg/week from the 2nd week onwards. The addition of Zn tended to counteract the effects of Cu on weight gains and food consumption during this period.

Table 2. *Expt* 1. *Effect of dietary supplements of zinc and copper on weight increase, food consumption and food conversion efficiency of pigs over a period of 7 weeks*

Dietary supplement (ppm air-dry food)	No. of pigs	Live-weight gain (kg)	Food consumption (kg)	Food conversion efficiency*
None	6	22·5	75·2	3·35
500 Zn	6	21·8	71·8	3·35
750 Cu	6	16·4	59·7	3·69
500 Zn + 750 Cu	6	19·5	69·8	3·83†
Residual SD		±4·7	±13·4	±0·67
Overall significance		NS	NS	NS

NS, not significant.
* Food consumed (kg)/live weight gain (kg).
† Poor value in this group was due largely to one individual result of 6·19.

Serum Cu and aspartate transaminase. The results given in Fig. 1 show that the concentration of Cu in serum rose rapidly for the first 4 weeks of the experiment in the group receiving 750 ppm Cu. In subsequent weeks, however, the concentration fell with similar rapidity, despite continued Cu supplementation. Compared with control animals, values for the group receiving 750 ppm Cu were significantly different ($P < 0{\cdot}01$) after 4 weeks, but similar after 6 weeks. The simultaneous addition of Zn to the diet largely prevented the increase in serum Cu levels between the 1st and 4th weeks, whereas Zn alone did not affect serum Cu levels.

The pattern followed by serum AST concentrations, illustrated in Fig. 2, resembles that described above for serum Cu. A severe but transient jaundice appeared in the group given 750 ppm Cu in the diet between the 3rd and 6th weeks when five out of six animals were affected. AST levels were only slightly elevated in the group receiving both Zn and Cu supplements, and jaundice was at no time evident in the serum.

No evidence of tissue damage was apparent from the results of AST assays on serum from pigs receiving the 500 ppm Zn supplement.

Although the protective effect of Zn was so apparent, the wide variation between animals prevented the demonstration of a significant effect on AST activity when Zn was added to the Cu-supplemented diet (Table 3). The variation was largely due to the complete absence of Cu toxicosis in one animal from the group given 750 ppm Cu, which showed serum concentrations of 223 μg Cu/100 ml and 1·42 log units AST activity/ml.

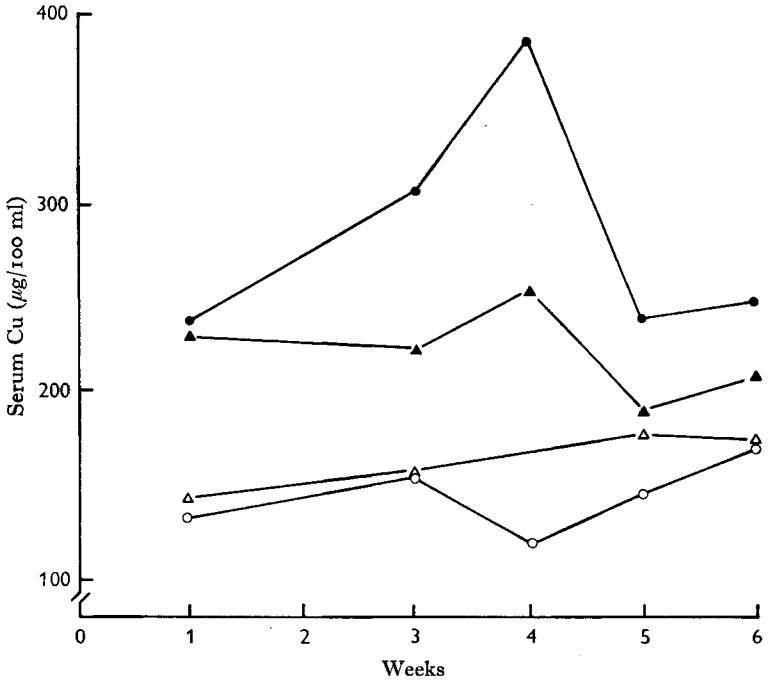

Fig. 1. Expt 1. Effect of dietary zinc and copper supplements on serum Cu concentration in the pig. o—o, none; ●–●, 750 ppm Cu; △–△, 500 ppm Zn; ▲–▲, 500 ppm Zn+750 ppm Cu.

Haematological findings. The addition of Cu to the diet caused a gradual decrease in haemoglobin level during the experiment, which was unaffected by the presence of Zn. The addition of Zn in the absence of Cu slightly depressed both haemoglobin levels and the erythrocyte count compared with the control values (Table 4). Mean cell volume and mean cell haemoglobin concentration were significantly lower in the anaemic Cu-supplemented groups compared with control animals, indicating that the anaemia was of a microcytic and hypochromic type.

Accumulation of Zn and Cu in liver tissues. The two groups receiving 750 ppm Cu showed similar and marked increases in liver Cu concentrations and total liver Cu content at the 7th week (Table 5). Retention of Cu in the liver was assessed by expressing the total liver Cu content as a percentage of the supplementary Cu intake. In making this assessment, the relatively small contribution to the increased liver Cu content, caused by increases in size of the liver in unsupplemented animals during the

experiment, was ignored. The group given 750 ppm Cu retained on average 2·07%, and the group given 500 ppm Zn plus 750 ppm Cu retained 1·64% of the supplementary Cu ingested. This difference was statistically significant ($P < 0.05$). In the absence of Cu, the Zn supplement did not affect the accumulation of Cu in the liver.

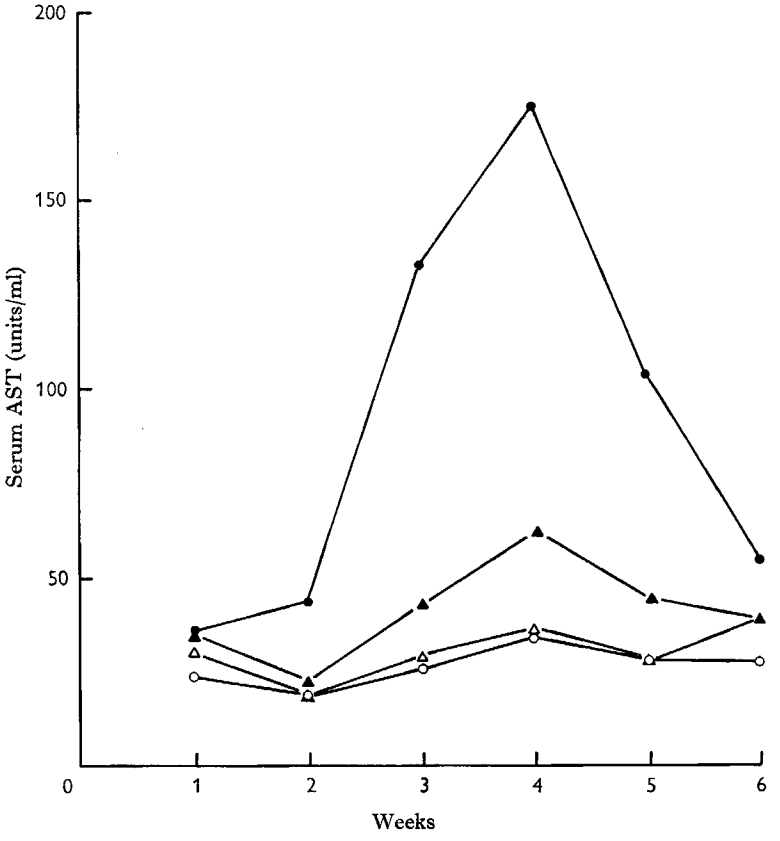

Fig. 2. Expt 1. Effect of dietary zinc and copper supplements on aspartate transaminase activity in the serum of the pig. ○–○, none; ●–●, 750 ppm Cu; △–△, 500 ppm Zn; ▲–▲, 750 ppm Cu + 500 ppm Zn.

Table 3. *Expt 1. Effect of dietary zinc and copper supplements on Cu and aspartate transaminase in serum of pigs after 4 weeks*

Dietary supplement (ppm air-dry food)	No. of pigs	Serum Cu content* (μg/100 ml)	Log serum aspartate transaminase activity† ($10^{-3}\Delta E_{340}$ of $NADH_2$/min ml)
None	6	117	1·52
750 Cu	6	388	2·12
500 Zn + 750 Cu	6	225	1·71
Residual SD		±88	±0·26
Overall significance		$P < 0.01$	$P < 0.01$

* Cu in serum of 500 Zn group not determined at 4th week.

† Three pigs sampled from 500 Zn group showed aspartate transaminase activities of 1·49, 1·49 and 1·60 units/ml serum.

On the other hand, the addition of Cu significantly ($P < 0.001$) increased the liver Zn concentration, and the effect on this of Cu was greater than that of adding 500 ppm Zn to the diet.

Table 4. *Expt* 1. *Effect of dietary zinc and copper supplements on haematological values of pigs after* 6 *weeks*

Dietary supplement (ppm air-dry food)	No. of pigs	Haemoglobin level (g/100 ml whole blood)	Erythrocyte count (10^6/mm^3)	Mean cell volume (μm^3)	Mean cell haemoglobin concentration (%)
None	6	10·4	5·19	64·0	31·5
500 Zn	6	9·6	4·65	67·6	30·6
750 Cu	6	7·8	5·82	46·8	29·1
500 Zn+750 Cu	6	7·6	5·81	45·0	29·4
Residual SD		±0·56	±0·66	±7·2	±1·2
Overall significance		$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$

Table 5. *Expt* 1. *Effect of dietary zinc and copper supplements on the accumulation of Zn and Cu in liver tissue of pigs after* 7 *weeks*

Dietary supplement (ppm air-dry food)	No. of pigs	Liver Cu concentration (ppm DM)		Total liver Cu content (mg)		Liver Zn concentration (ppm DM). True arithmetic mean
		Log mean	Derived mean	Log mean	Derived mean	
None	4	1·420	28	0·923	9	152
500 Zn	4	1·664	49	1·162	16	203
750 Cu	4	3·520	3502	2·887	847	292
500 Zn+750 Cu	4	3·493	3294	2·996	991	328
Residual SD		±0·146		±0·190		±41
Significance of Cu effect		$P < 0.001$		$P < 0.001$		$P < 0.001$

DM, dry matter.

Expt 2

Live-weight gain, food consumption and food conversion efficiency. The performance of the control group was severely affected by an outbreak of diarrhoea between the 4th and 6th weeks of the experiment, probably due to a coliform infection. One pig died and two others gained little weight in this period, despite the administration of antibiotics. The variable performance of pigs in the unsupplemented group is apparent from the results given in Table 6. A paired t test was used to compare the differences between litter-mates in the groups receiving Fe and in the groups receiving Cu supplements, and it was found that the addition of Fe to the Cu-supplemented diet significantly increased the growth rate ($P < 0.05$). The groups receiving Cu were, as in Expt 1, less affected by diarrhoea than pigs not receiving Cu. Variation in the Cu-supplemented groups was largely due to variation in the extent of Cu toxicosis developing.

Serum Cu and aspartate transaminase levels. An interrelationship between Cu and Fe was evident in the results for serum Cu and AST concentrations. In the 750 ppm Cu group Cu levels rose to a peak after about 4 weeks (Fig. 3), as in Expt 1. Although

Table 6. *Expt 2. Effect of dietary iron and copper supplements on live-weight gain, food consumption and food conversion efficiency of pigs over a period of 6 weeks*

Dietary supplement (ppm air-dry food)	Total live-weight gain (kg)		Total food consumption (kg)		Food conversion efficiency*	
	Mean	Range	Mean	Range	Mean	Range
None†	15·0	8·0–23·5	54·5	43·9–71·7	4·16	2·98–6·23
750 Fe	15·8	11·5–19·0	58·0	48·3–66·4	3·71	3·31–4·18
750 Cu	11·7	6·0–16·5	45·0	39·1–52·8	3·99	2·90–6·52
750 Fe+750 Cu	15·6	8·5–18·5	51·8	39·5–56·9	3·44	2·84–4·65

* Food consumed (kg)/live weight gain (kg).
† Results from mean of five control animals only; in other groups means are of six values.

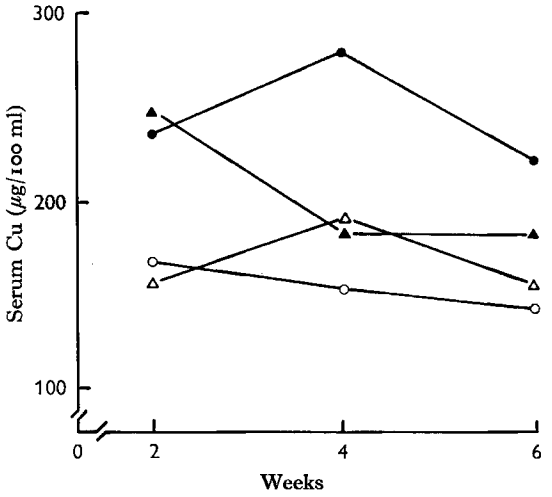

Fig. 3. Expt 2. Effect of dietary iron and copper supplements on the concentration of Cu in the serum of the pig. ○–○, none; ●–●, 750 ppm Cu; △–△, 750 ppm Fe; ▲–▲, 750 ppm Cu+750 ppm Fe.

the serum concentration in the group receiving supplementary Fe and Cu was higher than that of control animals 2 weeks after the start of the experiment, this difference was not statistically significant by the 4th week (Table 7). The addition of Fe alone was without effect on serum Cu values. Assays of AST activity at the 4th week showed similar treatment effects, in that the addition of Cu to the diet caused pronounced increases in activity in four of the six animals in that group and jaundice in two of these. Individual values from other groups were normal at that time. In two animals from the Cu-supplemented group, however, serum Cu and AST concentrations were normal after 4 weeks. In the absence of a normal distribution of responses, this group is represented by individual results in Table 7. It can be seen that the values for four animals differ considerably from those in other groups; they suggest the presence of a toxicosis that was eliminated by the simultaneous provision of an Fe supplement.

Haemoglobin levels. The results of haemoglobin determinations represented in

Table 7. *Expt 2. Effect of dietary iron and copper supplements on Cu content and aspartate transaminase activity in the serum of pigs after 4 weeks*

Dietary supplement (ppm air-dry food)	No. of pigs	Serum Cu (μg/100 ml)	Serum AST ($10^{-3}\Delta E_{340}$ of $NADH_2$/min ml)
None	6	155	29·8
750 Fe	6	192	34·5
750 Fe+750 Cu	6	184	30·0
Residual SD		±41	±4·1
Overall significance		NS	NS
750 Cu*		422†, 288, 255, 335†, 196, 179,	87, 85, 72, 71, 33, 25

* Individual results. † Jaundice evident.

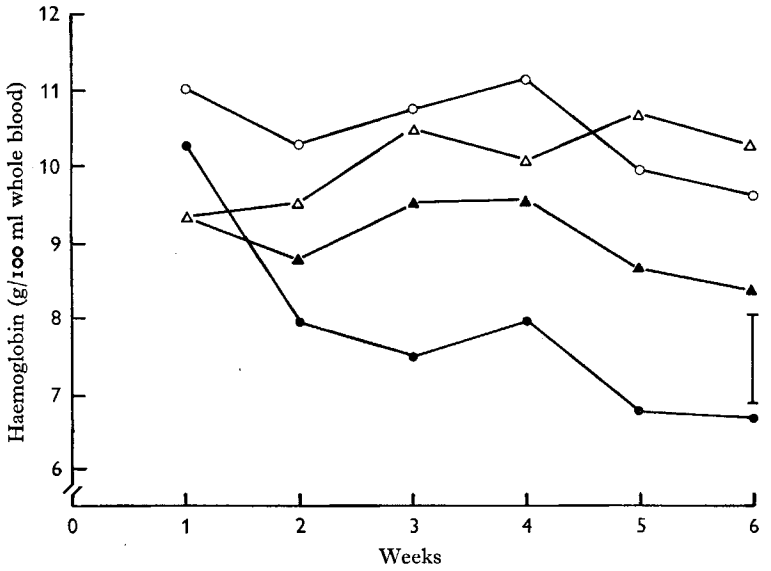

Fig. 4. Expt 2. Effect of dietary iron and copper supplements on haemoglobin concentration in the blood of the pig. o–o, none; ●–●, 750 ppm Cu; △–△, 750 ppm Fe; ▲–▲, 750 ppm Fe+750 ppm Cu. Vertical bar denotes least significant difference at the 5 % level of probability.

Fig. 4 show that the addition of Fe to the Cu-supplemented ration largely prevented the development of anaemia caused by the Cu supplement. The haemoglobin levels of pigs in the group receiving Fe alone did not differ from those found in pigs receiving no supplements.

Accumulation of Cu and Zn in liver tissue. The Cu supplements caused considerable increases in the concentrations of Cu and Zn in the liver (Table 8). Although the simultaneous provision of Fe limited the development of Cu toxicosis, it tended to increase the concentration and total accumulation of Cu in the liver in both the presence and absence of a Cu supplement. However, mean liver Cu retentions were not different, being 1·65 % and 1·66 % of the ingested Cu in the groups given 750 ppm Cu and 750 ppm Fe+750 ppm Cu, respectively; the residual standard deviation was

±0·52. It thus appears that in these two groups differences in the concentration and total Cu content of the liver were largely reflections of differences in food intake and therefore of Cu intake. Also Fe significantly increased the liver Zn concentration and the effects of Fe and Cu were partly additive.

Table 8. *Expt 2. Effect of dietary iron and copper supplements on the accumulation of Cu and Zn in liver tissue of pigs after 46 days*

Dietary supplement (ppm air-dry food)	No. of pigs	Liver Cu concentration (ppm DM)		Total liver Cu content (mg)		Liver Zn concentration (ppm DM). True arithmetic mean
		Log mean	Derived mean	Log mean	Derived mean	
None	5	1·199	17	0·546	4	173
750 Fe	6	1·421	28	0·807	7	290
750 Cu	6	3·438	2891	2·761	646	253
750 Fe+750 Cu	6	3·458	3027	2·840	775	333
Residual SD		±0·142		±0·206		±49
Significance: Cu effect		$P < 0·001$		$P < 0·001$		$P < 0·001$
Fe effect		$0·05 < P < 0·1$		$0·05 < P < 0·1$		$P < 0·001$

DM, dry matter.

DISCUSSION

The aetiology of Cu toxicosis, which developed in nine of the twelve pigs given a diet containing 750 ppm supplementary Cu in these two experiments, showed several features calling for comment. The steady increase in serum Cu concentrations until the 4th week of treatment suggests that Cu was entering the bloodstream after absorption at a rate exceeding that at which it was removed by storage and excretion. When the concentration exceeded 250–300 μg/100 ml, increases in serum AST level occurred that were of similar magnitude to the increases in Cu level.

The assay of AST and certain other enzymes in serum provides an indirect measure of the extent and sometimes the location of tissue damage (Abderhalden, 1961). AST is abundant in the kidney, liver and cardiac and skeletal muscle of the pig (Cornelius, Bishop, Switzer & Rhode, 1959), and elevated serum AST levels may result from damage to one or all of these tissues. A limited study of ornithine carbamoyltransferase (OCT) activity in the serum, which more specifically indicates liver damage (Wretlind, Orstadius & Lindberg, 1959), revealed elevated levels in pigs suffering from Cu toxicosis (Suttle, 1964). Post-mortem examinations of the carcasses of affected animals showed that gross degenerative changes were consistently present only in the liver. Histological examinations of this organ revealed a centrilobular necrosis and also some disruption of the bile canaliculi. It is probable, therefore, that the increases in AST activity resulted principally from damage to liver tissue.

The damage to liver tissue was not, however, caused directly by excessive storage of Cu, since the concentration of Cu in the liver of animals showing signs of toxicosis was similar to that in the liver of those Cu-supplemented animals remaining healthy. It is conceivable that there exists a maximum rate at which the liver can remove Cu from the bloodstream; above a certain level, increases in the serum Cu concentration

do not cause further increases in the accumulation of Cu in the liver. From our results it would seem that the critical level in serum is about 300 μg/100 ml. This level was frequently found but rarely exceeded in healthy animals, although it was frequently exceeded in pigs with Cu toxicosis. Above the critical level, liver tissue is probably damaged by the excessive amount of Cu arriving at the liver.

The relationship between degree of jaundice and elevated serum Cu and AST concentrations might indicate that the jaundice is largely of hepatic origin. In studies reported elsewhere (Suttle, 1964) there was no evidence of an increased fragility in erythrocytes from pigs given Cu in our Expts 1 and 2, which would be expected if the jaundice had a haemolytic origin. The anaemia occurring in the groups receiving additional Cu without Fe or Zn supplements is gradual in development, and it may be attributed initially to interference with Fe metabolism and later to gastro-intestinal haemorrhage rather than haemolysis.

The sudden decline in serum Cu and AST values that occurred after about 4 weeks of Cu supplementation presumably reflected a sudden change in the balance between the flow of Cu into and out of the bloodstream. The extent to which control of the intake and absorption of Cu on the one hand and endogenous and urinary excretions of Cu on the other contributed to this change could not be ascertained. It does seem, however, that the pig could in some way adapt itself to overcome the effects of a high Cu intake in the particular circumstances of our experiments. Although food intake and growth rate remained depressed, other signs of toxicosis had largely disappeared after 6–7 weeks of treatment.

The pattern of a gradual development of and recovery from Cu toxicosis in the pig contrasts with the sudden elevation of serum Cu concentrations and concomitant haemolytic crisis after an indeterminate period of Cu supplementation, which characterise Cu toxicosis in sheep (Marston, 1952; Todd & Thompson, 1963; Barden & Robertson, 1962). The methaemoglobinaemia and haemoglobinuria frequently observed in the sheep were not apparent in our pigs or those of Allen & Harding (1962) who produced a severe toxicosis with 1000 ppm Cu.

The development of Cu toxicosis was considerably affected by adding 500 ppm Zn or 750 ppm Fe to the Cu-supplemented diets. Both supplements reduced the accumulation of Cu in the serum and consequently almost eliminated tissue damage as reflected by the lower AST values. Jaundice was absent. Only the Fe supplement, however, gave effective protection against the development of anaemia.

These observations are in accordance with other recent evidence demonstrating interactions between Cu and Zn or Fe in the nutrition of various species. Thus, several workers have shown that adding large quantities of Zn to the diet of rats prevents an otherwise adequate dietary Cu supply from meeting the rat's requirement for Cu (Smith & Larson, 1946; Grey & Ellis, 1950; Van Reen, 1953; Grant-Frost & Underwood, 1958; Hill, Matrone & Starcher, 1963). Cox & Hale (1962) were, however, unable to demonstrate a similar antagonism in the pig. Ritchie *et al.* (1963) have reported a protective effect of a 100 ppm Zn supplement against the toxic effects of 250 ppm Cu on pigs. Their experiments differed from those described here in that the respective calcium contents of the diets were 1·3 and 0·8% and parakeratosis, usually

indicative of Zn deficiency, developed in their control animals. In their experiments Cu toxicity occurred when the dietary protein level was reduced at 45 kg live weight. The work of Bunch, Speer, Hays & McCall (1963) suggests that the growth response to Cu partly depends on the dietary Zn content. In other experiments in which Cu supplements have not produced toxicosis there has been no evidence of an interaction between Cu and Zn affecting the growth response to Cu (Wallace *et al.* 1960; Barber, Braude & Mitchell, 1960).

The microcytic and hypochromic nature of the anaemia which developed in the pigs in Expt 1 suggests a deficiency of Fe; several workers have shown that the addition of Cu to pig diets causes a depression in liver Fe stores (Cassidy & Eva, 1958; Bunch *et al.* 1963; Ritchie *et al.* 1963) or anaemia (Wallace *et al.* 1960; Bunch, Speer, Hays, Hawbaker & Catron, 1961). Bunch *et al.* (1963) have shown that a supplement of 141 ppm Fe improved blood haemoglobin contents in early-weaned pigs given 250 ppm supplementary Cu. Al-Ubaidi & Sullivan (1963) reported that raising the Cu content of a turkey diet from 2·8 to 6·8 ppm caused an increase in the degree of anaemia present when the diet contained only 18 ppm Fe. This finding is in contrast to many earlier ones that suggested a potentiating action of Cu on Fe in stimulating haemoglobin formation by many species at low dietary concentrations of Fe and Cu (Hart, Steenbock, Waddel & Elvehjem, 1928; Elvehjem & Hart, 1932; Copp & Greenberg, 1946; Hill & Matrone, 1961). The protective effect of Fe supplements against Cu toxicosis is probably related to the maintenance of body Fe stores as well as a decreased retention of Cu in the bloodstream.

Several investigations have shown that the addition of large Zn supplements to the diet of rats (Cox & Harris, 1960, 1962; Magee & Matrone, 1960; McCall, Mason & Davis, 1961) and pigs (Cox & Hale, 1962) causes a reduction in the Fe content of the liver, with or without the development of anaemia. Although the Zn supplement in our experiment decreased the general severity of Cu toxicosis, its failure to correct the anaemia might be due to its recorded capacity for interfering with Fe metabolism.

There are many alternative sites at which these mineral interrelationships may occur. Evidence has been obtained in in vitro studies that an excess of one element can cause the displacement of other elements from binding sites on protein molecules (Breslow & Gurd, 1963; Plocke & Vallee, 1962). Similar processes were thought by Kirchgessner & Weser (1963) to have contributed to the increase in the proportion of dialysable Zn resulting when Cu was added to an in vitro system containing protein. Several investigations have shown that metals such as Cu and Zn, with similar electron structures in the valency shell, behave similarly in inducing or preventing metal toxicities and deficiencies (Hill, Matrone, Payne & Barber, 1963; Hill, Matrone & Starcher, 1963; Britton & Hill, 1964).

The interrelationships between Cu, Zn and Fe demonstrated in Expts 1 and 2 might therefore involve competition between elements with similar affinities for binding sites on proteins in the digesta or in the tissues. By such mechanisms the addition of one element to the diet could affect the amount of other elements available for absorption, storage, transport and excretion. Competitive effects may also bring about the displacement of essential metals from functional sites on metallo-enzymes. The pre-

sence of Cu in toxic quantities might influence the metabolism of Fe and Zn by causing liver and kidney dysfunction and by generally altering the permeability of the cell wall. Similar disturbances to Fe and Zn metabolism have been reported in liver cirrhosis arising from agencies other than Cu (Gitlow, Beyers & Colmore, 1952; Vallee, Wacker, Bartholomoy & Hoch, 1957).

The ability of supplementary Cu and Fe to increase the retention of Zn in the liver requires elucidation. Several observers have noted a tendency for Cu supplements to exert this effect (Bellis, 1961; Bunch *et al.* 1963; Ritchie *et al.* 1963). It may be caused by abnormalities in the production of storage proteins, but it is unlikely that the mechanism underlying this effect will be elucidated until more is known about the nature of metal storage proteins in the liver.

In view of the innumerable possibilities involved, it is pointless to speculate as to the precise nature of the interactions produced by the manipulations of the Cu, Zn and Fe contents of the diet made in Expts 1 and 2. More fundamental knowledge of mineral metabolism and detailed factorial balance experiments are required to disentangle these complex interrelationships.

It is possible that variations in content and availability of Zn and Fe in the diet have in past studies contributed to the isolated instances of Cu toxicity in pigs when Cu was added to the diet in amounts up to 250 ppm. Diets low in Fe and Zn might predispose the pig to Cu toxicosis. The protection afforded by smaller Fe and Zn supplements, was investigated in experiments reported in the following paper (Suttle & Mills, 1966).

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