Copper Metabolism in Farm Animals

by

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A Review

In April and August, 1838, two deaths due to poisoning occurred near Amiens in France; one involved a woman poisoned by her husband and the other a man. Chemists examining the intestines came to the conclusion that the deceased persons were the victims of a 'copper compound'. The coincidence of the two cases led them to investigate copper in other persons who had died suddenly or naturally and 'to their surprise' they found small amounts of copper in men and women of different ages, in children of 15 years, 20 months, 20 days and even in a stillborn baby. The chemists, Alphonse Devergie and Osmin Hervy reported their findings (1838) and said that they proposed to continue their researches and to determine the copper and lead content of different tissues and organs.

By 1849 Deschamps was able to report that 'many works have been published on the question of normal, or physiological, copper'. In a paper entitled 'Cuivre normal dans le sang' (Deschamps, 1849) he described a technique for ashing and analysing blood for copper and speculated on the origins of this copper and stated his

belief that the element was 'necessary in the nourishment of plants, animals and man'.

Since 1816 the presence of copper in both plants and animals had been known, but Deschamps' remarks were probably the first to suggest that the copper was other than accidental. Even so, the essential nature of copper in nutrition was not demonstrated until the 1920's. First in the field was McHargue who attempted to show that rats on a synthetic diet grew less well than others on the same diet with added copper (McHargue, 1925; 1926). This was followed by a series of experiments which showed that rats maintained on cow's milk developed an iron-deficiency anaemia which did not respond to iron until copper was added to the diet; (Hart et al. 1928).

Since then work has been done in so many fields on the functions of copper in the animal body that it would seem more logical to discuss each aspect separately insofar as that is possible.

Copper in Tissues

All available analytical data points to the fact that copper is present in all tissues and organs of the animal body and in such varying amounts that normal values are often very difficult to quote. Cunningham (1931) presented a survey of the copper content of organs and tissues in different species and a perusal of his data makes it evident that the liver of all species contained the highest proportion of copper with heart muscle second in some species, then kidney and hair (or feathers). Further, it can be seen that while the liver of adult man, horse, pig, dog, cat and laboratory animals showed a range of 9.2 - 41.3 parts per million dry weight (p.p.m.D.M.) that of ox and sheep ranged from 77 - 263. A few young animals appeared to have higher levels than adults.

Beck (1956) studied the liver copper content of many species of mammals, marsupials, birds, reptiles, fish and insects. Most species had levels of less that 50 p.p.m. D.M. and he agreed with Cunningham that the ox and sheep, together with the duck and frog, had levels higher than other animals. Diet did not affect the level so much as species, in fact on high-copper pastures kangaroos and sheep had liver copper levels of 13 - 17 and 2700 p.p.m.

D.M. respectively. However, the levels found within a species did vary with intake, particularly in cattle and sheep. This agreed with the findings of Dick (1954) in sheep where, over the range of 3 to 20 mg. copper intake daily, the sheep liver stored a proportional amount of copper, equivalent to about 5.0 per cent of that in the diet. Beck suggested that those animals which stored higher amounts of copper had less ability to restrict storage than others, but his later work on the duck (Beck, 1961) led him to conclude that, in this species at any rate, the higher liver copper was under physiological control. However, he found it difficult to raise the duck's liver copper above certain levels, which is not the case with the sheep.

Recent work on the addition of copper to the diet of the pig (Barber et al. 1956) has shown that in this species also it is possible to produce copper levels of eight times normal in the liver. The connection between high liver copper and 'chronic' copper poisoning will be dealt with separately.

The form that copper took in the liver was the subject of study by Mann and Keilin (1938). They prepared a colourless, non-dialysable compound which they called 'hepatocuprein' and, since most or all of the

copper in normal liver was non-dialysable, they suggested that it might be in this form. Hepatocuprein contained about 0.34 per cent copper.

Our work in sheep (Barden and Robertson, 1962) has shown that copper in the normal liver, even with high levels, was distributed differently from that in the sheep liver after the haemolytic crisis of copper poisoning.

Porter, Wiener and Barker (1961) showed that 46 per cent of copper in immature bovine liver was in the mitochondrial fractions, 21 per cent in the washed nuclei, 6 per cent in microsome fractions and 24 per cent in the supernatant fluid. They said that the mitochondrial level suggested a specific enzyme function for this copper rather than its being just as a store.

Copper deficiency was shown by Gallagher, Judah and Rees (1956) to lead to severe depletion or absence of haemαand of cytochrome oxidase, of which haemαis probably the prosthetic group, showing the importance of the element in this enzyme. It is thought (Underwood, 1956) that many of the manifestations of copper deficiency can be attributed to lowering of cytochrome oxidase activity.

Most of the kidney samples examined by Cunningham (1931) had copper contents which lay between 10 and 20

p.p.m.D.M. and most quotations from normal samples have agreed with these findings. The amount was above the general level of the body, but there has been no work to show in what form the copper exists. There is normally only a very small amount of copper in urine compared with blood.

The relatively high copper levels found in heart muscle - 10 to 20 p.p.m.D.M. (Dunningham, 1931) - were also of interest. The high cytochrome oxidase activity of bovine heart found by Porter, Wiener and Barker (1961) suggested a clue to the function of the element at this site.

A brain copper level of between 10 and 20 p.p.m.D.M. has been confirmed by most analysts. The possible connection between cytochrome oxidase activity and phospholipin synthesis was discussed by Gallagher, Judah and Rees (1956) and the importance of this in copper deficiency in sheep is dealt with later.

It has been held that the normally high copper content of hair was related to its specific function in pigment formation (Marston, 1953; Lorincz, 1954) and in the keratinisation process (Marston, 1946; Rothman, 1954). It was suggested that hypocuprosis in cattle could be detected by the finding of lower values in hair

(van Koetsveld, 1954) and in fact that analysis of rump hair in spring could give an indication of the supply of copper and other trace elements in winter (Werner and Auke, 1960). So far as the relationship to pigmentation is concerned, any expected differences between coloured and white (or albino) hair have not been confirmed. Using the hair of rat, rabbit, guinea-pig, cat, dog, horse, sheep and man, Goss and Green (1955) found the copper content of white or light hair to be as great if not greater than that of black hair; only in the pig was it slightly less. Against this one must place the well-known phenomenon of achromotrichia in copper-deficient cattle (Jamieson and Allcroft, 1949) and rats (Hundley and Ing, 1951). In the latter animal, adrenalectomy or hypophysectomy caused a partial or complete return of the hair colour.

The effect of copper on keratinisation of hair and wool is also undoubtedly of major importance and will be dealt with in the section on copper deficiency. Perhaps the reason for the unreliability of hair analysis was hinted at in the statement of Schwartz (1960) that 'hair is an important channel for the excretion of .....copper'. An excretory path is probably not the best place to look for signs of deficiency.

Copper in Blood

Since blood is the most readily available tissue for analysis in a normal living animal, data on copper levels in blood naturally appeared early in the history of research in copper metabolism. With improvements in analytical techniques, however, the wide variations of results in earlier days are now less evident. Most authors agree that bovine blood contains about 90 to 100 µg per 100 ml. and ovine blood about 70 to 80 µg per 100 ml. The blood of the pig often contains higher levels, within the range 140 to 170 µg per 100 ml. Differences between the sexes have only been observed in man (Lahey et al. 1953). Variations outside these ranges are very common. Even so, Green (1951) felt it safe to quote levels below which 'copper deficiency' could be diagnosed. More recently, however, the evidence of Dick (1954), Beck (1956), Dempsey, Cartwright and Wintrobe (1958) and Schulz and Behrens (1960) showed that blood levels of copper were not related to liver levels or even to dietary levels and deficiency conditions; (see also P.11).

Underwood (1956) pointed out that there was a limited value in the quoting of whole blood copper levels and serum copper levels as if they were the same thing

in view of the recent research into the nature of copper in blood and its distribution between the cells and plasma.

Copper exists in both red cells and plasma. In normal man (Cartwright, 1950) and in normal sheep and cattle (Eden and Green, 1939) the plasma copper concentration was found to be slightly higher than red cell copper. This has since been confirmed by many workers. Outwith the normal range of whole blood copper, however, the proportion no longer remained the same and the difference may assume importance (Adams and Haag, 1957).

Copper in red blood cells exists mainly as a blue protein, first prepared from ox cells by Mann and Keilin (1938) and called by them 'haemocuprein'. Its molecular weight was over 35,000 and it had two atoms of copper per molecule. It was very similar to hepatocuprein, described by the same authors.

In the plasma, a large proportion of the copper present was found in the form of 'coeruloplasmin'. This blue protein contained eight atoms of copper per molecule, had a molecular weight of 151,000 and was first isolated by Holmberg and Laurell (1948). The same workers had previously described experiments (1947) in

which ammonium sulphate was used to precipitate globulins from the plasma and thus to show that most of the copper in human plasma was bound to globulin. It has since been shown (Lahey et al. 1953) that in human serum there is a good correlation between copper and α_2 and α_3 globulins. Gubler and others (1953) have shown that the proportion of globulin-bound copper in man, rat, dog and pig is 96, 99, 88 and 58 per cent., respectively. No data have so far been available concerning sheep. Holmberg and Laurell identified coeruloplasmin as a true oxidase and stated that its best substrate was para-phenylene diamine, a fact which has since been used to advantage in human clinical diagnosis, (Ravin, 1956).

The remainder of the copper in plasma is in a form which is loosely-bound to the albumin fraction. This statement is based on the observations of Gubler and others (op. cit.) that within the physiological pH range this copper was non-dialysable, yet it was not contained in the globulin fractions and could be made to react directly with a copper-detecting reagent (dithiocarbamate). Holmberg and Laurell (1947) stated, however, that at a physiological or slightly higher pH no part of serum copper reacted directly with dithio-carbamate, while small amounts of copper added to serum

would do so, showing that the serum copper-binding capacity was used up to 100 per cent.

Variations in Blood Copper

As stated above, the relationship between liver and blood copper levels has been studied by many workers and the facts seem to indicate that blood levels can give no real information as to the copper status of the liver and, therefore, of the animal. Usually, low blood or serum levels would give cause to suspect hypocuprosis but this condition may be present while blood samples yield apparently normal results. High levels of copper in blood can arise from a variety of causes.

In pregnancy there was a rise in serum copper in women (Nielsen, 1944; Fay, Cartwright and Wintrobe, 1949; Ventura and Klopper, 1950) and the level fell very quickly after parturition (Nielsen). A similar rise during pregnancy was observed in gravid sows (Moustgaard and Olsen, 1951) who showed that this was independent of the sow's diet. The blood copper levels of pregnant and lactating ewes were followed by Eden (1941) and MacDougall (1947) who found no differences characteristic of either state. So far, there is insufficient data on which to base an opinion about the copper levels in the gravid cow.

In connection with the changes in pregnancy, it is of interest to notice a few observations which have been made in newborn animals. Moustgaard and Olsen observed that the blood copper level in the baby pig was 23 to 49 per cent of that of the dam, but that it rose to normal levels in 20 days regardless of the diet. They noticed that serum albumin levels were similarly low in the newborn pig and suggest that 'the low copper content is due to the lower content of copper-binding protein in the blood'. Cantarutti and Panizon, (1954) noted a rise in infants' blood copper from 0 to 14 days, when it reached normal human levels.

These observations seem to agree with those made in humans by Fay, Cartwright and Wintrobe, where the placental blood copper was lower than that of the maternal blood. Their suggestion was that the higher maternal level was related to the mechanism of mobilisation for placental transfer. A further interesting observation was made by Neuweiler (1942) who observed that copper was higher in the serum from the placental vein than in serum from the placental artery by about 30 per cent; this suggested a very rapid uptake of copper by the foetus at term.

In sheep, MacDougall's observations showed that blood copper was low in the foetus, variable for a few hours after birth and higher than the ewe's some days later. This high level gradually declined over the next ten weeks to normal in the suckling lamb.

Apart from pregnancy, where the changes may be regarded as physiologically normal, blood, or serum copper levels have been shown to alter, sometimes remarkably, in various disease conditions. Unfortunately nearly all the information concerns human beings, there being few data on domestic animals in this respect. A rise in serum copper content was seen in subacute or chronic conditions such as tuberculosis, glomerulonephritis, arteriosclerosis and other diseases, (Carranza, 1953) neoplastic disease (Pirrie, 1952), cirrhosis (Gubler et al. 1957). Pirrie's work included the interesting observation that while serum iron fell in his patients, the serum copper rose and that there was a significant inverse relationship between the two. Lahey and others (1953) found a raised plasma copper level in iron-deficiency anaemia as well as many other conditions. The difference between normal levels and the raised levels seemed to be largely accounted for by a higher level of

the 'loosely-bound' fraction in the plasma but a very low coeruloplasmin level was found in nephrosis where coeruloplamin was lost in the urine (Markowitz et al. 1955).

These findings in pathological states have led to experiments to produce raised copper levels by inducing lesions in animals. Injection of rats (Gubler et al. 1952) with turpentine, culture of Staphylococcus aureus, milk, or typhoid vaccine caused a rise in plasma copper. Rises were greatest in the first two cases where there was a greater degree of local inflammation. The observation that 'stress' caused raised copper levels led to experiments involving the adrenal glands. In Gubler's rat experiments mentioned above, the copper level was not reduced by the limiting effect of cortisone on the degree of inflammation, while removal of the adrenals (Meyer, Meyer and Horwitt, 1958) made no difference to the increase in serum copper.

The condition of hepato-lenticular degeneration (Wilson-Uzman's Disease) in man, in which there are interesting changes in copper metabolism and excretion of large amounts in the urine does not appear to have any parallel in the domestic animals. It has been extensively studied in the work of the copper metabolism

team at Johns Hopkins Hospital, Baltimore.

Blood copper changes during the administration of high-copper diets and high-molybdenum diets are the subject of experimental work discussed fully later.

The Absorption, Storage and Excretion of Copper

It has become evident in recent years, as a result of increased work on the availability of dietary copper to animals and the factors which may affect it, that all manner of elements, compounds, clinical conditions and possibly other factors complicate the observations made upon absorption, storage and excretion of copper by the animal body. It is proposed first to discuss the position, as far as possible, in the 'normal', uncomplicated state.

Working on copper storage in the liver of sheep, Dick (1954) observed that liver uptake, when the diet contained between 3 and 20 mg copper per day, amounted only to some 4.5 to 5.0 per cent of the intake over a six-month period. Mylrea (1958) found that, over a twelve-week period, Shorthorn steers stored 4.6 per cent of ingested copper. In an acute experiment with pigs and using radioactive ('labelled') copper Bowland and others (1961) found very similar net absorption levels - 5.1 per cent of the amount administered orally.

The site of absorption of dietary copper has been investigated with labelled copper by van Koetsveld (1954) and Bowland and others (1961). The former stated quite

categorically that absorption was mainly in the upper small intestine. The latter authors showed that, in pigs, while transfer across the gut wall was mainly in the small intestine and colon, the site of greater transfer rate varied with the solubility of the copper compound used, more soluble compounds being found mainly in the small intestine wall and less soluble ones in the colon wall. Transfer in the colon almost certainly represented excretion of copper, since labelled copper appeared in the faeces within 2 hours after dosing. Experiments described later involving high-molybdenum diets support this view.

The fate of copper, once absorbed, was the subject of an early study by Eden and Green (1939) using rabbits and sheep. They drew attention to the importance of studying the distribution between the cells and the plasma and noted that the blood copper levels, having risen after one large dose of copper, returned to normal within 24 hours. They also showed that if the serum level was raised to 10 times normal it was fatal to the rabbit. Later work by Bearn and Kunkel (1954) showed that labelled copper given by mouth to rats first appeared in the albumin fraction of the blood and then shifted to the globulin

fraction - that is, it entered the blood as 'loosely-bound copper' and then was transferred to coeruloplasmin-copper. In the pigs of Bowland and others (op. cit.) much the same thing happened, the albumin copper remaining at a constant level and the globulin copper rising over a period of 48 hours.

As one might expect, the fate of copper given intravenously differs from that of orally administered copper. Bowland and others found that labelled copper injected into pigs was rapidly removed from the blood and that 24 hours after injection 44 to 51 per cent of it could be recovered from the liver. In the dogs of Mahoney and his colleagues (1955) 70 per cent of labelled copper injected intravenously was recovered in the liver. Gitlin, Hughes and Janeway (1960) injected mice with ^{64}Cu and the liver concentration rose for seven hours afterwards, the subsequent fall being associated with an increase first in the bile and later in the faeces.

The wide distribution of copper in the tissues of the animal body has already been discussed. It would seem reasonable to assume that this copper is, like other elements and compounds, in a dynamic state and that there is constant deposition and withdrawal from the cells.

Copper excretion has been studied by Schubert and Reizler (1947), Comar, Davis and Singer (1948) and Marston (1950) and all agreed that it was a very small proportion of excreted copper which appeared in the urine, while nearly all appeared in the faeces. When ^{64}Cu was fed to cattle 75 per cent appeared in the faeces and only 3 per cent in the urine (Comar, Davis and Singer) while Marston stated that the difference between output and intake in his animals was so small that it could not be accurately determined. He estimated the urinary output of copper to be less than 0.1 per cent of the intake. The missing 22 per cent of Comar, Davis and Singer is probably accounted for by the absorption rate described in the work of Bowland and others. The finding of these workers that bile contained enough of their labelled copper to account for up to 40 per cent of copper excreted lends weight to the bile excretion pattern described by Gitlin, Hughes and Janeway mentioned above. Similarly, 7 to 10 per cent of labelled copper injected into dogs (Mahoney et al.) appeared in the bile, while only 1.5 per cent was in the intestinal wall and 0.6 per cent in the urine. Ligation of the bile duct in these dogs increased excretion through the kidney and

intestine, though blood copper did not increase.

Urine does not appear to be important as a pathway of copper excretion in the normal animal. To the results quoted already which support this statement, one may add the quantities found in human urine by Porter (1951) - 3.5 to 14.7 µg in 24 hours, while 7 of his 11 people had amounts so small that he pronounced copper absent within the limits of his method. This would seem to fit in with statements already made, viz: that copper in blood is either (a) bound as coeruloplasmin or (b) loosely-bound to albumin but still non-dialysable and also that the serum copper-binding capacity is used up to 100 per cent.

The only other excretory pathway left to an element like copper would appear to be through the integumentary system. That hair and wool contain copper and form a pathway of excretion has already been mentioned. It is possible that skin and hair, as well as horn and hoof copper, account for a fair proportion of that part of the dietary copper absorbed and retained by animals, particularly sheep.

So much for what can be said about copper absorption and excretion in the normal animal and with the diet

uncomplicated by abnormal amounts of either copper or certain other substances. The whole picture becomes much more confused when these conditions do not prevail. It will be preferable to dwell on this subject in greater detail in a discussion of the experimental findings described later but a brief résumé of knowledge to date is included here.

Probably the first concrete evidence of a direct influence on copper metabolism by another substance was the discovery that molybdenum was responsible for the clinical condition of cattle on the now-famous 'teart' pastures of Somerset (Ferguson, Lewis and Watson, 1943). The cattle showed signs similar to those in copper deficiency (q.v.) and responded to treatment with oral copper sulphate. Comar, Singer and Davis (1949) investigated the possibility that the scouring in molybdenosis was due to bacterial causes and decided that it was not, and also that 'it was unlikely that the toxic action of molybdenum can be accounted for simply by a complex formation which renders other elements unavailable'. A great deal of work has been carried out on the effect of dietary molybdenum on copper metabolism by Dick and Bull (1945) and by Dick (1952, 1953, 1954) showing that

molybdenum could significantly reduce liver stores of copper so long as sufficient sulphate was also present. This occurred even when added copper was present in the diet, so that there seemed a possibility of reduced absorption as well as increased mobilisation and excretion. Wynne and McClymont (1955a; 1955b) confirmed the induction of hypocuprosis in sheep by molybdenum and sulphate, while naturally occurring 'molybdenosis', due to industrial contamination was reported by Buxton and Allcroft (1955) and Parker and Rose (1955). Arrington and Davis (1953) showed molybdenum to be toxic at high levels to rabbits, some of the conditions produced responding to copper supplementation of the diet.

The action of molybdenum remains something of a mystery, but investigations concerning its undoubted ability to deplete an animal of its copper reserves reached an exciting conclusion when Mills and Bell produced the first experimental 'swayback' lambs at Aberdeen (Mills and Bell, 1960).

The possibility that other substances might influence copper metabolism has led to work by Barnes (1959) which showed that when heifers were fed hay, molasses and urea, or hay and molasses the liver stores

of copper were significantly depleted.

Mylrea (1958) failed to show any significant effects due to manganese in Shorthorn steers, though he confirmed the effects of molybdenum and sulphate in the bovine animal.

The actions of molybdenum and sulphate were again confirmed by Harvey and his associates (1961) but they investigated a clinical interference with copper metabolism near Brisbane and concluded that it was not due to these substances.

It has been argued that where copper deficiency occurs in parts of Britain where herbage has not been shown to be lower than 5.0 p.p.m.D.M. there are 'other factors' involved. If there are, they have not been found.

The possibility that copper in plants may not necessarily be available to animals was the subject of a series of investigations by Mills. First he showed (1954) that about two-thirds of the total copper in herbage was insoluble in inorganic solvents or water, but that the difference between 'normal' and 'swayback' herbages was not great, though the latter contained rather less water-soluble copper. Then rats reared on

a low copper diet were fed herbage supplements or copper sulphate to the same copper level (Mills, 1955) and their response to dietary copper was assessed by their recovery from the signs of copper depletion. The herbage supplements gave a greater response and higher liver values than copper sulphate. The effect appeared to be associated with a water-soluble compound of copper which was more readily available than free copper. 'Normal' and 'swayback' pastures again showed no differences. When the aqueous extract of herbage was passed through an ion-exchange resin, stable copper complexes were obtained and when these were fed to copper-deficient rats they produced a greater physiological response than equivalent amounts of copper ions (Mills, 1956). It seemed likely that copper might pass through the intestine in the form of such complexes. The copper-binding properties of proteins were hinted at in the old treatment of copper poisoning; Moussu (1911) advises 'oeufs crus, blancs d'oeufs battus ...'. MacCall (1958) demonstrated that with high protein levels in the diet less copper was absorbed by rats than when more normal levels were present.

Amino-acids containing an -SS- or -SH group were found to have the greatest copper-binding activity,

(Deijs and Bosman, 1959). Aqueous extracts of fresh grass cut in August had strong copper-binding properties, varying according to type of grass and declining through Autumn. Cocksfoot grass had the highest copper-binding activity of 10 grasses examined; hay had less than fresh grass.

Copper Deficiency

When the physiological functions of a mineral element or a vitamin have been investigated in the past, the knowledge gained has frequently been due to research into the conditions caused by a deficiency. In the case of copper we have come to know more of its nutritional properties in just this way. The first good demonstration of the essential nature of copper to animals was in the classic experiments of Hart and his colleagues mentioned on Page 2. (Waddell et al. 1928a; Waddell et al. 1928b; Hart et al. 1928). Pigs maintained on a cow's milk diet with added manganese, iodine and iron, with vitamins A and D, developed an anaemia with very low haemoglobins, (2 to 3 g. per 100 ml.) and a dystrophic bone condition. The addition of copper to the basal diet prevented the anaemia and the leg condition and in some cases was curative. (Teague and Carpenter, 1951).

The rat experiments of Hart were repeated by Chase et al. (1952) with the refinement of a labelled iron supplement and different levels of copper supplementation. The quantity of ^{59}Fe absorbed was increased by each additional increment in copper intake but as little as 0.05 mg copper per day gave the maximum increase in total body iron and

in haemoglobin levels. The copper need not be in the diet it was just as effective when in the body though not obtained from the food.

Having produced a rise in haemoglobin and red cell count in anaemic patients by administration of copper, de Vries (1952) concluded that copper acted 'as a catalyst' in blood formation.

The iron content of the liver could be increased in anaemic pigs but while they were still copper-deficient their haemoglobin levels did not rise (Lahey et al. 1952). Then administration of copper produced a rise in haemoglobin. The same work showed that copper deficiency reduced the absorption of iron from the gut.

In the dog, copper deficiency decreased the actual number of red blood cells in the blood, while iron deficiency only reduced their haemoglobin content, (van Wyk et al. 1953). This effect of copper deficiency was evident in the bone marrow. This finding was confirmed when Brooksbank (1954) showed that circulating reticulocytes were fewer in pigs with copper deficiency receiving iron supplement. He also found an abnormally high lymphocyte:neutrophil ratio which responded to copper but not to iron.

When investigating hypocuprosis in Eastern England,

Blakemore and Venn (1950) described an anaemia which suggested interference with normal haemopoiesis.

The importance of giving copper in 'intractible' cases of iron-deficiency anaemia in man was demonstrated by Hasegawa and Ito (1954) and hypocupraemia complicating anaemia treatment in children was discussed by Zipursky (1958). Lahey (1957) and Schubert and Lahey (1959) suggested that iron-deficiency anaemia in children led to protein deficiency and that this in turn impaired the retention of copper.

It seems, then, that we can safely say that copper increases the absorption of iron from the gut, whether it is present in the food or already in the intestine it helps the formation of liver stores of iron and is necessary for their effective mobilisation; it is also necessary for the formation and maturation of red blood cells in the bone marrow.

The effects of copper depletion on bone growth mentioned above were also seen by Baxter and van Wyk (1953) in dogs where their purified diet contained less than 1 part per million. The bones were deformed and some fractured easily and were seen to have thin cortices, reduced cancellous bone and wide epiphyseal gaps. These abnormalities were not considered to be typical of rickets

and no abnormalities in calcium, phosphorus, phosphatase or vitamin D were found. Copper supplementation produced a clinical improvement in a few weeks. The skeleton was not affected by iron deficiency, even when the dogs were severely anaemic. The bones were examined histologically (Baxter, van Wyk and Follis, 1953) but no gross disturbance of calcification was found. However, it is evident that copper plays some fundamental part in the formation of bone tissue.

Early in the work on copper deficiency in rats it was noticed that black rats when copper-deficient became red or grey. Hundley and Ing (1951), investigating this, showed a connection with adrenal secretions, as adrenalectomy prevented the achromotrichia developing in the majority of cases. When rats whose coats were already grey on a purified diet were subjected to adrenalectomy (or, in some cases, hypophysectomy) there was a partial or complete return of colour to the hair. Achromotrichia has also been described in sheep (Lee and Moule, 1947) and in cattle (Cunningham, 1954) when copper deficiency was present and has been produced in sheep with molybdenum (Dick, quoted by Underwood) but an interesting feature here is that kemp fibres and facial hair were not affected in the same way as the wool.

A more economically serious effect of copper depletion on sheep's wool is the loss of the characteristic 'crimp'. This has been described in Australia, (Lee and Moule, 1947; Lee, 1950; Lee, 1956) but not in Britain, although it occurred in British breeds of sheep on Australian copper-deficient pastures, (Lee 1956). A thorough study of the properties of wool taken from Merino sheep grazed on copper-deficient pastures and supplemented with varying amounts of copper was undertaken by Palmer (1949). He found that with increasing supplement there was an increase in fibre diameter, in tensile strength and in 'behaviour during processing'. Burley and Horden (1959) showed that normal wool contains an 'inter-molecular cementing substance' of low molecular weight which was lacking in wool from copper-deficient sheep.

A feature of copper deficiency about which our knowledge is more nebulous is the cachexia and 'ill-thrift' of cattle whose body reserves of copper are low. The first record of bovine 'hypocupraemia' in Britain is that of Allcroft (1946). She reported low blood copper levels in cattle which had a 'severe chronic scouring disorder'. The pasture copper was normal (More than 10 p.p.m.) and there was no reason to suppose

that the molybdenum level (5 to 9 p.p.m.) was high. In East Anglian fen pastures (Blakemore and Venn, 1950) the first sign of hypocuprosis in cattle was depression of appetite, affected cattle not thriving and some heifers becoming infertile. Copper and molybdenum levels in the pasture were in the accepted normal range. Jamieson and Allcroft (1949) described a condition in the North of Scotland in which cattle had low blood copper levels and where clinical improvements resulted from copper administration. Allcroft suggested (1952) that molybdenum was not the only factor interfering with copper metabolism and, in view of the normal levels of copper in the pastures concerned, chose to call the effects 'conditioned copper deficiency'. This name was used in a review by Stableforth (1953). The view was further upheld by Field (1957a; 1957b) after several years' work in the East Anglian area where, in spite of the level of copper always being 'adequate for grazing stock', he was able by administering copper to cattle to improve their weight gains and produce recovery in animals actually showing clinical signs of deficiency. It is probably not a coincidence that hypocuprosis in ruminants appears on reclaimed peaty or hill land. Apart from the fens and

hill farms in Britain, it has been described in Friesland on reclaimed land subject to flooding (Hartmans, 1960), in New Zealand on peaty ground (Cunningham, 1950), in Norway on hill farms (Havre, Dynna and Ender, 1960) and in Ireland (Senior, Sheehy, O'Sullivan and O'Donovan, 1954).

Enzootic Neonatal Ataxia

A disease involving inco-ordination of gait in newborn or very young lambs was connected with copper deficiency by Bennetts and Chapman (1937) and its pathology described by Innes and Shearer (1940). While the pastures where it occurs in Australia are low in copper content (or high in molybdenum) no such conditions prevailed where cases occurred in England. More recently, the condition has been investigated in Scotland (Barlow, 1956) on land not dissimilar to the English farms in character - hilly districts where sheep farming predominates - but in 62 per cent of cases, copper in pasture samples was below 5 p.p.m. The pathology as described by Innes and Shearer was typified by demyelinated tracts in the spinal cord and corresponding lesions in the brain which sometimes involved vacuolation. Barlow, however, described far less severe lesions macroscopically, but microscopic examination

revealed changes in individual cells in brain and cord. Many of his cases were lambs which had appeared normal at birth and had developed ataxic signs later.

Neonatal ataxia has been described in South Africa (Schulz et al. 1951), Iceland (Pálsson and Grimsson, 1953) Germany (Schulz and Behrens, 1960) and many other parts of the world.

In an attempt to show that copper depletion affected adversely the development of the brain, Frick and Lampl (1953) showed that in rats deprived of copper there were no nerve lesions and the brain and spinal cord showed no abnormality on histological examination. They concluded that another influence, additional to copper deficiency, must operate in lambs.

Gallagher, Judah and Rees (1956) found a relationship between cytochrome oxidase activity and phospholipin synthesis. They pointed out the possible relevance of this to demyelination of the central nervous system.

The prevention of swayback has presented no great problem. The early methods were by drenching ewes with a weak solution of copper sulphate sufficient to give about 1.5 g. $CuSo_4 \cdot 5H_2O$ twice during the pregnancy - about 8 weeks and 4 weeks before lambing being ideal. Dunlop (1951) claimed that if swayback occurred in

lambs at the beginning of the lambing season there was still time to prevent it in others by drenching immediately those ewes still to lamb.

More recently, commercial interests have favoured the use of copper injections parenterally as a glycinate in a suitable cerate carrier. There are dangers to this practice (Cunningham, 1957) such as the introduction of the spores of clostridial organisms or the production of a cold abscess at the site. Allcroft, Clegg and Uvarov (1959) demonstrated a satisfactory rise in blood copper to normal levels in sheep injected with copper glycinate.

The Effect of Copper on Growth Rate

Ever since the earliest copper deficiency experiments of McHargue (1925; 1926) showing that copper-deprived rats grew less well than when copper was added to their diet, there has been an interest in the growth-rate effect of copper supplementation. In ruminants the ability of copper to improve growth rate would seem to be limited to cases where the animals would otherwise be relatively copper-deficient and suffering to some degree from the cachexia and scouring already discussed above. Interesting results were obtained in West Suffolk among beef animals raised on reclaimed land (Field, 1953); control calves gained 1.5 lbs per day while calves from cows dosed with 10 g. copper sulphate every 14 days during pregnancy gained 2.2 lbs per day. Growing cattle dosed with copper sulphate were clinically normal and had good weight gains from 12 to 30 months of age, while controls scoured severely during their third summer and had poor gains.

Where cattle are <u>not</u> copper-deficient, added copper does not appear to improve their performance. Range Hereford cattle in the U.S.A. did not benefit from copper

glycinate injections (Raleigh and Wallace, 1962).

In pigs there was an interesting effect to be seen. Copper sulphate added to the diet produced an enhanced growth rate. Barber, Braude and Mitchell, (1960) produced evidence that when the diet contained 250 p.p.m. copper (0.1 per cent $CuSO_4.5H_2O$) the growth rate increased by 10.4 per cent, food conversion efficiency by 4.5 per cent, food consumption increased by 5.8 per cent and the dressing percentage improved by 1.7 per cent. The carcass length was reduced by 2 per cent and some carcasses 'graded' one class lower because of this.

The site of action of the added copper is probably to be found in the alimentary tract. This view is supported by the findings of Barber et al. (1956) that copper was as effective in significantly increasing the growth rate of pigs as oxytetracycline and aureomycin when these were fed at levels now common in pig diets. The postulated reduction of 'subclinical disease' was probably similar, and a reduced weight of gut tissue was found in pigs on high copper diets, supporting this idea.

The fact that pigs fed these high levels of copper sulphate showed no ill effects, while similar levels have

been fatal to sheep, is remarkable. Very high levels must be fed to produce copper poisoning in pigs and most pigs will refuse them anyway (Allen and Harding, 1962).

Copper Poisoning in Ruminants

It is well-known that large amounts of copper salts are fatal to animals and man. Apart from the malicious cases of poisoning of humans mentioned in Page 1, copper poisoning in animals was well-known by the late nineteenth century. Ellenberger and Hofmeister (1883) are reputed to have recognised copper poisoning as a distinct clinical entity, and early experiments were carried out by Baum and Seeliger (1898). They observed icterus and haemoglobinuria to be constantly present as clinical manifestations of poisoning with copper in sheep. One assumes that they were describing the 'chronic' type of toxicosis seen with ingestion of copper over long periods by sheep, as when a text-book of animal diseases was later to include a description of the conditions it outlined the sources of copper (Moussu, 1911) as 'vases de cuivre (vert-de-gris, sous-acetate de cuivre) onguent égyptiac, ou les feuilles de vignes impregnées de sulfate de cuivre durant les années de disette'. The last mentioned treatment of grape-vines has found a parallel as recently as 1952, when Muth reported deaths in sheep with the characteristic lesions

of copper poisoning after they had grazed pasture contaminated with the same Bordeaux mixture. Moussu's text-book also included mention of the urine 'containing haemoglobin in solution'.

Eden and Green (1939) followed the fate of large amounts of copper administered to rabbits and sheep. They found that less than ten times the normal serum level was fatal to the sheep.

The subject of 'chronic' copper poisoning was reviewed by Boughton and Hardy in 1934 and by Bull (1949) in the light of a large-scale investigation made into 'enzootic jaundice' in Australia. A review covering many aspects of the subject has just appeared (Todd, 1962).

Clinical occurrences of copper poisoning have continued to appear in sheep (Ogilvie, 1954; Clegg, 1956; Pearson, 1956; Bracewell, 1958; Pryor, 1959; Berwyn-Jones, 1960; Barden and Paver, 1961) and in cattle (Todd and Gracey, 1959; Shand and Lewis, 1957) where, usually by accident, the animals have eaten diets containing high levels of copper.

The effects of molybdate supplement on sheep with high liver stores was tried by Pierson and Aanes (1958) and has been followed up in the present work, as described in Section III.

Section II

The Determination of Copper in Biological Material

The Determination of Copper in Biological Material

The amounts of copper in animal tissues, plants and soil are so small that an accurate method of determination is necessary before useful investigations can be carried out. Before the experiments which are described in Section III were performed, some time was spent in selecting a method which was accurate enough and yet also simple and quick enough to allow the maximum number of determinations to be made during the course of the trials.

Colorimetric methods for estimating copper include the use of several reagents, some of them specific for copper, others necessitating the isolation of the copper from other elements before determination. Three established reagents were tried out for their usefulness in this work; sodium diethyl-dithiocarbamate, biscyclohexanone oxalyldihydrazone, and 2:2'-diquinolyl. The methods employing each are briefly described here and a few results shown to compare them.

Sodium Diethyl-dithiocarbamate - Method I

The first method described is based on those of Eden and Green (1940), Clare, Cunningham and Perrin (1945) and Kuang and Bray (1953).

Procedure

Place in a boiling tube 5 ml. of blood, serum or plasma, or sufficient tissue to contain about 1 to 15 µg Cu. Standard solutions should be run alongside. Add 1 ml. sulphuric acid, 2 ml. perchloric acid and 2 ml. nitric acid. In the case of blood the latter may be left until most of the water has been boiled off.

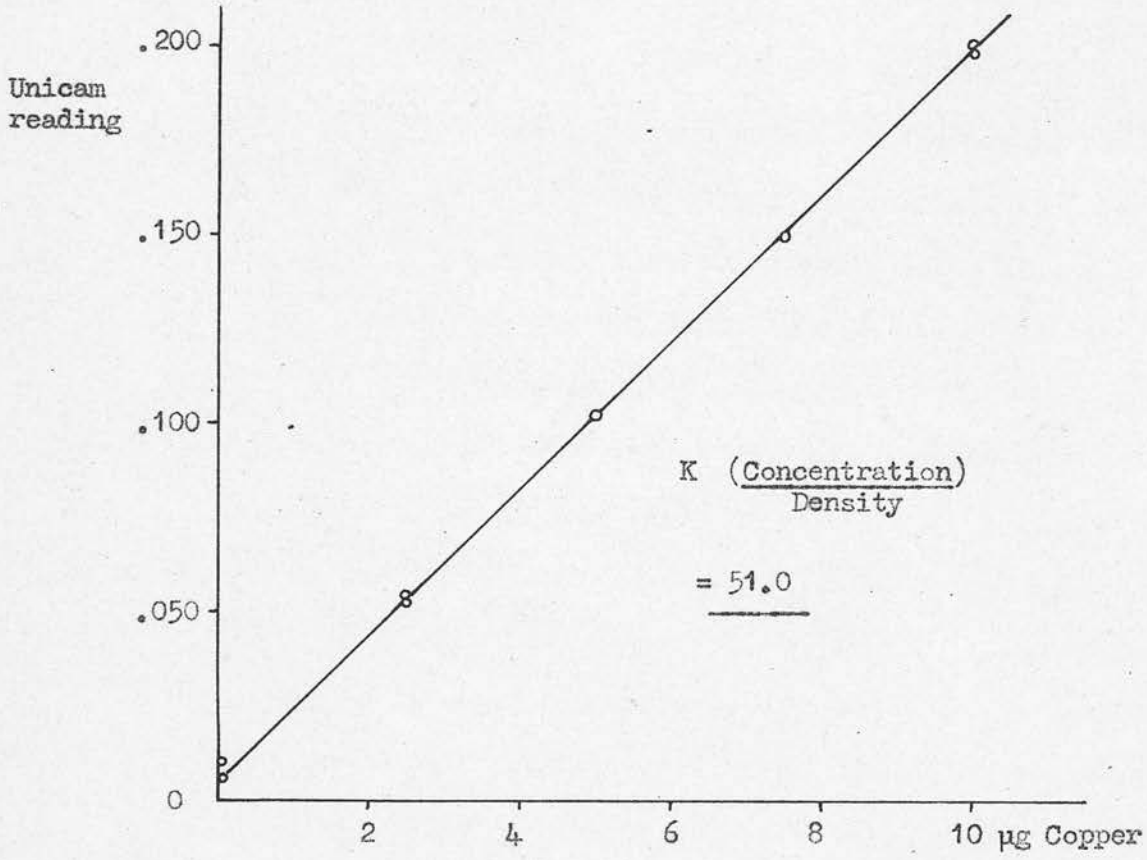
Heat on a hotplate or over a microburner flame until a small volume of clear acid is left, with rings of acid rising around the sides of the tube. This is the end of the combustion.

Remove the tube from the heat and cool slowly. Dilute the contents with about 12 ml. water. Add sufficient ammonia solution from a burette to make sure that the pH is alkaline.

Development of Colour

Place the solution in a suitable separating funnel. Add 10 ml. of a solution containing 20 per cent ammonium citrate and 5 per cent versenate (the disodium salt of ethylene diamine tetra-acetic acid) and mix. Add 2 ml. of a 1 per cent solution of sodium diethyl dithiocarbamate, followed by exactly 10 ml. of carbon tetrachloride. Stopper the funnel and shake vigorously for more than two

Figure 1.



Calibration curve obtained by using "wet-ash" technique on standard copper solutions and developing colour with Sodium diethyl-dithiocarbamate

minutes. After allowing the mixture to settle out, run off the carbon tetrachloride layer into a dry tube. Repeat the extraction with another 5 ml. carbon tetrachloride and add the two extracts together. The second should be almost colourless. If they are at all cloudy they should be centrifuged.

The intensity of colour of the extracts is read in any suitable photometer at a wavelength of 440 mμ. With readings from standard solutions a graph may be prepared or calculations of unknown samples made by allowing correction for a blank and finding the amount of copper present from the formula:

$$\mu g \text{ copper} = \text{reading} \times K, \text{ where } K = \frac{\text{concentration}}{\text{density}}.$$

A standard graph prepared by this method is shown in Figure 1.

Liver samples from ruminants may give readings well outside the range of standards used. A suitable dilution of the ash solution should be prepared and the calculation adjusted accordingly.

Sodium diethyl-dithiocarbamate - Method II

Cubler and others developed a method for estimating copper in blood (1952) which was based on a technique

described by Tompsett (1934).

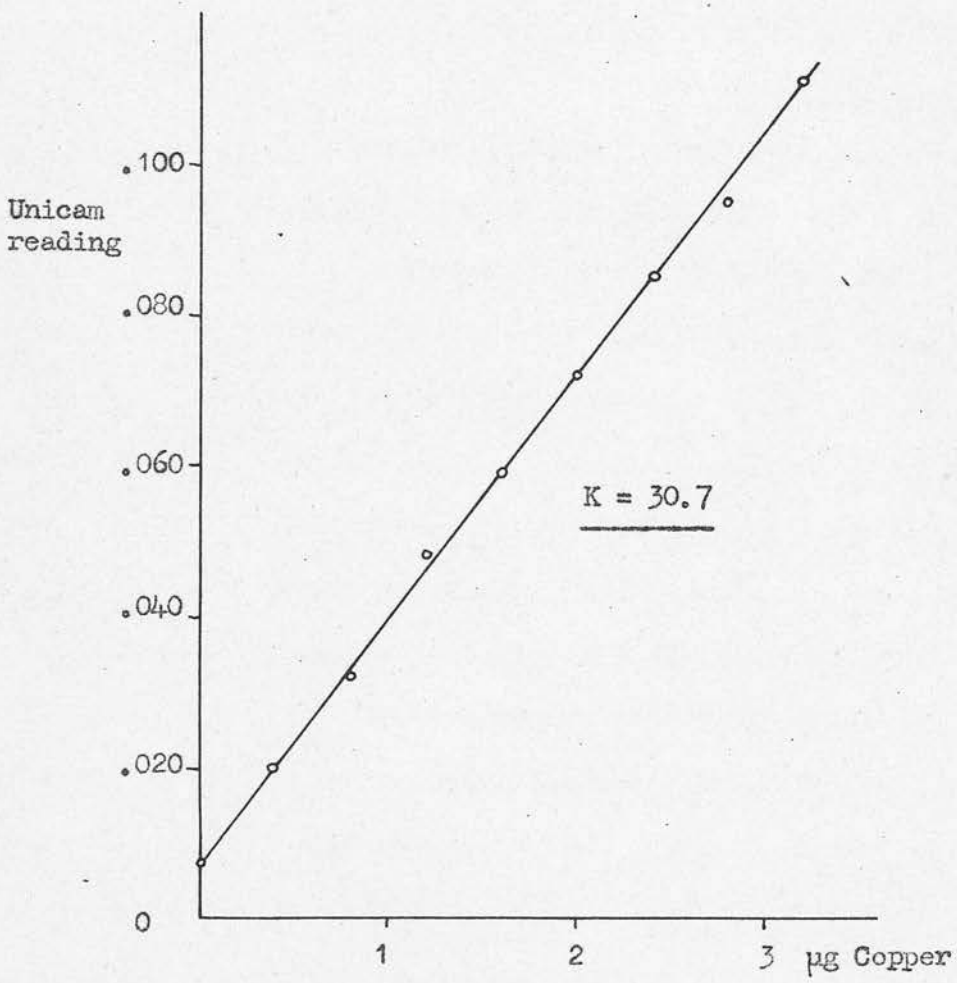
Procedure for Plasma or Serum

To 1 ml. of plasma or serum in a small centrifuge tube add 1 ml. of 2N HCl, stir well and allow to stand for 10 mintues. Then add 1 ml. of 20 per cent trichloroacetic acid, stir well and allow to stand for a further 10 minutes. Cover the tubes and centrifuge at 3,000 r.p.m. for 30 minutes to ensure maximum volume of supernatant, which is then poured off into a small tube. Pipette a 2.4 ml. aliquot into a small graduated tube and add 0.2 ml. of a saturated solution of sodium pyrophosphate, 0.2 ml. of saturated sodium citrate solution, 0.4 ml. of 20 per cent ammonia solution and water to make the volume 3.3 ml. The density is then read against a reagent blank containing no carbamate or against distilled water. After this, 0.2 ml. of 0.1 per cent dithiocarbamate is added and the density read again.

Procedure for Whole Blood or Red Cell Suspensions

To 1.0 ml. of blood, or red cell suspension in saline, add 0.5 ml. of water followed by 1.0 ml. of 2N HCl. Stir well and after 10 minutes add 1 ml. trichloroacetic acid and stir again. After centrifugation

Figure 2.



Calibration curve prepared for use with method for estimation of copper in serum by direct reaction between the trichloroacetic acid filtrate and Sodium diethyl-dithiocarbamate

the procedure above is followed again.

Calculations

Since the density is read directly and 1.0 ml. samples are used, the copper concentration of the original material in microgrammes per 100 ml. is calculated as follows:

$$\text{Plasma Cu} = (D_2 - D_1 f) \times K \times \frac{3.0}{V} \times 100 \ \mu g \text{ per cent.}$$

Whole blood or red cell

$$\text{Cu} = (D_2 - D_1 f) \times K \times \frac{3.5}{V} \times 100 \ \mu g \text{ per cent.}$$

where D_1 = density without carbamate

D_2 = density after adding carbamate.

$f = \frac{\text{volume before adding carbamate}}{\text{final volume}}$

K = slope of standard curve

V = volume of supernatant aliquot used.

Red cell copper must be reported as µg per 100 ml. of packed cells, calculated from the packed cell volume of the suspension used.

A standard curve prepared for this method is shown in Figure 2, and a comparison of results using Methods I and II is given in Table I. The two methods were used to determine the copper content of (a) whole blood, (b) plasma and (c) red cells in two different

Table I

Packed Cell Volume (P.C.V.) and Copper Content of Blood Fractions (in µg/100 ml.) in two animals.

		Plasma		Whole Blood		Red Blood Cells		
	I	II	III	IV	V	VI	VII	
Cow	P.C.V.	Wet ash	T.C.A.	Wet ash	T.C.A.	Wet ash	T.C.A.	Calculated from Cols. I, II & IV.
A	31%	51.1 48.9 50.0	97.50 41.25 56.25 48.75 60.00 48.75 60.00 41.25 41.25 82.50 57.75	67.3 68.7 68.0	81.25 67.50 41.25 60.00 38.75 56.25 56.25 73.75 63.75 41.25 58.00	106.6 109.4 108.0	216.0 276.0 408.0 444.0 408.0 0.0 108.0 468.0 372.0 492.0	108.6
B	26%	31.1 30.1 30.6	25.00 41.25 33.75 27.50 27.50 31.25 31.04	52.5 51.1 51.8	56.25 48.75 60.00 43.75 63.75 42.50 52.50	112.9 111.1 112.0		112.6

Two methods of copper estimation.

Method I – 'Wet-ash' technique; estimation on ash solution.

Method II – 'T.C.A.'; precipitation by trichloroacetic acid and estimation on the supernatant obtained.

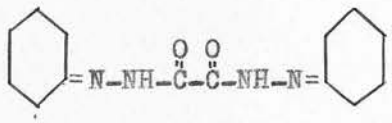
samples of blood. The red cell copper content was also calculated from the formula:

$$\text{Red cell Cu } (\mu g/100 \text{ ml. packed cells}) = \frac{(\text{Whole blood Cu} \times 100) - \text{Plasma Cu}(100 - \text{PCV})}{\text{PCV}}$$

The results demonstrate the difficulty encountered in the 'trichloroacetic acid' method. The technique was considered unsuitable for experimental work.

Method Using Biscyclohexanone Oxalyldihydrazone

Peterson and Bollier (1955), after offering reasons why dithiocarbamate was unsuitable for trichloroacetic acid filtrates of serum, described a method employing biscyclohexanone oxalyldihydrazone (O.D.H.) which Nilsson (1950) had shown to react with copper in the cupric form in alkaline solution to give a blue colour. It gave no colour with any other ions commonly encountered in biological materials.

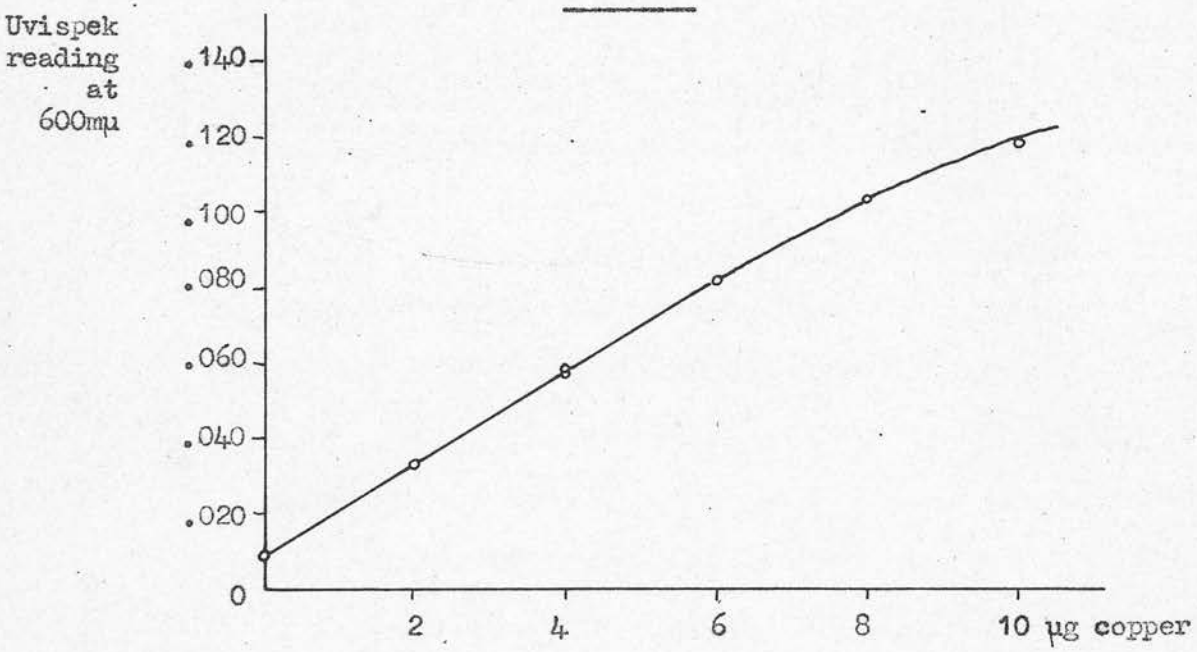


Biscyclohexanone Oxalyldihydrazone

Procedure using O.D.H.

To 1 ml. serum in a 10 ml. test tube add 0.7 ml. of

Figure 3.



Calibration curve for method of copper estimation using Biscyclohexanone Oxalyldihydrazone

Table II

Sheep	Plasma Copper µg/100 ml.	
	Wet ash & Carbamate	O.D.H. Method
A	97.0 96.0 96.5	87.0 83.0 62.0 75.0 76.8
B	73.0 71.0 72.0	63.0 41.0 56.0 43.0 50.8
C	108.0 108.0 108.0	92.0 83.0 102.0 77.0 88.5

Comparison of results obtained by analysis of plasma samples using the wet-ash and sodium diethyl-dithiocarbamate method and that using biscyclohexanone oxalyldihydrazone.

2N hydrochloric acid and after allowing it to stand for 10 minutes at room temperature add 1 ml. of 20 per cent trichloroacetic acid; mix with a thin stirring rod and centrifuge at 3,000 r.p.m. for 30 minutes.

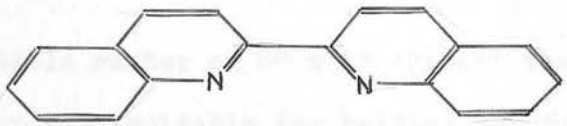
Pipette 2 ml. of the supernatant into a graduated tube and add one drop of phenolphthalein indicator. Mixing all the time, add saturated tribasic potassium phosphate drop by drop until the solution just turns pink. Add 2N hydrochloric acid in the same way until the solution returns to colourless; this should bring the solution to a range of pH 7.5 to 7.9. Add 0.2 ml. of O.D.H. (saturated solution in 50 per cent ethyl alcohol and make up to 3.5 ml. with water. Mix and read at wavelength 600 mμ in a spectrophotometer after five minutes but within an hour. Blanks and standards are carried at the same time and a standard graph prepared. Such a standard graph is shown in Figure 3. All the graphs prepared in this method showed the same characteristic of being linear up to values of about 6 μg, and then falling into a curvilinear form.

Results of serum and plasma analyses using this method were very disappointing. Readings were haphazard and usually low (see Table II). The adjustment of the pH was extremely difficult and it soon became obvious that it

had to fall within very narrow limits, so that the time taken to make it correct was too long and the method made tedious. Wood and Clark (1958) pointed out that colour development was hindered by high salt concentration, which imposed limits on the amounts of acid and sample that could be used; a high concentration of ammonium ions also caused low results.

Method Using 2:2' -diquinolyl ('Cuproin')

Hoste (1950) showed 2:2' -diquinolyl ('Cuproin', 2:2' -D)



to be a specific reagent which was useful for the accurate determination of copper. The spectrophotometric properties of the cuproin-copper complex, the stability of the colour, its partition coefficient between water and iso-amyl alcohol and the influence of temperature, foreign ions and pH were studied by Hoste, Eeckhout and Gillis (1953) who outlined procedures for the estimation of copper in plant and animal tissues, water, lampblack and steel.

It has been found practicable to use one container for the preparation of a 'wet ash' as described already

and for the subsequent development of the coloured complex. It is not necessary to adjust the solution to a narrow pH range, provided that it lies between pH 2 and 9. A small piece of litmus paper is useful at this stage without affecting accuracy. The use of an air pump assisted in mixing uniformly a large number of samples. The modified method finally used was as follows. It is given in full as it has not been described elsewhere.

Apparatus

A suitable number of 8" x 1" 'Pyrex' test tubes.

A water-bath suitable for holding the tubes.

A pump delivering air through Pasteur pipettes. (A suitable pump which was found ideal is the 'Hy-Flo' aquarium pump manufactured by Medcalf Bros., Florence Street, London, N.1.)

Reagents

GLASS DISTILLED WATER must be used throughout.

Sulphuric acid, nitric acid and 60 per cent perchloric acid. Suitable grades of these acids are prepared by Messrs. Hopkin & Williams for the estimation of lead in foodstuffs.

Tartaric acid. Prepare a 50 per cent solution.

Hydroxylamine hydrochloride. Prepare a 15 per cent

solution and purify by shaking with a few ml. of the cuproin solution (see below).

Sodium Hydroxide, 30 per cent solution or Ammonium Hydroxide, diluted 1:1 with water. (The latter is more easily prepared as a copper-free reagent).

Cuproin. 2:2' -diquinolyl (May and Baker). Prepare a 0.02 per cent solution, in amyl alcohol.

Procedure

The sample is ashed in the usual way with sulphuric, perchloric and nitric acids. Cool the tube and residue slowly, dilute with a little water and add 2 ml. tartaric acid and 2 ml. hydroxylamine hydrochloride. Neutralise with sodium or ammonium hydroxide until a very small piece of litmus paper placed in the solution turns mauve. Place the tube in a water-bath at 25°C. When the temperature has been steady for some minutes add exactly 5 ml. of the cuproin solution and bubble with air, mixing well for 2 minutes. Allow the layers to settle out and draw off the lower, aqueous phase with a Pasteur pipette attached to a filter pump. Discard this layer. If the alcohol layer is turbid it may be centrifuged or left overnight before reading. The copper-cuproin complex is lilac in colour and the extinction is read at 546 mμ on a suitable spectrophotometer. A calibration

Figure 4.

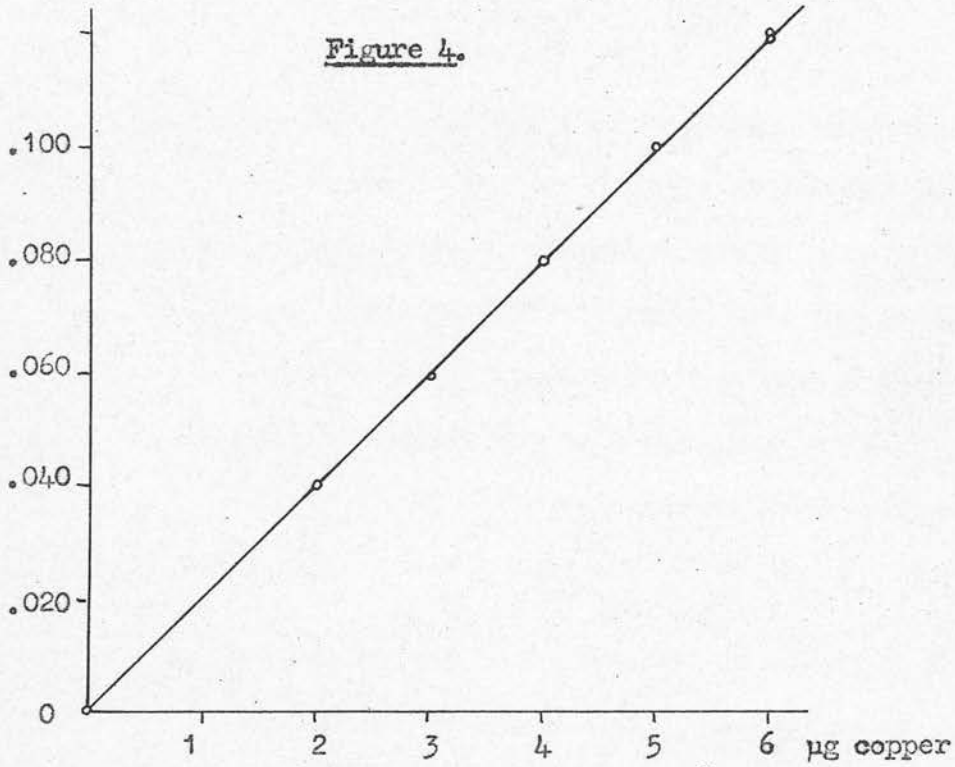
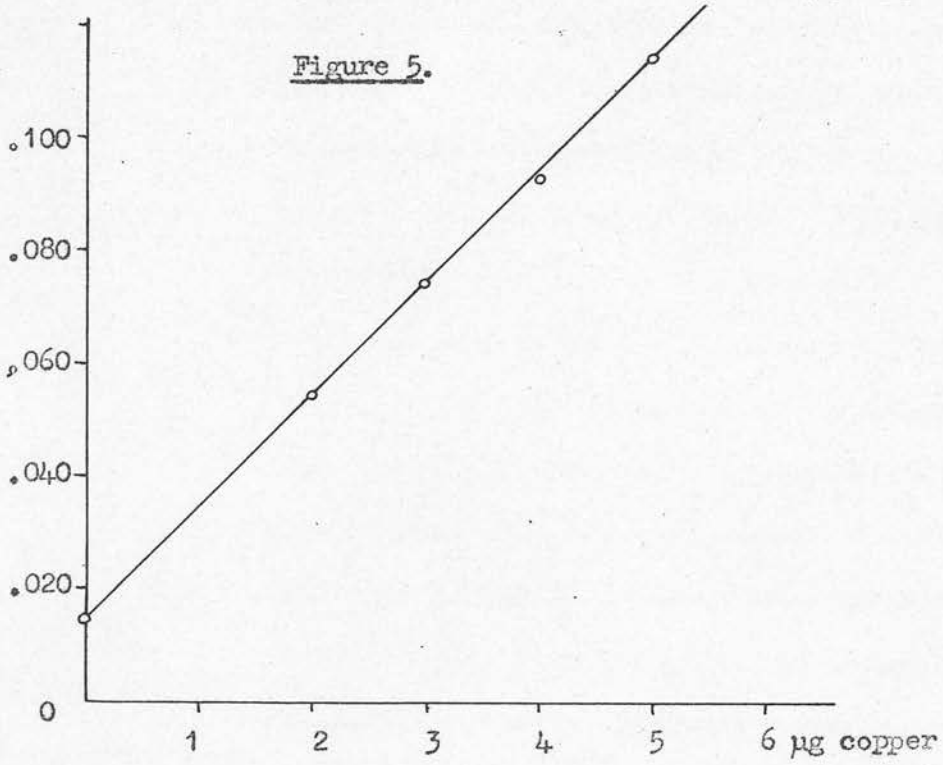


Figure 5.



Calibration curves for Cuproin (2:2'-diquinolyl) using

(Fig.4) - Standard solution and reducing agent only,

(Fig.5) - All reagents as in the full method.

curve is prepared from blanks and standards. 5 ml. of this solution of cuproin will read up to 50 µg copper with accuracy. Figure 4 shows a calibration graph prepared using only standard copper solutions and hydroxylamine hydrochloride. This gives an accurate indication of the slope with the minimum of blank reading. The blank reading, even with the best and cleanest reagents, is always appreciable when the full method is carried through, as shown in Figure 5.

Advantages of the Cuproin Method

The method employing 'Cuproin' as the colouring reagent for copper was chosen for all the work which is described in Section III, as it had distinct advantages over other methods:

(a) Only one container needs to be used for the whole procedure. The washing-up of apparatus is cut down to a minimum and therefore the chance of using copper-contaminated glassware is reduced.

(b) The organic phase of the final mixture is not so readily evaporated, so accuracy is not lost because of the mixing process.

(c) The high partition coefficient of the coloured complex means that only one extraction is necessary for accuracy, (Hoste, Eeckhout and Gillis, 1953).

Table III

Extinction of Copper-Cuproin Complex in 5 ml. Amyl Alcohol Extract from Standard Copper Solutions and Serum with copper added.

Hilger 'Uvispek' readings at 546 mμ

Original Tube Contents	Reading	Estimated copper content of tube (μg)	Copper content of tube corrected for blank reading (μg)	Recovered copper %
Blanks	.014	0.693		
	.015	0.743		
2 μg Cu	.053	2.62	1.91	95.3
	.054	2.67	1.96	97.8
5 μg Cu	.116	5.74	5.02	100.5
	.115	5.69	4.97	99.5
10 μg Cu	.217	10.74	10.02	100.2
	.215	10.64	9.93	99.3
5 ml serum I	.073	3.61	2.90	
	.075	3.71	3.00	
5 ml serum I & 2 μg Cu	.114	5.64	4.93	98.8
	.114	5.64	4.93	98.8
5 ml serum I & 5 μg Cu	.172	8.51	7.80	96.9
	.171	8.47	7.75	95.9
5 ml serum II	.088	4.36	3.63	
	.087	4.31	3.59	
5 ml serum II & 2 μg Cu	.127	6.29	5.57	97.8
	.125	6.19	5.47	92.8
5 ml serum II & 5 μg Cu	.189	9.36	8.64	100.5
	.192	9.50	8.79	103.4

(d) The stability of the colour allows for delay for centrifuging of turbid samples or saving up samples to be read in one batch later. (Hoste, Eeckhout and Gillis, 1953).

(e) The accuracy of the method is satisfactory, (see Table III).

Section III

(i)

Copper Poisoning in Sheep

Experimental Copper Poisoning in Sheep

The first experimental production of 'chronic' copper poisoning in sheep appears to have been carried out by Baum and Seeliger (1898), who stated that icterus and haemoglobinuria were constant findings. Sutter and others (1958), after conducting their own experiments, said that the haemolytic syndrome was not an essential criterion for diagnosis, which could be confirmed from blood levels of copper.

In the present work it was decided to feed high-copper rations to a group of normal sheep, to follow the levels of copper in their blood, both in the plasma and red-cell fractions, and to determine as accurately as possible the clinical course of copper poisoning and the pathological findings after death.

Materials and Methods

Six yearling cross-bred sheep, 2 castrated males and 4 females, were kept indoors and fed on a basic diet of good hay and crushed oats. Weekly sampling of venous blood was performed by jugular puncture using stainless steel needles, samples always being taken in the morning before food was offered. Copper analysis was carried out on whole blood and plasma. In order to determine that

part of the plasma copper which was 'loosely-bound' (non-coeruloplasmin copper), the globulins in the plasma were precipitated with ammonium sulphate and analysis carried out on the supernatant fluid, simulating the conditions of analysis described by Holmberg and Laurell (1947) and Earl, Moulton and Selverstone (1954). The method of analysis was that modified from Hoste, Eeckhout and Gillis described in Section II.

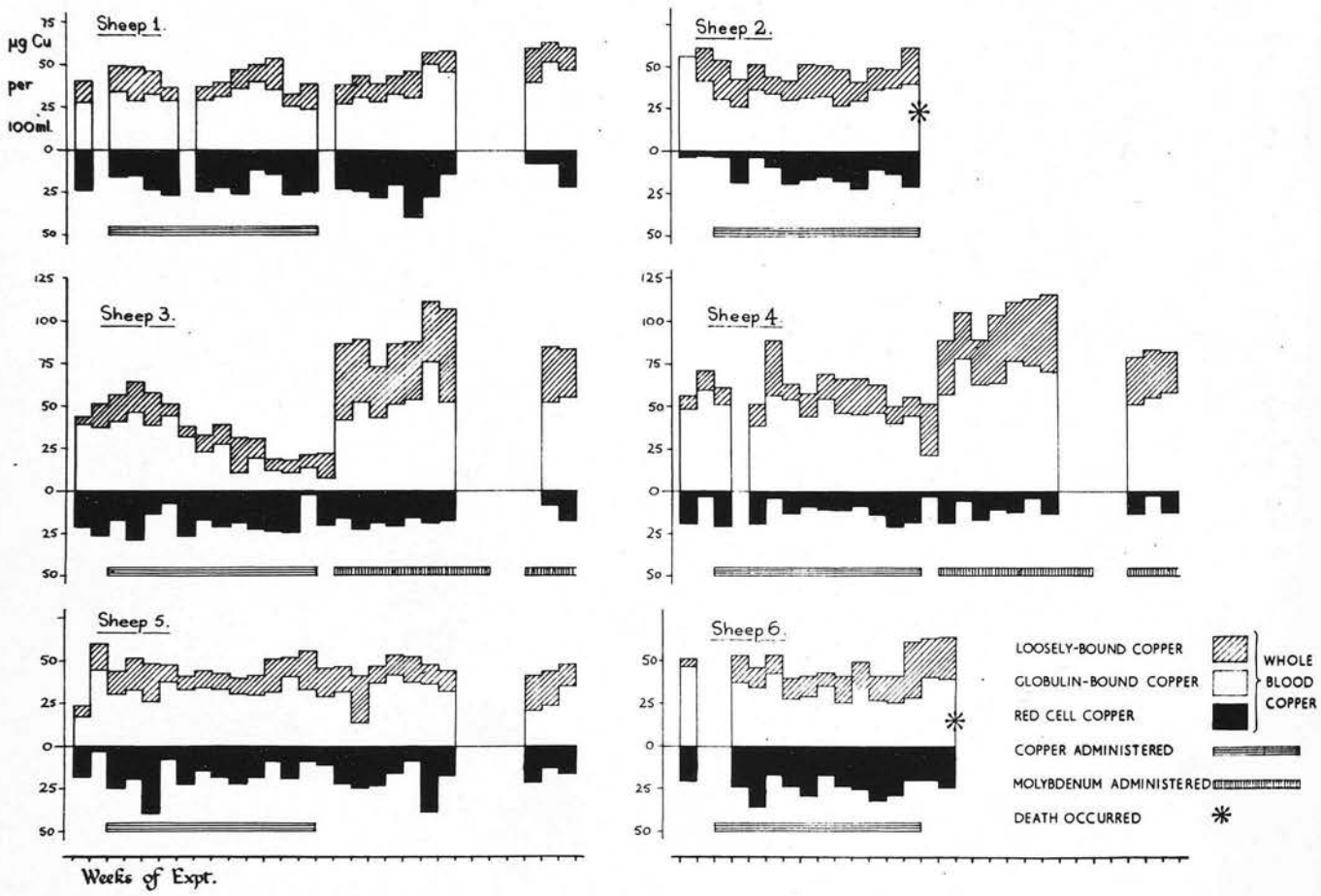
After a preliminary settling-in period the rations of all the sheep were supplemented with one gramme of hydrated copper sulphate per day, by mixing a copper sulphate solution with the food for each individual sheep. This was continued until one death had occurred from copper poisoning.

Results

The copper levels found in whole blood and plasma, with the calculated levels in each fraction, are set out in Table XII(see Appendix). These levels were found, using the Packed Cell Volume of each sample, by the formula on Page 44. They are also represented by the first part of the graph on Plate 1. The results discussed here are those of the first 16 weeks.

The first point of interest is the proportion of globulin-bound copper in the plasma of the normal sheep.

Plate 1.



Copper in three fractions of blood from sheep during administration of copper supplement - Section III (i) - and during administration of molybdenum supplement - Section III (ii).

In the first two samples quoted for each animal, that is before any copper supplementation had begun, the proportion of globulin-bound copper varied between 70 and 100 per cent of the plasma copper with most of the values around 75 per cent. This is a higher value than that quoted for the pig by Gubler et al. (1953) but lower than their values for man, rat and dog.

During the period when copper was added to the diet of the sheep each day there was no great change in the blood copper levels which could be ascribed to the extra dietary copper. The figures relating to sheep 3 show that the blood copper level actually fell gradually, though this was mainly due to the fall in the globulin-bound fraction.

In the thirteenth week of the experiment three sheep (Nos. 1, 2 and 3) became dull and had swollen lips. They were otherwise clinically normal and recovered in two days. The signs at first suggested that the sheep concerned were exhibiting photosensitisation. However, they had been subjected to no more light than was usual in the house. An attempt to demonstrate the presence of phylloerythrin in the plasma of these animals by the method of Perrin (1958) failed.

In the fifteenth week sheep 2, a wedder which had been normal at the time of the previous blood sampling

Table IV

Sheep 2 - Blood Analyses During Haemolytic Crisis

µg Copper per 100 ml.

Time before death	P.C.V. per cent	Direct Analysis			Calculated distribution per 100 ml. whole blood		
		Whole Blood	Plasma	Plasma less globulin	R.B.C.	Globulin-bound	Non-Globulin
* 4 days	28.5	82	86	30	20.5	40	21.5
21 hours	15.0	427	362	240	119	104	204
15½ hours	15.0	445	381	250	121	111	213
5½ hours	9.0	380	372	268	41	95	244
2 hours	6.0	332	323	248	28	71	233

* Last samples from the clinically normal animal included for comparison.

I am indebted to Mr. K.W. Head, Department of Veterinary Pathology, University of Edinburgh, for some of the haematological results in this Table and Table V.

suddenly became dull and listless, but without any signs of thickening of the lips. Within 3 hours of the dullness being noticed a very mild icterus developed. This became more intense after a further four hours, but was of an unusually dirty colour, quite unlike the usual golden colour of jaundice. The animal occasionally gritted its teeth as if with abdominal pain. Blood samples were taken at intervals and the results from these are shown in Table IV. Since such analyses of fractionated samples of blood have not been reported before, in this condition, they are of considerable interest. The last normal blood sample taken from this animal is included in the Table for comparison.

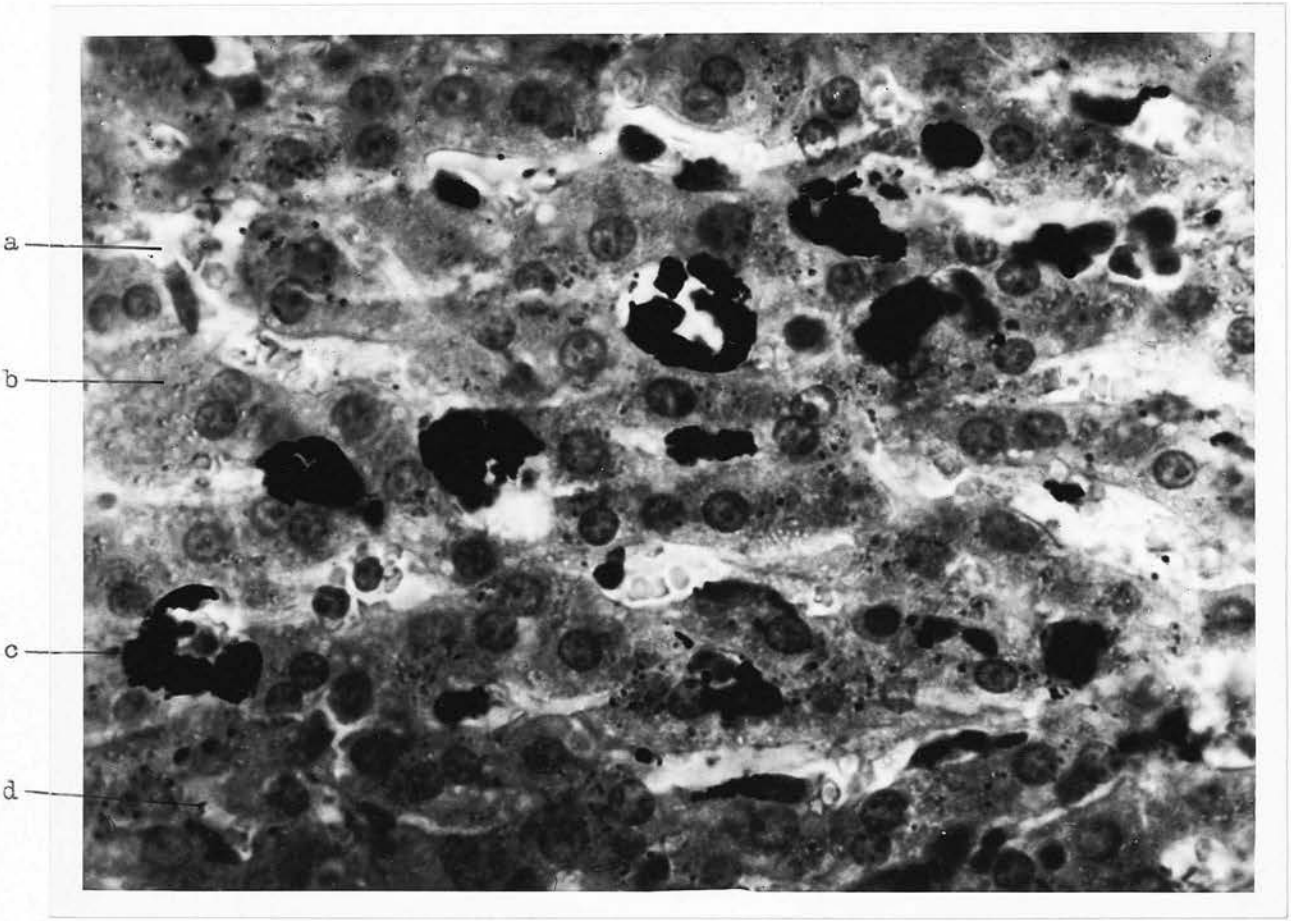
The most obvious change was the great increase in blood copper. In the first sample taken, 21 hours before death, the rise is seen to have been in both red cells and plasma and in the plasma two-thirds of the copper was of the 'loosely-bound' type. In other words the ratio of globulin-bound copper to 'loosely-bound' copper was completely reversed. Haemolysis had already begun when this sample was taken but was progressing slowly, the packed cell volume after a further 5½ hours remaining the same. By this time there had been a slight increase in the copper level of the plasma fraction.

After a further 10 hours the haemolysis had progressed and the packed cell volume had dropped to 9 per cent. Red

Red cell copper had fallen proportionately rather further, while globulin-bound copper was only slightly less than in the previous sample. Loosely-bound copper had increased so that it constituted five-sevenths of the plasma copper. The proportion increased in the next sample, taken 2 hours before the sheep died, when the packed cell volume had fallen to 6 per cent and the animal was in severe respiratory and circulatory distress. The blood samples showed a progressively darker brown colouration of the plasma until in the last sample it was difficult to distinguish cells from plasma.

Sheep 2 became comatose and died 30 hours after the onset of clinical signs. Post-mortem examination showed yellowish brown discolouration of all the tissues except the central and peripheral nervous system. The liver was bright yellow in colour, with indistinct lobulation, suggestive of widespread fatty change. The kidneys were dark in colour and rather soft, but with no evidence of capsular adhesions. The kidney tended to bulge out when the capsule was cut. The bladder contained dark brown cloudy urine. The spleen was black and slightly enlarged. Carcass lymph nodes were all enlarged and oedematous, and yellowish brown in colour. Histological examination of the kidney showed considerable glomerular leakage of red blood cells, some lysed, into the tubules, where

Plate 2.



Section of Liver from Sheep 2, which died of Copper Poisoning

Stain: Rubeanic acid x 600

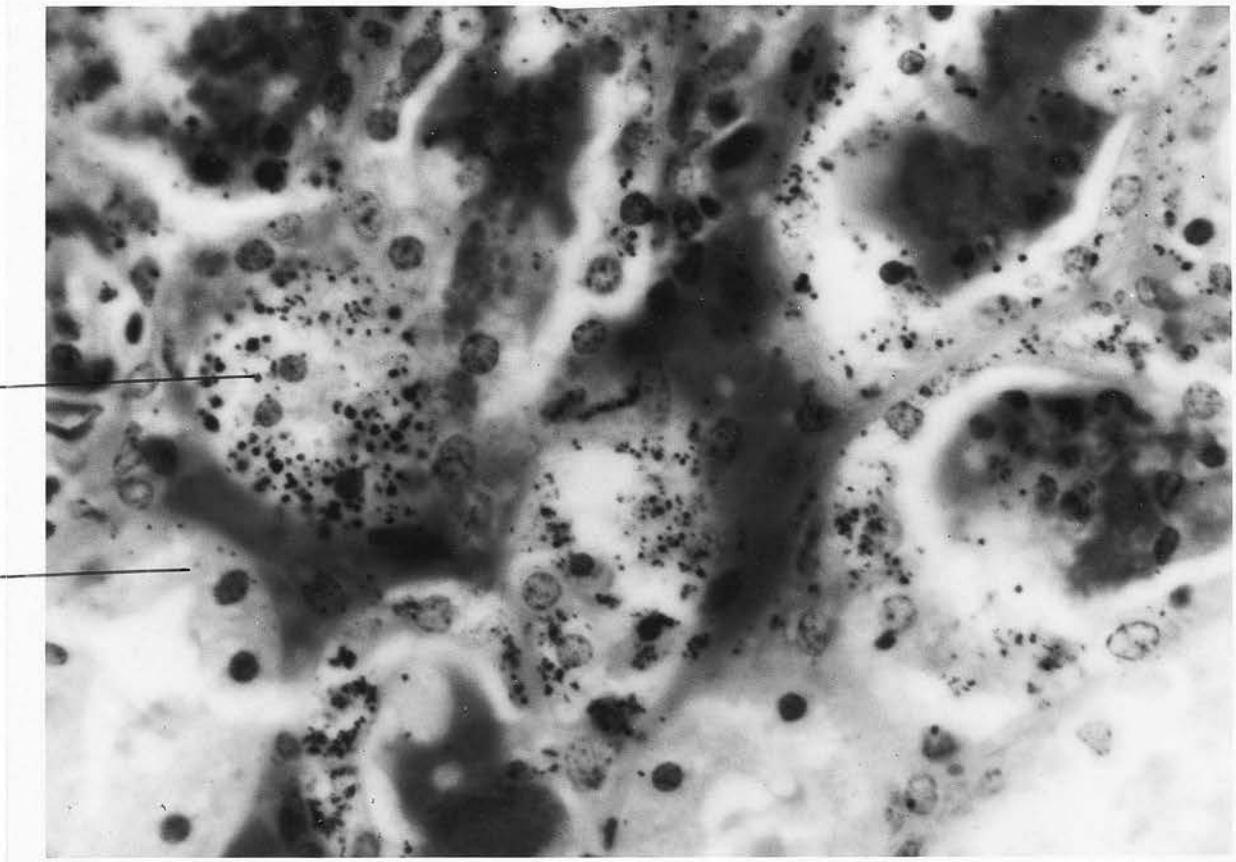
Note: (a) small size of sinuses because of

(b) swelling of hepatic parenchyma cells

(c) large quantities of copper in Kupffer's cells

(d) smaller amounts of copper in parenchyma cells.

Plate 3.



Section of Kidney from Sheep 2, which died of Copper Poisoning

Stain: Rubeanic acid x 1000

Note: (a) Copper granules present in epithelium of proximal tubule.

(b) very little copper present in distal tubule

(c) healthy state of epithelial nuclei (cf. Plate 6)

protein was also present. This evidence of increased permeability is consistent with anoxia. The spleen had intensely engorged red pulp but showed no evidence of extra-medullary haemopoiesis.

Some tissues were subjected to staining by the rubeanic acid technique of Uzman (1956). This revealed large quantities of copper in the liver, particularly in Kupffer's cells (see Plate 2), in the macrophages of the lymph nodes, in renal epithelial cells (Plate 3) and moderate amounts in the splenic macrophages. No copper was detected histochemically in the central nervous system or in the peripheral nerves. The results of the histochemical technique have not been reported before in copper poisoning.

The lesions described are indistinguishable from those described in field cases of copper poisoning, with the possible exception of the dark colour of the icterus. This was probably due to the fact that the haemoglobin released into the plasma by the massive haemolysis did not have time to break down completely by the time that the animal died. The pigments were examined in the Biochemistry Department by Dr. Ramsay, who stated that less than 10 per cent was bilirubin.

Immediately after the death of sheep 2, copper supplementation of all the sheeps' rations was stopped

Table V

Sheep 6 - Blood Analyses During Haemolytic Crisis

µg Copper per 100 ml.

Time before death	P.C.V. per cent	M.C.V. c.u.	Direct Analysis				Calculated Distribution per 100 ml. whole blood	
			Whole Blood	Plasma	Plasma less globulin	R.B.C.	Globulin-bound	Non-Globulin
* 6 days	34.0	-	88	96	36	24.6	39.6	23.8
40 hours	36.5	38.0	728	504	412	408	58.0	262
27 hours	27.0	41.6	810	505	356	441	109	260
24 hours	23.5	43.4	786	386	289	491	74	221
20 hours	21.0	44.2	747	420	308	415	89	243
3 hours	10.5	39.4	466	320	274	180	41	245

* Last sample from clinically normal animal included for comparison.

and two of the sheep (Nos. 3 and 4) were given a molybdenum supplement as described in Section III (ii).

Two weeks later sheep 6 (female) was noticed to have slightly greener urine than usual. A sample was taken through a stainless steel catheter and was found to contain 78 µg copper per 100 ml. as compared with about 10 - 20 in normal ovine urine. By the evening of the same day this sheep had become listless and mild icterus was seen in the sclera. Mucous membranes were generally pale and the animal was inappetent. A blood sample showed discolouration of the plasma, evidence that haemolysis had begun. Next morning the icterus was severe and the animal stood with her eyes half closed but still offered resistance to handling. She began to show slight staggering by mid-day but was not actually recumbent until the following morning. By then the jaundice was very severe and the animal was unable to stand without assistance. There was some gritting of the teeth and occasional soft bleating. Sheep 6 died 42 hours after the onset of dullness. Serial blood samples had been taken and the results are shown in Table V . Again, the last normal sample is included for comparison.

The packed cell volume of the first abnormal sample (taken after haemolysis was evident) was actually greater than in the normal sample. The red blood cells seemed

Table VI

Organ	Copper Analyses of Organs µg Cu. per g. Dry Weight.	
	Sheep	
	2	6
Liver - central	2,241	1,928
right	1,846	3,113
left	2,660	2,740
caudate	3,047	2,685
Kidney cortex	700	1,670
Spleen	197	237
Heart Muscle	162	165
Bone Marrow	17	23
Brain	12	21
Skeletal Muscle	8	14
Thyroid gland	4	-
Lymph node	-	40

Copper Analysis of Tissues from two Sheep
which died of Copper Poisoning

to be swelling throughout the course of the crisis, as shown by the mean corpuscular volume figures. The packed cell volume fell gradually, as in sheep 2, until death.

The circulating whole blood copper was higher in this animal than in the previous one, with the interesting feature that in the early samples more than half was in the red cells. Again in the plasma the 'loosely-bound' copper was higher than the globulin-bound fraction, viz: by 4 times as much at first, with an increase in the globulin-bound portion in 13 hours. Loosely-bound copper thereafter remained more than three-quarters of the plasma copper until death.

Post-mortem examination and histological investigations of sheep 6 revealed lesions similar to those seen in sheep 2, the only difference being that the kidney was quite black with a greenish sheen.

Copper analyses of various tissues and organs of both sheep 2 and 6 are shown in Table VI . These show that the liver copper was very high and that the kidney cortex contained levels much higher than normal, confirming the findings of Eden (1940) though Todd et al. (1962) found that when sheep did not die in less than 3 - 4 days this was not the case. In this connection, it may be of interest to record that when a field case of copper

poisoning was investigated earlier in this laboratory, livers were found to contain 800 to 2330 parts per million copper and kidneys 83 to 190 parts per million copper, in dry weight.

The high levels of copper found in other organs are probably associated with the large amounts of copper circulating in the blood stream immediately before death. Organs such as the spleen and lymph nodes which contain reticulo-endothelial tissue were noticeably higher in copper content on histochemical examination.

Summary of Findings

1. When a group of sheep were fed 1 gramme of copper sulphate daily there was no demonstrable effect on their blood copper levels.
2. Three of the six showed signs suggestive of photosensitisation, which may have been a premonitary indication of copper poisoning.
3. After 16 weeks, one sheep developed the typical signs of copper poisoning and died in 30 hours. Blood analyses made during the crisis indicated that:
   (a) there was a considerable rise of 'loosely-bound' copper in the plasma, and
   (b) there was a progressive haemolysis which took place so rapidly that only a small proportion of the

liberated haemoglobin was broken down to bilirubin. The discolouration of the tissues seen both before and after death was consequently brownish-yellow rather than yellow.

4. Post-mortem findings included changes in the liver and kidney, while analyses showed that these organs contained large amounts of copper.

5. Histochemical evidence showed that the liver copper was largely deposited in Kupffer's cells after death.

6. A second sheep died two weeks later with similar clinical signs and post-mortem findings.

Section III

(ii)

The Effects of Molybdenum on Copper Metabolism

The Effect of Molybdenum on Sheep which have received High-Copper Diets

When Ferguson, Lewis and Watson (1943) showed that a high molybdenum content in pasture was associated with the clinical signs in cattle on 'teart' pastures, they started a series of investigations which have led to interesting results. Dick and Bull (1945) investigated liver copper levels in cows which had received a high molybdenum intake over a period of three years, and in sheep which had received 10 or 100 mg. molybdenum per day, and found that the copper content was significantly reduced. When no additional copper was given, 10 mg. molybdenum a day to a sheep resulted in a loss of copper already stored in the liver (Dick, 1952). Showing a quantitative relationship between the two elements, Dick was nevertheless sure that other factors were still involved. He found that when an oats diet was fed, the reduction in copper storage following molybdenum supplements was less than when a lucerne diet was fed. He then showed that the difference was due to the sulphate contents of the two rations (Dick, 1953). When sulphate intake was about 2 grammes per day, as little as 0.5 mg. molybdenum per day would significantly reduce liver copper. In later experiments Dick (1954) found that blood levels of

molybdenum and copper rose with molybdenum administration and that increased sulphate in the diet affected this also. In sheep receiving 8 grammes sulphate, 80 mg. molybdenum and 4.6 mg. copper per day, the blood copper rose to 125 µg per 100 ml. At the same time the wool lost its crimp which rather suggested that the circulating copper was not physiologically available for enzymic functions.

The fall in liver copper produced by molybdenum and sulphate supplement was also found by Wynne and McClymont (1955a) in Merino sheep receiving 0.4 per cent sulphate in their diet with either 0.7 p.p.m. or 5.0 p.p.m. molybdenum. The effect of either molybdenum or sulphate alone was less than both together, (Wynne and McClymont, 1955b).

The findings were confirmed in cattle by Cunningham, Hogan and Lawson (1959) with the interesting note that diarrhoea did not occur on the molybdenum supplement.

It is but a short step to conjecture on the usefulness of molybdenum in sheep where dangerously high copper levels are thought to exist, such as in a flock where a few sheep have already died of copper poisoning. Pierson and Aanes (1958) reported trials they had carried out where there had been clinical cases of copper poisoning in two flocks of sheep. They sprayed molybdate and sulphate solutions on to the food fed to the survivors

and claimed success by this method.

The possibility of successful preventive action seemed sound, so, in order to obtain further fundamental data on the action of molybdenum in this connection, an extension of the experiment described in Section III (i) was undertaken. The first was on sheep known to have received large amounts of copper in their diet.

Materials and Methods

As soon as one sheep had died from copper poisoning in the previous experiment the copper supplement to the other five was stopped and after six days two sheep (Nos.3 and 4) were given 100 mg. ammonium molybdate in aqueous solution by mouth, i.e. sufficient to give 54 mg. molybdenum each day, while Nos. 1, 5 and 6 remained untreated. Blood samples were taken as before and the same analyses were carried out on them. At the end of 14 weeks all the surviving sheep were subjected to laparotomy under pentobarbitone anaesthesia and part of the caudate lobe of the liver was removed for analysis and histological examination.

Results

TableXIII(see Appendix) shows the blood analyses obtained in this experiment and the fractional analyses are illustrated in Plate 1 . (Opposite Page 54).

Sheep 6 (untreated) died a fortnight after sheep 2, as has already been described. Of the remaining four, those given supplementary molybdenum showed an almost immediate rise in the blood copper level. While there was no significant change in red cell copper the plasma copper in both animals increased, in sheep 3 by about 300 per cent, and in sheep 4 by about 100 per cent. Unlike the change seen in the cases of copper poisoning, however, the 'loosely-bound' copper remained less than half the plasma copper throughout the period.

There was no clinical effect on the animals whatsoever; no scouring was seen at any time during the experiment, a confirmation of the findings in cattle of Cunningham, Hogan and Lawson (1959). There were no further deaths from copper poisoning in either treated or untreated sheep. Sheep 5 died in the course of the laparotomy, mainly because of the inhalation of rumen contents which were expelled violently when the liver was handled. (This was prevented in the other three sheep by endotracheal intubation at the start of the operation). The accident was not entirely without profit, however, as the organs of this sheep which had received the high-copper diet with no other treatment and had <u>not</u> died of copper poisoning make an interesting comparison with those of the sheep which died in the first

Table VII

Copper Analyses of Organs

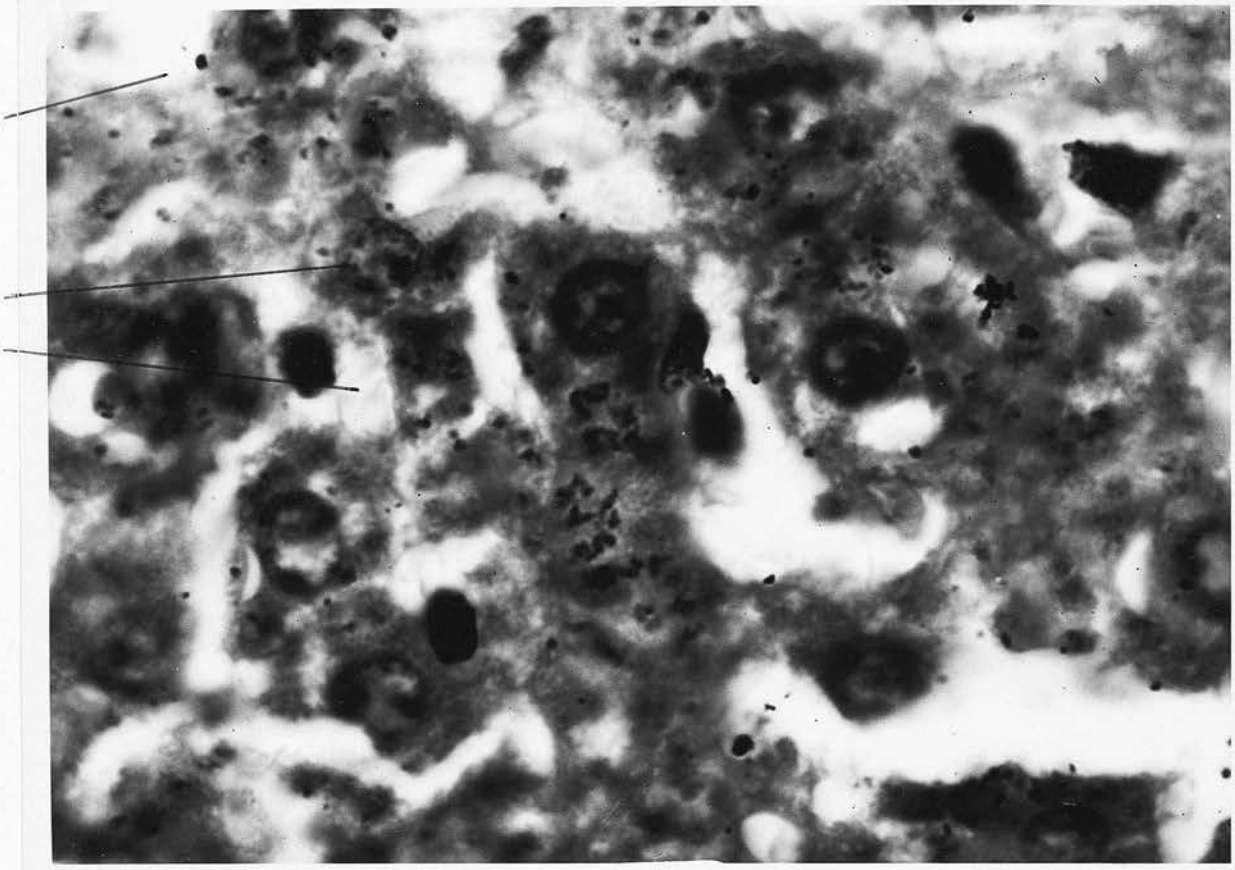
µg Cu. per g. Dry Weight

Organ	Sheep					
	1	2	3	4	5 *	6
Liver - central	-	2,241	-	-	1,135	1,928
right	-	1,846	-	-	987	3,113
left	-	2,660	-	-	1,178	2,740
caudate	†678	3,047	†846	†467	1,342	2,685
Kidney cortex	-	700	-	-	12	1,670
Spleen	-	197	-	-	9	237
Heart muscle	-	162	-	-	13	165
Bone marrow	-	17	-	-	3	23
Brain	-	12	-	-	-	21
Skeletal muscle	-	8	-	-	4	14
Thyroid gland	-	4	-	-	3	-
Lymph node	-	-	-	-	11	40

* Died under Anaesthesia.

† Biopsy Specimen.

Plate 4.



Section of Liver from Sheep 3, which received Molybdenum Supplement

Stain: Rubeanic acid x 1000

Note: (a) Large sinuses (cf. Plate 2)

(b) copper granules in parenchyma cells

(c) no copper in Kupffer's cells.

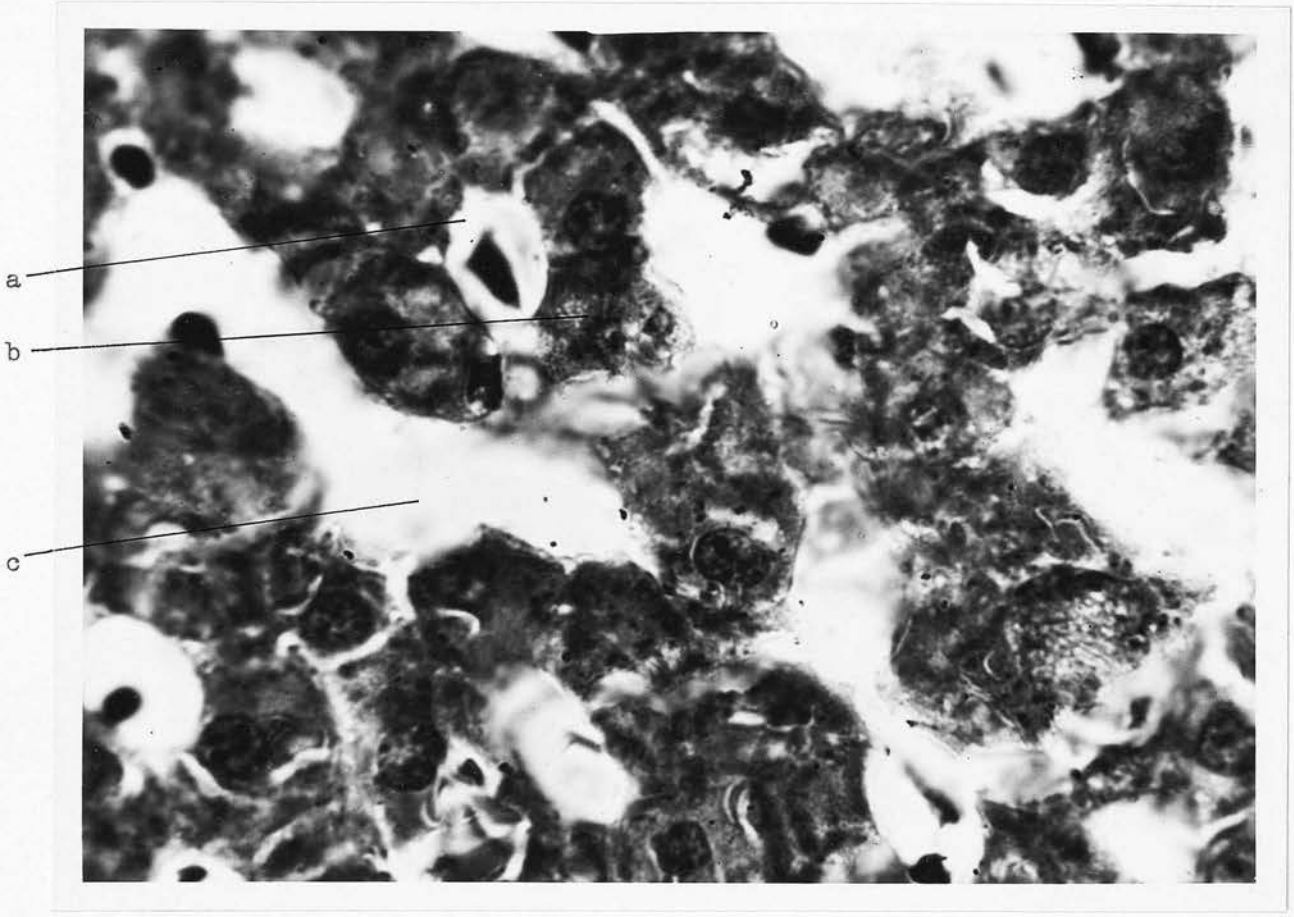
experiment. The analyses of tissues of sheep 5 and the copper levels found in the biopsy specimens from sheep 1, 3 and 4 are shown in Table VII.

Of the two untreated sheep, one (No.5) showed a very high liver copper at laparotomy, viz: nearly 50 per cent of that in the sheep which died of copper poisoning. Sheep 1 had a level about half that of sheep 5 and twice the normal. Of the treated animals, sheep 4 had reached the lowest of all, while sheep 3 retained a level actually in excess of that of sheep 1, which was not treated. The mean caudate lobe copper levels in pairs of animals were:

Copper poisoning (post-mortem)	2,866 µg. per G.
Treated with Mo for 10 weeks	656 µg. per G.
Untreated sheep after the same period	1,005 µg. per G.

Histological examination of the liver samples from sheep 3 and sheep 1 revealed no significant degenerative parenchymatous changes or evidence of macrophage activation or infiltration. Histochemically, sections treated by the rubeanic acid method revealed large quantities of copper in a finely granular form in hepatic parenchymatous cells and to a much smaller extent in Kupffer's cells. The granules were, however, so small as to be identified only under oil immersion examination (see Plate 4).

Plate 5.



Section of Liver from Sheep 5, which received no treatment but did not die of copper poisoning

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Stain: Rubeanic acid x 1000

Note: (a) no copper in Kupffer's cells

(b) fine copper granules in parenchyma cells

(c) large sinuses.

In comparing the organs of sheep 5 with those of sheep 2 and 6 in the previous experiment there is a striking difference mainly in the kidney and spleen copper levels, these being in the normal range in sheep 5. Examination of the liver of sheep 5 by the rubeanic acid staining method showed the distribution of copper to be similar to that in sheep 3 and 1 (see Plate 5). Thus all the sheep which had not died from copper poisoning, whether they had been treated with molybdenum or not, had markedly different copper distribution in the liver than those which did die of copper poisoning, (compare Plates 4 and 3).

Summary of Findings

1. Two sheep which had been on a high-copper diet were given 54 mg. molybdenum daily per os, while three similar animals were used as untreated controls.
2. Weekly examination was made of whole blood copper, plasma copper and globulin-free plasma copper.
3. No deaths occurred in the molybdenum treated group while one occurred in the controls.
4. Molybdenum produced no clinical effect in the animals, but raised the plasma copper levels by 100 to 300 per cent. The proportions of globulin-bound to 'loosely-bound' copper remained approximately the same, in contrast to the situation when the

blood copper rose in the haemolytic crisis of copper poisoning.

5. Liver biopsy samples, taken after 14 weeks, showed that (a) the liver copper content had fallen in the treated sheep in comparison with controls and (b) the liver copper was mainly to be found in parenchymatous cells, and not in Kupffer's cells, in all sheep which had not died of copper poisoning.

Copper Balance Trial in a Sheep on a Normal Diet and on a Normal Diet plus Molybdenum

Materials and Methods

A cross-bred female sheep (sheep A) was placed in a metabolism crate which was fitted for the collection of faeces and urine. She was fed a ration of good hay, chopped short so as to avoid spilling, each day's ration being carefully weighed and the weight of any left over being noted. Glass-distilled water only was allowed for drinking.

A settling-in period of 5 days was allowed before the experiment proceeded.

The daily output of faeces and urine was measured and samples of each were analysed for copper and the mean copper level for the whole batch of food was used to calculate the daily copper intake. Blood samples were taken at 48 hour intervals and the copper level in the serum determined.

After 6 days, an aqueous solution of ammonium molybdate was given to the animal daily by mouth, sufficient to give 54 mg. molybdenum per day, and the sampling and analysis were continued for a further period of 6 days.

Results

There was no clinical effect on the animal. The

Figure 6.

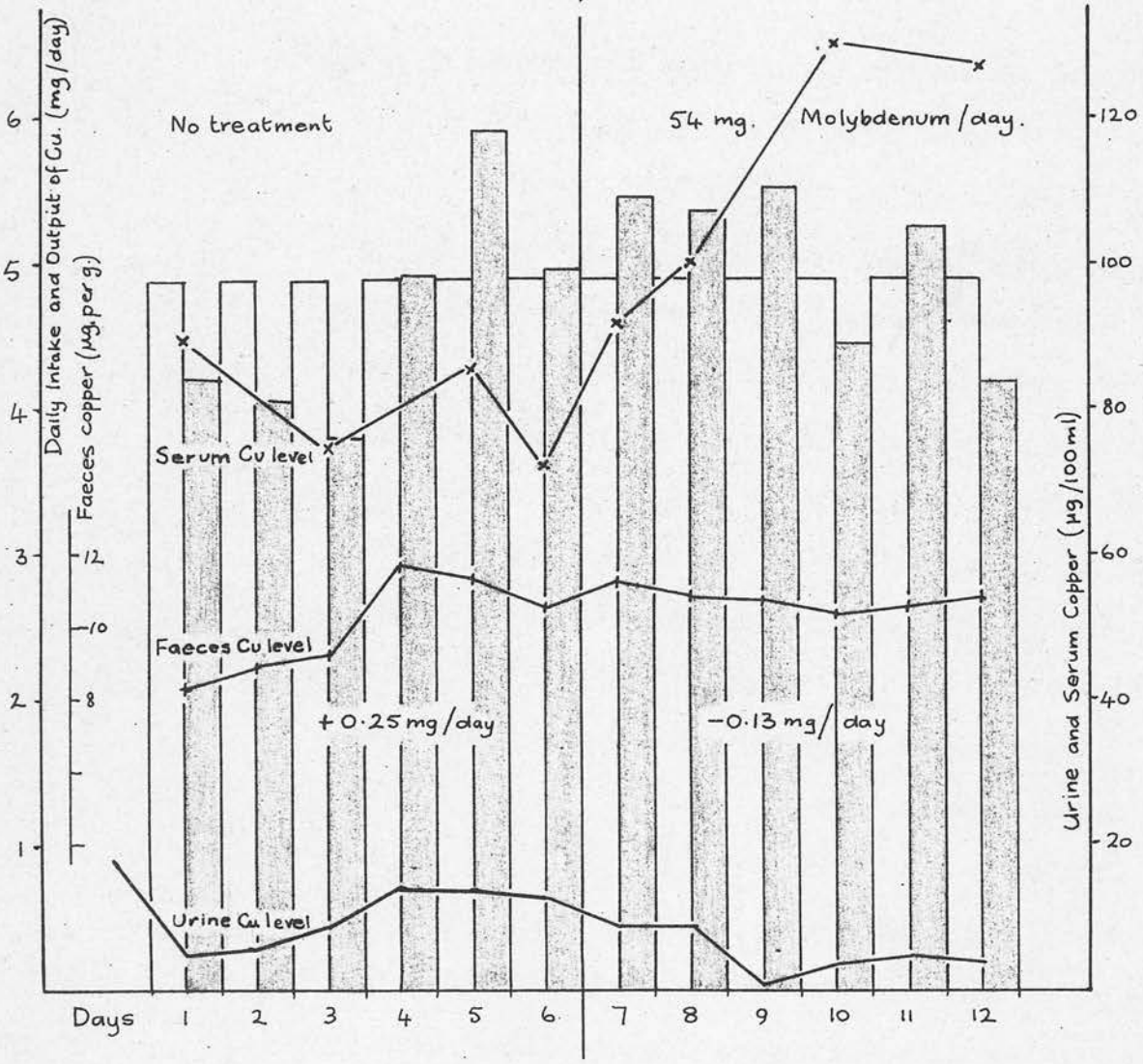


Figure 6.

Diagrammatic representation of results obtained in copper balance trial with Sheep A.

Note: In this and the following four figures, the daily copper intake and output are represented by the unshaded and shaded portions of the histograms.

The mean daily gain or loss of copper over each period of the experiment is stated in figures superimposed on each diagram.

The results obtained from analysis are set out in Table vi (see Appendix) and are also shown graphically in Figure 6.

On administration of molybdenum, there was a rise in serum copper similar to the rise seen in the previous experiment. Parallel with this there was an overall decrease in the copper content of the urine. Faeces copper remained at almost the same level but whereas before treatment there was a slight positive balance, some 5.1 per cent of the intake, this was changed to a negative balance, about 2.7 per cent when molybdenum was supplied. This represented a loss of copper to the sheep at the rate of 0.13 mg. per day instead of a gain of 0.25 mg. per day.

Copper Balance Trials in Sheep on Normal Diets and with Molybdenum and Sulphate Supplements

As mentioned in Section I, Dick (1952; 1953; 1954) showed that the effect of molybdenum in reducing liver copper was enhanced by the presence of sulphate in the diet and this has been confirmed by several workers. In order to see whether the effect of sulphate could be shown in a balance experiment such as the one described above, similar experiments were performed in four sheep using both molybdenum and sulphate supplements.

Materials and Methods

For each experiment a sheep was placed in a metabolism crate as described previously and fed the same diet throughout the trial. Food intake was measured, distilled water only was offered and the urine and faeces were collected. After a preliminary settling-in period the copper intake and output were measured over a period on normal food, then in two of the sheep (B and C) a molybdenum supplement was added to the diet in the same way as in the previous trial and finally a daily supplement of sulphate was given by dosing the sheep with a solution of sodium sulphate sufficient to give 2.0 grammes of sulphate per day. In two other sheep (D and E) the same supplements were given in the reverse order, so that they

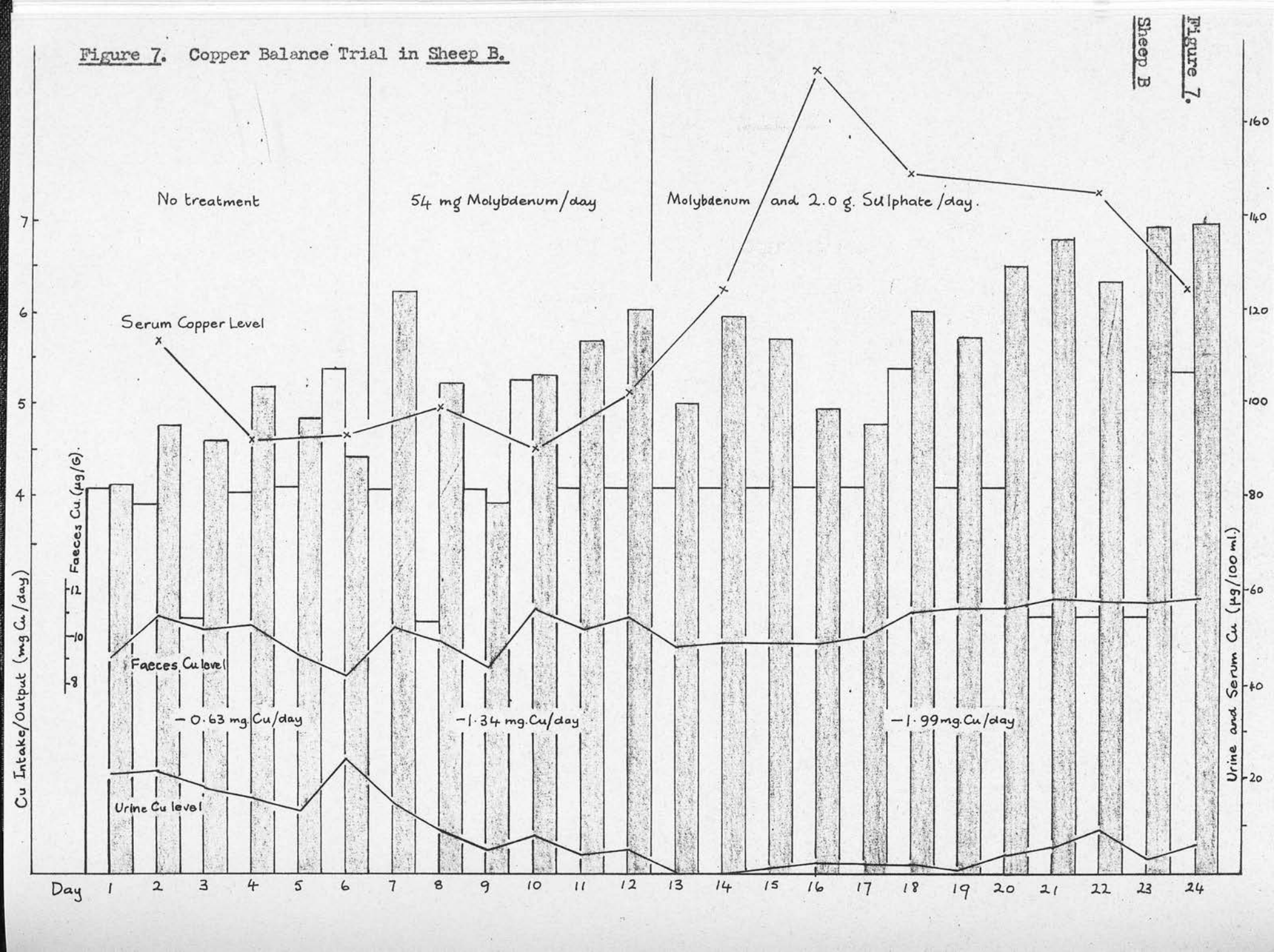


Figure 7. Copper Balance Trial in Sheep B.
Figure 7.
Sheep B
No treatment
54 mg Molybdenum/day
Molybdenum and 2.0 g. Sulphate/day.
Serum Copper Level
Faeces Cu level
Urine Cu level
−0.63 mg. Cu/day
−1.34 mg. Cu/day
−1.99 mg. Cu/day
Cu Intake/Output (mg Cu/day)
Faeces Cu. (µg/G).
Urine and Serum Cu (µg/100 ml.)
Day
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
7 6 5 4
12 10 8
160 140 120 100 80 60 40 20

received first sulphate alone and then molybdenum with sulphate.

Results

Sheep B

See Figure 7, and Table viii in the Appendix.

In sheep B the effect of molybdenum alone on the serum copper level was not so great as in Sheep A. However, urine copper fell over the six days by three-quarters. The effect of molybdenum on the faeces copper level was only slight but the total faeces copper output accounted for the animal increasing its negative balance from 0.63 mg. per day to 1.34 mg. per day.

When sulphate was added to the molybdenum supplement the serum copper level reached 171 µg. per 100 ml. in four days and during the remainder of the period was always higher than normal. Urine copper actually fell to zero immediately after the sulphate was added, while the faecal copper level rose by some 10 per cent and the output increased proportionately so that the negative balance was further increased to 1.99 mg. per day. Considering the relative insignificance of the total urinary copper output, this negative balance is entirely accounted for by faecal copper. The hay used in this trial was of poor quality so far as copper content was concerned,

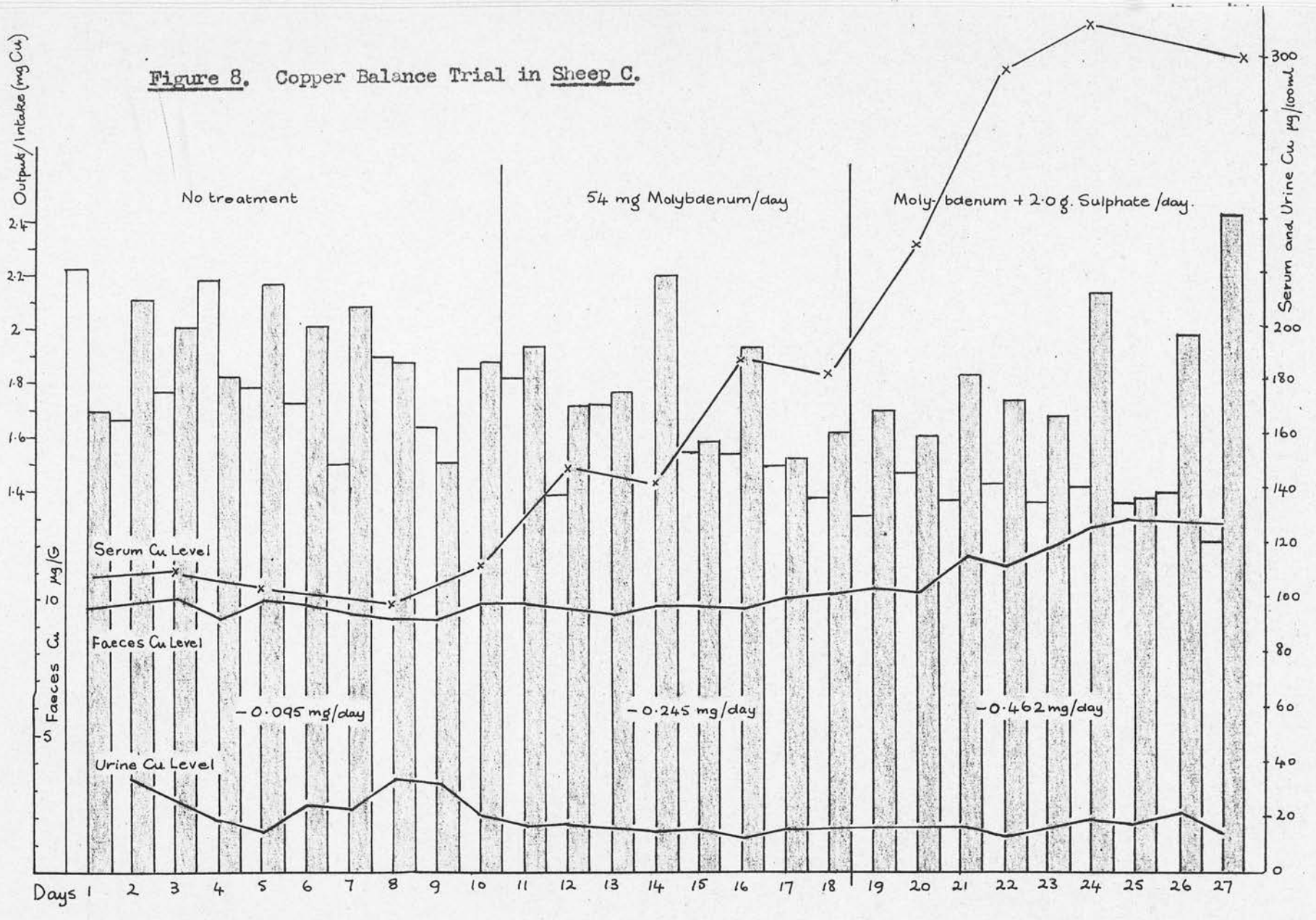


Figure 8. Copper Balance Trial in Sheep C.
No treatment
54 mg Molybdenum/day
Moly- bdenum + 2·0 g. Sulphate /day.
Outputs/Intake (mg Cu)
Serum and Urine Cu µg/100ml
Faeces Cu µg/G
Serum Cu Level
Faeces Cu Level
Urine Cu Level
−0·095 mg/day
−0·245 mg/day
−0·462 mg/day
Days
2·4
2·2
2
1·8
1·6
1·4
10
5
300
200
180
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

containing only 3.0 parts per million, and this, coupled with the variable food consumption, would perhaps explain why the sheep was in negative balance at the start of the experiment.

Sheep C

See Figure 8 and Table viii in the Appendix.

During the 'normal' 10 days at the start of this trial, sheep C was in almost a state of copper balance, losing only about 0.1 mg. per day.

Molybdenum alone produced in this sheep a rise of serum copper up to 188 µg. per 100 ml. Urine copper fell by about half and faecal copper level rose very slightly so that the total output was increased sufficiently to increase the negative balance to 0.25 mg. per day.

When sulphate was given in addition to molybdenum the serum copper level rose to 300 per cent of normal. This was the most spectacular rise in serum copper obtained in these experiments and it is of interest to note that the animal remained clinically normal throughout and that this high level of copper in blood was not associated with any haemolysis.

The urine copper in this sheep did not fall any further under the influence of molybdenum and sulphate together than it did under molybdenum alone, but after a few days the faecal copper level rose by some 20 per cent sufficient to raise the total output so that the negative

Table VIII

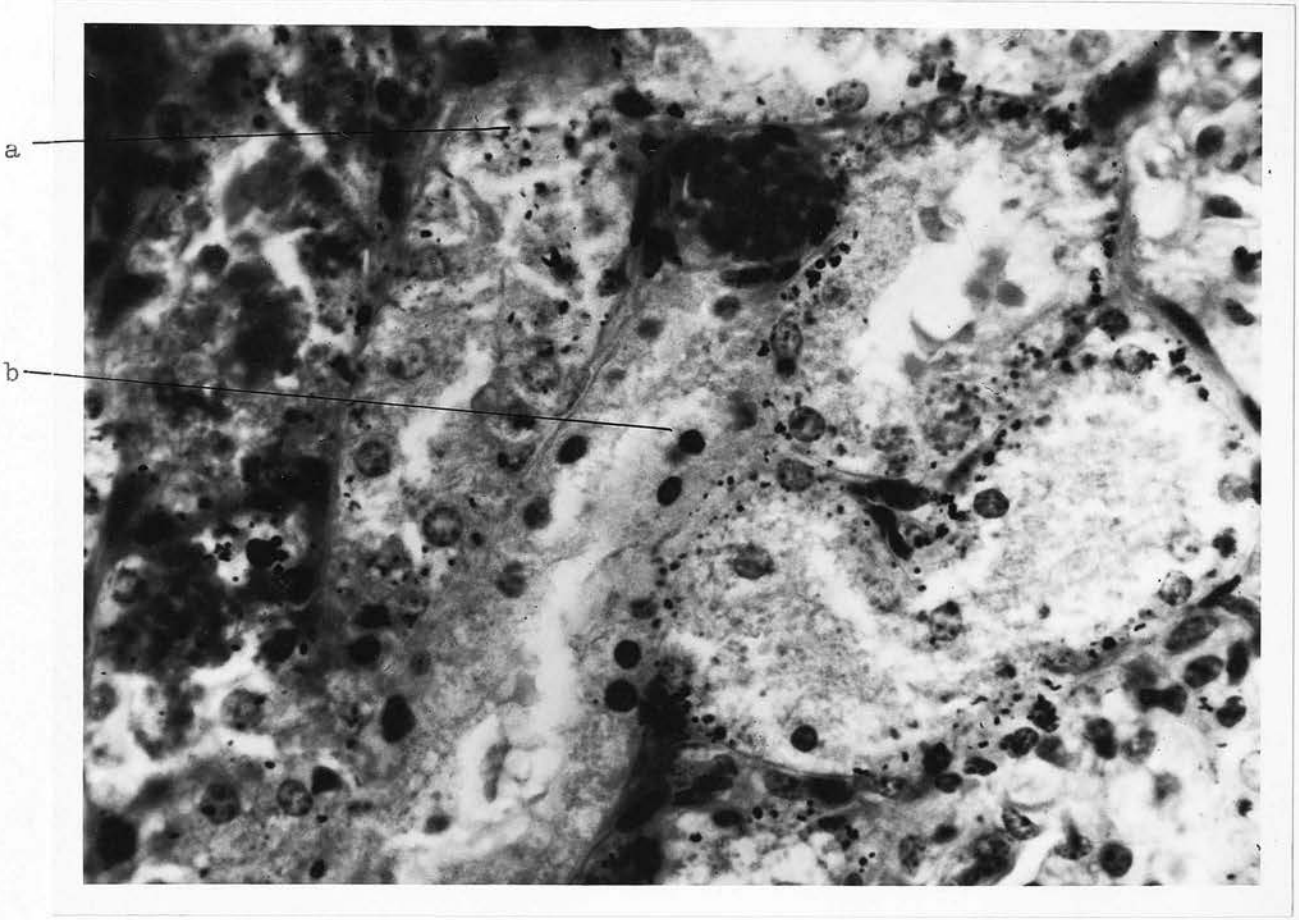
Sheep C.

Copper content of Tissues immediately after treatment with Molybdate and Sulphate Supplements

Parts per million of dry, fat-free tissue

Liver, right	444
central	420
left	511
caudate	451
Kidney cortex	183
Heart muscle	18
Spleen	14
Pancreas	5
Submax. salivary gland	9
Submax. lymph node	18
Adrenal gland	18
Thyroid gland	8
Retropharyngeal lymph node	27
Mesenteric lymph node	22
Skeletal muscle	9
Bone Marrow	3
Aorta	8
Brain	-

Plate 6.



Section of Kidney from Sheep C, which received Molybdenum and Sulphate supplements

Stain: Rubeanic acid x 1000

Note: (a) copper in tubular epithelial cells

(b) dying nuclei of tubular epithelial cells

Table IX

Sheep C.

Copper analysis of gut contents immediately after treatment with Molybdate and Sulphate supplements

Part of Tract		Copper µg/g D.M.	Silica % D.M.	Copper: silica
Rumen		6.6	1.87	3.54
Reticulum		6.3	1.51	4.18
Omasum		6.6	2.02	3.26
Abomasum		7.3	4.08	1.80
Small intestine	1	33.3	2.04	16.32
	2	10.2	1.93	5.30
	3	9.5	1.50	6.33
	4	11.8	2.82	4.18
	5	11.4	2.79	4.09
	6	9.6	—	—
Caecum		14.8	3.57	4.15
Colon	1	15.9	3.65	4.36
	2	12.7	3.44	3.68
	3	13.4	3.53	3.80
	4	14.3	3.52	4.08
Rectum		12.6	3.40	3.70

balance was 0.46 mg. per day.

Sheep C was killed by barbiturate euthanasia at the end of the third phase of the trial and organs and tissues were analysed, along with samples from the entire alimentary canal. Results of these analyses are shown in Tables VIII and IX.

The copper content of the tissues of sheep C follow the pattern seen in normal sheep (Cunningham, 1931) except for the remarkably high level of copper in the kidney which was about ten times normal. Histological examination of this organ showed that copper, in a very fine granular form, was present in the tubular tissue in the proximal part of the nephron. This suggests tubular reabsorption of copper in a form not dissimilar to that found in the liver. Degenerative changes had taken place in the nuclei of the tubular epithelium (see Plate 6).

It is very difficult to interpret analyses of gut contents for an individual element because of the movement of water and digestible substances into and out of the alimentary lumen. However, an attempt has been made to relate the copper level to the silica level, each calculated on the dry matter content. There are one or two odd results but in the main it is felt that there is some validity in the figures quoted. There was a sharp difference between the copper levels in the

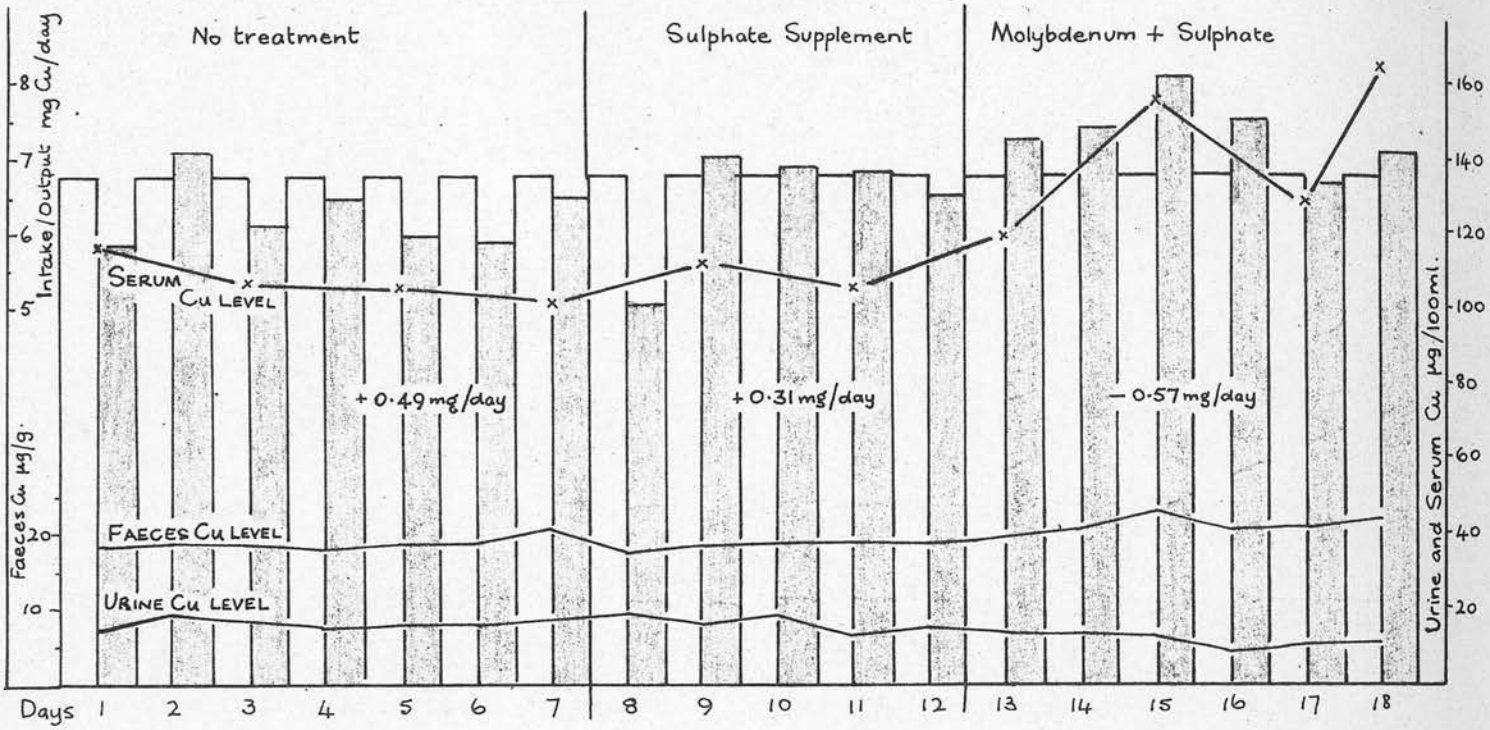


Figure 9.

Copper Balance Trial in Sheep D.

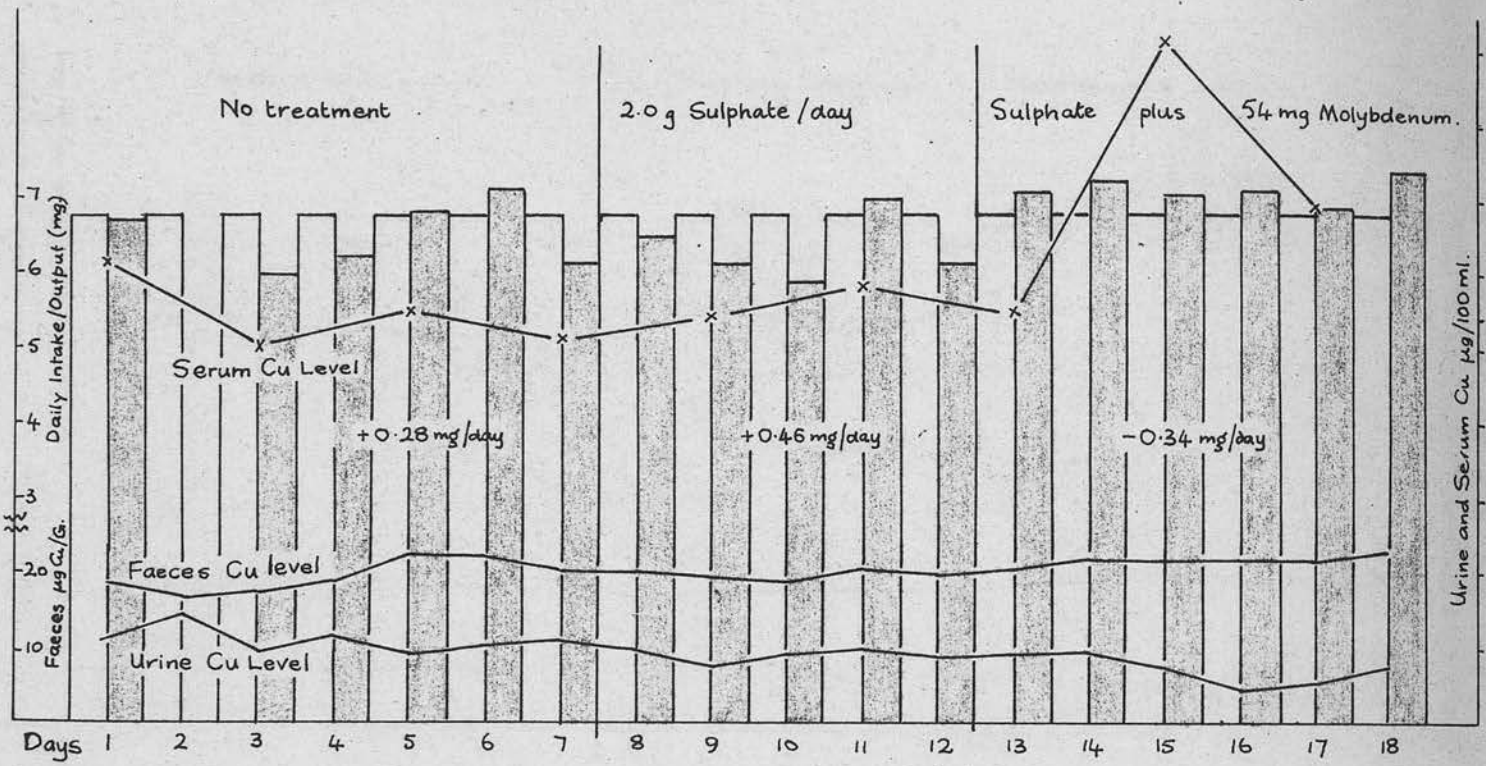


Figure 10.

Copper Balance Trial in Sheep E.

abomasum and in the first part of the small intestine, which makes it seem probable that copper was being added here by way of the bile. Copper seems to have been absorbed gradually as it passed along the small intestine and small changes to and fro took place in the large intestine.

Sheep D and E

In the trials with these two sheep the order of adding the supplements was reversed. To make feeding easier than before and to produce a constant intake of food they were also given grass in prepared nuts. This increased the daily copper intake but seemed unlikely to alter materially other relevant conditions of the experiment.

The results of the various analyses are shown in Figures 9 and 10 , and Tables viii and ix in the Appendix.

Both animals were in slight positive balance during the first phase of the trial.

The supplement of sulphate to the diet produced no significant changes in either animal. The serum copper level did not rise and urine and faeces copper remained substantially the same so that while sheep D retained slightly less copper, sheep E retained slightly more. When molybdenum was added, however, the pattern of the

Table X

Sheep D.

Copper content of tissues immediately after treatment with Sulphate and Molybdate supplements

Parts per million of dry, fat-free tissue

Liver, right	282
central	238
left	235
caudate	269
Kidney cortex	37
Heart muscle	21
Spleen	3
Pancreas	-
Submax. salivary gland	5
Adrenal gland	14
Thyroid gland	5
Retropharyngeal lymph node	20
Mesenteric lymph node	11
Skeletal muscle	8
Bone Marrow	3
Aorta	1
Brain	28

Table XI

Sheep D.

Copper analysis of gut contents and gut wall immediately after treatment with sulphate and molybdate supplements

		Copper µg/g D.M.	Silica % D.M.	Copper: Silica	Copper in wall µg/g D.M.
Rumen		7.0	1.71	4.09	--
Reticulum		7.2	1.74	4.14	--
Omasum		9.7	2.70	3.60	--
Abomasum		7.5	2.50	2.98	--
Small intestine	1	11.9	2.00	6.00	9.17
	2	10.8	1.86	5.81	11.90
	3	9.3	1.10	8.43	8.54
	4	12.4	1.61	7.71	6.43
	5	13.1	2.55	5.14	10.17
	6	13.4	2.60	5.20	10.02
	7	12.6	2.64	4.78	11.30
Caecum		14.1	3.39	4.15	6.45
Colon	1	12.4	2.94	4.22	14.63
	2	11.9	2.94	4.04	18.55
	3	12.9	3.16	4.09	11.06
	4	15.9	3.20	4.97	7.24
Rectum		20.8	3.58	5.70	5.12

previous trials was established. Urine copper fell, though not by so great a margin. Faeces copper level rose so that the total output was increased and both animals passed into negative copper balance over the third phase of the experiment. Again, this was associated with a significant increase in copper circulating in the serum.

Sheep D was killed by barbiturate euthanasia at the end of the third phase of the experiment and tissues and the alimentary tract were analysed for copper. In this animal, the gut wall was included in the analysis. Table X shows the levels of copper found in the tissues. All these are within the normal range except possibly the kidney which, while it did not contain the same high level as that of sheep C (Table VIII) it was almost twice normal.

Table XI shows levels of copper and silica ratios found in the alimentary contents, with the level in each appropriate part of the gut wall alongside. Again there appears to have been addition of copper by the bile since the copper in the proximal small intestine increased.

The pattern in the rest of the tract also follows roughly that of sheep C. The gut wall values may indicate to some extent movement of copper either into or out of

the lumen and in this case the higher levels in the colon are of interest, especially since mucous secretion is added to the material at this level, and the findings agree somewhat with those of Bowland et al. (1960)

Summary of Findings

1. 5 sheep were kept on a constant diet and their intake and output of copper was measured. Regular blood samples were taken for analysis.
2. The effect of daily oral administration of 54 mg. molybdenum was to:
   (a) raise the serum copper level
   (b) depress the urine copper level and output and
   (c) raise the faecal copper level and output.
3. When 2.0 grammes sulphate was added daily to the diet, the effects of molybdenum were enhanced.
4. When sulphate was given alone no effect was seen on serum copper, urine copper or faecal copper, but when molybdenum was added it appeared to be effective more quickly.

Section IV

Discussion

Discussion

Absorption of Copper

The metabolism trials described here confirmed the views discussed in Section I that there was very little difference between dietary intake and faecal excretion of copper, in all the sheep studied. The maximum retention of dietary copper in these trials occurred in sheep A and here only 5 per cent of the copper was retained.

The first experiment confirmed that when the dietary intake of copper was high, the excess copper which was absorbed was stored in the liver. The daily administration of 1 gramme of hydrated copper sulphate would mean that the intake was in the region of 250 mg. copper per day. In 88 days therefore, sheep 2 had ingested 22 grammes copper, and after the haemolytic crisis and death its liver contained about 0.63 g. or only 3 per cent of the intake. Thus even when readily soluble copper was administered retention was at the same low level.

The analysis of tissues and organs in all these experiments confirmed that the organ which stored most of the copper retained in the body was the liver. From the histological findings it appeared that liver

stores, even under high-level conditions, were to be found within the hepatic cells in a diffuse form - possibly as the 'hepatocuprein' of Mann and Keilin (see Section I).

Copper Poisoning

Eden (1940) showed that when sheep were fed copper supplement in sufficiently large amounts to lead eventually to copper poisoning, the 'latent' or symptomless phase of the condition could be divided into two parts. In the first of these there was no change detectable either clinically or by blood copper estimations. The second was characterised by an increase in blood copper level, though not great, which could be regarded as a premonitory sign of copper poisoning. When the rate of supplementation was constant, these two periods of the latent phase were of approximately equal duration in Eden's cases. Sutter and other (1958) also reported that a rise in blood copper occurred during ingestion of supplementary copper and they concluded that the raised levels could be used in diagnosis. Neither of these views was borne out in the work described here. With the exception of the last sample taken in each case from the sheep which died, while they were still clinically

normal, it could not be said that any result of blood copper analysis could have given any warning that the animals were consuming excessive copper and were likely to die from copper poisoning. Even allowing for the exceptional case of sheep 3, where the level slowly fell, no sample gave a result far outside the normal range for ovine blood.

The events of the thirteenth week are of interest. Although photosensitisation could not be experimentally proved when three of the sheep showed thickening of the lips, the possibility that this was present gives cause for conjecture, particularly in view of the recent report of Todd and Thompson (1962) that hepatic dysfunction was present at and probably before the haemolytic crisis of poisoning. Under other circumstances (Bull, 1949) it has been shown that photosensitisation occurs when certain plants which contain high copper levels are eaten, and that this has been associated with liver damage. It may well be that this brief but easily recognised sign was a premonitary indication of copper poisoning.

The first animal to show signs of copper toxicosis was a wedder, showing that the recognised effects of

prolonged copper administration are not confined to the female, as was suggested by Sutter and others (1958). These authors also disagreed with Marston (1953) who stated that there was a sudden outpouring of copper from the liver at the crisis stage of the disease. They suggested that the rise they found earlier simply continued until death. The results obtained in this work bear out Marston's opinion in view of the very sudden rise in blood copper and particularly considering the histological differences between the livers of sheep 5 and sheep 2 and 6. In the animal with high liver copper but which did not die from copper poisoning the copper was distributed in the hepatic parenchyma in a very diffuse form (Plate 5). In the sheep which had died after the haemolytic crisis the copper had largely left the hepatic cord cells and had settled in Kupffer's cells (Plate 2) as well as being present in the spleen and other organs at levels far higher than normal. This could only be explained reasonably through the supposition that the high level of circulating copper had originated in an outflow from the liver. Again the hepatic dysfunction reported by Todd and Thompson (1962) may have some bearing on this aspect of the condition.

The question arises, just why the massive haemolysis

should take place. Its nature appears to have been straightforward; the mean corpuscular volume figures from sheep 6 (Table V) show that the red cells were swelling during this phase, much as they do when lysed by hypotonic solutions. If the copper in the blood were the direct cause of this, there would also have been a danger of haemolysis occurring when molybdenum supplementation raised the blood copper. There was a distinct difference here, however, The effect of molybdenum (see Plate 1) was to raise the plasma copper in both the globulin-bound and loosely-bound copper fractions, so that the loosely-bound copper was always less than half the globulin-bound, coeruloplasmin copper. In the haemolytic crisis however, (see Tables IV and V) the loosely-bound copper was considerably in excess of the globulin-bound fraction. In fact in both sheep there was more than ten times the loosely-bound copper in the plasma than in the same animal when the last normal sample was taken. This tenfold increase agrees exactly with the amount of free copper, when introduced intravenously, required to kill rabbits and sheep in the work of Eden and Green (1939). Thus it appears likely that in the living animal free copper in the blood at this level of ten times the normal has a simple lytic effect on red

blood cells.

Another hypothesis arises out of the work of Todd, (1962). He suggested that in copper poisoning the liver damage may be accompanied by a reduction in blood glutathione. Jocelyn (1958) showed that glutathione was necessary for the protection of the red blood cell against haemolysis though the action remained obscure. However, Todd, Gracey and Thompson (1962) tried to relieve the symptoms in the haemolytic stage of copper poisoning by injecting glutathione and this was unsuccessful. The illness was still fatal in every case.

Among the early metabolic changes which were observed was the high urine copper level of 78 µg per 100 ml. in the sample taken when sheep 6 first showed signs of ill-health. One of the reasons that copper is not normally found in the urine in such quantities is no doubt due to the forms which it takes in the blood. That part which is joined to globulins would not normally pass into the urine and only a fraction of that part loosely-bound to albumen appears there. It seems possible, therefore, that much more of the copper is free and dialysable, even at the onset of the crisis. Later the increased permeability of the glomerulus due to developing anoxia allows cells, proteins and presumably copper to pass into the tubules

(as seen in the histology of the kidney in sheep 2 - see Page 57). This would account for the extremely high level of copper found in the kidneys immediately after death.

On the present evidence, therefore, it would seem reasonable to hold the view that in the haemolytic crisis of copper poisoning great quantities of copper leave the liver, possibly due to complete liver failure, and that this copper in some physico-chemical way is responsible for the massive haemolysis, which eventually leads to death.

Molybdenum and Copper Metabolism

The point of major importance concerning the administration of molybdenum to two of the sheep which had received copper supplement has already been touched upon. There was a distinction between the high blood copper levels found in the haemolytic crisis and those found associated with the molybdenum supplement. In the latter the main increase was in the plasma fraction and there it was due to a rise in both globulin-bound copper and loosely-bound copper, so that they remained in approximately the same proportions to each other.

The second point arising from this experiment was that the sheep which had received molybdenum had together

a lower liver copper than the pair which had no further treatment when the high-copper diet was stopped. Clearly with this small number of animals it would be unwise to place too much significance upon this result, but it does partly corroborate the claims of Pierson and Aanes (1958) for success with molybdenum in two field outbreaks of copper poisoning.

The balance trials described here confirmed that, under the influence of molybdenum, sheep were depleted of copper by excreting more than their intake. There is ample evidence to show that the urine was not the pathway by which this increased excretion took place. Molybdenum appeared to act rather by increasing the faecal excretion of the element; there was not simply a reduction of the retention of copper but an excess of excretion through the faeces over the dietary intake. In every case this was associated with a rise, of quite considerable proportions, in the copper circulating in the bloodstream. This raises the question of how the faecal excretion is increased.

The interpretation of gut content analysis for one element is always notoriously difficult. There are three main variables; (a) exchange of water (b) change and absorption of digestible nutrients and the excretion of

solids in solution and (c) the absorption and excretion of the element under investigation. No matter how one tries to relate the element to the remainder of the contents therefore, error which may be quite considerable is necessarily involved. However, from the data concerning sheep C and D (Tables IX and XI) it is fairly evident that there was a rise in intestinal copper at the first part of the small intestine. In sheep C the magnitude of this rise can perhaps be explained by the fact that there was very little food in this part of the gut at slaughter, so that any added copper would be diluted far less than in sheep D where food was present in this region. Even in sheep D however, there was a twofold increase in the copper/silica ratio, which certainly suggested an inflow of copper at this site. The most obvious source of this copper would be the bile. If this were so, it would present two possible alternative actions for molybdenum in this aspect of copper metabolism (a) an increase of biliary excretion of copper so that the net effect was an outflow of the element (b) a decrease in the intestinal absorption of copper so that the normal bile copper was not reabsorbed but lost to the animal. Gitlin, Hughes and Janeway (1960 - see Section I) showed that bile was an important

route of copper excretion in the normal animal so the possibility of molybdenum acting there is quite strong.

The results of the gut wall analysis were in the main disappointing. Where there were the greatest changes in the copper level of the gut contents in sheep D, there were some of the lowest levels in the related tissues. One might have expected that where copper was entering or leaving the lumen of the intestine one could have recognised this by the presence of higher levels in the wall, as did Bowland and others (1961) in their work with labelled copper in pigs. Bremner (1961), working with calves, obtained somewhat similar conflicting results to those described here.

The rise in serum copper observed in these experiments may have been caused by a release of copper from the liver when molybdenum was introduced. This in turn may have led to increased biliary copper excretion and also possibly increased copper excretion in all the alimentary secretions. The rise in serum copper may, on the other hand, have been the result of reabsorption of some copper excreted by the bile in excess of the normal amount. The latter is the more unlikely for two reasons; (a) the rise in serum copper usually preceded any increase in faecal copper excretion in the balance trials and (b)

consideration of the feeding of high-copper diets as in the first experiment shows that this alone did not raise blood or plasma copper levels at all.

It seems reasonable, therefore, to conclude that the greatest effect of molybdenum is a release of copper from the liver into the circulation and that this, through an increase in copper output through some or all of the alimentary secretions, leads to increased excretion of copper in the faeces.

The Rôle of Sulphate

It is of particular interest to compare the results from sheep B and C with those from sheep D and E as far as the effects of adding sulphate to the diet were concerned. When molybdenum supplementation was followed by molybdenum and sulphate together (sheep B and C) the effects of molybdenum were increased, with a slight delay. When the order of supplementation was reversed (sheep D and E) there appeared to be little change due to sulphate alone but when molybdenum was added its effect was more immediate than when it was given alone or when sulphate was added second.

These findings suggest that molybdenum has its greater effect due to sulphate after the latter has been absorbed.

It is well-known that sodium sulphate is somewhat slowly absorbed (Wilson and Schild, 1952) and this may explain the relatively larger amount which has to be given to increase the action of molybdenum.

The Effect of Molybdenum on Urinary Copper Excretion

One might have supposed that if molybdenum caused a loss of copper to the whole organism, an increase in the urinary excretion of the element might have been seen. In fact, though the effect was less pronounced in some animals than in others, the urinary copper output fell, in some cases to almost nothing at all. Had the copper released from the liver been readily dialysable it would almost certainly have appeared in the urine, as did the copper released in the haemolytic crisis, unless it was reabsorbed.

While the urine copper output was low under the influence of molybdenum, there was the remarkably high kidney copper in sheep C at the end of the trial. Histological examination of this kidney showed that copper, in a very fine granular form is present in the tubular tissue in the proximal part of the nephron. This suggests tubular reabsorption of a copper compound not dissimilar to that found in the normal liver. If such

a substance passed the glomerular filtration process, one might suspect epithelial damage in the glomeruli, though none was evident in this case.

When all these points are considered, it seems evident that a molybdenum and sulphate supplement to a normal diet has various effects on the animal body, of which depletion of the copper reserves is only one manifestation.

General Summary

When a group of sheep were fed 1 gramme of copper sulphate daily there was no demonstrable effect on their blood copper levels. Three out of six may have shown a premonitary sign of poisoning in a form of photosensitisation. It was sixteen weeks before one died with the typical signs and post-mortem findings of copper poisoning, but this was followed by another in two weeks. Blood analyses indicated that there was a considerable rise of loosely-bound copper in the plasma in the terminal crisis.

When two of the survivors were given 54 mg. molybdenum daily by mouth, their blood copper level rose while that of controls remained the same. This increase was accounted for mainly in the plasma fraction but, in contrast to the findings in the haemolytic crisis

of copper poisoning, the proportions of globulin-bound and loosely-bound copper remained approximately the same. The animals were not clinically affected and at the end of the period there was some evidence that their liver copper had been reduced. The distribution of copper within the liver was found to be different, in animals which had not died from copper poisoning, from that in animals which had.

In a further experiment, five sheep were kept on a constant diet and their intake and output of copper was measured. 54 mg. molybdenum per day, given by mouth, was found to raise serum copper levels, depress urine copper and raise faecal copper output. The effect of 2.0 g. sulphate per day was to enhance these effects, but sulphate alone did not produce the same results.

Post-mortem examination of the kidneys of two of these animals revealed tubular reabsorption of copper and damage to tubular epitheluim.

Appendix

Details of Blood Analyses in

Copper Poisoning Experiment

Molybdenum Administration Experiment

and

Balance Trials

Table XII

Copper Poisoning Experiment

Section III (i)

Results of Blood Copper Analyses

Sheep 1

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		64	73	65	74	70	63
Plasma copper, µg/100 ml.		67	-	75	73	71	56
Non-globulin copper, µg/100 ml. plasma		21	28	23	30	21	12
100 ml. blood contain	R.B.C. copper	23.8		15.5	15.1	23.7	26.6
	All plasma copper	40.2		49.5	48.9	46.3	36.4
	Non-globulin copper	12.6	18.2	15.1	20.1	13.7	7.8

7	8	9	10	11	12	13	14	15	16
69	62	62	73	62	68	59	64	46	61
-	56	58	67	70	75	45	54	72	53
15	12	12	15	14	25	10	21	24	16
	24.5	22	25.4	11.6	14	25.7	24.6		22.3
	37.5	40	47.6	50.4	54.0	33.3	39.4		38.7
9.6	8.0	8.3	10.7	10.1	18.0	7.4	15.3	17.5	11.7

Sheep 2

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		59	64	57	66	65	54
Plasma copper, µg/100 ml.		109	86	74	71	73	62
Non-globulin copper, µg/100 ml. plasma		0	27	32	28	22	14
100 ml. blood contain	R.B.C. copper	3.4	2.9	3	23.4	3.2	9.4
	All plasma copper	55.6	61.1	54	42.6	51.8	44.6
	Non-globulin copper	0	19.2	23.4	16.8	15.6	10.1

7	8	9	10	11	12	13	14	15
61	68	66	67	63	60	62	82	
59	69	69	66	55	69	67	86	
16	27	26	30	15	18	16	30	Died
19.1	16.2	14.9	17.5	21.7	10.3	12.8	20.5	
41.9	51.8	51.1	49.5	41.3	49.7	49.2	61.5	
11.4	20.3	19.2	22.5	11.3	13.0	11.8	21.5	

Table XII (continued)

Sheep 3

Week		1	2	3	4	5	6
Whole Blood copper, µg/100 ml.		65	77	73	93	71	58
Plasma copper, µg/100 ml.		71	73	79	95	80	70
Non-globulin copper, µg/100 ml. plasma		7	19	21	26	26	8
100 ml. blood contain	R.B.C. copper	21	25.9	16.9	28.9	13.3	7.6
	All plasma copper	44	51.1	56.1	64.1	57.7	50.4
	Non-globulin copper	4.3	13.3	14.9	17.6	18.7	5.8

7	8	9	10	11	12	13	14	15
65	50	60	50	54	42	43	23	42
53	46	54	43	42	25	24	28	29
9	14	16	29	16	9	10	10	19
26.3	16.4	20.6	18.2	22.5	23.2	24.6	2	20
38.7	33.6	39.4	31.8	31.5	18.8	18.4	21	22
6.6	10.2	11.7	21.5	12.0	6.8	7.7	7.5	14.4

Sheep 4

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		75	74	81	75	71	93
Plasma copper, µg/100 ml.		116	118	110		79	141
Non-globulin copper, µg/100 ml. plasma		15	19	18	24	19	51
100 ml. blood contain	R.B.C. copper	19.3	3.2	20.5		19.6	4.2
	All plasma copper	55.7	70.8	60.5		51.4	88.8
	Non-globulin copper	7.2	11.4	9.9	17.3	12.4	32.1

7	8	9	10	11	12	13	14	15
76	67	80	77	75	76	71	74	55
103	94	103	99	97	93	72	83	77
15	21	22	30	30	24	14	20	45
12.7	9.7	11	11.2	9	13.7	21	18.8	3.4
63.3	57.3	69.0	65.8	66	62.3	50	55.2	51.6
9.2	12.8	14.7	20.0	20.4	16.1	9.7	13.3	30.2

Table XII (continued)

Sheep 5

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		42	63	69	71	88	55
Plasma copper, µg/100 ml.		58	95	70	80	78	75
Non-globulin copper, µg/100 ml. plasma		16	24	22	29	37	16
100 ml. blood contain	R.B.C. copper	18.2	3.1	24.9	19.8	39.6	8.0
	All plasma copper	23.8	59.9	44.1	51.2	48.4	48
	Non-globulin copper	6.7	15.1	13.9	18.6	22.9	10.2

7	8	9	10	11	12	13	14	15	16
63	59	61	62	60	60	71	65	56	68
65	65	65	59	62	76	76	83	67	68
11	15	14	14	17	29	17	33	24	21
22.7	14.8	18.1	22.5	18.8	9.1	19.3	9.4	10.8	21.8
40.3	44.2	42.9	39.5	41.2	50.9	51.7	55.6	45.2	46.2
6.8	10.2	9.2	9.4	11.3	19.4	11.6	22.1	16.2	14.3

Sheep 6

Week		1	2	3	4	5	6
Blood copper, µg/100 ml.		71	68	91	78	82	70
Plasma copper, µg/100 ml.		92	107	-	92	76	88
Non-globulin copper, µg/100 ml. plasma		7	32	-	28	19	18
100 ml. blood contain	R.B.C. Copper	20.4			24.6	36	16.3
	All plasma copper	50.6	68.5		53.4	46	53.7
	Non-globulin copper	3.9	20.5		16.2	11.5	11.0

7	8	9	10	11	12	13	14	15	16
64	71	60	65	75	73	70	81	83	88
66	66	67	65	79	65	64	94	97	96
20	20	13	25	-	23	21	50	35	36
24.4	29.4	16.4	23.7	25.2	31.4	28.4	19.9	19.9	24.6
39.6	41.6	43.6	41.3	49.8	41.6	61.1	61.1	63.1	63.4
12.0	12.6	8.5	15.9		14.7	13.7	32.5	22.8	23.8

Table XIII

Sheep 1

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		46	61	68	67	64	86
Plasma copper, µg/100 ml.		72	53	62	52	57	61
Non-globulin copper, µg/100 ml. plasma		24	16	18	14	14	20
100 ml. blood contain	R.B.C. copper		22.3	24.0	27.7	19.8	39
	All plasma copper		38.7	44.0	39.3	44.2	47.0
	Non-globulin copper	17.5	11.7	12.8	10.6	10.9	15.4

7	8	13	14	15
85	73	67	71	82
80	76	83	85	82
9	16	27	15	18
26.6	13.7	6.4	6.4	20.5
58.4	59.3	60.6	64.6	61.5
6.6	12.5	19.7	11.4	13.5

Sheep 3

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		42	102	112	91	107	103
Plasma copper, µg/100 ml.		29	111	119	93	110	114
Non-globulin copper, µg/100 ml. plasma		19	57	50	37	46	44
100 ml. blood contain	R.B.C. copper	20	15.4	22.7	18.5	20.1	15.2
	All plasma copper	22	86.6	89.3	72.5	86.9	87.8
	Non-globulin copper	14.4	44.5	37.5	28.9	36.3	33.9

7	8	13	14	15
130	124	89	93	101
143	139		115	111
46	71	60	44	38
18.5	17		7.9	16.6
111.5	107		85.1	84.4
35.9	54.7	45.0	32.7	28.9

Table XIII (continued)

Sheep 4

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		55	108	111	106	115	123
Plasma copper, µg/100 ml.		77	131	162	128	147	165
Non-globulin copper, µg/100 ml. plasma		45	48	41	38	57	51
100 ml. blood contain	R.B.C. copper	3.4	18.9	5.7	16.4	10.6	11.6
	All plasma copper	51.6	89.1	105.3	89.6	104.4	111.4
	Non-globulin copper	30.2	32.6	26.7	26.6	40.5	34.4

7	8	13	14	15
117	128	93	86	95
157	170	119	124	125
53	66	43	43	37
4	12.4	13.3	1.7	11.9
113	115.6	79.7	84.3	83.1
38.2	44.9	28.8	29.2	24.6

Sheep 5

Week		1	2	3	4	5	6
Whole blood copper, µg/100 ml.		56	68	66	80	69	61
Plasma copper, µg/100 ml.		67	68	66	70	79	77
Non-globulin copper, µg/100 ml. plasma		24	21	44	16	18	22
100 ml. blood contain	R.B.C. copper	10.8	21.8	24.7	23.1	15.3	8.6
	All plasma copper	45.2	46.2	41.3	46.9	53.7	52.4
	Non-globulin copper	16.2	14.3	27.5	10.7	12.2	15.0

7	8	13	14	15
87	62	62	57	64
69	64	58	63	67
16	18	29	29	18
38.7	17.2	20.8	12.6	15.8
48.3	44.8	41.2	44.4	48.2
11.2	12.6	20.6	20.4	13.0

Sheep 6

1	2	
83	88	
97	96	
35	36	Died
19.9	24.6	
63.1	63.4	
22.8	23.8	

Appendix vi

Balance Trial - Sheep A

Results of Analyses

Day	Treatment	Serum Cu µg/100ml	Food copper (mg)	Urine copper level (µg/100ml)	Urine volume (ml)	Faeces copper level (µg/g dry)	Wt. of faeces dry (g)	Total copper output (mg)	Daily gain or loss of copper (mg)	
1		89.3	4.89	5.0	568	8.3	502	4.21	+0.68	
2			4.89	6.0	432	9.0	448	4.05	+0.84	
3	None	74.5	4.89	9.0	520	9.2	408	3.80	+1.09	+0.25
4			4.89	14.0	485	11.7	414	4.91	-0.02	
5		86.0	4.89	14.0	610	11.3	513	5.90	-1.01	
6		72.0	4.89	13.0	566	10.5	465	4.95	-0.06	
7		91.5	4.89	9.0	666	11.2	480	5.45	-0.56	
8	54 mg	100.0	4.89	9.0	580	10.7	495	5.35	-0.46	
9	Mo		4.89	1.0	582	10.6	518	5.51	-0.62	-0.13
10	/	130.0	4.89	4.5	580	10.3	429	4.43	+0.46	
11	day		4.89	5.0	535	10.5	493	5.23	-0.34	
12		127.0	4.89	4.0	478	10.8	385	4.16	+0.73	

Balance Trial - Sheep B

Results of Analyses

Day	Treatment	Serum Cu µg/100ml	Food copper (mg)	Urine copper level (µg/100ml)	Urine volume (ml)	Faeces copper level (µg/g dry)	Wt. of faeces dry (g)	Total copper output (mg)	Daily gain or loss of copper (mg)	
1			4.08	21	540	9.2	436	4.12	-0.04	
2		113	3.90	22	290	10.9	430	4.75	-0.85	
3	None		2.69	18	490	10.3	334	4.58	-1.89	
4		92	4.03	16	610	10.5	483	5.17	-1.14	-0.63
5			4.08	13	810	9.3	508	4.88	-0.80	
6		93	5.35	24	720	8.3	509	4.42	+0.93	
7			4.06	15	285	10.4	590	6.18	-2.12	
8	54 mg	99	2.66	9	435	9.8	524	5.20	-2.54	
9	Mo		4.08	5	390	8.7	448	3.92	+0.16	
10	/day	90	5.24	8	710	11.2	469	5.29	-0.05	-1.34
11			4.08	4	800	10.3	546	5.65	-1.57	
12		102	4.08	5	645	10.8	552	5.99	-1.91	
13			4.08	0	340	9.6	521	4.98	-0.90	
14	54 mg	124	4.08	0	650	9.7	610	5.92	-1.84	
15	Mo		4.08	1	1085	9.7	585	5.68	-1.60	
16	&	171	4.08	2.5	1100	9.7	507	4.94	-0.86	
17			4.08	2	760	10.0	477	4.77	-0.69	
18		149	5.36	2	990	11.0	543	5.99	-0.63	-1.99
19	2 g.		4.08	1	735	11.2	510	5.71	-1.63	
20	$-SO_4$	-	4.08	4	350	11.2	579	6.47	-.2.39	
21			2.72	5.5	1510	11.6	578	6.76	-4.04	
22	/day	145	2.72	9	200	11.5	546	6.30	-3.58	
23			2.72	3	1180	11.5	598	6.89	-4.17	
24		124	5.34	6	780	11.6	590	6.92	-1.58	

Appendix viii

Balance Trial - Sheep C

Results of Analyses

Day	Treatment	Serum Cu µg/100ml	Food copper (mg)	Urine copper level (µg/100ml)	Urine volume (ml)	Faeces copper level (µg/g dry)	Wt. of faeces dry (g)	Total copper output (mg)	Daily gain or loss of copper (mg)	
1		109	2.22	-	0	9.6	176	1.69	+0.53	
2			1.66	34	570	9.8	195	2.10	-0.44	
3		110	1.76	26	480	10.0	203	2.01	-0.25	
4			2.18	19	360	9.3	188	1.82	+0.36	
5	None	104	1.78	15	545	9.9	210	2.16	-0.38	-0.095
6			1.71	24	375	9.7	199	2.02	-0.31	
7			1.49	23	390	9.4	211	2.08	-0.59	
8		98	1.89	34	490	9.2	183	1.86	+0.03	
9			1.63	33	220	9.2	155	1.51	+0.12	
10		112	1.85	20	320	9.8	185	1.87	-0.02	
11			1.81	17	480	9.8	189	1.93	-0.12	
12	54 mg	149	1.38	17	735	9.6	179	1.71	-0.33	
13			1.71	-	0	9.4	187	1.76	-0.05	
14	Mo	142	1.42	14.5	425	9.7	220	2.19	-0.77	-0.245
15			1.54	15	350	9.7	157	1.58	-0.04	
16	/day	188	1.53	12.5	750	9.6	191	1.92	-0.39	
17			1.49	16	360	10.0	146	1.52	-0.03	
18		182	1.37	-	0	10.2	158	1.61	-0.24	
19			1.30	16	675	10.3	153	1.69	-0.39	
20	54 mg	230	1.46	-	0	10.2	157	1.60	-0.14	
21	Mo		1.36	16.5	675	11.5	149	1.82	-0.46	
22		295	1.42	13.5	435	11.2	149	1.73	-0.31	
23	&		1.35	-	0	11.8	142	1.68	-0.33	
24		313	1.41	19.5	380	12.6	163	2.13	-0.72	-0.462
25	2.0 g		1.35	18.0	660	12.9	97	1.37	-0.02	
26	$-SO_4$		1.39	21	460	12.8	146	1.97	-0.58	
27		300	1.21	14	56	12.7	191	2.42	-1.21	

Appendix ix

Balance Trial - Sheep D

Results of Analyses

Day	Treatment	Serum Cu µg/100ml	Food copper (mg)	Urine copper level (µg/100ml)	Urine colume (ml)	Faeces copper level (µg/g dry)	Wt. of faeces dry (g)	Total copper output (mg)	Daily gain or loss of copper (mg)	
1		116	6.73	15	420	18.3	313	5.83	+0.90	
2			6.73	19	385	18.6	374	7.06	-0.33	
3		106	6.73	17	360	18.4	326	6.10	+0.63	
4	None		6.73	15	390	17.9	354	6.43	+0.30	+0.49
5		105	6.73	16	515	18.7	313	5.95	+0.78	
6			6.73	16	400	18.9	304	5.84	+0.89	
7		101	6.73	17	365	20.6	308	6.45	+0.28	
8			6.73	19	540	17.9	275	5.02	+1.71	
9	2.0 g	112	6.73	16	405	18.3	377	7.00	-0.27	
10	$-SO_4$		6.73	18	380	18.8	359	6.85	-0.12	+0.31
11	/day	105	6.73	13	380	18.9	353	6.78	-0.05	
12			6.73	15	465	18.5	344	6.47	+0.26	
13	2.0 g	119	6.73	14	320	19.8	359	7.20	-0.47	
14	$-SO_4$		6.73	14	500	20.4	357	7.38	-0.65	
15	&	155	6.73	13	420	22.9	346	8.02	-1.29	
16	54 mg		6.73	9	345	20.7	360	7.49	-0.76	-0.57
17	Mo	128	6.73	11	340	20.8	316	6.62	+0.11	
18	/day	164	6.73	11	410	21.3	329	7.07	-0.34	

Appendix X

Balance Trial - Sheep E

Results of Analyses

Day	Treatment	Serum Cu µg/100ml	Food copper (mg)	Urine copper level (µg/100ml)	Urine volume (ml)	Faeces copper level (µg/g dry)	Wt. of faeces dry (g)	Total copper output (mg)	Daily gain or loss of copper (mg)	
1		122	6.73	23	415	18.9	348	6.68	+0.05	
2			6.73	29	320	L.S. *	-	-	-	
3		100	6.73	19	455	16.9	347	5.97	+0.76	
4	None		6.73	23	465	17.2	353	6.18	+0.55	+0.28
5		109	6.73	19	365	18.7	355	6.75	-0.02	
6			6.73	21	425	22.3	312	7.06	-0.33	
7		102	6.73	22	420	22.0	271	6.07	+0.66	
8			6.73	20	316	20.1	316	6.45	+0.28	
9	2.0 g	108	6.73	16	375	19.2	310	6.06	+0.67	
10	$-SO_4$		6.73	19	340	18.7	305	5.81	+0.92	+0.46
11	/day	116	6.73	20	450	20.5	334	6.94	-0.21	
12			6.73	18	385	19.7	305	6.10	+0.63	
13	2.0 g	109	6.73	19	565	20.4	339	7.02	-0.29	
14	$-SO_4$		7.73	19	420	21.6	328	7.19	-0.46	
15	&	181	6.73	15	400	21.5	321	7.00	-0.27	-0.34
16	54 mg		6.73	9	512	21.5	324	7.06	-0.33	
17	Mo	137	6.73	11	445	21.4	314	6.81	-0.08	
18	/day		6.73	15	320	22.9	317	7.35	-0.62	

* Lost sample

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Experimental Copper Poisoning in Sheep

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SUMMARY.—This paper describes an experiment in which "chronic" copper poisoning was induced in sheep by copper supplementation of the normal diet. The development of "jaundice," and the rapid rise in blood copper during the terminal stages of the disease are described. The effect of molybdate administration to survivors was to produce a rise in blood copper which was shown to occur mainly in the plasma fraction. Further, analysis of liver biopsy samples suggests that the effect of molybdate was to reduce the liver copper to safer levels.

CLINICAL occurrences of "chronic" copper poisoning have been described in sheep (Clegg, 1956; Pearson, 1956; Muth, 1952; Bracewell, 1958; Berwyn-Jones, 1960), and in young cattle (Shand & Lewis, 1957; Todd & Gracey, 1959). Experimental copper poisoning of sheep has been described by Sutter, Rawson, McKeown and Haskell (1958) who, having followed blood copper levels through a period of administration of copper supplement, concluded that a definitive diagnosis of copper poisoning could be made from the blood copper level and that the haemolytic syndrome was not an essential criterion. Experiments concerning the effect of other elements on the storage of copper in the liver of the sheep were performed by Edgar (1942) and Dick (1954). The latter author showed that under conditions of high molybdenum and sulphate intake, not only is copper retention reduced but the rate of loss of copper is increased. These findings were confirmed in heifers by Cunningham, Hogan and Lawson (1959). A report of treatment in clinically occurring copper poisoning in sheep was given by Pierson and Aanes (1958), the method being to supply ammonium molybdate and sodium sulphate, either in a mineral mix or as a spray on hay.

Copper in blood is distributed between the red cells and the plasma, the concentrations in each being approximately the same, with plasma levels usually slightly higher than red cell levels. While this applies to normal animals, it should not be assumed that the proportions remain the same in situations where blood copper levels are changing, and the indiscriminate quoting of whole blood, plasma, or serum copper levels as if they were interchangeable cannot be justified (Underwood, 1956).

The distribution of copper in plasma has been studied in species other than sheep. It has been shown that most of the copper is in a form bound to a globulin as caeruloplasmin or a related compound (Holmberg & Laurell, 1947, 1948). The remainder is in a loosely-bound form and can be made to react almost directly with copper-detecting reagents; it accounts for about 4 per cent. of the plasma copper in man, about 12 per cent. in the dog and up to 40 per cent. in the pig (Gubler, Lahey, Cartwright & Wintrobe, 1953). There is a lack of data on the proportion in sheep.

The following experiment was carried out firstly to investigate further the clinical events in copper poisoning in the sheep, and secondly to test the protective effect of molybdenum supplementation in sheep which were known to have been exposed to high doses of copper over a long period.

Materials and Methods

Six yearling cross-bred sheep, 2 castrated males and 4 females, were kept indoors and fed on a basic diet of good hay and crushed oats. Weekly sampling of venous blood was performed by jugular puncture, samples always being taken in the morning before food was offered. Copper analysis was carried out on the whole blood, plasma and plasma treated with ammonium sulphate to remove globulin (Earl, Moulton & Selverstone, 1954; Holmberg & Laurell, 1947). The analysis was performed, after wet combustion with nitric, perchloric and sulphuric acids, by a method modified from that of Hoste, Eeckhout and Gillis (1953).

After a preliminary settling-in period the rations of all the sheep were supplemented with one gramme of hydrated copper sulphate per day, by mixing a copper sulphate solution with the food for each individual sheep.

When one death from chronic copper toxicosis had occurred, administration of copper to the remainder was stopped. Two of the remaining 5 sheep were given 100 mg. ammonium molybdate (about 54 mg Mo) in aqueous solution by mouth daily. Blood sampling and copper analysis were continued. One of the untreated sheep died in the following week.

In the case of the 2 sheep which died, the events during the haemolytic crisis were noted and data concerning blood changes were collected. Analysis of copper content was also made on several organs at autopsy. Four months after the commencement of the molybdenum supplement the 4 sheep remaining in the experiment were subjected to laparotomy and part of the caudate lobe of the liver was removed for copper analysis.

Results

The copper levels in whole blood and plasma during the course of the experiment are set out in Table I. The copper level in each fraction was calculated, using the Packed Cell Volume of each blood sample, and the results are set out diagrammatically in Fig. 1. Table I and Fig. 1 do not include the detailed observations in the terminal stages of copper poisoning.

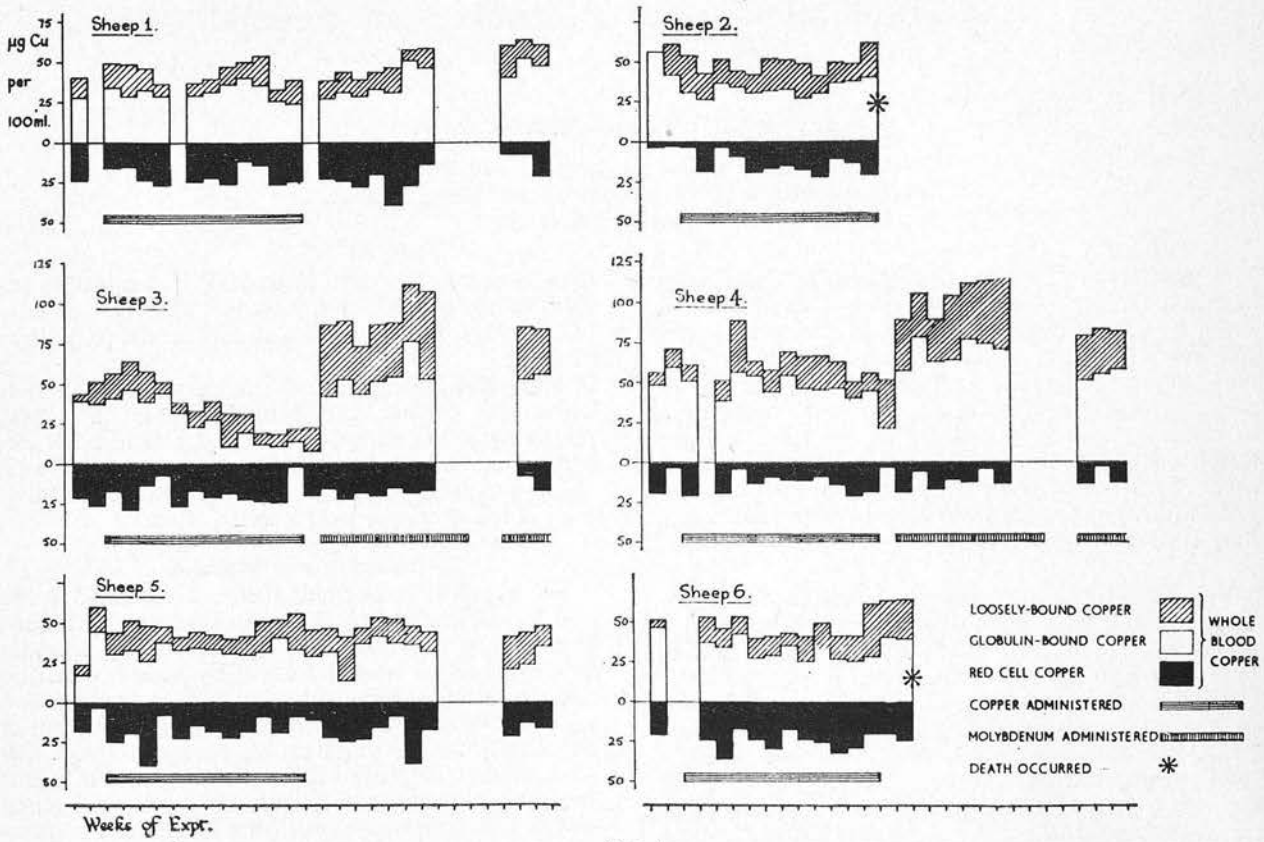

FIG. 1

TABLE I

COPPER LEVELS IN WHOLE BLOOD AND PLASMA
µg per 100 ml.

Sheep No.	Week of Expt.	1	2	*3	4	5	6	7	8	9	10	11	12	13	14	15	†16	17	18	19	20	21	22	27	28	29
1	Whole blood	64	73	65	74	70	63	69	62	62	73	62	68	59	64	46	61	68	67	64	86	85	73	67	71	82
	Plasma	67	—	75	73	71	56	—	56	58	67	70	75	45	54	72	53	62	52	57	61	80	76	83	85	82
2	Whole blood	59	64	57	66	55	54	61	68	66	67	63	60	62	82											
	Plasma	109	86	74	71	73	62	59	69	69	66	55	69	67	86											
3	Whole blood	65	77	73	93	71	58	65	50	60	50	54	42	43	23	42	†102	112	91	107	103	130	124	89	93	101
	Plasma	71	73	79	95	80	70	53	46	54	43	42	25	24	28	29	111	119	93	110	114	143	139	—	115	111
4	Whole blood	75	74	81	75	71	93	76	67	80	77	75	76	71	74	55	†108	111	106	115	123	117	128	93	86	95
	Plasma	116	118	110		79	141	103	94	103	99	97	93	72	83	77	131	162	128	147	165	157	170	119	124	125
5	Whole blood	42	63	69	71	88	55	63	59	61	62	60	60	71	65	56	68	66	80	69	61	87	62	62	57	64
	Plasma	58	95	70	80	78	75	65	65	65	59	62	76	76	83	67	68	66	70	79	77	69	64	58	63	67
6	Whole blood	71	68	91	78	82	70	64	71	60	65	75	73	70	81	83	88									
	Plasma	92	107		92	76	88	66	66	67	65	79	65	64	94	97	96									
Treatment		None			*Copper supplement to all sheep											None	†Molybdenum supplement to sheep 3 and 4									

In the thirteenth week of copper supplementation 3 sheep (Nos. 1, 2 and 3) became dull and had swollen lips. They were otherwise clinically normal and recovered in 2 days. In the fifteenth week sheep 2, a wedder which had been normal at the time of the previous blood sampling, became very dull and list-

TABLE II

SHEEP 2—BLOOD ANALYSES DURING HAEMOLYTIC CRISIS
μg Copper per 100 ml.

Time before death	P.C.V. per cent.	Direct analysis			Calculated distribution per 100 ml. whole blood		
		Whole blood	Plasma	Plasma less globulin	R.B.C.	Globulin-bound	Non-globulin
*4 days	28·5	82	86	30	20·5	40	21·5
21 hours	15·0	427	362	240	119	104	204
15½ hours	15·0	445	381	250	121	111	213
5½ hours	9·0	380	372	268	41	95	244
2 hours	6·0	332	323	248	28	71	233

*Last samples from the clinically normal animal included for comparison.

less 3 days later. There was no thickening of the lips on this occasion. Within 3 hours of the dullness having been noted a very mild icterus developed. This became more intense after a further 4 hours, but was of an unusually dirty colour, quite unlike the golden colour of other jaundices. The animal occasionally gritted its teeth as if with abdominal pain. Blood samples were taken at intervals and the results from these are shown in Table II. Sheep 2 died 30 hours after the onset of clinical signs.

Two weeks later sheep 6 (female) was noticed to have slightly greener urine than the other sheep. A sample was taken and found to contain 78 μg of copper per 100 ml., compared with normal urines which contain about 2 to 5 μg per 100 ml. By the evening of the same day sheep 6 had become listless and mild icterus was seen in the sclera. Mucous membranes were generally pale and the animal was inappetent. A blood sample showed discoloration in the plasma; evidence that haemolysis had begun.

Next morning the icterus was severe and the animal stood with her eyes half closed but still offered resistance to handling. She began to show slight staggering by midday but was not actually recumbent until the following morning. By then jaundice was very severe and the animal was unable to stand without assistance. There was some gritting of the teeth and occasional soft bleating. Sheep 6 died about 42 hours after the onset of dullness. Serial blood samples had been taken and the results are shown in Table III.

Post-mortem examination of Sheep 2 and 6 revealed in each case lesions indistinguishable from those encountered in field cases of copper poisoning, the lesions present being those associated with acute severe haemolysis, while the immediate cause of death was anoxia.

The most striking macroscopic abnormalities included gross brownish black pigmentation of the kidneys, particularly of the cortices and brownish yellow pigmentation of almost all other body tissues including the remaining thoracic and abdominal viscera, bone, bone marrow, sclerae, pituitary and lymph nodes, but excluding the central nervous system and peripheral nerves.

Subsequent histological examination confirmed the presence of lesions basically similar to those described by other authors. Using a modified rubeanic acid method (Uzman, 1956), an attempt was made to assess histochemically tissue variations in copper content. This revealed large quantities of copper in the liver, particularly within Kupffer's cells, in the macrophages of the mesenteric lymph nodes, in renal epithelial cells and the elastic fibres of the aorta, moderate amounts in splenic macrophages and bone marrow but only traces in heart, adrenal, carcase lymph nodes, pituitary and the lung. No copper was detected histochemically in the central nervous system or peripheral nerves.

Both of the sheep which were given molybdenum supplement from the sixteenth week on showed an immediate rise in blood copper levels (Table I; Fig. 1.)

There were no further deaths in either molybdenum treated or untreated sheep. Sheep 5 died in the course

TABLE III

SHEEP 6—BLOOD ANALYSES DURING HAEMOLYTIC CRISIS
μg Copper per 100 ml.

Time before death	P.C.V. per cent.	M.C.V. c.u.	Direct analysis			Calculated distribution per 100 ml. whole blood		
			Whole blood	Plasma	Plasma less globulin	R.B.C.	Globulin-bound	Non-globulin
*6 days	34·0	—	88	96	36	24·6	39·6	23·8
40 hours	36·5	38·0	728	504	412	408	58·0	262
27 hours	27·0	41·6	810	505	356	441	109	260
24 hours	23·5	43·4	786	386	289	491	74	221
20 hours	21·0	44·2	747	420	308	415	89	243
3 hours	10·5	39·4	466	320	274	180	41	245

*Last sample from clinically normal animal included for comparison.

TABLE IV

COPPER ANALYSES OF ORGANS

μg Cu per g. dry weight

Organ	Sheep					
	1	2	3	4	5*	6
Liver—central	—	2,241	—	—	1,135	1,928
—right	—	1,846	—	—	987	3,113
—left	—	2,660	—	—	1,178	2,740
caudate-	†678	3,047	†846	†467	1,342	2,685
Kidney cortex	—	700	—	—	12	1,670
Spleen	—	197	—	—	9	237
Heart muscle	—	162	—	—	13	165
Bone marrow	—	17	—	—	3	23
Brain	—	12	—	—	—	21
Skeletal muscle	—	8	—	—	4	14
Thyroid gland	—	4	—	—	3	—
Lymph node	—	—	—	—	11	40

*Died under anaesthesia. †Biopsy specimen.

of laparotomy, mainly because of inhalation of rumen contents, which were expelled violently when the liver was handled. The results of copper analysis of organs appear in Table IV.

Histological examination of the liver samples from Sheep 3 and 1 revealed no significant degenerative parenchymatous changes or evidence of macrophage activation or infiltration. Histochemically, sections treated by the rubeanic acid method revealed large quantities of copper in a finely granular form in hepatic parenchymatous cells and to a much smaller extent in Kupffer's cells. The granules, however, were so small as to be identified only under oil immersion examination.

Discussion

The conclusion of Sutter *et al.* that blood copper levels rise during ingestion of supplementary copper and that these high levels can be used for diagnosis was not borne out in this experiment. None of the sheep showed what could be described as anything more than normal variation. In fact, in the case of Sheep 3 there is a decided fall in whole blood and plasma copper levels throughout the course of the first phase of the experiment.

The events during the thirteenth week are of interest. The clinical signs suggested strongly that the sheep concerned were exhibiting photo-sensitisation. However, they had been subjected to no more light than was usual in the house, and at this stage no explanation can be offered for the appearance of the signs in 3 animals at the same time. An attempt to demonstrate the presence of phylloerythrin in the plasma of these animals by the method of Perrin (1958) failed.

The first animal to show signs of copper toxicosis was a wedder showing that the recognised effects of prolonged copper administration are not confined to the female, as was suggested by Sutter *et al.* These authors further suggest that blood copper levels rise gradually until death, and that there is no sudden outpouring of copper from the liver as stated by Marston (1953). The terminal events in the fatal cases in this experiment support the latter's view, the rise in the last 3 days of life being upwards of 500 per cent. (see Tables II and III). The difference between the histological findings in those sheep which died after the haemolytic crisis and the remainder also show how different is the distribution of the copper in the liver following the crisis.

Progressive lysis of the red blood cells is demonstrated in the packed cell volume figures in the terminal stages. It is of interest to notice the preliminary *rise* in P.C.V. in Sheep 6. This may indicate swelling of the red blood cells before lysis, a supposition at least partially borne out by the mean corpuscular volume of the samples from Sheep 6. The statement of Sutter *et al.* that "death occurs from acute liver failure with secondary jaundice" seems unnecessary, since a sudden onset of severe haemolysis would explain all the signs seen; breakdown of haemoglobin produces icterus while the haemoglobinaemia gives the dirty appearance to the jaundice; the haemoglobinuria which is so marked a feature of the crisis in copper poisoning would follow naturally from wholesale haemolysis.

The distribution of copper in the tissues of the animals which died confirms the findings of previous workers. The liver contained very high concentrations while the kidneys had levels much higher than kidneys from normal sheep (17.8 p.p.m., Cunningham, 1931), and from Sheep 5 in this experiment which did not die from copper poisoning. Among other tissues only spleen and heart muscle are outstandingly different from normal, while the remainder of those analysed show an increase over the normal which is probably consistent with the high levels of copper circulating in the blood.

The concentration of copper in the liver in animals where the liver copper has reached dangerously high levels varies considerably from one part to another (Table IV). It was for this reason that no biopsies were taken during the course of the experiment by the "stab" technique; it was thought that the results so obtained would be of limited value, incommensurate with the risk involved to the animal. Since, in previous chemical evaluations made in this laboratory upon livers from cases of copper poisoning, the caudate lobe of the liver seemed to be among the parts higher in copper concentration and because of the relative ease with which it can be removed surgi-

cally this part was chosen for sampling the liver of surviving animals.

The administration of molybdenum in 2 sheep produced an immediate rise in total blood copper, and this was reflected in the plasma copper, red blood cell copper remaining normal; both globulin-bound copper and "loosely-bound" copper in plasma were increased. There was no clinical effect on the sheep whatsoever; no scouring was seen at any time during molybdenum treatment.

Of the 2 untreated sheep, 1 (No. 5) showed a very high liver copper at laparotomy, *viz.* nearly 50 per cent. of that in the sheep which died. The other (No. 1) had a level which was about half that in sheep No. 5 and twice the normal. Of the treated animals, sheep 4 had reached the lowest level of all and might almost be pronounced normal, while sheep 3 retained a level actually in excess of that of sheep 1 which was not treated. The mean caudate lobe copper levels in the 3 pairs of animals were:

Copper poisoning (*post-mortem*)	2866 μg. per g. D.M.
Treated with molybdenum for 10 weeks	656 μg. per g. D.M.
Untreated sheep after the same period ...	1005 μg. per g. D.M.

With such small numbers of sheep involved, it cannot be claimed that the findings of this experiment are significant, but the effect of molybdenum therapy on the blood copper levels and the apparent reduction of liver copper offer further evidence that molybdenum therapy might be worth trying in flocks where deaths from copper poisoning have recently occurred.

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