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SYMPOSIUM ON 'METAL TOXICITIES'

The toxicity of cadmium, zinc and molybdenum and their effects on copper metabolism

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The classical approach to studies on the toxicity of metals has generally been to establish dose-response relationships and to search for a connexion between the intake of the metal, its accumulation in the body and especially in target organs, and the development of particular symptoms of toxicosis. On the basis of the results obtained, attempts have been made to establish maximum safe or permissible levels of the metal in water and foodstuffs. In the case of cadmium, for example, it is generally assumed that the first signs of Cd poisoning will normally be the development of renal failure and that this will only occur if renal Cd concentrations are around 200 μg/g.

It is now apparent, however, that this approach does not take sufficient cognizance of the considerable influence of a wide range of nutritional and physiological factors on the susceptibility of animals to heavy metal toxicities (Bremner, 1974, 1978). The dietary intake of essential metals, including calcium, iron, zinc and copper, is of particular importance in this regard. For example, absorption of Cd is enhanced in animals receiving diets which are low in Fe or Ca (Valberg *et al.* 1976; Washko & Cousins, 1976). The lesions which develop under these circumstances can be especially severe and, moreover, are not always the same as those normally associated with chronic Cd poisoning. Thus, signs of Fe deficiency have appeared in Japanese quail within days of their receiving a diet with only 2·5–5 mg Cd/kg and long before any significant increase in renal Cd concentrations occurred (Jacobs *et al.* 1974).

It follows, therefore, that it can sometimes be misleading to rely solely on the measurement of the concentrations of the toxic metals in tissues or body fluids in predicting the risk associated with a particular level of exposure to the metals. Moreover, one of the earliest signs that toxic metals may be adversely affecting the health of man or animals may be a disturbance in the metabolism of an essential element. In this paper particular attention will be given to the importance of the

changes in Cu metabolism which occur when intakes of Zn, Cd or molybdenum are increased.

Effects of Zn on Cu metabolism

Although Zn is not generally regarded as a very toxic metal (Ott *et al.* 1966), signs of Cu deficiency can develop in rats if their diet contains about 5000 mg Zn/kg (e.g. Grant-Frost & Underwood, 1958). Because of the large amounts of Zn required to produce this effect it appeared at one stage that Zn toxicity was of limited biological importance. However, there is increasing evidence that this effect of Zn could be of some significance in both medicine and animal production. Oral administration of large amounts of Zn for the treatment of coeliac disease (Porter *et al.* 1977) and sickle cell anaemia (Prasad *et al.* 1978) has caused hypocupraemia and neutropenia in humans. At the other extreme, oral Zn has been used to reduce tissue accumulation of Cu and improve the clinical condition of patients with Wilson's disease, which is a form of Cu poisoning of genetic origin (Hoogenraad *et al.* 1978). Similarly, dietary supplementation with Zn can afford protection against the development of Cu toxicosis in sheep, by reducing liver Cu concentrations (Bremner *et al.* 1976).

It is also possible that Zn-induced disturbances in Cu metabolism could be of importance in cases of environmental exposure of grazing animals to Zn. Herbage Zn concentrations in the vicinity of certain industrial plants can range from 160–3000 mg Zn/kg, depending on the season and distance from the source of the Zn-emission (Mills & Dalgarno, 1972). Although these concentrations are less than those generally found necessary to produce Cu deficiency in rats, the studies of Hill & Matrone (1970) show that the susceptibility of these animals to Zn is much greater if they are fed a low-Cu diet. Dietary Zn contents of only 200–400 mg/kg will then induce clinical signs of Cu deficiency.

It is evident, therefore, that careful attention should be paid to the control of the Cu intake of animals in assessing the hazard associated with environmental exposure to Zn and other metals. In recent investigations of this topic at this Institute, the dietary Cu intake has deliberately been set at a level which was only just sufficient to meet the estimated requirement for this metal. Preliminary studies on rats showed that plasma caeruloplasmin activity could then be reduced by 40% in rats fed diets with only 300 mg Zn/kg (Campbell & Mills, 1974). Increasing the Zn intake to 1000 mg/kg further decreased the activity of this Cu-containing enzyme and in addition reduced growth rate, affected skeletal development and decreased Cu concentrations in both liver and kidney. All these changes are indicative of Cu deficiency.

A severe Cu deficiency could also be induced in pregnant ewes by feeding them a diet with 750 mg Zn/kg (Campbell & Mills, 1979). This was associated with a large reduction in growth rate and food intake. Moreover, the abortion rate was extremely high and the viability of the lambs which were alive at birth was low. The susceptibility of pregnant ewes to Zn toxicosis appears, therefore, to be much greater than that of growing or adult male sheep, which can tolerate Zn intakes of

400–1000 mg/kg diet with at most only a slight reduction in growth, food intake or haematological status (Ott *et al.* 1966; Bremner *et al.* 1976).

It was initially suspected that the lower tolerance of the pregnant ewes to Zn was a result of their Cu deficient state but further studies showed that correction of the Cu deficiency had no beneficial effect on the growth of the ewes or survival of the lambs (Campbell & Mills, 1979). It seems likely that in this instance the greatly increased concentrations of Zn in the tissues of the ewes and their offspring was a major factor in producing these lesions of Zn toxicosis. It is known that the efficiency of Zn absorption is increased during pregnancy in the rat (Davies & Williams, 1977) and this may have contributed to the excessive accumulation of Zn in the ewes and its subsequent transfer to the foetus.

Considerable interest has been shown in the mechanism whereby Zn can cause Cu deficiency and reduce tissue accumulation of Cu in animals. The interaction appears to occur principally at the intestinal level, as injection of large amounts of Zn into a segment of the intestine of the rat significantly reduced ^{64}Cu absorption (Van Campen & Scaife, 1969). It was suggested that this resulted from displacement of Cu from a specific intestinal protein which played an important role in Cu absorption (Starcher, 1969; Evans *et al.* 1970). This protein was later shown to be metallothionein (Evans & Johnson, 1978). It is difficult, however, to reconcile this suggestion with subsequent findings that (a) this protein is probably involved in the homoeostatic regulation of Zn absorption and serves, therefore, to prevent rather than promote metal absorption (Richards & Cousins, 1975, 1976), (b) synthesis of this protein in the intestinal mucosa and liver can be induced by Zn (Richards & Cousins, 1975, 1976; Bremner & Davies, 1975), and (c) Cu has a greater binding affinity for the protein than does Zn (Bremner & Marshall, 1974; Rupp & Weser, 1978).

In recent studies, designed to establish more clearly whether intestinal metallothionein is indeed involved in the antagonistic effect of Zn on Cu metabolism, it was confirmed that absorption of dietary ^{64}Cu was decreased in rats receiving a diet containing 900 mg Zn/kg (Hall *et al.* 1979). Although distribution of the absorbed ^{64}Cu within the carcass was unaffected by Zn, the accumulation of ^{64}Cu in the intestinal mucosa was increased threefold and this appeared to be at least partly responsible for the decrease in ^{64}Cu absorption. Most of the additional ^{64}Cu in the mucosa was incorporated into metallothionein, concentrations of which were greatly increased in the Zn-supplemented rats. It appears, therefore, that the decrease in Cu absorption and therefore the development of Cu-deficiency in Zn-poisoned animals is a consequence of the induction of synthesis of intestinal metallothionein by Zn and the subsequent displacement of Zn from the protein by Cu. The ultimate fate of the Cu which is incorporated into metallothionein in the Zn-treated rats has yet to be established. It has been suggested (Richards & Cousins, 1975, 1976) that this protein acts as a Zn-sequestering agent in the mucosa, thus limiting the transfer of Zn to the plasma and allowing excretion of Zn via the desquamation of mucosal epithelial cells. It is quite possible that it may, in some circumstances, limit Cu absorption in the same way.

Effect of Cd on Cu metabolism

A similar mechanism has been proposed to explain the antagonistic effect of Cd on Cu metabolism, as the reduction in ^{64}Cu absorption in rats receiving a Cd-supplemented diet is associated with increased mucosal binding of ^{64}Cu and incorporation of some of the ^{64}Cu into an analogous protein (Davies & Campbell, 1977). However, Cd is a much more potent inhibitor of ^{64}Cu absorption than is Zn, and there was nearly a 100-fold difference in the molar concentrations of dietary Zn and Cd required to reduce ^{64}Cu absorption by half in these separate studies (Davies & Campbell, 1977; Hall *et al.* 1979). It is significant that there was a similar difference in the ability of Cd and Zn to induce synthesis of intestinal metallothionein, as equivalent amounts of this protein were produced in the mucosa of rats receiving 0·16 mmol Cd or 6·9 mmol Zn/kg diet. This gives further support to the view that the reduction in Cu absorption in Cd- and Zn-treated rats is related to increased production of intestinal metallothionein.

The greater potency of Cd as an antagonist of Cu is also shown in studies where rats, pregnant ewes and their lambs have received diets containing low levels of Cd (Mills & Dalgarno, 1972; Campbell & Mills, 1974). Thus, liver and plasma Cu concentrations can be greatly reduced at dietary Cd contents ranging from only 1·5 to 12 mg/kg, with, in some cases, signs of skeletal rarefaction and reductions in growth rate. Transfer of Cu to the developing foetus can also be inhibited when pregnant ewes and rats receive Cd-supplemented diets (Mills & Dalgarno, 1972; Choudhury *et al.* 1978) and this may be partly responsible for the poor viability of the offspring and the decreased birth weights which are sometimes observed under these circumstances. The concentrations of Cd used in these studies are in the range of those encountered in the vicinity of Zn smelting plants (Mills & Dalgarno, 1972) and it is possible that the poor condition of animals grazing in such areas could arise, at least partly, from the development of Cu deficiency. However, as in the experiments with Zn, the appearance of these disturbances in Cu metabolism at low Cd intakes is a reflection of the composition and in particular the Cu content of the diet, as this was only marginally adequate to meet normal demands. It is significant that the adverse effects of Cd could be prevented by increasing the Cu content of the diet (Campbell *et al.* 1978). This treatment had an additional beneficial effect as it decreased Cd concentrations in the rat kidneys by 50%, suggesting that in long-term exposure to Cd, the onset of renal damage could perhaps be delayed by Cu supplementation.

Similar observations have been made in experiments with Japanese quail, at dietary Cd concentrations of only 20–1000 µg/kg, which is in the range found in human diets. Under these circumstances liver and kidney Cd concentrations could be decreased by about 30% merely by increasing the dietary intakes of Cu, Zn and manganese from requirement levels to double these quantities (Jacobs *et al.* 1978). When it is considered that dietary intakes of Cu and Zn by humans may often be close to or even below the current estimates of requirement, it is evident that the types of interaction between Cd, Cu and Zn described in this paper could be of considerable importance.

Effect of Mo on Cu metabolism

There is no doubt about the importance of the antagonistic effect of Mo on Cu metabolism in ruminants, as this is responsible for the development of conditioned Cu deficiency in cattle in many parts of the world. The symptoms of molybdenosis include poor growth, reduced food intake, diarrhoea, anaemia, achromotrichia and joint and bone abnormalities. In most cases these are accompanied by changes in tissue and blood Cu concentrations and can be abolished by Cu administration.

Two of the principal features of molybdenosis which have attracted particular attention are (a) the extreme susceptibility of ruminants and especially cattle to Mo and (b) the potentiation of the effects of Mo in ruminants by both sulphate and organic forms of S. This is illustrated by the reduction in the availability of Cu in both sheep and calves receiving up to 5 mg Mo or 4 g S/kg diet or both (Suttle & MacLaughlan, 1976; Mills *et al.* 1977). Compared with sheep, the calves appeared to be more affected by the Mo and less affected by the S but in both species the combination of Mo and S had the greatest effect. As a rule monogastric animals can tolerate Mo intakes much greater than this and in these species SO_4^{2-} generally has a protective effect against molybdenosis.

The extreme susceptibility of ruminants is believed to be related to the pathway of S metabolism in the rumen and perhaps to the effect of Mo on S^{2-} generation (Mills, 1960). There has been a lack of agreement as to whether Mo stimulates or inhibits the accumulation of S^{2-} in rumen contents but it now appears that the concentration of Mo in the rumen influences the specific response (Mills, 1960; Bryden & Bray, 1972; Bremner, 1975). Moreover, Mo can inhibit the rate of both S^{2-} production and S^{2-} removal from the rumen (Gawthorne & Nader, 1976). Since the latter is the more affected in sheep receiving 50 mg Mo/d, the net effect under these circumstances is that S^{2-} concentrations are increased.

It is possible that this could lead to increased conversion of ruminal Cu into CuS, with a reduction in both the solubility and availability of the Cu (Bird, 1970). However, there is not always a direct relationship between rumen S^{2-} concentrations and either the solubility of Cu in rumen contents or the apparent availability of the dietary Cu (Bremner, 1975; Mills *et al.* 1977). In addition this hypothesis does not readily explain the effects of Mo on the distribution of Cu in both plasma and kidneys.

Although dietary Mo intakes in the range 2–5 mg/kg can decrease plasma Cu concentrations in ruminants, consistent with the development of Cu deficiency (Mills *et al.* 1977), this is not always so at greater Mo intakes. Thus, increased plasma and kidney Cu concentrations have frequently been found in sheep receiving diets with about 25 mg Mo/kg, even though liver Cu concentrations are reduced (Smith & Wright, 1975; Bremner & Young, 1978). The Cu which accumulates in the plasma is not present as caeruloplasmin but is associated partly with albumin and partly with another protein with molecular weight of about 90 000, which also appears to bind Mo (Bremner & Young, 1978). Similarly, Cu and Mo tend to accumulate in the same chromatographic and subcellular fractions in the kidney, in an atomic ratio of about 2:1. The metals are bound very firmly in

both plasma and kidneys and cannot be released by the addition of trichloroacetic acid solutions. Moreover, Cu only accumulates in these forms in the plasma and kidneys if the dietary S intake is also increased, suggesting that S, or more specifically S^{2-}, is involved in the binding (Bremner & Young, 1978; Cardin et al. 1976). As the rate of clearance of ^{64}Cu from the plasma of sheep receiving Mo- and S-supplemented diets is relatively slow (Smith et al. 1968) the Cu which accumulates in the plasma and also in the kidneys is probably not available for normal metabolic processes.

Recent investigations have produced considerable evidence that many aspects of the Cu–Mo interaction could be explained by the formation in the rumen of thiomolybdate derivatives (Dick et al. 1975; Mills et al. 1978). Thus, intravenous or intraduodenal injection of ammonium tetrathiomolybdate in sheep caused an increase in plasma Cu concentrations, with the Cu accumulating in the same chromatographic fractions as Mo (El-Gallad et al. 1977). When included in the diet of rats at low concentrations (2–6 mg Mo/kg), this same compound decreased intestinal ^{64}Cu absorption, plasma caeruloplasmin activities and liver Cu concentrations (Mills et al. 1978). Moreover, it caused Cu to accumulate with Mo on the same proteins and in acid-insoluble form in both plasma and kidneys.

The clinical effects of these low concentrations of dietary tetrathiomolybdate are also similar to those found in cattle with molybdenosis. The growth rate of the rats is decreased and they develop anorexia, anaemia, hair depigmentation, diarrhoea and severe bone lesions. The protective effect of oral Cu against the development of these lesions was shown to arise from inhibition of the absorption of thiomolybdate or some derivative thereof. Parenteral Cu was equally effective in improving the clinical condition of the rats, suggesting that Cu can also overcome the systemic effects of thiomolybdate, arising when intestinal absorption of thiomolybdate is promoted by a low dietary content of copper.

These observations are all consistent with the view that thiomolybdates could be involved in the Cu–Mo interaction in ruminants. However, it still has to be shown that these compounds are formed within the rumen under normal physiological conditions, although there is spectral evidence that tetrathiomolybdate can be produced in vitro by cultures of rumen micro-organisms containing large amounts of Mo and S (Mills et al. 1978). Our understanding of this complex interaction is still incomplete and it would be premature to claim that all the toxic effects of Mo in ruminants can be ascribed to thiomolybdate formation.

The types of investigation reported here, involving detailed study of the behaviour and binding of the elements at the molecular level are of great benefit in explaining how the toxic effects of one metal can be related to a disturbance in the metabolism of another. Moreover, they indicate other possible antagonisms yet to be discovered and future areas of research. For example, other metals which can induce synthesis of intestinal metallothionein can be regarded as potential inhibitors of copper absorption. In the same way, other anions which can form thioderivatives may also influence the availability of copper in ruminants. It has already been demonstrated that ammonium tetrathiotungstate can inhibit the

intestinal absorption and hepatic uptake of Cu in the rat (Bremner *et al.* 1979). It will be of considerable interest to establish whether increased dietary tungsten intakes in ruminants result in the development of Cu deficiency.

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