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COMMENTARY

FUNCTIONS OF METALLOTHIONEIN*

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Cadmium is a natural component of the Earth's minerals and it is inevitable, therefore, that both animals and man will absorb the metal through the food chains. The metal once absorbed is retained within the body with very little excretion and thus, even in uncontaminated environments, there is a 'cumulative' absorption of the metal, which can lead to appreciable body burdens. The major part of the body burden is found in the liver and kidney and, in these organs, over 80 per cent of the cadmium is bound to the metallo-protein, metallothionein. This unusual protein was first isolated from horse kidney by Margoshes and Vallee [1] and later characterised by Kägi and Vallee [2, 3]. The latter authors established that the protein was of low molecular weight, water soluble, bound cadmium, zinc and copper, and had an exceptionally high content of cysteinyl sulphur. These characteristics prompted Kägi and Vallee [2] to name the protein "metallothionein". Later studies by Piscator [4] demonstrated that the repeated dosing of rabbits with cadmium led to a progressive accumulation in the livers and kidneys of not only the metal, but also the metallothionein. Piscator [4] proposed that the protein was synthesized in response to the presence of cadmium as a 'protective detoxifying mechanism'. At the same time there was evidence that environmental cadmium pollution was a causative factor in the Japanese Itai–Itai disease (see Tsuchiya [5]) and was a possible hazard to human health in other industrialised countries (see Friberg *et al.* [6]). These observations stimulated a great deal of biochemical and chemical research into the toxicological importance of metallothionein, specifically with regard to its function as a defence mechanism, at least against low level chronic cadmium exposure.

It soon became apparent, however, that other metals, in particular zinc and copper, could also induce metallothionein synthesis, and currently it seems to be accepted that the primary function of metallothionein is in the homeostasis of these essential elements. Important in the development of this concept were two discoveries. Firstly, Bremner *et al.* [7] showed that hepatic metallothionein synthesis occurred once a critical concentration of whole liver zinc (approximately 30 ppm) was exceeded. Secondly, Richards and Cousins [8] demonstrated that zinc administration increased the incorporation of both the cation and labelled amino acids into the metallothionein of intestinal mucosal cells, as well as hepatocytes. From these and later studies, particularly by Cousins and his group (see Ref. 9), dence accumulated that zinc metabolism is regulated by the synthesis of intestinal and hepatic metallothioneins which, respectively, control the efflux of the cation from the mucosal cell and its uptake from the blood by the liver. In the model proposed by Cousins [9] the size of the intracellular zinc pool determines the content of the zinc metallothionein in the mucosal cell. Thus an increased zinc uptake results in more metallothionein synthesis and less absorption into the plasma; conversely a decreased intracellular zinc content results in less metallothionein zinc and a greater zinc absorption into the plasma.

Similar mechanisms have been proposed for the regulation of copper [10] and cadmium absorption [11, 12]. As the mucosal cells turnover rapidly, metals retained in the dose-dependent metallothionein pool are considered to be eliminated by desquamation. It may be emphasized, however, that these models are reversible and can function in the removal of cations from the plasma. In the rat after ingestion of high dietary levels of zinc, for example, the reverse transfer from the serosa to the mucosa exceeds the transfer from the mucosa to the serosa [13]. Intestinal cadmium–thionein is present after the parenteral administration of cadmium [14]; in much of their work on the intestinal zinc-thionein, Richards and Cousins [15–17] induced the metalloprotein by the intraperitoneal injection of zinc. The intestinal mucosa is not a simple structure; its lining epithelium, for example, contains both columnar absorbing cells and secretory goblet cells. Also the Paneth cells, which occur in the crypts of Lieberkühn are rich in zinc and, according to Elmes and Gwyn Jones [17], these cells as well as enterocytes and goblet cells may contain metallothionein. Obviously more work is needed to integrate the biochemical studies with the (immunochemical) localization of metallothionein in the intestine. Thus, whereas Hall, Young and Bremner [18] find a direct correlation between zinc intake over a period of 7 to 8 days and zinc binding to intestinal metallothionein, a number of other recent observations, particularly on zinc absorption over shorter periods of time, do not support the mechanism proposed by Cousins [9]. Jackson *et al.* [19], for example, found that in normal rats, absorption of zinc occurs predominantly by a slow carrier-mediated process and is proportional to dietary intake, whilst the body level is controlled by regulation of the amount excreted. When dietary

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levels of zinc are low, absorption increases; not as a result of decreased mucosal binding, but by the activation of a second high-affinity mechanism. Also the presence of cadmium–thionein in the mucosa does not interfere with the intestinal uptake and transfer of either zinc, or cadmium [14, 20]. Indeed, according to Kello *et al.* [14], metallothionein is not a determinant in cadmium absorption. Hall *et al.* [18] also believe that, under normal conditions, mechanisms other than incorporation into metallothionein regulate the absorption of copper and they suggest that induction of copper–thionein in the rat intestine may occur only when the concentration of copper in the mucosa is increased by some other means.

These "other means", which have yet to be identified, may be significant with regard to the excessive accummulation of thionein-bound copper in the gut, as well as the kidneys of certain mottled mouse mutants (see e.g. Ref. 21). In these mutants, which provide animal models of Menkes' disease, the rate of copper uptake by gut segments is normal [22]. It seems likely, therefore, that the increased metallothionein synthesis in these mice which, perhaps, limits both the intestinal transport and renal metabolism of copper, is due to factors other than the intracellular cation concentrations. One possibility is that thionein synthesis precedes metal uptake. As discussed later, Karin and Herschman [23] conclude that the dexamethasone-induced synthesis of thionein in cultured rat hepatocytes can occur in the absence of increased metal uptake. Under certain conditions, therefore, zinc accumulation may be a consequence of thionein synthesis, not its cause. A similar mechanism could regulate copper uptake, for example in the mottled mouse mutants. This suggestion, however, has a number of obvious limitations. Hormonal control mechanisms, for example, would be expected to act systemically and thus to affect thionein synthesis in the liver as well as the intestine. Also in ruminants, which are particularly susceptible to copper toxicosis [24, 25] increased dietary levels of copper can lead to excessively high hepatic accumulation of the metal without concomitant metallothionein synthesis [25]. Furthermore, zinc supplementation not only is protective, but also increases the hepatic concentration of thionein-bound copper. Induction of the synthesis of this protein, either by a hormonal process, or as a direct response to copper, therefore, would not seem to be a significant regulatory mechanism of copper absorption in these animals. If this is true, it could explain why, as in human-beings with Wilson's disease, 2,3-dimercaptopropanol (BAL) and certain other chelating agents are effective in removal of excess copper [26] but, at best, have only limited therapeutic activity in the treatment of cadmium toxicity when, as in chronic exposure, most of the accumulated metal is bound as the metallothionein.

Hepatic metallothionein synthesis in the rat in response to the injection of the appropriate dose of zinc begins between two and four hours after dosing and requires the induction of m-RNAs [27]. Whilst the latter may be a direct response to elevated intracellular concentrations of zinc, there are also other possibilities. In the model of Cousins [9] the major factor affecting metallothionein synthesis is the cytosolic zinc concentration, which is regulated by the level of zinc in the plasma. An elevated zinc plasma level produces an increase in cytosolic zinc and stimulates the production of metallothionein m-RNA and subsequently metallothionein. Significantly, a variety of physiological stress conditions (Table 1), such as exposure to low temperature, food restriction, bacterial infection, adrenalectomy, hepatectomy and sham operation, can lead to profound changes in plasma zinc level. Apart from the sham operation experiments of Brady [34], pronounced hypozincaemia, accompanied by an increased hepatic zinc–thionein concentration is a consistent finding in these studies (Table 1). As, however, sham operation increases both the blood and liver concentrations of zinc, it is possible that elevation of plasma zinc is common to different stresses but, with some of them, it is exceeded by the liver uptake.

The reasons for the hepatic metallothionein synthesis under conditions of stress may be due to changes in the levels of adrenal and pituitary hormones. Various glucocorticoids have been shown to stimulate zinc uptake and zinc-thionein formation in isolated heaptocytes [23, 36] and HeLa cells [37]. Of these, dexamethasone is the most potent. There is some disagreement whether the increased zinc influx precedes or follows the production of thionein. Failla and Cousins [36] maintain that synthesis is induced by zinc and that the glucocorticoid action results in the synthesis of other m-RNAs which are encoding for proteins involved in zinc transport across the hepatocyte membrane. In contrast Karin and Herschman [23] consider that dexamethasone

Table 1. Zn–MT synthesis in response to physiological stimuli

Stimulus	Hepatic zinc	Zn–MT	Plasma zinc	Author
Stress (cold, exercise, CCl_4)	No change	↑	↓	Oh *et al.* [28]
Food restriction	↑	↑	NT*	Bremner and Davies [29]
Bacterial infection	NT	↑	↓	Sobocinski *et al.* [30]
Endotoxin	↑	↑	↓	Sobocinski *et al.* [30]
Endotoxin	↑	↑	—	Suzuki [32]
LEM (Leukocytic endogenous mediators)	↑	↑	↓	Sobocinski *et al.* [31]
Adrenalectomy	↑	↑	NT	Brady *et al.* [33]
Sham operation	↑	↑	↑	Brady *et al.* [34]
Hepatectomy	↑	↑	↓	Ohtake *et al.* [35]
Sham hepatectomy	↑ ↓	↑ ↓	↑ ↓	Ohtake *et al.* [35]

* NT: not tested.

is a primary inducer of metallothionein m-RNA, which leads to the synthesis of the metal free apoprotein, thionein. This protein binds any available zinc, thereby stimulating zinc uptake. The latter hypothesis is supported by the observation that dexamethasone increases metallothionein synthesis in the liver of the adrenalectomised rat and thereby results in a redistribution of zinc in the cytosol [39]. This pattern of changes is observed also after low temperature exposure, or excessive exercise [28] and thus may indicate the involvement of hormones in the stress-response. The finding that binding of dexamethasone to receptor sites in the nucleus precedes the synthesis of metallothionein in HeLa cells [37] also indicates a direct effect of glucocorticoids on the production of thionein m-RNA. Various other steroids also stimulate this synthesis, and have different affinities for the dexamethasone binding site. This is apparent particularly with the intermediate effectors such as progesterone and aldosterone, which inhibit the activity of dexamethasone by competition for this binding site [38].

Irrespective of the precise induction mechanism, it is clear that zinc–thionein synthesis responds readily to variations in zinc level and thus complies with an essential requirement for a homeostatic mechanism. Another criterion for such a function is that the metal should be readily available. A physiological role for zinc–thionein in cation insertion has been inferred from observations that, *in vitro*, this metallothionein is able to donate its bound cation to apoproteins of various zinc-dependent enzymes and thereby restore activity to the latter [40, 41]. Similar findings have been reported for copper–thionein and the metal-free forms of certain copper proteins [42]. It is interesting and, perhaps, contrary to expectations from current theories of the structural arrangements at the metal binding sites, that the presence of cadmium in the metallothionein does not seem to have any significant effect on zinc exchange [41]. There is, of course, no evidence that these observations on *in vitro* systems are relevant to the living animal and, *in vivo*, it is possible that the metal is made available by thionein degradation.

In vivo zinc–thionein not only has a short half-time, but the rates of zinc turnover and protein-breakdown are the same. When cadmium is incorporated in the metallothionein molecule the half-time of the latter increases with the Cd:Zn ratio [43]. This could affect zinc-homeostasis and may be relevant to some of the toxic effects of cadmium. As yet, however, there is no direct evidence that degradation of zinc–thionein does provide a source of zinc for other cellular functions. Nevertheless, Ohtake *et al.* [44] believe that zinc–thionein synthesis precedes and is essential for the synthesis of DNA in regenerating liver after partial hepatectomy. These authors claim that only isometallothionein II is produced in the regenerating liver and suggest that this form has a specific role in the regulation of DNA synthesis. As it is difficult to understand why the hepatocyte needs two, apparently very similar major isometallothioneins, this hypothesis is attractive as it proposes a specific function for one of them. Unfortunately, it is not true; both isometallothioneins are synthesized after partial hepatectomy although, as shown in Fig. 1, not at the same rate. Thus synthesis, or accummulation, of isometallothionein II is greater than that of isometallothionein I until 18 hr, when the concentrations of both forms begin to decrease. The same synthetic pattern is observed after partial hepatectomy in cadmium-treated rats and it seems that in these animals also zinc–thionein II is synthesized in excess of zinc–thionein I but, because of this additional synthesis, there is redistribution of cadmium between the two forms. This, of course, may reflect nothing more than the normal post-operation changes in metabolism. The apparent increased general protein synthesis in the liver after partial hepatectomy is known to be due to a reduction in the rates of cellular protein degradation [45, 46]. Thus the greater accumulation of metallothionein II under these conditions probably is a complex interaction, due to both increased synthesis and decreased degradation.

Another system in which to investigate the functions of metallothioneins in cell growth is provided by the foetal or newborn animal. Metallothioneins occur in high concentrations in the livers of newborn pigs, foetal and newborn rats and both human and lamb foetuses (see e.g. Ref. 47). In the liver of the human foetus, as in the foetal lamb, the concentration of the metallothionein, which contains both zinc and copper, is maximal during gestation [48, 49]. In the foetal and postpartal rat, however, the copper content of the hepatic metallothionein is very low, although it does increase with age after birth [50]. The concentration of thionein-bound metals is below the limit of detection at 16 days of gestation, but increases rapidly thereafter to a maximum at 2 days *post partum*. Treatment of the dam on or after the 16th day of pregnancy with an acute dose of cadmium inhibits the placental transport of zinc [51] and thus, at birth, the pups of these treated dams are zinc-deficient (Fig. 2). As, however, birth eliminates the block to transport, the hepatic concentrations of thionein-bound and total zinc in these pups then increase rapidly to reach maxima similar to, but about 5 days later than those in the normal newborn. It seems, therefore, that either there is a requirement

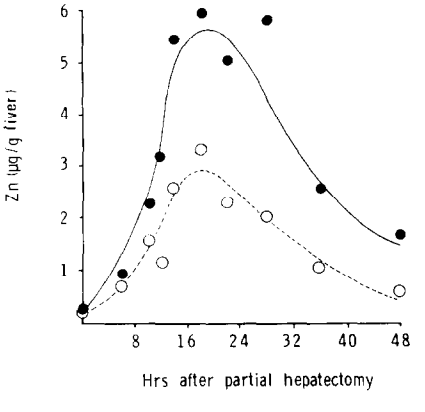

Fig. 1. Changes in the liver concentrations of Zn^{2+} bound to metallothionein-I (○---○) and metallothionein-II (●——●) with time after partial hepatectomy (K. Cain and B. Griffiths, unpublished results).

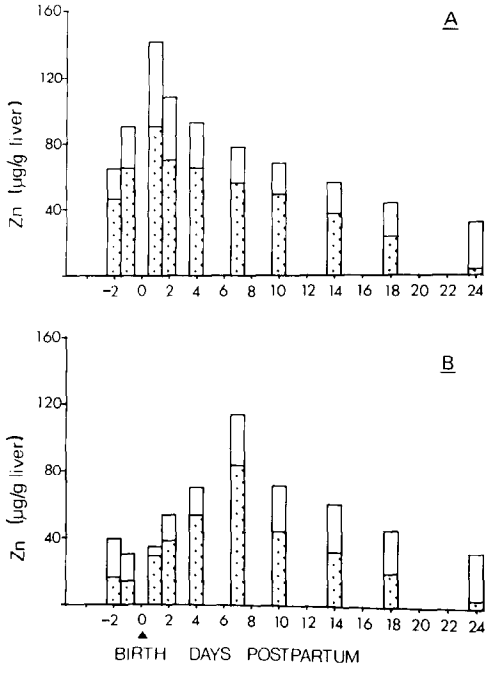

Fig. 2. Changes in the hepatic concentrations of total (unshaded columns) and thionein-bound Zn^{2+} (dotted columns) in foetal and newborn rats from (A) normal dams and (B) dams after treatment with Cd^{2+} (1.0 mg/kg body weight, i.v.) on the 18th day of gestation (figure redrawn from Ref. 46).

for zinc metallothionein during this period of development, or the metalloprotein is produced as a result of some other essential metabolic process.

Between 2 and 26 days *post partum* both body weight and liver weight increase exponentially with age. Mason *et al.* [50] have shown that, throughout this period, the total hepatic zinc content in the normal rat increases steadily, whereas the total zinc-concentration declines progressively to the adult level at 26 days. The content of thionein-bound zinc, however, remains constant for the first 16 days and then decreases. As from birth to 26 days of age, the concentration of zinc in the non-thionein fraction of the cytosol remains constant, it has been suggested that the metallothionein may provide a major reserve of zinc for the maintenance of this concentration. The hepatic concentration of metallothionein-bound copper, in contrast with that of zinc, remains low from birth to 6 days of age and then increases to a maximum at 15 days, at which time dramatic changes occur in the distribution of this metal in the intestine [51].

In the rat gastrointestinal tract the concentration of copper increases from about 17 μg just before birth to 140 μ/g wet weight tissue at 2 days *post partum*, apparently because at least 60 per cent of the copper ingested from the maternal milk is retained therein [52]. Over the same period, most of the ingested zinc is absorbed and only about 7.5 per cent remains in the intestinal mucosa. With the growth of the pup, the intestinal copper concentration falls, at first slowly between 2 and 13 days of age and then rapidly, from about 80 to 11 μg/g tissue during the next 4 days. At all times, however, about 60 per cent of this copper is in the soluble fraction of the tissue. The intestinal concentration of zinc not only is much lower than that of copper but also a smaller percentage of the total is in the soluble fraction of the tissue, mainly in association with proteins of high molecular weight; zinc–thionein is conspicuous by its absence. In contrast, when the mucosal extracts are submitted to gel filtration, most of the soluble copper is found in an extremely heterogeneous, polydisperse fraction (Fig. 3). This copper complex, at least in the intestine of the Wistar rat, is difficult to isolate and purify on a preparative scale and, thus far, its identity has not been established. At this stage even the tentative assumption that it is a mixture of polymeric copper–thioneins is unjustified. Nevertheless, the analogous complex from the intestine for the 5 day-old Long–Evans rat, which contains zinc in addition to copper, has been resolved by Johnson and Evans [53] into two main fractions, the amino acid compositions of which characterize them as metallothioneins.

Most of the copper is lost from the intestinal copper complex of the Wistar rat between the 13th and 15th day after birth, and at 21 days, this cation is associated only with two clearly defined fractions, the second of which also contains zinc and has the properties of a metallothionein. From these observations it seems possible that, before the closure of the intestine in these rats, a mechanism exists to limit the absorption of copper but not of zinc. In consequence zinc metabolism is regulated by the liver, specifically by the synthesis of metallothionein, until the intestine acquires the functional capacity of the adult.

This hypothesis has limitations in that foetal and/or newborn rabbits, Syrian and Chinese hamsters, two strains of mice and the human infant, have hepatic metallothioneins which vary greatly in metal composition and content according to species, as well as age [47]. The hepatic metallothionein of either the newborn rabbit, or Chinese hamster, for example, is a zinc metalloprotein, whereas that of the Syrian hamster contains appreciably more copper than zinc. In the last of these species, however, no polydisperse copper complex is detectable in the

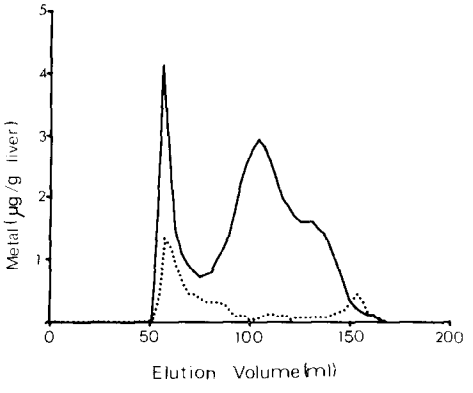

Fig. 3. Distribution of copper (———) and zinc (– – – –) in the soluble fraction from the intestine of the 6-day-old rat (previously unpublished data).

intestine. Possibly, therefore, the newborn of some species lack an intestinal control mechanism of copper absorption and, in consequence, the metabolism of this cation, as well as that of zinc, is regulated by the liver.

Ion exchange chromatography of these different metallothionein preparations revealed that, whereas isometallothionein I is the predominant species in the liver of a human full-term still-birth [47], the concentration of isometallothionein II exceeds that of isometallothionein I in the liver of the newborn rat [50]. Also essentially only one isometallothionein seems to be present in either the kidney or testis of the male rat before 35 days of age [54]. Thus the relative proportions of the isometallothioneins not only vary with tissue and species but, in the same tissue and species, differ between the newborn and the adult. At present the reason for this difference is unknown. It may be significant, however, that when cell proliferation in the liver of the adult rat is stimulated through partial hepatectomy, the ratio of the two isoforms of the metallothionein also changes with time (Fig. 1).

In the newborn Wistar rat the decrease in hepatic zinc–thionein content occurs at the time when the growth pattern begins to change from increase in cell number to increase in cell size. Ohtake *et al.* [35, 44] would regard this as evidence of the association between the metallothionein concentration and DNA synthesis. If, however, this association exists, a high concentration of zinc–thionein might be expected in most organs of the newborn rat, which grow by cell proliferation until 16 to 20 days of age. Evidence for this is lacking. In the kidney and testis [54], for example, the contents of thionein-bound zinc as well as copper, remain low for at least 30 days after birth, i.e. during growth by cell proliferation.

Both the intestinal copper complex and the hepatic zinc–thionein of the neonatal rat provide additional, immediately available, high-affinity binding sites for foreign cations, such as cadmium and mercury. Retention and distribution of these cations, therefore, differ in the newborn and adult. Binding of cadmium to the copper complex probably explains the observations of Sasser and Jarboe [55] that this cation persists in the gastrointestinal tract of the neonate as a "metallothionein-like-protein" until excreted through extrusion of the mucosal cell into the intestinal lumen. Mercury also is retained in this complex [56] and it is not surprising, therefore, that newborn rats are more resistant than adults to orally administered cadmium or mercury. Their whole body retention of these cations may be greater, but the amount absorbed into the systemic circulation will be less.

As previously stated it is well established and generally accepted that the inducible synthesis of metallothioneins in the liver and kidney of the adult animal provides a detoxification mechanism against chronic exposure to cadmium and, perhaps, to other metals. It is probable, however, that it may be due to nothing more than the fortuitous interactions of these foreign cations with the normal homeostatic mechanisms for zinc and copper. Also metallothionein synthesis in response to cadmium, mercury or gold is not necessarily protective. Renal tubular damage, which is a frequent manifestation of heavy metal toxicity, for example, can result from a primary action of the cation on the brush border membrane. Under these conditions, intracellular metallothionein synthesis can occur after the damage has been done. Thus in the rat parenteral administration of cadmium together with excess cysteine leads to the rapid renal uptake of a toxic amount of cadmium and to appreciable synthesis of metallothionein between 2 and 7 hr [57]. The animals, however, die from renal tubular damage which, even by light microscopy, is clearly apparent by 4 hr and by 7 hr is extensive. Also administration of gold leads to the accumulation of a metallothionein, which contains both gold and copper, in the kidneys of the rat and guinea pig, but not of the hamster and rabbit [58]. Renal damage, however, is common to and similar in all four species, irrespective of gold binding to metallothionein.

At chronic exposure to cadmium the kidney is the critical organ and it seems a common assumption that the onset of damage is correlated with a renal concentration of about 200 μg cadmium/g wet wt (see, e.g. Ref. 51). More probably, however, damage is related to the concentration of non-thionein-bound cadmium and, according to Nomiyama [59] this is not a fixed percentage of the total cadmium, but varies with the species and method of dosing. Nevertheless the existence of a critical concentration of cadmium in some form suggests that damage results from intracellular interactions. Thus far, however, the intracellular targets of toxicity have not been identified.

A contributory factor to the renal accumulation of cadmium at chronic exposure is the slow transfer of the cation from the liver to the kidney. Some years ago Piscator [3] proposed that this transfer occurred through the renal reabsorption of cadmium–thionein released into the blood from the liver. As isolated cadmium–thionein, when injected or infused into normal animals is accumulated selectively by the kidney and can be nephrotoxic [60] it has been suggested that such transport could have special significance in the development of renal dysfunction. The experiment described by Webb [61] provides an example of this liver to kidney transfer in rats which, at 16 weeks after dosing, had a hepatic content of 150 μg cadmium. During the next 54 weeks, the hepatic cadmium decreased by 56 μg whilst the renal content increased by 30 μg; the average rates of renal uptake and liver loss being 0.06 ng cadmium/min and 0.1 ng cadmium/min, respectively. Thus even if this transfer occurred by the release of hepatic cadmium–thionein, the concentration of the metalloprotein in the blood at any time probably would be too low, either to be detectable, or to produce any specific toxic response in the kidney.

In summary, it seems that thionein is a normal foetal protein, which functions in zinc and probably copper homeostasis during gestation and early post-natal life. The capacity for the synthesis of this protein is retained in the adult and can form a control mechanism when serious disturbances occur in the metabolism of cations *In vitro* zinc– and copper–

thioneins can act as cation donors and restore functional activity to the apoproteins of appropriate metalloenzymes. It is not known, however, whether this is relevant to *in vivo* systems, in which degradation of the metallothioneins could provide a mechanism for cation release. Induction of metallothioneins by cations such as cadmium, mercury and gold, the last of which seems to be determined in some way by alterations in copper metabolism, probably is an expression of the homeostatic function. The selective renal accumulation of cadmium–thionein in the kidneys is compatible with but does not prove, a role of the metalloprotein in the transport of cadmium, or any other cation, from the liver to the kidneys.

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