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Properties of cadmium-binding proteins in rat testes

Characteristics unlike metallothionein

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Since the exposure of rats to cadmium causes zinc to accumulate in metallothionein in liver and kidney but not in a similar protein in the testes, the properties of the low-M_r cadmium-binding proteins were investigated in rat testes. Weanling rats that had been given dietary cadmium for 6 weeks were injected with $^{109}CdCl_2$ and subsequently killed, and the ^{109}Cd-labelled low-M_r proteins from testes were purified. The pooled low-M_r cadmium-containing fractions from the gel-filtration (Sephadex G-75) columns were eluted through DEAE-Sephacel columns, yielding two peaks. Each of the individual peaks from this Sephacel column was further purified by rechromatography on DEAE-Sephacel and on Bio-Gel P-10 columns. Amino acid analysis of the two purified proteins revealed a low cysteine (about 3%) content, with aspartate, glutamate and glycine as the predominant amino acids. Thus these low-M_r cadmium-binding proteins induced by cadmium in rat testes do not appear to be metallothionein.

INTRODUCTION

Cadmium has been shown to be bound to metallothionein (MT) in liver and kidney of animals (Kägi & Vallee, 1961; Winge & Rajagopalan, 1972; Squibb & Cousins, 1974; Chen *et al.*, 1975; Shaikh & Smith, 1976). Even though cadmium is bound to predominantly one protein, MT, in liver and kidney (Webb, 1972), this metal is bound to three different low-M_r proteins in the testes (Chen *et al.*, 1974*a*; Deagen *et al.*, 1980*a*, *b*). Since the smallest cadmium-containing peak is eluted at the same position as MT, this metal has been assumed to be bound to MT in the testes (Wisniewska-Knypl *et al.*, 1971; Chen & Ganther, 1975).

While studying cadmium binding to tissue protein, we found evidence that cadmium did not cause an accumulation of zinc in what was assumed to be testes MT as it does in liver or kidney MT (Whanger *et al.*, 1980). Cadmium treatments do not cause an accumulation of zinc in what was assumed to be MT in tissues of the American oyster (Frazier, 1979). Other workers have shown that this latter cadmium-binding protein does not appear to be MT since it has a low cysteine (7.6 mol/100 mol) content (Ridlington & Fowler, 1979). Singh *et al.* (1974) partially purified the cadmium-binding protein from rat testes and found that it differed from the low-M_r proteins in liver and kidneys in absorption spectra, M_r and behaviour on disc-gel electrophoresis. Zinc did not accumulate in what was thought to be testes MT as it did in liver, kidneys and pancreas MTs when a bull was given excess dietary zinc (Whanger *et al.*, 1981). In the light of these observations, we became suspicious that the cadmium-binding proteins in testes were not MTs, and the present investigation was undertaken to study the properties of these proteins. While the present paper was in preparation, a publication appeared confirming our suspicion that the low-M_r cadmium-binding proteins in testes were not MT (Waalkes *et al.*, 1984*a*). Since we found slightly different results, it was decided to proceed with our plans to publish our work. A preliminary report of some of the present studies has been presented (Deagen *et al.*, 1980*a*).

METHODS

Long Evans weanling male rats were fed on a basal casein diet composed of casein, cerelose, corn oil, salt mixture and vitamins as previously described (Chen *et al.*, 1974*b*), with addition of 100 p.p.m. of cadmium, for 6 weeks. Feeding rats with dietary cadmium was done because our earlier work showed that this would cause a much greater uptake of ^{109}Cd in the low-M_r fraction of rat testes (Deagen *et al.*, 1980*b*). The low-M_r cadmium-binding proteins were purified from 30 of these rats that had been injected with 26 μCi of $^{109}CdCl_2$ per rat and killed 6 days later. The testes were removed, yielding about 88 g from all rats, and immediately homogenized on ice in 170 ml of 50 mM-Tris/HCl buffer, pH 8.4, containing 0.85% NaCl. This was centrifuged at 10000 *g* for 20 min, and the supernatant was subsequently centrifuged at 105000 *g* for 90 min to obtain the cytosol.

The ^{109}Cd-labelled low-M_r cadmium-binding protein was purified by gel filtration and ion-exchange resin chromatography. The cytosol was first eluted through a column of Sephadex G-75 (obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.), and the fractions containing the low-M_r cadmium-binding proteins were combined, dialysed against 3 mM-Tris/HCl buffer, pH 8.4, and chromatographed on DEAE-Sephacel columns (Sigma Chemical Co.). The individual peaks were subsequently dialysed against 3 mM-Tris/HCl buffer, pH 8.4, and rechromatographed on DEAE-Sephacel columns. Finally, the pooled fractions from each peak were dialysed against water and freeze-dried. The freeze-dried samples were dissolved in 50 mM-Tris buffer, pH 8.4, and chromatographed in this same buffer on columns of Bio-Gel P-10 (Bio-Rad Laboratories,

Abbreviation used: MT, metallothionein.

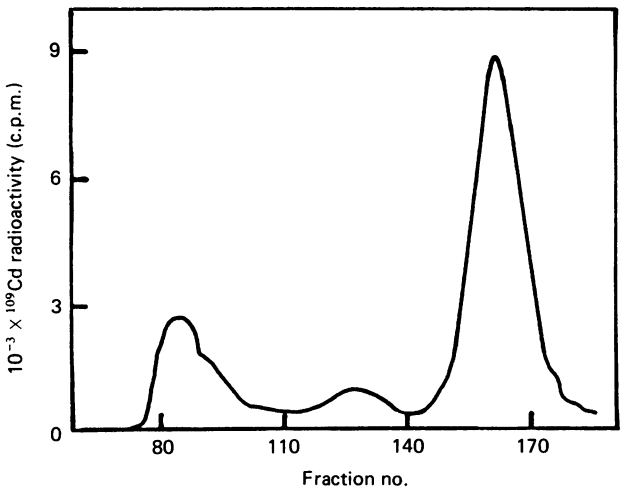

Fig. 1. Gel filtration of cytosol from rat testes

Cytosol (175 ml) was applied to a Sephadex G-75 column (5.0 cm × 150 cm) and eluted with 50 mM-Tris/HCl buffer, pH 8.4, at 4 °C. From 12 to 15 ml was collected per tube, at a flow rate of 120 ml/h.

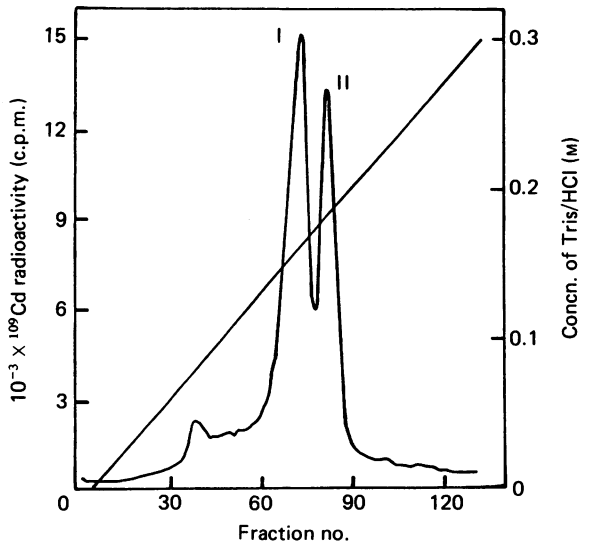

Fig. 2. DEAE-Sephacel chromatography of the major ^{109}Cd-labelled peak from the gel-filtration step

Fractions 148–177 from the gel-filtration step were pooled, dialysed and eluted through a column (2.5 cm × 20 cm) of DEAE-Sephacel. A gradient from 3 mM- (400 ml) to 300 mM- (400 ml) Tris/HCl buffer, pH 8.4, was used to elute the sample through this column. The Tris concentration at which the peaks were eluted was measured from the gradient profile. Fractions (6.3 ml) were collected at a flow rate of 50 ml/h at 4 °C.

Richmond, CA, U.S.A.). Each sample was subjected to polyacrylamide-gel electrophoresis (Bio-Rad model 150 apparatus) to assess the purity of the final products (Davis, 1964). The gels were fixed and stained in a solution of Coomassie Blue in 7% (v/v) acetic acid for 4 h. They were destained in 10% (v/v) acetic acid with a Bio-Rad model 172A diffusion destainer apparatus. The purified samples were subjected to performic acid oxidation (Moore, 1963), and subsequently hydrolysed with constant-boiling 6 M-HCl at 100 °C for 24 h. Amino acid analyses were performed on these hydrolysates with a Beckman Instrument Co. model 120B amino acid analyser on columns of Dionex DC6A ion-exchange resins, with sodium citrate buffers, pH 3.22, 4.12 and 6.17 (Moore & Stein, 1963).

RESULTS

The elution pattern for the testes cytosol through the Sephadex G-75 column is shown in Fig. 1. Three peaks of cadmium were eluted from the column, and the third peak (fractions 148–177), which is the low-M_r one eluted at the position of MT, was pooled for the next step. Three zinc peaks also co-chromatographed with each of the three ^{109}Cd-labelled peaks, but cadmium exposure had no effect upon the relative distribution of zinc between these peaks (results not shown).

The pooled peak from the gel-filtration step was dialysed against 3 mM-Tris/HCl buffer, pH 8.4, and eluted through a column of DEAE-Sephacel (Fig. 2). Two major ^{109}Cd-labelled peaks and a minor peak (fractions 34–44) were obtained from this step. Peaks I and II, which also contained zinc (results not shown), were eluted respectively at 160 mM- and 180 mM-Tris/HCl. Peaks I and II were individually pooled (fractions 63–77 and 78–90) and rechromatographed on columns of DEAE-Sephacel (Fig. 3). Peak I was eluted as a symmetrical peak (91 mM-Tris), with only traces of cadmium elsewhere (Fig. 3*a*). The fractions (29–35) of the main peak were pooled and chromatographed on a column of Bio-Gel P-10. As expected, since peaks I and II were eluted so close together on the first DEAE-Sephacel column, there was further separation of peak I from peak II (130 mM-Tris) when peak II pooled fractions were rechromatographed on DEAE-Sephacel (Fig. 3*b*). The fractions (41–47) of peak II were pooled and chromatographed on a column of Bio-Gel P-10.

Slightly further purification was obtained when the two individual peaks were chromatographed on Bio-Gel P-10 columns (results not shown). A small ^{109}Cd-labelled peak was eluted ahead of each of the major peaks. Both of the major peaks were eluted in a symmetrical fashion.

Disc-gel electrophoresis (Fig. 4) indicated that both peaks I and II were nearly pure, consistent with the symmetrical peaks obtained from these columns. One predominant band was obtained for each of the two testis proteins. There was a small amount of foreign protein in the peak I preparation, but the peak II protein appeared to be homogeneous. The respective R_m values for the peak I and peak II proteins were calculated to be 0.14 and 0.62. Bromophenol Blue (0.1%) was used as the tracking dye, which migrates towards the positive pole. Shown for comparison are the two respective MT species from rat liver, which were purified as indicated previously (Ridlington *et al.*, 1983). The respective R_m values of 0.39 and 0.61 are very close to previously reported values. The R_m for liver MT II is very similar to that for the testis II cadmium-binding protein.

As indicated in Table 1, these cadmium-containing proteins possess a low cysteine content. This content was less than 3%, with aspartate, glutamate, glycine and alanine being the predominant amino acids. Included for comparison are the amino acid compositions of the cadmium-binding protein from oysters (Ridlington &

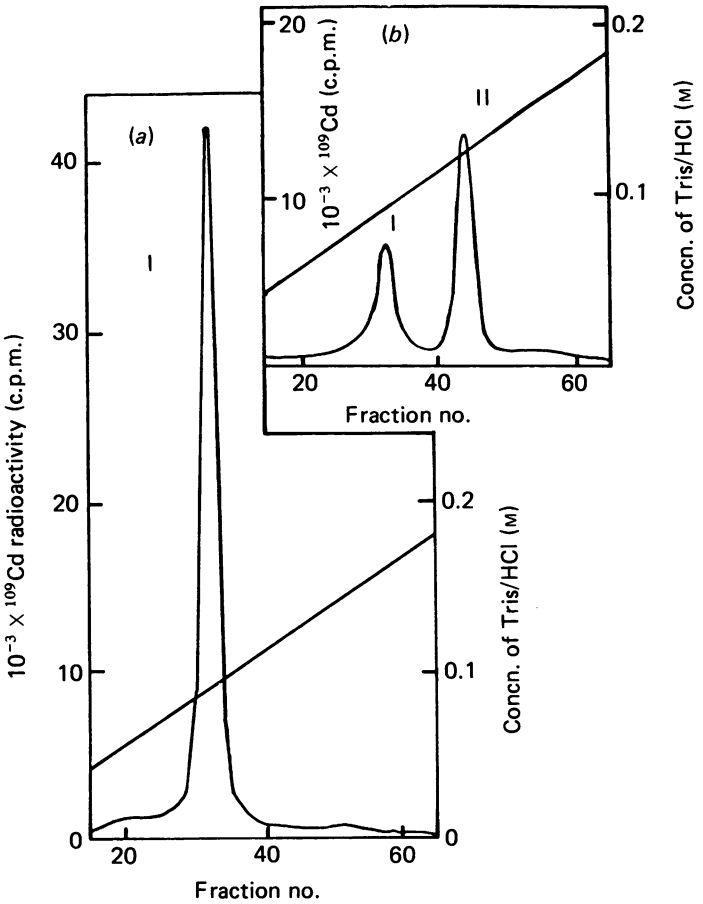

Fig. 3. Rechromatography of the cadmium-binding proteins from rat testes on DEAE-Sephacel

The peaks from the DEAE-Sephacel column (Fig. 2) were individually rechromatographed on DEAE-Sephacel columns (1.5 cm × 12 cm). A gradient from 3 mM- (200 ml) to 300 mM- (200 ml) Tris/HCl buffer, pH 8.4, was used. The Tris concentration at which the peaks were eluted was determined as indicated in Fig. 2. About 4.3 ml was collected per fraction, at a flow rate of 21.5 ml/h at 4 °C.

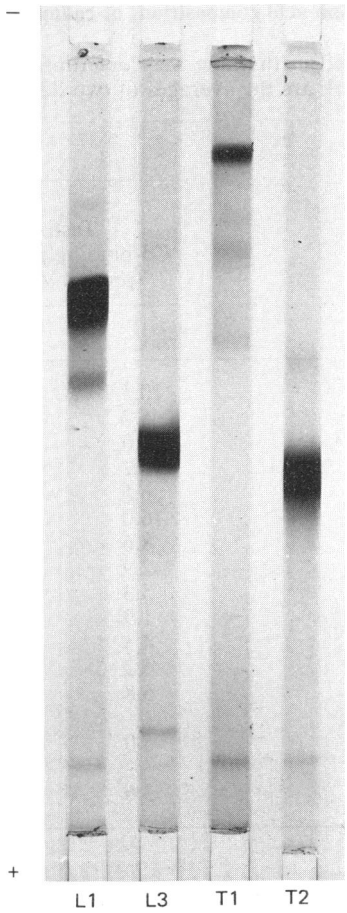

Fig. 4. Polyacrylamide-gel electrophoresis of the two rat liver MT species and the two rat testis cadmium-binding proteins

The respective R_m values for the rat liver MT I and MT II and for rat testis proteins I and II are 0.38, 0.61, 0.14 and 0.62. L1, L2, T1 and T2 represent respectively liver MT I and MT II and testis proteins I and II. The tracking dye is at the bottom of the gels, which is the positive pole.

Fowler, 1979), of cadmium-binding proteins from rat testes reported by another research group (Waalkes *et al.*, 1984*a*) and of those from rat liver that were purified (Whanger & Deagen, 1982) by a procedure similar to that used in the present work. The amino acid compositions of our preparations are remarkably similar to those reported by others for rat testes, except for serine, glycine, leucine, cysteine and histidine (Waalkes *et al.*, 1984*a*). There are some slight differences as well as many similarities in amino acid composition between the oyster protein and the rat testis proteins. In comparison with the amino acid composition of MT, these results indicate that the major cadmium-binding proteins in testes of rats are not MT.

DISCUSSION

The present results support our earlier suspicions (Whanger *et al.*, 1980, 1981; Deagen *et al.*, 1980*a*) and are consistent with the work of Waalkes *et al.* (1984*a*) showing that the cadmium-binding proteins in rat testes are not MT, on the basis of amino acid composition. However, our results differ slightly from the data of Waalkes *et al.* (1984*a*), and may be due either to different animal preparations or to different cytosol treatments. In their work, testicular cytosol was prepared from unexposed rats and saturated with non-radioactive cadmium before subjection to the purification procedures. In contrast, our rats were given dietary cadmium for 6 weeks in order to induce a greater uptake of a subsequent radioactive dose of ^{109}Cd (Deagen *et al.*, 1980*b*) before they were killed for the isolation of the proteins. Both of the cadmium-containing proteins isolated by Waalkes *et al.* (1984*a*) from rat testes had an R_m very similar to that of liver MT II, whereas only peak II in our preparation had a similar value to that of MT II (Fig. 4). The testes peak I protein had an R_m value (0.14) markedly less than those of either of the hepatic MT species. It may be possible that saturation *in vitro* of the cytosol with cadmium may have caused binding to other proteins besides the ones binding this element *in vivo*.

In other work from this same laboratory (Waalkes *et al.*, 1984*b*), evidence was presented to indicate intercon-

Table 1. Amino acid compositions of cadmium-binding proteins in rats testes and liver, and in oysters

Cysteine and methionine were determined respectively as cysteic acid and methionine sulphone. The values for testes in the present work are the averages of two determinations.

	Amino acid composition (mol/100 mol)						
	Testis Cd-binding proteins (present work)		Testis Cd-binding proteins (Waalkes *et al.*, 1984*a*)		Rat hepatic MT (Whanger & Deagen, 1982)		Oyster Cd-binding protein (Ridlington & Fowler, 1979)
Amino acid	I	II	I	II	I	II	
Aspartate	10.5	11.3	6.8	9.4	6.8	6.8	12.9
Threonine	5.2	5.4	5.5	4.6	6.7	2.8	5.8
Serine	9.0	11.0	8.0	5.6	15.2	18.5	7.3
Glutamate	10.2	11.2	10.4	11.5	2.1	4.6	9.7
Proline	6.5	3.2	4.3	6.8	3.2	3.9	4.4
Glycine	16.0	16.7	11.9	11.2	9.1	6.3	9.5
Alanine	8.6	9.4	8.6	6.2	5.1	7.6	6.6
Cysteine	2.9	2.6	7.2	5.4	34.5	32.0	7.6
Valine	5.3	3.9	5.9	7.1	3.3	1.6	6.1
Methionine	1.9	—	2.5	1.4	1.4	1.7	1.5
Isoleucine	4.3	2.2	4.6	2.6	0.2	1.4	3.9
Leucine	5.2	3.3	6.7	9.0	0.3	0.4	4.5
Tyrosine	5.8	4.0	1.5	2.3	—	0.3	2.6
Phenylalanine	—	2.8	2.3	2.7	—	—	3.5
Lysine	5.0	7.0	7.5	7.7	12.0	11.9	8.9
Histidine	—	4.3	2.5	2.7	0.1	0.2	2.3
Arginine	3.6	1.7	3.9	3.7	—	—	2.9

version between the cadmium-binding proteins in testes. Cadmium saturation *in vitro* may have affected these interrelations. In support of this, the greatest amount of cadmium was present in peak I in samples subjected to gel filtration (see Fig. 1 in Waalkes *et al.*, 1984*a*), whereas the greatest amount of cadmium was present in peak III in our samples subjected to gel filtration (Fig. 1). The major difference in purification procedure was the use of Bio-Gel P-10 in our studies. However, if the proteins purified by us or by Waalkes *et al.* (1984*a*) are homogeneous, the differences in purification procedure should not account for the variance in the final products.

Except for serine, glycine, leucine, cysteine and histidine, the amino acid compositions of our preparations are very similar to those found by the Kansas group (Waalkes *et al.*, 1984*a*). We found higher amounts of serine, glycine and tyrosine but lower amounts of cysteine and leucine. They found equal amounts of phenylalanine, methionine and histidine in both forms of the testes protein, whereas we found phenylalanine and histidine only in the peak II protein and methionine only in the peak I protein. Except for glycine and cysteine, the cadmium-binding proteins of rat testes are remarkably similar to the cadmium-containing protein in oysters (Ridlington & Fowler, 1979). These results support our earlier contention (Whanger *et al.*, 1980; Whanger & Deagen, 1982) that similar elution positions of metals from a gel-filtration column cannot be taken as evidence for binding to MT. The proteins must be purified and the amino acid compositions determined to have assurance of the type of protein binding the metal. Several metals in many different organisms have been suggested to be bound to MT on the basis of the elution patterns on gel filtration (see Kägi & Nordberg, 1978), but the present results indicate that this is not sufficient, and identification by amino acid analysis is the ultimate test.

The present work and that of Waalkes *et al.* (1984*a*) are consistent with some other work on mice testes (Durnam & Palmiter, 1981). Except for liver, the MT mRNA content was highest in testes, of several tissues examined, in unexposed mice. However, exposure of mice to cadmium did not cause a significant increase of MT mRNA in testes, but marked increases (6–60-fold) occurred in all other tissues examined (Durnam & Palmiter, 1981). This suggests that low MT concentrations may be present in testes, but that cadmium exposure does not elevate its concentration but instead increases those of other cadmium-binding proteins. When this is done, this makes the MT concentrations appear even lower in comparison with the cadmium-binding proteins in testes. The difficulty in detecting MT in testes even though the MT mRNA content is high is analogous to the situation with the liver, another tissue with high MT mRNA contents in unexposed rats (Durnam & Palmiter, 1981). MT concentrations are essentially undetectable in liver of unexposed rats, but, in contrast with the testes, become readily detectable in exposed rats (Squibb & Cousins, 1974; Chen *et al.*, 1974*b*, 1975, 1977; Leber & Miya, 1976; Kägi & Nordberg, 1978; Deagen *et al.*, 1980*b*). Therefore one of the minor cadmium-containing peaks from the testes preparations may be MT. The first peak (fractions 35–45) from the DEAE-Sephacel column (Fig. 2) could be this protein.

The two larger-M_r cadmium-binding proteins (peaks I and II from the gel-filtration step) have also been characterized (Waalkes *et al.*, 1984*b*). The amino acid compositions of these two groups of proteins are similar to those of the low-M_r ones discussed in the present work

and in their other work (Waalkes *et al.*, 1984*a*). The major difference appears to be twice the cysteine content (13.5 mol/100 mol) in one of the proteins as compared with the low-M_r ones of 5–7 mol/100 mol.

Prior exposure of rats to cadmium makes them more tolerant to subsequent doses of this element (Leber & Miya, 1976), and treatment of rats with a half-threshold dose of cadmium decreases the testicular damage due to subsequent cadmium doses (Chen *et al.*, 1974*a*). Since prior administration of cadmium causes increased uptake of subsequent doses of cadmium in the low-M_r fraction of rat testes (Deagen *et al.*, 1980*b*), this was the reason the rats in the present study were fed with non-radioactive cadmium for 6 weeks before injection of $^{109}CdCl_2$. This provided greater quantities of protein for purification. Thus, if the concentrations of these binding proteins are increased by cadmium treatment, this testicular response may offer some protection against cadmium damage analogous to that by MT in liver and kidney (Winge & Rajagopalan, 1972).

The present results indicate that cadmium is bound to three different M_r groups of proteins, as indicated by gel filtration in the testes, which is in agreement with the work of others (Chen *et al.*, 1974*b*; Deagen *et al.*, 1980*b*; Waalkes *et al.*, 1984*a*, *b*). Without prior cadmium exposure, the cadmium is bound to these three proteins at about equal amounts when examined by gel filtration, but prior exposure to the metal causes a 3–4-fold greater uptake in the low-M_r fraction, peak III (Deagen *et al.*, 1980*b*). This is in contrast with the binding of cadmium to proteins in liver and kidney, where cadmium is predominantly bound to one protein, MT (Winge & Rajagopalan, 1972; Deagen *et al.*, 1980*b*; Whanger & Deagen, 1982).

Cadmium causes an accumulation of zinc in MT of liver and kidney, but no effect upon the basal amount of zinc in this fraction of the testes (Whanger *et al.*, 1980). Feeding with high concentrations of zinc also causes an accumulation of this element in MT of rat liver and kidney (Chen *et al.*, 1977), but did not alter the zinc content in this fraction of rat testes (J. T. Deagen & P. D. Whanger, unpublished work). Similar patterns have been noted for sheep and cattle (Whanger *et al.*, 1981), where, even though zinc accumulated in liver and kidney MT, it did not accumulate in this fraction in sheep or bovine testes when high concentrations of dietary zinc were given. Waalkes *et al.* (1984*a*) also found zinc-binding properties with their low-M_r cadmium-binding proteins from testes. However, our results indicate that the zinc contents of these proteins are not markedly altered *in vivo* by elevated cadmium or zinc exposure of animals. Since the present results indicate that the major cadmium-binding proteins, and presumably the zinc-binding ones, in testes are not MT, this offers a possible explanation for the different behaviour of testes compared with other tissues.

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