

Physiological zinc-binding proteins of medium molecular weight in the rat gut

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(Received 20 May 1985 - Accepted 8 October 1985)

1. Gel filtration on Sephadex G 75 was used to separate the medium-molecular-weight zinc-binding proteins from the soluble fractions from the duodenal and jejuno-ileal segments of the rat gut at 30 min after the intragastric administration of a tracer dose of ^{65}Zn . These proteins were resolved by ion-exchange chromatography on DEAE cellulose.
2. In both the duodenum and jejuno-ileal segment an appreciable fraction of the total soluble Zn was bound in a protein fraction that resembled metallothionein (MT) in its behaviour on gel filtration. These fractions, however, were not homogeneous, but contained several medium-molecular-weight Zn-binding proteins. In the duodenum, but not in the jejuno-ileal segment, two of these proteins appeared to be the isometallothioneins, ZnMT-I and ZnMT-II.
3. These results suggest a possible role for MT in the binding of newly-absorbed Zn in the duodenal mucosal cells. They also show that gel filtration alone is insufficient for the identification of MT in the intestine.

The recognition of human pathological disorders, such as acrodermatitis enteropathica (Moynahan, 1974) and coeliac disease (Elmes *et al.* 1976), in which defects in zinc absorption may play an important role, has led to many attempts to elucidate the absorptive mechanisms and pathways in animals and man (Hurley *et al.* 1977; Cousins, 1979; Davies, 1980; Starcher *et al.* 1980; Jackson *et al.* 1981; Bonewitz *et al.* 1982; Sugawara, 1982). One major area of contention has been the possible role of the medium-molecular-weight (MMW) protein, metallothionein (MT), in the process of absorption. The two isomers of this metalloprotein have been isolated from the small intestine of the adult rat after either parenteral injection of Zn or oral administration of cadmium, and characterized unequivocally by their amino acid compositions (Richards & Cousins, 1977; Taguchi & Nakamura, 1982). The observation of an inverse relation between the intestinal MT concentration and the transfer of Zn to the portal circulation led Richards & Cousins (1975, 1976; see also Cousins, 1979) to suggest that this metalloprotein regulates the transport of newly-absorbed Zn through the mucosal cells to the carrier-proteins in the plasma. This hypothesis was rejected by Starcher *et al.* (1980) who concluded that the rate of Zn absorption is related directly to the intestinal concentration of MT, which functions as a transport protein. This conclusion, however, now seems untenable since, according to Flanagan *et al.* (1983), Zn absorption in the mouse is unaffected by the concentration of MT in the intestine.

As much of the above-mentioned work was done with non-physiological doses of Zn, it is possible that the accumulation of mucosal ZnMT is not (part of) a homeostatic mechanism, but is due either to saturation or to the breakdown of normal regulatory control

(Bremner, 1982). Whilst it is clear that either parenteral or oral administration of a single dose of Zn leads to the temporary accumulation of ZnMT in the intestine (see e.g. Richards & Cousins, 1975; Menard *et al.* 1981), and there is a direct correlation between elevated dietary Zn intake and mucosal MT content (Hall *et al.* 1979; Olafson, 1983), it remains to be established whether the low concentration of the metalloprotein in the intestine of unsupplemented rats (Hall *et al.* 1979) is relevant to the absorption of Zn under normal physiological conditions. To investigate this, a study has been made of the binding of ^{65}Zn to the MMW proteins of the mucosal layers of the duodenum and jejunum-ileal segment of the rat gut at 30 min after an oral dose. Since the transfer of Zn from the lumen to the wall of the small intestine occurs very rapidly (Jackson *et al.* 1981), whereas the induced synthesis of MT is not immediate (see e.g. Menard *et al.* 1981), it was considered that only the endogenous Zn-binding proteins would be labelled in significant amounts at this time.

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats were used for all experiments. They were maintained on a standard laboratory diet and were fasted for 24 h before and throughout the experiments to ensure that the stomach and small intestine were relatively free of digesta. For studies on the physiological Zn-binding MMW proteins, each animal received $4\ \mu\text{Ci } ^{65}\text{Zn}$ (Amersham International plc, Amersham, Bucks) supplemented with zinc chloride ($0.25\ \mu\text{mol}$). At 30 min after the administration of the dose in 0.5 ml distilled water via a stomach-tube, each rat was killed by exsanguination under diethyl ether anaesthesia. The entire gut was removed immediately, cleaned of extraneous tissue and dissected to separate the duodenal and jejunum-ileal segments. These were washed with 1–2 ml cold isotonic saline (9 g sodium chloride/l) to remove the gut contents and then cut lengthwise with scissors. The mucosal cells were removed with a glass slide and either processed immediately or frozen in liquid nitrogen (-197°) until required. ZnMT was induced in the liver and intestine by the intraperitoneal injection of ZnCl_2 ($25\ \mu\text{mol}$), the animals being killed by decapitation after 18 h (Richards & Cousins, 1975). The livers and mucosal cells, separated from the isolated mucosal segments as described previously, were stored frozen in liquid N_2 .

Analytical methods

The mucosal cells from the appropriate intestinal segments from at least four rats were pooled, suspended in ice-cold 10 mM-ammonium formate buffer, pH 8.0 (5 ml or 3 vol. for tissue weights < 1 g and > 1 g respectively), and homogenized in a motor-driven Potter-Elvehjem homogenizer. The soluble fractions were separated by centrifugation (140 000 g for 45 min at 5°) and applied to columns of Sephadex G 75 (Pharmacia Ltd, Milton Keynes). The columns were eluted at 5° with 10 mM-ammonium formate buffer, pH 8.0, at a flow-rate of either 8–10 ml/h or 13–14 ml/h (see p. 372), fractions (either 3 ml or 4 ml in volume) being collected automatically. After analysis (see p. 374), the MMW fractions for each column were combined and lyophilized. The residual solid was dissolved in the same buffer (10 ml) and fractionated (fraction vol. 3 ml) on a column (300 mm \times 15 mm) of Whatman DEAE cellulose (Uniscience Ltd., Cambridge) with a linear gradient of 10–200 mM-ammonium formate buffer, pH 8.0, at a flow-rate of 28–30 ml/h as eluant. In one experiment, in which protein-bound Zn was replaced by Cd under anaerobic conditions, N_2 -purged buffers were used for the preparation and fractionation of a duodenal homogenate. The MMW fraction from the gel-filtration column was transferred directly to a DEAE cellulose column, pre-equilibrated with N_2 -saturated 10 mM-ammonium formate buffer.

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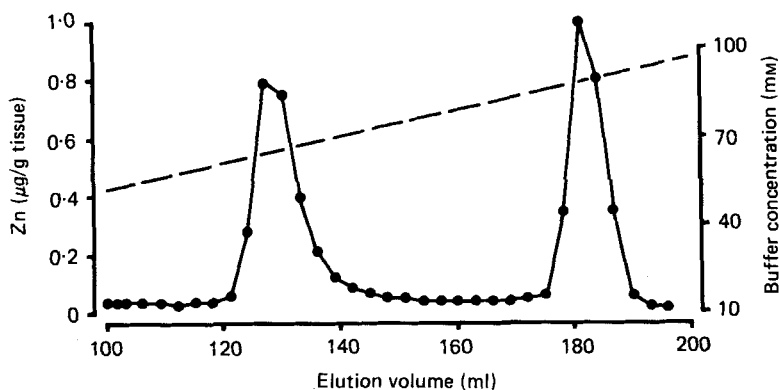


Fig. 1. Separation of the isomeric forms of zinc metallothionein (MT) induced in the intestine of the male rat by the intraperitoneal injection of zinc chloride ($25 \mu\text{mol}$). The ZnMT, isolated from the soluble fraction of the mucosal cells by gel filtration on a column ($900 \text{ mm} \times 15 \text{ mm}$) of Sephadex G 75, was fractionated by ion-exchange chromatography on a column ($300 \text{ mm} \times 15 \text{ mm}$) of DEAE-cellulose with a linear gradient (---) of 10–200 mM-ammonium formate buffer, pH 8.0, as eluant. The MT isomers, ZnMT-I and ZnMT-II, were eluted at buffer concentrations of 65 mM and 88 mM respectively.

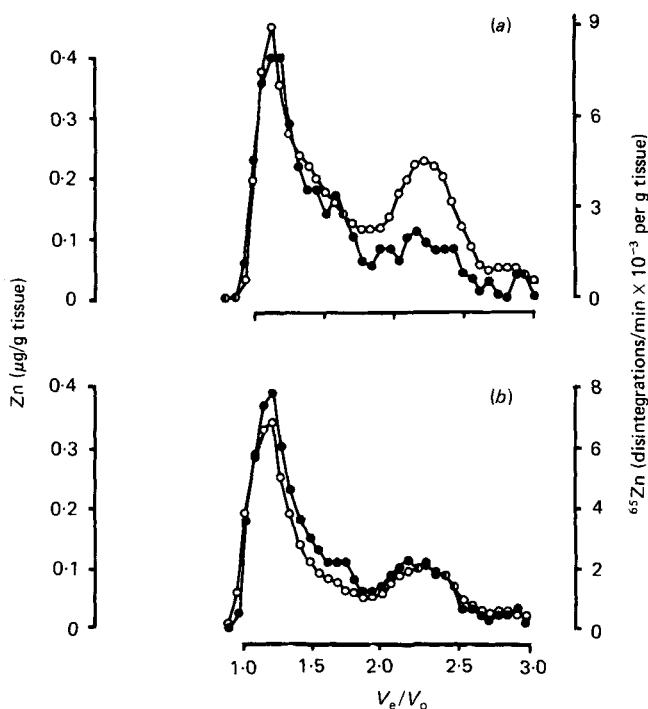


Fig. 2. Elution profiles (elution volume (V_e)/void volume (V_0)) of zinc (●) and ^{65}Zn (○), obtained by gel filtration on columns ($900 \text{ mm} \times 15 \text{ mm}$) of Sephadex G 75, of the soluble fractions from the mucosa of (a) the duodenum and (b) the jejuno-ileal segment of the intestine of the male rat at 30 min after the intragastric administration of a tracer dose of ^{65}Zn ($4 \mu\text{Ci}$; $16 \mu\text{Ci}/\mu\text{mol}$ zinc chloride).

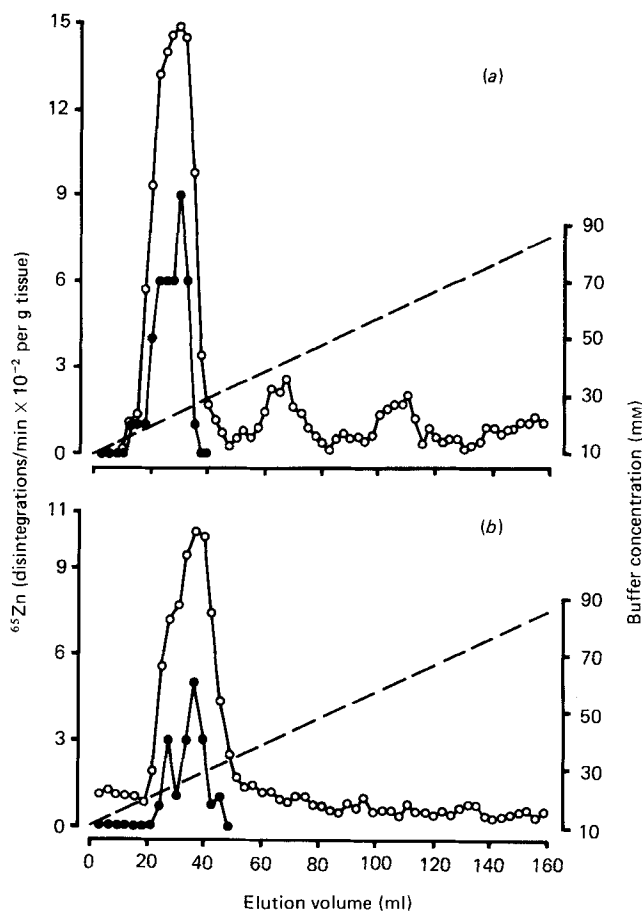


Fig. 3. Ion-exchange chromatography on a DEAE-cellulose column (300 mm \times 15 mm), with a linear gradient (---) of 10–200 mM-ammonium formate buffer, pH 8.0, as eluant, of the physiological medium-molecular weight, ^{65}Zn -labelled, Zn-binding-protein fractions (see Fig. 2) from (a) the duodenum and (b) the jejunio-ileal segment of the rat intestine. Zn, (●); ^{65}Zn , (○).

Column fractions were analysed for Zn and Cd by atomic absorption spectrophotometry and for ^{65}Zn in an Auto-gamma Scintillation Spectrometer (Model 5330; Packard Instruments Ltd., Caversham, Berks).

RESULTS

The ZnMT that was induced in the rat intestine by the intraperitoneal injection of ZnCl_2 (25 μmol) was isolated as a single peak (elution volume (V_e)/void volume (V_0) 1.9–2.0) from the soluble fraction of the mucosal tissue by gel filtration on a column of Sephadex G 75 (900 mm \times 15 mm) with 10 mM-ammonium formate buffer, pH 8.0, at a flow-rate of 8–10 ml/h as eluant. The crude metalloprotein was resolved by ion-exchange chromatography or a DEAE-cellulose column as described previously into the isometallothioneins, ZnMT-I and ZnMT-II, which eluted at buffer concentrations of 65 mM and 88 mM respectively (Fig. 1).

On gel filtration, under identical conditions, of the soluble fraction of the mucosal cells

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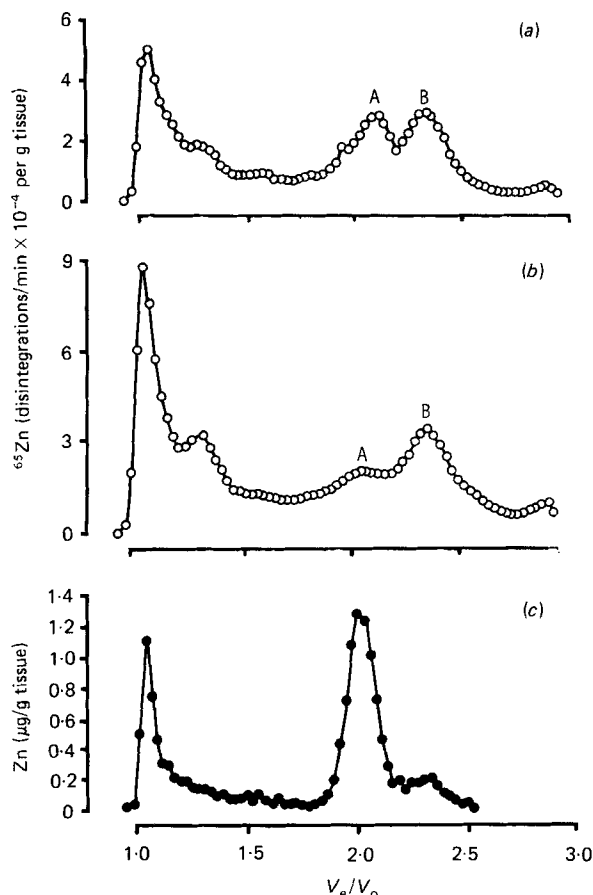


Fig. 4. Elution profiles (elution volume (V_e)/void volume (V_0)) of ^{65}Zn (○) or Zn (●) obtained by gel filtration on columns (900 mm \times 25 mm) of Sephadex G 75 with 10 mM-ammonium formate buffer, pH 8.0, at a flow-rate of 13–14 ml/h as eluant, of the soluble fractions from the mucosa of (a) the duodenum and (b) the jejunum-ileal segment of the rat intestine at 30 min after the intragastric administration of ^{65}Zn (4 μCi ; 16 $\mu\text{Ci}/\mu\text{mol}$ zinc chloride) and (c) the duodenum at 18 h after the intraperitoneal injection of ZnCl_2 (25 μmol).

from either the duodenum (Fig. 2(a)) or the jejunum-ileal segment (Fig. 2(b)) at 30 min after the oral administration of ^{65}Zn , both stable and radioactive Zn were found to be located in the high (H)- medium (M)- and low (L)-molecular-weight (MW) regions of the elution profile (V_e/V_0 of the column being 1.0–1.8, 1.8–2.7 and 2.7–3.0 respectively).

The LMW fraction (possibly the free cation or amino acid complexes thereof) was not resolved into subfractions by gel filtration on Sephadex G 75. Neither this fraction nor the HMW fraction was investigated further. The MMW fraction, which bound a greater proportion of the total soluble ^{65}Zn in the duodenum (Fig. 2(a)) than in the jejunum-ileal segment (Fig. 2(b)), resembled the Zn-induced ZnMT in its elution characteristics. Hepatic ZnMT, added at a concentration of 65 μg MT-bound Zn to appropriate volumes (equivalent to 1 g initial wet weight of tissue) of the soluble fractions from these intestinal segments, was recovered without loss of Zn in the MMW fraction at V_e/V_0 2.

Ion-exchange chromatography of each of these MMW fractions on DEAE cellulose, however, yielded only one major peak of protein-bound Zn (or ^{65}Zn), which eluted at low

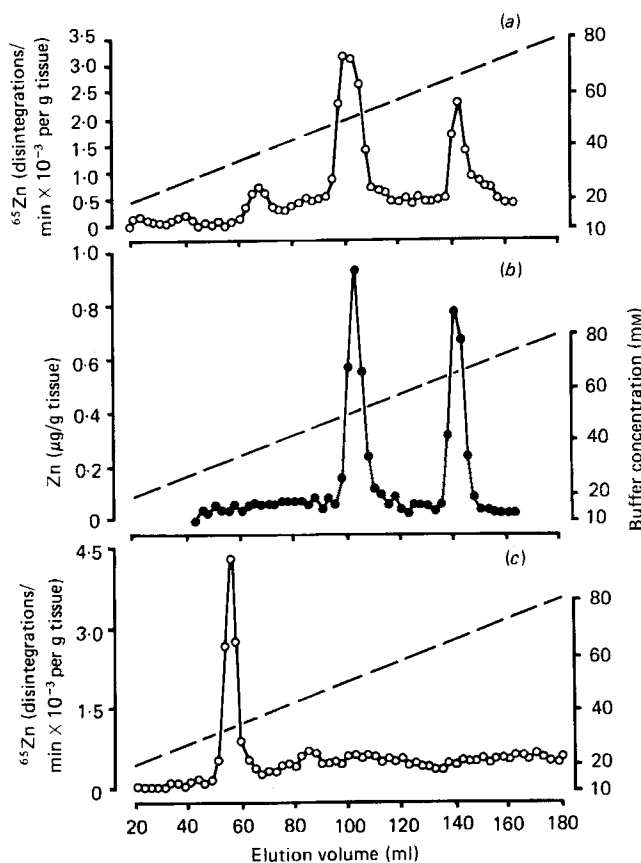


Fig. 5. Ion-exchange chromatography on DEAE-cellulose columns (300 mm \times 15 mm) with a linear gradient (---) of 10–200 mM-ammonium formate buffer, pH 8.0, as eluant, of the ^{65}Zn -labelled medium-molecular-weight, physiological Zn-binding protein fractions A (a) and B (c) from the duodenum (see Fig. 4(a)) and Zn metallothionein (MT) (b) from the duodenum of the rat injected intraperitoneally with Zn (see Fig. 4(c)). Zn, (\bullet); ^{65}Zn , (\circ). In (b) the MT-isomers, ZnMT-I and ZnMT-II, eluted at buffer concentrations of 50 mM and 65 mM respectively.

ionic strength (Fig. 3(a, b)). A similar weakly-charged metal-binding protein fraction was obtained by fractionation of an anaerobically-prepared duodenal homogenate after the addition of sufficient Cd (sixfold molar excess over the endogenous Zn) to displace the bound Zn and to block any free thiol groups. In the MMW fraction of the duodenum of the ^{65}Zn -dosed rat, two small additional peaks, the Zn contents of which were below the limit of detection, were identified by their ^{65}Zn contents (Fig. 3(a)).

The soluble MMW Zn-binding-protein fraction from either the duodenum or the jejuno-ileal segment of the normal rat intestine, which was recovered as a single peak from a 900 mm \times 15 mm column of Sephadex G 75 (Fig. 2), was separated into two peaks (A and B) by filtration through a larger column (900 mm \times 25 mm) of the same gel-matrix at a reduced flow-rate (13–14 ml/h; i.e. approximately half the usual flow-rate for a column of this size). The relative proportions of the two peaks differed in the soluble fractions from the duodenum (Fig. 4(a)) and the jejuno-ileal segment (Fig. 4(b)). Under the same conditions the ZnMT that was induced in either of the intestinal segments by the parenteral injection of Zn, remained as a single peak (Fig. 4(c)); the elution patterns for the soluble

fractions from the duodenum and jejunio-ileal segment were identical. Ion-exchange chromatography of fraction A from the normal duodenum yielded two major Zn-containing components (Fig. 5(a)) which, in their elution characteristics, resembled the isoMT that were resolved by the same method from the Zn-induced ZnMT of the duodenum (Fig. 5(b)) and jejunio-ileal segment. In contrast, most of the protein-bound Zn in fraction B was eluted from the DEAE-cellulose column as a sharp peak at low ionic strength (Fig. 5(c)) and the presence of the MT isomers (or similarly-charged proteins) was not detected. Fraction B from the jejunio-ileal segment also yielded a Zn-binding protein that eluted at a low, but apparently slightly higher ionic strength (42 mM-ammonium formate) than that from fraction B of the duodenum (i.e. 36 mM-ammonium formate; Fig. 5(c)). Fraction A from the jejunio-ileal segment, however, gave a diffuse distribution pattern (values not shown), possibly indicative of the separation of ionic Zn during chromatography, with the resolution of only one small peak which, from its position in the elution profile, may have been ZnMT-I.

DISCUSSION

From previous studies (Jackson *et al.* 1981) it is probable that, at 30 min after the oral administration of ^{65}Zn under the conditions of the present experiments, about 60% of the dose had been removed from the intestinal lumen and the majority of the absorbed ^{65}Zn remained within the gut wall. At this time the elution profile of ^{65}Zn that was obtained by gel filtration of the soluble components of the duodenum on a 900 mm \times 15 mm column of Sephadex G 75 was qualitatively similar but quantitatively different from that of the endogenous Zn (Fig. 2(a)). Thus although the largest amounts of both stable and radioactive Zn were present in the HMW-protein fraction, which probably contained the proteins of 10⁶ MW that Kowarski *et al.* (1974) consider to be concerned with Zn transport, the specific activity (total counts/total Zn) of this fraction (20 500 disintegrations/min per μg Zn) was appreciably less than that of the MMW fraction (44 900 disintegrations/min per μg Zn). In the jejunio-ileal segment (Fig. 2(b)), in which the distribution of stable Zn in the soluble proteins was similar to that in the duodenum, the specific activities of the HMW and MMW fractions were about the same (17 900 and 19 800 disintegrations/min per μg Zn respectively). It seems, therefore, that although the latter fraction of the duodenal mucosa contained only about 20% of the total soluble Zn, it was particularly avid in the binding of lumen Zn and, therefore, may be important in the process of Zn absorption.

Under the above-defined conditions of gel filtration the elution characteristics of the duodenal MMW fraction were similar to those of the MT, which was induced in both the duodenum and the jejunio-ileal segment of the rat by parenteral administration of ZnCl_2 (25 μmol). However, the assumption, made by Starcher *et al.* (1980) and by Sugawara (1982), that the physiological MMW Zn-binding fraction from the whole rat intestine can be identified as MT on the basis of its behaviour on gel filtration is erroneous. The MMW Zn-binding fractions from both the duodenum and the jejunio-ileal segment of the intestine of the normal rat, which were obtained as single peaks (Fig. 2(a, b)) under these conditions, were heterogeneous. Both yielded at least four Zn-containing protein fractions on high-performance liquid chromatography (D. Holt, M. J. Jackson and M. Webb, unpublished results) and were resolved by gel filtration on a larger column at a reduced flow-rate into two metalloprotein fractions (Fig. 4(a, b)). The ZnMT, induced in the intestine by excess Zn, however, remained as a single peak under these conditions (Fig. 4(c)). Nevertheless, the change in the dimensions or flow-rate of the initial Sephadex G 75 column, or both, affected the elution characteristics of the isometallothioneins, ZnMT-I and ZnMT-II, when these were separated by subsequent ion-exchange chromatography on a 300 mm \times 15 mm column of DEAE cellulose (see Figs 1 and 5(b)).

The first fraction (peak A), which was isolated from the duodenum of the normal rat by the modified gel-filtration procedure, was separated by ion-exchange chromatography into two major Zn proteins (Fig. 5(a)) which, in their elution characteristics, were identical to the isometallothioneins, ZnMT-I and ZnMT-II (Fig. 5(b)). The second peak (B) from the duodenum, which had a lower relative MW than that of peak A and thus could not be a MT-polymer, yielded only one major Zn protein of low charge on ion-exchange chromatography (Fig. 5(c)). This weakly-charged species remained after treatment of an anaerobically-prepared homogenate, before fractionation, with a metal (Cd) of much higher binding affinity for thionein than Zn (Webb, 1979) and thus seems unlikely to have been an artifact, due to the intramolecular oxidation of a Zn-undersaturated MT during the isolation procedures. Furthermore, on ion-exchange chromatography, peak B from the jejuno-ileal segment also yielded a Zn-protein fraction, similar to that from peak B of the duodenum. Analyses by Dr I. Bremner, according to the method of Mehra & Bremner (1983), however, showed that the MMW fraction from the jejuno-ileal segment, in contrast to that from the duodenum, did not have immunoreactivity against an antibody to rat liver ZnMT-I.

Analysis of the heat-stable (90°/4 min) components of homogenates of intestinal segments by pulse polarography led Flanagan *et al.* (1983) to conclude that, in the mouse, the duodenum contains the largest concentration of MT. The results of the present work also suggest that MT isomers are located mainly in the duodenum of the normal rat and form two of several MMW Zn-binding proteins which are rapidly labelled after the administration of ⁶⁵Zn by gavage. This suggests that they may be functional in Zn absorption although the presence of non-specific metal binding ligands which do not play a role in Zn absorption cannot be discounted. Nevertheless, comparison of Figs. 3(a) and 5(a) suggests that the content of MT in the duodenal mucosa may be very variable. This variation seems unlikely to be due to differences in proteolysis during the preparative and fractionation procedures, since hepatic ZnMT, added to the soluble fractions from the intestinal segments, was recovered unchanged in the subsequent gel filtration. This is in contrast to the observation of Cousins *et al.* (1978) that high proteolytic activity of the rat intestinal mucosa leads to the rapid hydrolysis of ZnMT and other Zn-binding proteins in the isolated cytosol with the formation of LMW (< 2000) Zn complexes. Restriction of food intake in the rat is known to increase the Zn and ZnMT contents of the liver (Bremner & Davies, 1975) and it is therefore possible that the concentrations of the MT isomers in the duodenum are also influenced by starvation. In the present work some experiments were undertaken using fed animals (results not shown), but quantitative comparison of the results with those for starved rats was not possible, since the presence of digesta in the intestines interfered with the absorption of ⁶⁵Zn. Nevertheless, it was clear from these studies that the presence of ZnMT-I and ZnMT-II in the duodenum was not an artifact due to starvation.

The authors are extremely grateful to Dr Ian Bremner (Rowett Research Institute, Aberdeen) for the radioimmunoassay of the MMW fractions from the duodenum and jejuno-ileal segment for MT-I.

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